No Evolutionary Response to Four Generations of Laboratory Selection on Antipredator Behavior of *Aedes albopictus*: Potential Implications for Biotic Resistance to Invasion

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ABSTRACT *Aedes albopictus* (Skuse) is an invasive container-dwelling mosquito and an important disease vector that co-occurs with the native mosquito, *Aedes triseriatus* (Say), and the predatory midge, *Corethrella appendiculata* (Grabham). Larval *Ae. triseriatus* show significantly greater antipredatory responses when compared to larval *Ae. albopictus* in the presence of predation cues from *C. appendiculata*. The potential for evolution of antipredatory behavioral responses to *C. appendiculata* in *Ae. albopictus* is unknown. We used a controlled laboratory selection experiment to test whether *Ae. albopictus* could evolve antipredatory behavioral responses to *C. appendiculata* predation. We subjected replicate *Ae. albopictus* populations to four generations of predation by *C. appendiculata* or a predator-free control treatment and compared the behavior and life history of *Ae. albopictus* in the two treatments in each generation. There were no differences in *Ae. albopictus* behavioral responses between predation and control lines in any of the four generations. There was also no evidence of differences in life histories between predation and control lines. *Ae. albopictus* is superior as a competitor compared with *Ae. triseriatus*, which it has replaced in areas where *C. appendiculata* are rare. Our results suggest limited potential for *Ae. albopictus* to evolve stronger antipredatory behavioral responses to *C. appendiculata* predation and imply that *C. appendiculata* will continue to act as an impediment to invasion by *Ae. albopictus* and replacement of *Ae. triseriatus* and to promote coexistence of these competitors.

KEY WORDS *Aedes albopictus*, *Aedes triseriatus*, *Corethrella appendiculata*, antipredatory behavior, controlled selection

Although the ecological impacts of invasive species are increasingly well studied (Mack et al. 2000, Sakai et al. 2001), evolutionary effects occurring with invasions are much less well documented (Cox 2002). In an invasion, species encounter new environments and new interacting species. Lee (2002) and the references therein emphasize the importance of natural selection on invasive species in adapting to novel environments. In reviewing evolutionary effects involving invasive species, effects of invaders on resident competitors (Kitajima et al. 2006), effects of novel physical environments on invaders (Huey et al. 2000, Roy et al. 2000), and effects of invasive predators on native prey (Freeman and Byers 2006) have been documented. However, we know of no controlled tests for evolutionary effects of newly encountered, resident predators on invading prey species. Invading prey sometimes do not initially show appropriate responses to novel predators (Kesavaraju and Juliano 2004), so that invasive species may be selectively preyed on by the native predators, thereby contributing to the biotic resistance of a native community and potentially limiting impacts of the invader on native species (DeRivera et al. 2005). This component of biotic resistance may be temporary if natural selection by native predators on invasive prey causes evolution of improved responses by invasive prey, thus changing the outcome of native–invasive interactions (Mooney and Cleland 2001, Lee et al. 2003).

Adaptive responses of prey to cues from predators may include behavioral, morphological, or life history changes (Lima and Dill 1990). Behavioral adaptations seem to predominate in aquatic systems (Sih 1984). Understanding these behavioral adaptations is vital in evaluating the proximate causes of invasion success (Holway and Suarez 1999). Prey that alter their behavior in response to cues to predation risk have been shown to be less vulnerable to predation (Kats et al. 1988, Buskirk et al. 1997, Relyea 2002b). Consider a system where a native prey alters its behavior in response to cues from a native predator. The success of an invader entering that system as a competitor of the native prey may depend on whether it already shows some level of appropriate behavioral modifications or other facultative changes in response to cues from the novel native predators it encounters as it invades a

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new area. If there is heritable variation in responses to such cues within the population of invaders, there is potential for the invader population to evolve responses to predation cues because of selection imposed by the novel native predators.

