The Importance of Oxidases in the Tolerance of Deciduous Leaf Infusions by Aedes (Stegomyia) aegypti and Aedes (Stegomyia) albopictus (Diptera: Culicidae)

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ABSTRACT  Aedes (Stegomyia) aegypti (L.) and Aedes (Stegomyia) albopictus (Skuse) larvae rely on oxidases to reduce toxicity of water soluble toxins from some senescent tree leaf infusions. The mortality of third instar Ae. aegypti larvae in live oak and pin oak leaf infusions increased significantly in the presence of piperonyl butoxide (PBO), a broad inhibitor of cytochrome P450s (CYPs). In contrast, PBO treatment did not increase mortality in water controls or infusions of northern red oak or sugar maple leaf infusions for Ae. aegypti larvae. A similar pattern was observed for Ae. albopictus larvae, that is, an increase in mortality when CYPs were inhibited in live oak leaf infusions and no increase in sugar maple leaf infusions or water controls. However, the fresh live oak leaf infusion (5 d old) was the most toxic infusion to Ae. aegypti, but appeared less toxic to Ae. albopictus than the older infusions. A direct comparison of survival between the two Aedes species revealed Ae. aegypti exhibited a greater mortality than Ae. albopictus in PBO-treated live oak leaf infusions. These findings suggest that toxic components of some leaf litter in larval habitats may impose cryptic energy costs (detoxification).

KEY WORDS  Aedes albopictus, Aedes aegypti, leaf litter, toxicity, cytochrome P450

The Asian tiger mosquito, Aedes (Stegomyia) albopictus (Skuse), and the yellowfever mosquito, Aedes (Stegomyia) aegypti (L.), are two invasive mosquito species whose close association with humans has facilitated their spread across several continents (O’Meara et al. 1995, Juliano and Lounibos 2005). Both species share similar life histories including the ability to colonize artificial container habitats in urban and periurban areas. Ae. aegypti is the primary vector of dengue, yellow fever, and Chikungunya viruses in many parts of the world, whereas Ae. albopictus is a major nuisance species that can also be an important vector, as illustrated by recent outbreaks of Chikungunya virus in Italy and French Island of La Reunion and LaGrosse encephalitis virus in southern areas of eastern United States (Mackenzie et al. 2004, Josseran et al. 2006, Angelini et al. 2007, Medlock et al. 2012). Ae. albopictus is widely considered one of the most invasive container mosquito species worldwide, with a broader latitudinal range than Ae. aegypti, primarily due to the resistance of diapausing eggs to desiccation and cold temperatures (Juliano and Lounibos 2005, Benedict et al. 2007, Urbanski et al. 2010, Lounibos et al. 2011).

In the United States, Ae. albopictus was first detected in Houston, TX, in 1985, probably introduced in waste tires from southeast Asia, and is now widely distributed throughout the southeastern states with perennial populations as far north as Illinois, Ohio, and New York (Hawley 1988, Benedict et al. 2007). The rapid dispersal of Ae. albopictus through Florida coincided with regional declines and local extinctions of Ae. aegypti, a species introduced to the Americas from Africa several times since the 15th century (Moore and Mitchell 1997). However, the two species have established a stable coexistence at some sites, whereas Ae. albopictus has been unable to establish resident populations in some coastal sites (O’Meara et al. 1995, Juliano et al. 2004, Yee et al. 2013). The mechanisms underlying these disjunctive distribution patterns are poorly understood, despite their potential importance for estimation of disease risk and improving vector management strategies.

Context-dependent interspecific competition is considered to be one of the most plausible mechanisms accounting for the variable distribution patterns of Ae. aegypti and Ae. albopictus in Florida based on field and laboratory observations that Ae. albopictus larvae outcompete those of Ae. aegypti under most natural detritus conditions (Moore and Fisher 1969, Black et al. 1989, Daugherty et al. 2000, Murrell and Juliano 2008). In general, slow decaying detritus is associated with lower microbial growth, which yields a competitive advantage for the more starvation-resistant Ae. albopictus larvae. In contrast, rapidly decaying detritus, which is associated with greater microbial growth, often reduces, eliminates, or even reverses the competitive advantage of Ae. albopictus in...