Water-filled small containers such as natural depressions in tree trunks (tree holes) support a diverse invertebrate community (Kitching 2000) that provides an ideal experimental system in which to test for evolution of an invasive species in response to native predators. These container systems can be natural (e.g., tree holes) and man made (e.g., cemetery vases and discarded tires). *Aedes albopictus* is a container-dwelling invasive mosquito that invaded the United States in the 1980s from Asia (Hawley et al. 1987) and has established itself in many of the southeastern parts of the United States (O'Meara et al. 1995). *Ae. albopictus* is a vector of dengue (Hawley 1988) and is a competent laboratory vector for diseases such as West Nile (Ibanez-Bernal et al. 1997, Turell et al. 2005). Larvae of the midge *Corethrella appendiculata* occur in some of the same habitats as invasive *Ae. albopictus* in Florida (Kesavaraju et al. 2008), and *C. appendiculata* prey readily on early instars of *Ae. albopictus* and other container-dwelling mosquitoes (Griswold and Lounibos 2005b). *C. appendiculata* larvae use mechanoreceptors to detect their prey and predominantly hunt at the bottom of the containers (Kesavaraju et al. 2007). Prey that move at the bottom of containers are at a higher risk of being captured by *C. appendiculata* than are motionless prey at the surface of the water (Kesavaraju et al. 2007). Second instars are more vulnerable to predation than are later life cycle stages (Kesavaraju et al. 2007). Although predatory *Corethrella* species occur in Asia, species from the native range of *Ae. albopictus* primarily occur in ground water habitats and not the containers occupied by larval *Ae. albopictus* (Borkent 2008). Hence, *Ae. albopictus* has no evolutionary history with *C. appendiculata* and probably has no evolutionary history with any predatory *Corethrella.*

Aedes albopictus larvae are typically competitively superior to other mosquitoes such as *Aedes triseriatus* (Livdahl and Willey 1991, Teng and Apperson 2000, Aliabadi and Juliano 2002, Juliano and Lounibos 2005). Despite *Ae. albopictus*'s apparent competitive superiority to *Ae. triseriatus* under laboratory conditions, invasion of south Florida by *Ae. albopictus* has not resulted in reduced abundances of *Ae. triseriatus* in the field, especially in tree holes (Lounibos et al. 2001). Compared with *Ae. albopictus*, *Ae. triseriatus*, which has a history of exposure to *C. appendiculata*, shows more extensive plasticity in behavioral responses to *C. appendiculata* predation risk cues (Kesavaraju et al. 2007). In Florida, *Ae. albopictus* abundances are negatively correlated with *C. appendiculata* abundances, whereas *Ae. triseriatus* abundances seem to be independent of *C. appendiculata* (Kesavaraju et al. 2008). Classic studies (Morin 1981) and more recent work (Ciros-Perez et al. 2004) showed that coexistence of a competitively inferior species with a superior species can be aided by selective predation on the competitively superior species. The superior competitor always seems to be less effective in antipredator responses in this keystone predation system (Leibold 1996). Treeholes invaded by *Ae. albopictus* seem to function as a keystone predator system, with the inferior competitor, *Ae. triseriatus*, less vulnerable to predation, in this case because of more effective antipredator responses. Antipredator responses are postulated to be costly because such responses often reduce the time and energy that can be devoted to other important activities such as foraging (Relyea and Werner 1999, Van Buskirk 2000, Relyea 2001, Eklov and Svanback 2006). In aquatic systems, many studies have shown that there is a tradeoff between adaptive behavioral responses to predation risk cues and competitive ability (Werner 1991; Werner and Anholt 1993; Werner and Anholt 1996; Kats and Dill 1998; Relyea 2000, 2002a; Relyea and Auld 2004). It is possible that *Ae. albopictus* shows more limited antipredator responses (i.e., relatively small reductions in movement in response to predation cues) because the costs of strong antipredator responses in reduced foraging and competitive ability are too great.

Strong behavioral responses of *Ae. triseriatus* to cues from predation by *C. appendiculata* suggest that such responses could also be adaptive in invasive *Ae. albopictus* (Kesavaraju et al. 2007). Second instars of *Ae. triseriatus* show significantly greater reductions in activity levels at the bottom of the containers in response to water-borne cues from *C. appendiculata* predation compared with *Ae. albopictus.* The result is that *Ae. albopictus* is more vulnerable to predation by *C. appendiculata* than is *Ae. triseriatus* (Griswold and Lounibos 2005a, 2006). In the field, *Ae. albopictus* dominates in areas were *C. appendiculata* are rare or absent, whereas in areas where *C. appendiculata* abundances are higher, there is coexistence between *Ae. triseriatus* and *Ae. albopictus*, suggesting that this predator contributes to the biotic resistance of this community to invasion and impact by *Ae. albopictus* (Kesavaraju et al. 2008). *Ae. triseriatus* increases its frequency of antipredatory behaviors to another native predator, *Toxorhynchites rutilus* (Coquillett). Distribution of *C. appendiculata* is restricted toward the southeastern parts of the United States, but *Ae. triseriatus* co-occurs with *Tx. rutilus* in many areas of the United States. *Ae. triseriatus* from *Tx. Rutilus*–abundant areas (Vero Beach, FL) show a higher frequency of antipredatory behaviors compared with those from areas were *Tx. rutilus* populations are low (Normal, IL) (Juliano and Reminger 1992).

Aedes albopictus have clearly undergone adaptive evolution since arriving in North America in the 1980s (Lounibos et al. 2003). *Ae. albopictus* from northern latitudes in the United States diapause as eggs to avoid unfavorable cold conditions. However, after their spread from the north to southern subtropical areas such as Florida, populations of *Ae. albopictus* have lost their diapause response, indicating that egg diapause can evolve rapidly in *Ae. albopictus* (Lounibos et al. 2003). Thus, evolution in response to novel agents of

selection can occur in this species in a relatively short time. Whether similar rapid evolution in response to novel biotic agents, such as predation by *C. appendiculata,* is possible is unknown. If there is evolution of improved antipredator responses in *Ae. albopictus,* biotic resistance of tree hole communities may decline after initial invasion by *Ae. albopictus,* and this species may be more likely to exclude *Ae. triseriatus* even in *C. appendiculata*–abundant areas.

Adaptive behavioral responses of *Ae. albopictus* to novel predators is only one possible response to new selection pressures after invasion. Second instars of *Ae. albopictus* are more vulnerable to predation than later instars (Kesavaraju et al. 2007). In size-selective predator–prey systems, prey may evolve faster development to reduce their encounter rate with predators when they are at their most vulnerable stages (Spitze 1991, Wellborn 1994). Thus, another hypothesis for evolutionary responses of invasive *Ae. albopictus* is that consistent predation may select for accelerated development to minimize predator impact.

Replicate populations of this species were subjected either to consistent high levels of predation by *C. appendiculata* or to no predation. In each generation, we compared treatments for the behavioral responses to predation cues and development rates. This approach enables us to study the selective effect of *C. appendiculata* predation on *Ae. albopictus* and to determine the potential for adaptation of this invader to this native predator.

Materials and Methods

Larvae of *Ae. albopictus* were collected from water-filled vases in cemeteries (Oak Hill, Bartow, FL; Rose Hill, Tampa, FL; Joshua Creek, Arcadia, FL; White City, Ft. Pierce, FL), used tires in auto salvage yards (M&K Used Auto Parts, Vero Beach, FL; A & A Auto Salvage, Ft. Pierce, FL; Snake Road Auto Salvage, Stuart, FL; Action Auto Salvage, Okeechobee, FL), and tree holes in forested areas (Indrio Road, Ft. Pierce, FL; Sherwood Hammock, Ft. Pierce, FL; Highlands Hammock State Park, near Sebring, FL) between May 2004 and October 2005 (see Kesavaraju et al. 2008 for a map of the locations and for data on the abundances of *C. appendiculata* at these sites). At these cemetery and tire sites, *C. appendiculata* are rare or absent, but at the tree hole sites, they are often abundant (Kesavaraju et al. 2008). Larvae from all sites were pooled, and the emerging adults were propagated in a single cage in the laboratory at a 14:10-h daylight cycle. If there is any genetic variation among *Ae. albopictus* populations, pooling adults from the different sites should enhance the genetic variability in the resulting laboratory population. If that variation includes variation in behavior and responses to predator cues, this pooling can provide more variation on which our laboratory selection can operate. Adults were provided with 20% sucrose solution, blood fed with anesthetized guinea pigs, and provided with 250-ml plastic beakers lined with paper towels for oviposition. Eggs were hatched with 0.33 g of nutrient broth in 750 ml deionized water, larvae were reared in enamel pans with 1:1 ratio of lactalbumin and brewers yeast as a food substrate, at 25°C, 14:10-h daylight cycle, and resulting adults propagated in cages for one more generation to reduce possible maternal effects. Eight control lines and eight predation lines were established with F_3 generation larvae. Each line started with 500 newly hatched *Ae. albopictus* larvae and was held in a 1,000-ml plastic beaker with 900 ml of deionized water. These controlled experiments test both whether an organism can evolve in response to a specific agent of selection, and how they evolve in response to that agent under controlled conditions, thus showing what trait or traits change and in what directions (Conner 2003, Fry 2003).