A common stress for aquatic organisms is the presence of natural and synthetic xenobiotics (Snyder 2000; Boyer et al. 2006a,b), thus mosquito distribution and abundance may partially reflect species-specific detoxification capabilities (Reiskind et al. 2012). Plants produce a wide array of allelochemicals that impact the survival and fitness of arthropods typically in a dose-dependent manner (Regnault-Roger et al. 2008, Silva et al. 2008, Pohl et al. 2011). In fact, natural products extracted from fruits, leaves, stems, barks, or roots are considered potential sources of future mosquito management agents (Hardin and Jackson 2009, Rahuman et al. 2009, Ghosh et al. 2012). Larval development and survival and the subsequent adult fitness may be dependent upon their adaptation to groups of toxins in decaying leaf litter. For example, decaying alder leaf detritus is high in polyphenols and species typically associated with polyphenol-rich habitats tended to have a greater tolerance to lignin-like compounds in the leaf litter than mosquito species not associated with alder habitats (David et al. 2000a,b,c). Assays with third instar *Ae. aegypti*, a sensitive non-adapted reference species, indicated the toxicity of the crude alder leaf litter increased with detritus age (up to 10 mo) and was not related to the toxicity of short-term leachates of alder leaves (David et al. 2001). An important component in the differential tolerance of *Aedes* species to alder leaf litter was their species-specific detoxification capabilities, especially the difference in oxidation potential of midgut cytochrome P450s (CYPs; David et al. 2000b,c; Tilquin et al. 2004; Poupardin et al. 2008). Different types of leaf detritus leachates induced CYP genes in *Ae. aegypti* and *Ae. albopictus*, and the leachate appeared to have costs for both larval and adult fitness (Kim and Muturi 2012).

The goal of our study was to evaluate the role of CYP oxidases in the tolerance of third instar larvae of *Ae. aegypti* and *Ae. albopictus* to common leaf infusions. Acute mortality studies measure the impact of short-term exposure, typically of a late instar, whereas resource utilization studies measure the impact of chronic exposure to detritus types (Alto and Lounibos 2013). We compared the larval mortality of two *Aedes* species in infusions of *Quercus* and *Acer* species and water controls with and without the addition of piperonyl butoxide (PBO), a CYP inhibitor. Three hypotheses were addressed: 1) aqueous infusions of oak and maple leaves, known to contain phenolic compounds, are toxic to *Ae. aegypti* and *Ae. albopictus* when oxidative enzymes are suppressed, 2) the two exotic *Aedes* species exhibit similar patterns of larval mortality in response to native leaf infusions, and 3) the length of time leaf material is allowed to leach and decompose in water increases infusion toxicity to mosquito larvae.

**Materials and Methods**

**Bioassay 1: Toxicity of Oak and Maple Leaf Infusions to *Ae. aegypti* in the Presence of a CYP Inhibitor.** The first bioassay exposed third instar larvae of *Ae. aegypti* to 155-d-old infusions made from senescent, air dried leaves of sugar maple (*Acer saccharum* Marshall), live oak (*Quercus virginiana* Miller), northern red oak (*Quercus rubra* L.), and pin oak (*Quercus palustris* Münchhausen). Infusions were made by fermenting 20 g of powdered leaves in 10 liters of deionized water. Coarse ground leaf material was used in the first assay to maximize the surface area for microbial metabolism and to mimic the natural long-term processing of detritus decay to fine organic particulates (Kim and Muturi 2012). The subsequent bioassays in our study included leachate of whole leaves and large leaf fragments, which was meant to mimic the earlier stages of detritus decay. Toxicity in the aged ground leaf material in bioassays 1, 2, and 3 probably represents the combined extended extraction of water soluble toxins or microbial byproducts associated with decomposition, whereas the younger, whole, or partial leaf infusions probably reflect the sensitivity of the *Aedes* species to water-soluble toxins only.

Negative controls for the first bioassay were freshly prepared deionized water. For each infusion and water control replicate, 370 ml was added to a 400-ml plastic Tri-pour graduated beaker (United States Plastic, Lima, OH). Twenty F9 generation of *Ae. aegypti* larvae, originally collected from Jacksonville, FL, and maintained in colony at the Illinois Natural History Survey in Champaign (Illinois), were added to Tri-pour beakers containing 0.1, 0.5, or 0.0 ppm of PBO (Sigma-Aldrich, St. Louis, MO). Subsequent bioassays used a standardized toxicity bioassay protocol with 100 ml of infusion or water in paper cups (Eckenbach et al. 1999, Lampman et al. 2000). Analytical grade PBO was diluted in acetone such that 100 μl added to the container yielded the appropriate PBO concentration. The “0 ppm PBO” treatment containers received a 100-μl sham treatment of acetone. This method was repeated in all subsequent bioassays.