Predation Culling. Three fourth-instar *C. appendiculata* were added to each predation line to subject larvae (1 d old) to predation until the total number of larvae in each predation line was reduced to ≈50% of their original number. This reduction required ≈24 h. After the reduction by culling, predators were removed from the predation lines. The mean number of larvae eaten from the eight predation lines was determined, and this mean was used as the target number of larvae to be removed from each control line.

Control Culling. A device consisting of eight hollow cylinders (2.54-cm-diameter PVC pipe couplers) was constructed by gluing them together (openings vertical) in a two cylinder by four cylinder array. Each cylinder was numbered. When the device was placed into a tray of control larvae, groups of larvae were isolated in each cylinder. A random number (1–8) was generated, and larvae from the corresponding cylinder were removed. This process was repeated until the target number to be removed was reached.

Larval Rearing. Remaining larvae from control and predation lines were fed with 0.04 g of 1:1 lactalbumin: brewers yeast mix and reared at 25°C in a 14:10-h daylight cycle. They were fed every 2 d from the 1st day to the 7th day, every 3 d from the 8th day to the 26th day, and finally every week from the 33rd day until all larvae had pupated. Water in the rearing containers was replaced with fresh deionized water by sieving (106 μm, U.S. std. no. 140; Fisher Scientific, Pittsburgh, PA) on the seventh day for all the lines. This feeding regimen provides enough food for the larvae to develop to the pupal stage with minimal intraspecific competition (B.K., unpublished data). Pupae from each line were isolated in 250-ml plastic cups and were housed separately by line in cages, and the resulting adults were provided with 20% sucrose solution. Females were blood fed with anesthetized mice twice a week and provided with 250-ml plastic beakers with paper towels for oviposition. Eggs from each line were hatched, and the next generation of 500 larvae (F_4 generation) for each line was established. Predation lines and the control lines were subjected to culling as described above. This cycle was repeated for three generations (F_4, F_5, and F_6). In a similar experiment on laboratory evolution of behavioral responses of *Ae. triseriatus* to a different predator, two generations of culling was sufficient to cause significant di-

vergence in behavioral responses of control and predation lines (Juliano and Gravel 2002).

Behavior. A sample of the larvae from each line in each generation was tested for behavioral changes in response to water-borne cues from *C. appendiculata* predation. Behavior of larvae was recorded in control and predation water. Following Kesavaraju et al. (2007), predation water was prepared by feeding three fourth-instar *C. appendiculata* with 10 second-instar *Ae. albopictus* in 10-ml disposable cups with 10 ml of deionized water. Cups were checked daily for 5 d, and dead, eaten, and pupated larvae were replaced daily. Control water was prepared similarly except that there were no predators in the cups. Test larvae (i.e., larvae for which behavior was to be recorded) were hatched and held individually in glass vials with 5 ml of water and fed 1 ml of liver powder suspension (LPS) prepared by adding 0.3 g of liver powder to 1,000 ml of water. Food was transferred using a pipette while the suspension was stirred constantly on a stir plate to ensure homogeneous delivery of food (Juliano and Gravel 2002). Test larvae were fed only once, which was sufficient for development to second instar, at which time they were transferred to 10-ml cups with 10 ml of water and starved for 24 h to standardize hunger. Test larvae were transferred to the treatment cups to record behavior. Thus, the rearing of test larvae ensured no experience with predator cues until they reached the experimental cups. Before transferring the test larvae, both the predator and prey larvae were removed from the treatment cups, leaving behind only dissolved chemical cues and solid cues (e.g., uneaten body parts, feces) from predation. Behavior was recorded using a Winfast XP 2000 PCI computer card (Leadtek, Walnut, CA) in MPEG-2 format for 15 min. Each video clip had four cups: two control and two predation treatments. Only 24 clips were recorded in a day because of time constraints, so behavior for each generation was recorded over a period of 5 d. A sample of eggs from the F_2 generation (the source generation with which the 16 lines were started) was also tested for their behavior in response to predation risk cues. This group served to quantify behavior in larvae before creation of separate control and predation lines. The treatments were prepared in the manner described above.