In the first bioassay, there were three replicates of each PBO-treated infusion (1.0 and 5.0 ppm) and PBO-treated water controls. The untreated infusions and water controls (i.e., no PBO added) had four replicates each. This bioassay had 50 Tri-pour beakers (i.e., five treatments lacking PBO with four replicates each and five treatments with two PBO concentrations and three replicates of each). The beakers were monitored for mortality at 24 and 48 h posttreatment. Mosquito larvae that did not move away when prodded with a wooden applicator stick were graded as dead or moribund for all assays.

The first bioassay was conducted in a walk-in incubator at 27 ± 2°C with a photoperiod of 16:8 (L:D) h. Because of the subsequent lack of availability of space
in the incubator, the three following bioassays were conducted in an insectary at a lower temperature (21.1 ± 2°C), but same long-day photoperiod. We did not systematically explore the role of temperature in the pattern of response. Thus, the results of the first bioassay were used to narrow the number of leaf treatments in the other assays, so we could explore other sources of variation, such as infusion age. *Aedes* eggs were hatched and transferred within 8 h to enamel pans with 1.0 liters of deionized water. The first instar larvae were fed 3–5 drops of fish food slurry and a rabbit chow pellet was added after 24 h. At 72 h posthatching, the larvae were third instars, which were used in all bioassays.

**Bioassay 2: Impact of Infusion Type (Live Oak and Sugar Maple Leaf Infusions) and Age of Infusion on Survival of *Ae. aegypti*** Ten third instar F9 *Ae. aegypti* larvae were added to 100 ml of infusion or water in 296-ml paper drink cups (No. 410, Solo Cups, Urbana, IL). The bioassay exposed *Ae. aegypti* to two infusions (sugar maple and live oak) of three ages (251, 12, and 5 d), water controls of two ages (12 and 5 d), and two PBO concentrations (0 and 5 ppm) in infusions and water. There were four replicates of each treatment for a total of 64 containers. The live oak and sugar maple leaves were chosen for subsequent comparisons with *Ae. albopictus* and *Ae. aegypti* because they represented infusions with strong and weak interactions with PBO in the first bioassay. The 12- and 5-d-old leaf infusions were made by adding 20 g of whole leaf and large leaf fragments of the appropriate species to 10 liters of deionized water in 18.9-liter plastic buckets. The 251-d infusion was the same as the one used in the first bioassay from powdered leaf material. The original infusion was meant to mimic long-term processing of leaf material in an ecosystem, whereas the other infusions are more indicative of aquatic leachates in early stages of detritus formation. The deionized water treatments (with and without PBO) were from water samples held in 18.9-liter plastic buckets for 12 and 5 d before the start of the bioassay. No water control equivalently aged at 251 d was available. Mortality in all beakers was measured at 24, 48, and 72 h after treatment with PBO.

**Bioassay 3: Impact of Live Oak Leaf Infusion and Age of Infusion on Survival of *Ae. albopictus*** The third bioassay exposed 10 F9 generation third instar *Ae. albopictus* from Florida to live oak and sugar maple leaf infusions of two ages (258 and 12 d) in the absence or presence of PBO (0 vs. 5 ppm PBO). These infusions were from the same stock infusions used in the previous bioassay, but a week older. Each infusion and water control had a series with 0.0 and 5.0 ppm PBO. The bioassay included two infusions, two ages for each infusion, and two PBO treatments with four replicates of each. Each of the two infusion age groups had a set of four PBO-treated and four untreated water samples (all 12 d old; i.e., a total of eight PBO and eight untreated water controls for the experiment). There were 64 containers in this assay and mortality was measured at 24, 48, and 72 h as in the second bioassay.

**Bioassay 4: Impact of Live Oak Leaf Infusion and Age of Infusion on Survival of *Ae. albopictus* and *Ae. aegypti* in the Presence and Absence of PBO** The fourth experiment simultaneously compared the response of the two *Aedes* species to PBO-treated and untreated live oak leaf infusions of two ages (20 and 5 d) with water controls (all 5 d old) for each species. Each infusion and water control had a 0.0 and 5.0 ppm PBO treatment, with the 0.0 ppm PBO treatment consisting of the addition of 100 μl of acetone only. Each treatment combination was replicated four times for a total of 48 containers. Mortality was recorded as in the previous two bioassays.

**Statistical Analyses.** For each bioassay, the influence of the main effects and their interactions on mean mortality at the longest exposure period, either 48 h (bioassay 1) or 72 h (bioassays 2–4), were evaluated by multiway ANOVAs (SPSS 11.0.2, IBM SPSS Inc., Somers, NY). Mortality in the PBO-treated and untreated water replicates were used to correct for control mortality in the PBO-treated and untreated infusions. Water treatments were excluded from the multiway ANOVAs because we lacked water controls with complementary periods of aging as in the infusions. Most bioassays exhibited significant interactions of main effects in the multiway ANOVAs; therefore, the means and SEM are presented for the uncorrected mortality of each unique combination of treatment factors, including treated and untreated water controls. This provided means for comparison even when main effects significantly interacted. Differences in mortality due to these mixed treatments were identified by post hoc separation of mortality using Bonferroni’s method of multiple comparisons (SPSS 11.0.2).
different from controls with the addition of PBO, and that the magnitude of mortality of Ae. aegypti larvae differed in a nonlinear fashion with the age of the oak infusion (Fig. 2). In the presence of PBO, the 5-d-old live oak infusion had greater larval mortality compared with 12- and 251-d-old live oak infusions. This effect was not observed in untreated controls. The mortality in the 12-d-old sugar maple infusion with PBO was equal to the mortality in the treated live oak infusion of 251 d, but the treated maple infusion was not significantly different from the untreated sugar maple control of the same age. The 12-d-old PBO-treated sugar maple infusion had significantly greater mortality than the untreated sugar maple infusions of 5 and 251 d in this assay, but it was not significantly different from PBO treated sugar maples aged 5 or

**Fig. 1.** Mean percent mortality (±SEM) of third instar larvae of Ae. aegypti at 48 h after treatment with 0, 1, or 5 ppm of PBO in water and leaf infusions of four deciduous trees (live oak, northern red oak, pin oak, and sugar maple). Means with the same letter are not significantly different at α = 0.05 by Bonferroni pairwise multiple comparison (ANOVA: df = 14, 35; F = 11.5; P < 0.001).

**Fig. 2.** Mean percent mortality (±SEM) of third instar larvae of Ae. aegypti at 72 h after treatment with 0 or 5 ppm of PBO in water and leaf infusions of live oak and sugar maple that were aged 5, 12, or 251 d before the addition of mosquito larvae. Means with the same letter are not significantly different at α = 0.05 by Bonferroni pairwise multiple comparison (ANOVA: df = 15, 48; F = 9.3; P < 0.001).
251 d, which in turn were not different from their untreated controls. Addition of PBO tended to slightly increase mortality above the untreated control, but the increase was seldom significant.

Bioassay 3: Impact of Infusion Type (Live Oak and Sugar Maple), Infusion Age (12 and 258 d), and Presence of CYP Inhibitor (PBO) on Survival of Ae. albopictus. The mean mortality of third instar Ae. albopictus larvae at 72 h was significantly affected by an interaction between infusion type and PBO concentration (df = 1.24; F = 25.73; P < 0.001). The presence of PBO in live oak leaf infusion resulted in ≈10-fold increase in mortality of Ae. albopictus larvae compared with untreated live oak infusion, but these effects were not observed in sugar maple infusion (Fig. 3). In contrast, age of infusion (258 or 12 d) did not affect the mean mortality of Ae. albopictus larvae (P = 0.766).

Bioassay 4: Impact of Live Oak Leaf Infusion and Age of Infusion on Survival of Ae. albopictus and Ae. aegypti in the Presence and Absence of CYP Inhibitor. The final bioassay simultaneously compared the response of the two Aedes species to live oak leaf infusion, aged 5 and 20 d and with or without PBO (i.e., 0 or 5 ppm). Larval mortality was significantly influenced by an interaction between PBO presence, age of infusion, and mosquito species (df = 1.24; F = 9.87; P = 0.01). Larval mortalities of Ae. aegypti in live oak infusions treated with 5.0 ppm of PBO were significantly greater than controls for both infusion ages (5 and 20 d; Fig. 4). Furthermore, mortality in Ae. aegypti was significantly greater than that of Ae. albopictus. The two species also differed in response to infusion age; Ae. albopictus mortality was only significantly different from untreated control infusion in the 20-d-old infusion. The changes in mean mortality suggested an incremental decrease with infusion age for Ae. aegypti, and an increase with infusion age for Ae. albopictus.