Video clips were viewed, and the activity and position of each *Ae. albopictus* larva was determined every 30 s for 15 min by instantaneous scan censuses (Juliano and Reminger 1992) using Observer software (Noldus, Leesburg, VA). Four activities (1, resting—larva not feeding or moving; 2, browsing—larva propelled along the surfaces of cup by movement of mouthparts; 3, filtering—larva floating in the water column, propelled by movement of mouthparts; 4, thrashing—vigorous lateral movements of the body of the larva, propelling it through the water) and four positions (1, surface—spiracular siphon of the larva in contact with water-air interface; 2, bottom—larva within 1 mm of bottom; 3, wall—larva within 1 mm of the cup walls; 4, middle—larva >1 mm from surfaces of cup and not in contact with the water–air interface) were identified (Juliano and Reminger 1992). Resting at the surface is associated with the least risk and thrashing at the bottom with the most risk of predation by *C. appendiculata* (Kesavaraju et al. 2007).

Activities and positions were converted to proportions and arcsine square root transformed to satisfy assumptions of normality and equal variance. Principal components analysis (PCA) was used to obtain uncorrelated descriptors of activity and position and to reduce the number of variables for analysis (SAS Institute 1990). Data were analyzed by nested multivariate analysis of variance (MANOVA) with the principal component scores (PCs) as response variables and generation (F_3–F_6), culling (control, predation), line (1–8 nested within control and predation), water (control, predation), and recording day (1–5, to account for any daily variation within each generation) as independent categorical variables. Between the F_4 and F_5 generations, control line 8 was lost because of insufficient oviposition and so they were dropped from the analysis. For *F*-tests for the culling effect, line nested within culling was used as the error term. For *F*-tests for generation and generation-culling interaction effects, generation-line nested within culling was used as the error term. For water, water-culling interaction, and water-generation interaction effects, water-line nested within culling was used as the error term (Potvin 2001). Standardized canonical coefficients (SCCs) were used to interpret the relative contribution of PCs to significant multivariate effects (Scheiner 2001). Behavior of the larvae from F_2 generation (i.e., before culling and before separation of replicate lines) was also coded and analyzed by one-way MANOVA on PCs testing for a water (control, predation) effect before any controlled selection. Because the data for the F_2 generation were structured differently, without lines and culling treatments, this analysis was done separately.

Life History. A sample of the larvae from each line in each generation was tested for their life history traits. Larvae from each line/generation were hatched and held individually in glass vials with 5 ml of deionized water at 25°C and a 14:10-h daylight cycle. They were fed with 1 ml LPS every 2 d as described above until they reached pupation. At pupation, days to pupation and sex of each pupa were recorded.

Males and females were analyzed separately because males have a shorter time to pupate and a smaller mass than females (Hawley 1988). Independent variables were generation (F_3–F_6), culling (control, predation), and line (1–8 nested within control and predation). Mass (dry weight, Cahn Microbalance; Thermo Fisher, Waltham, MA) and days to pupation ($\log_{10}$-transformed to satisfy assumptions of equal variance and normality) for each adult were analyzed separately by nested analysis of variance (ANOVA). Significant effects were further analyzed with multiple comparisons (Tukey's method; Sokal and Rohlf 1995).

Table 1. Rotated factor pattern scores

Variables	PC1	PC2	PC3
Resting	**97**	−15	−3
Browsing	**−94**	−28	−4
Thrashing	3	**98**	−11
Filtering	4	6	**99**
Surface	**97**	−15	-3
Wall	**−84**	−29	2
Middle	8	**96**	23
Bottom	**−68**	−5	−12
Interpretation	Resting, surface versus browsing, wall, bottom	Thrashing, middle versus other	Filtering, versus other

Values >40 are in bold. The three PCs explained 88% of the variation.

Results

The F_3 generation had *Ae. aegypti* contamination in five of the lines (three control and two predation lines). In total, there were eight *Ae. aegypti* adults (among ≈3,337 *Ae. albopictus* adults), with a maximum of two in a container. They were eliminated in the F_3 generation, and the subsequent generations (F_4–F_6) had no contamination. A laboratory colony of *Ae. aegypti* was reared in the same environmental chamber as *Ae. albopictus*, which could be the source of the contamination. We monitored the *Ae. albopictus* adults that were eclosing for each line in the F_3 generation after the culling, and the number of females that eclosed from each line ranged from 50 to 150.

Behavior. PCA reduced the number of variables to three uncorrelated PCs with eigen values >1 (Table 1). The three PCs explained 88% of the variation in larval behavior. For PC1, larvae with larger positive scores spent more time resting at the surface, whereas larvae with larger negative scores spent more time browsing at the wall and bottom. For PC2, larvae with larger positive scores spent more time thrashing in the middle; larvae with larger negative scores devoted more time to other behaviors. For PC3, larvae with larger positive scores spent more time filtering; larvae with larger negative scores devoted more time to other behaviors (Table 1).

The interaction between generation (F_3, F_4, F_5, F_6) and water (control, predation) was significant, but all other effects were not significant (Table 2). Most importantly, the culling (control, predation) by water interaction was not significant, indicating that culling had no significant effect in the way the larvae responded to the two water treatments (Fig. 1). SCCs indicated that all PCs contributed strongly to the significant interaction between generation and water (Table 2).

For F_2 generation larvae, there was a significant water effect, indicating that larvae in control and predation water behaved differently before treatments (Pillai's trace = 0.681, df = 3,38, $P \leq 0.0001$). SCCs indicated that PC1 was the main contributor (PC1 = 1.159, PC2 = 0.748, PC3 = −0.514) to the significant water effect. *Ae. albopictus* in the control water browsed at the bottom of the containers more often (control PC1: mean ± SE = −0.5274 ± 0.1447) but those in predation water reduced activity at the bottom of the containers and increased resting at the surface (predation PC1: mean ± SE = 0.6709 ± 0.1448). *Ae. albopictus* from the F_2 generation spent more time resting in predation water (predation resting: mean ± SE = 0.6889 ± 0.0521) than control water (control resting: mean ± SE = 0.3284 ± 0.0583).

Life History

Development Time. *Females.* Generation (F_3, F_4, F_5, F_6) and the interaction between culling (predation, control) and generation were significant (Table 3). Despite the significant interaction of culling and generation, no pairwise differences between predation and control lines within any of the generations could be identified as statistically significant (Fig. 2a). The direction of the difference between predation and control lines reversed between the F_3 and F_4 generations and again between the F_4 and F_5 generations (Fig. 2a). Multiple comparisons showed that females from both control and predation lines in F_3 generation had longer development times to pupation than did all other generations (Fig. 2a).

Males. Generation, culling, and their interaction were significant (Table 3). In broad outline, multiple comparisons for males were similar to those for females, with control and predation lines in F_3 generation having a longer development time to pupation than all other generations (Fig. 2b). For males in the F_5 generation, predation lines took a significantly shorter time

Table 2. Results of MANOVA for the behavior experiment

Variables	NumDF	Den df	Pillai's trace	*P*	Standardized canonical coefficients		
					PC1	PC2	PC3
C	3	12	0.105	0.7093	−0.755	−0.353	1.009
G	**9**	**117**	**1.0811**	**<0.0001**	**0.764**	**−0.343**	**0.721**
W	**3**	**11**	**0.995**	**<0.0001**	**1.653**	**0.539**	**−0.313**
G × C	9	117	0.292	0.1945	0.795	−0.697	0.387
C × W	3	11	0.096	0.7621	1.105	1.105	0.726
G × W	**9**	**39**	**1.709**	**<0.0001**	**1.260**	**0.705**	**−0.729**
G × C × W	9	4,602	0.009	0.1150	1.191	0.689	−0.559

Significant effects are in bold. Standardized canonical coefficients of only the significant effects are bold to understand the relative contribution of the dependent variables in producing the significant effect.

C, culling; G, generation; W, water.

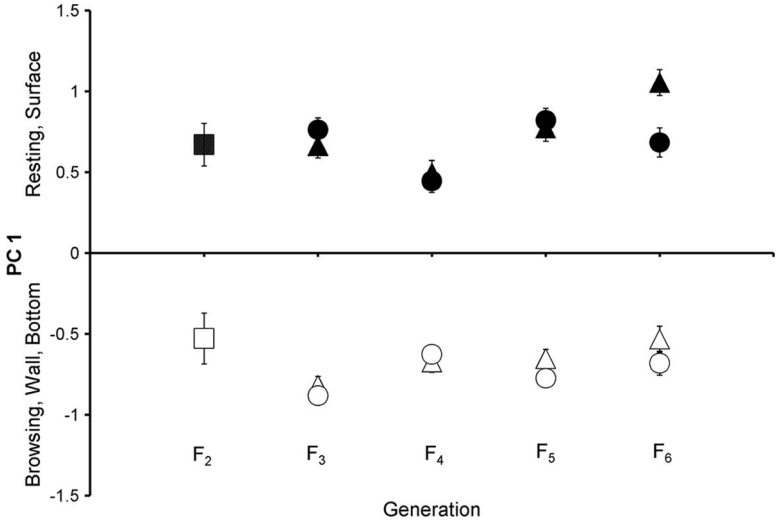

Fig. 1. Mean ± SE of PC1 for the behavior experiment. Squares are preculling generation F_2 (included for reference), triangles are control lines, and circles are predation lines. White figures are control and dark figures are predation water.

to develop compared with control lines, but that divergence disappeared in the F_6 generation (Fig. 2b).