Discussion

Our study was designed to quantify the acute toxicity of different ages of oak and maple leaf infusions for third instar larvae of Ae. aegypti and Ae. albopictus in the presence and absence of an inhibitor of CYP oxidases (i.e., PBO). The first hypothesis that all oak and maple deciduous leaf infusions have toxic principles that require oxidative metabolism by CYPs was rejected. The mean mortality of Ae. aegypti third instars was 68.5% after 72 h in PBO-treated live oak infusions, 35.0% for PBO-treated pin oak, and between 10 and 13% for the other leaf treatments and untreated controls (Fig. 1). For third instar Ae. albopictus, we observed about a 20% increase in mortality with the addition of 5 ppm PBO in live oak infusions. 35.0% for PBO-treated pin oak, and between 10 and 13% for the other leaf treatments and untreated controls (Fig. 1). For third instar Ae. albopictus, we observed about a 20% increase in mortality with the addition of 5 ppm PBO in live oak infusions. Neither species exhibited a consistent increase in mortality in PBO-treated sugar maple infusions. The absence of toxicity in sugar maple and northern red oak leaf infusions in our study was unexpected because leaves of both trees are known to contain tannins, simple phenolics, and polyphenols (Lovett et al. 2004, Muturi et al. 2012), which are toxic to Aedes species in the presence of esterase and oxidase metabolic inhibitors (David et al. 2000b, Poupardin et al. 2008). Furthermore, we had conjectured that sugar maples would be toxic because aqueous infusions induce P450 genes (Kim and Muturi 2012), presumably in response to xenobiotics. Chronic exposure studies indicated a low survival of Ae. aegypti and Ae. albopictus to adulthood in sugar maple infusions, which is often anecdotally related to potential tannin and phenolic content of sugar maple leaves (Palik et al. 2006, Kim and
Muturi 2012, Muturi et al. 2012). However, the suppression of oxidative metabolism with PBO did not increase acute toxicity of sugar maple infusions to either *Aedes* species. Xenobiotics in aquatic ecosystems may also act indirectly by altering microbial growth and composition, thereby reducing energy reserves (Lovett et al. 2004; Murrell and Juliano 2008, 2012). Our results indicate that 1) CYPs are important for both *Aedes* species to tolerate toxic principles in live oak leaf infusions and 2) sugar maple and northern red oak infusions lack toxic levels of allelochemicals in presence of PBO. In the absence of CYP suppression, none of the infusions exhibited significant toxicity to third instar larvae of either mosquito species.

Our bioassays yielded mixed results for our second hypothesis that *Ae. albopictus* and *Ae. aegypti* exhibit equivalent trends in toxicity to the leaf infusions when CYPs were suppressed. Both species had an increase in mean mortality with the addition of 5 ppm PBO to live oak infusions, and both species did not exhibit an increase in mortality to sugar maple infusions under CYP suppression. In the absence of PBO suppression, none of the infusions exhibited significant toxicity to third instar larvae of either mosquito species.

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Our third hypothesis that the length of time leaf material is allowed to leach and decompose in water increases toxicity was also not supported by the data. We had anticipated older infusions would be more toxic because of a greater time to extract toxic principles and the buildup of microbial decomposition by-products as observed in aged alder leaf detritus (David et al. 2000b, Poupardin et al. 2008). Instead, we found considerable variability in toxicity of live oak leaf litter with age of infusion. For *Ae. aegypti*, the 5-d-old live oak leaf infusion consistently had higher mean mortalities in CYP-suppressed *Ae. aegypti* than the older infusions, although the difference was not always significant (Figs. 2 and 4). We did not compare the chemical composition of the aged infusions; therefore, we can only report that the toxic factors seem to rapidly leach from live oak leaves for *Ae. aegypti*. In contrast, *Ae. albopictus* exhibited no difference in mortality between infusions of 12 and 258 d (Fig. 3), and the 5-d-old infusion was not toxic to *Ae. albopictus* (Fig. 4). Thus, the two species have a different response pattern to the toxins or different chemicals vary in toxicity to the two species. Our experimental design does not allow us to differentiate between these possibilities. Furthermore, nutritional stress may have played a role as the youngest infusion undoubtedly had the lowest concentration of microbes. The competitive superiority of *Ae. albopictus* larvae over *Ae. aegypti* larvae under poor quality detritus may include both nutritional (Arrivillaga and Barrera 2004, Barrera 2011, Murrell and Juliano 2008, Reiskind and Lounibos 2013) and toxicological components (our study). Further investigations are needed to partition...
nutritional and toxicological components in species interactions.

Induction of metabolic enzymes can have negative effects on mosquito performance (Rivero et al. 2011), similar to those observed under limiting food conditions (Juliano 2009). Our results support the conclusion that species-specific patterns of mosquito distribution and abundance may be related to tree leaf toxins in the aquatic habitat, as speculated by Reiskind et al. (2012); however, the presence of toxins is not always easily detected, as both *Aedes* species in our study showed no acute mortality in the absence of a CYP inhibitor.

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