Mass. There were no significant effects in females, but for males, generation (F_3, F_4, F_5, F_6) and culling (predation, control) were significant (Table 3). Multiple comparisons showed that males from the F_4 generation were smaller compared with the F_3 generation, but there was no other difference between generations (Fig. 3). Males from controls were significantly smaller (mass: mean ± SE = 0.17620 ± 0.0012 mg) compared with predation (mass: mean ± SE = 0.1839 ± 0.0012 mg).

Discussion

Aedes albopictus larvae from control and predation culling reduced their activity in predation water, but their responses did not diverge over three generations of culling (Fig. 1). This suggested that either individuals showing reduced activity are not favored sufficiently for selection by *C. appendiculata* predation to outweigh the effects of random processes such as genetic drift or that there is insufficient additive genetic variation for this behavior for there to be a response to any selection imposed by *C. appendiculata.* The ineffectiveness of laboratory culling to change behavior contrasts with results from previous work with another container-dwelling mosquito. Larvae of both *Ae. albopictus* and *Ae. triseriatus* show some reduced activity at the bottom of containers in the presence of cues from predation by *C. appendiculata* (Kesavaraju et al. 2007). Larvae of *Ae. triseriatus* that were subjected to two generations of culling by predation (using a different predator, *T. rutilus*) in an experiment similar to the one in this study showed a consistent pattern of reduced activity at the bottoms of containers, and greater resting at the surface even in control water treatments (i.e., even in the absence of predation cues, they adopt the low risk pattern of behavior; Juliano and Gravel 2002). Thus, even though initial behavioral responses of *Ae. albopictus* and *Ae. triseriatus* are qualitatively similar, their respective responses to laboratory selection regimens are not.

We may have failed to detect selection effects on behavior because predation primarily selects for other characters. In some systems, prey that are vulnerable to predation during younger, smaller stages become invulnerable to predation when they become larger than the predator (Lundvall et al. 1999, Nilsson and Bronmark 2000). Second-instar *Ae. albopictus* larvae are equivalent in size to a fourth-instar *C. appendiculata,* so second instars are more vulnerable to predation by *C. appendiculata* compared with later stages (Kesavaraju et al. 2007). Under this scenario, larvae

Table 3. Results ANOVA for the effects culling on the life history of *Ae. albopictus* across generations

Variables	Num df	Development time						Mass					
		Female			Male			Female			Male		
		Den df	*F* value	*P*	Den df	*F* value	*P*	Den df	*F* value	*P*	Den df	*F* value	*P*
Generation	**3**	**39.72**	**38.33**	**<0.0001**	**39.53**	**102.5**	**<0.0001**	40.83	1.76	0.1706	**39.81**	**4.54**	**<0.0079**
Culling	1	13.16	3.42	0.0869	**13.06**	**11.79**	**0.0044**	13.30	3.94	0.0682	**13.11**	**6.98**	**0.0202**
Generation × culling	**3**	**39.74**	**4.40**	**0.0092**	**39.52**	**10.29**	**<0.0001**	40.85	0.64	0.5965	39.81	0.87	0.4656

Significant effects are in bold.

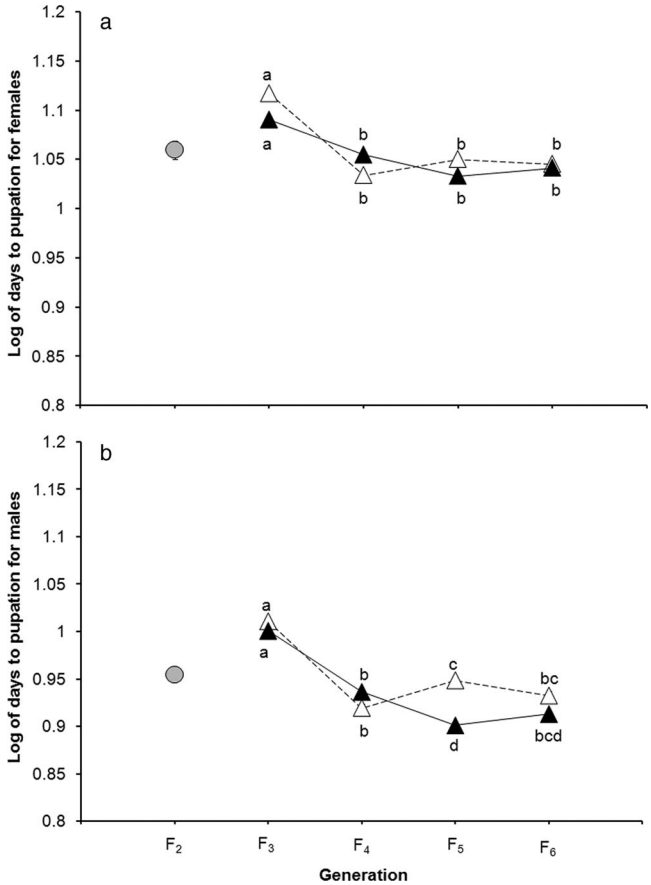

Fig. 2. (a) Mean ± SE log values of days to pupation for females in the life history experiment. (b) Means ± SE log values days to pupation for males in the life history experiment. Triangles are postculling generations from F_3 to F_6, and the circle is preculling generation F_2 (included for reference). White figures are control lines and dark figures are predation lines. SEs are too small to be visible on the graph. Means with similar letters are not significantly different from each other.

that have faster growth and development rates, with a relatively short time in the most vulnerable stages such as first and second instars, will have reduced fatal encounter rate with the predator *C. appendiculata.* Thus, if larvae are being selected for rapid growth and development rates, we would expect a significant reduction in the developmental time across generations of selection for larvae from the predation culling lines compared with those in the control culling lines. F_3 generation female and male larvae of both control and predation culling lines took a longer time to develop compared with subsequent generations (Fig. 2a). However, more importantly, the development times of females from the predation lines were not significantly different from those of the control lines, and the predation lines did not differ between F_4 and F_6 generations, indicating development time was not changing in response to selection by *C. appendiculata* predation (Fig. 2a). In the F_5 generation, males from predation lines took a shorter time to develop compared control lines, as predicted, but this difference disappeared in the F_6 generation (Fig. 2b), suggesting that some of the variation from generation to generation is a result of random processes and that any selection was not strong enough to override effects of random drift. The F_2-generation larvae for males and females had developmental times similar to those of the F_4–F_6 generations, which suggests that longer time to develop in F_3 could be in some unintentional way favored by or caused by our laboratory-rearing conditions (Fig. 2). Developmental times and growth rates often affect the mass, but there was no interaction between water and culling for mass. Males from F_4 generation were smaller than the F_3 generation, but that change disappeared in subsequent generations, which again could be caused by our rearing conditions. Males from control culling were significantly smaller compared with the predation culling, which indicates that predation selected for bigger and probably healthier males.

Prey may use different types of cues to detect the presence of predators in their environment and show appropriate responses to escape from predation (Lima and Dill 1990). The most common types of cues for aquatic systems are the water-borne chemical cues from the actual act of predation, such as the cues tested in this study, and physical cues, where prey show adaptive responses to the physical presence of

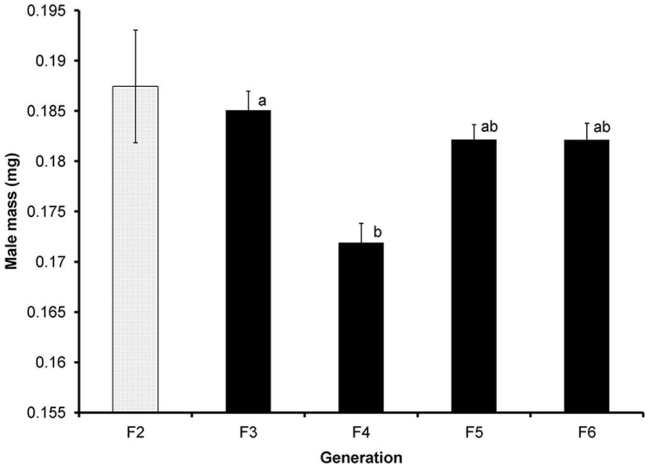

Fig. 3. Mean ± SE of male mass (mg) for the life history experiment. Dark bars are postculling generations and the gray bar is preculling generation (included for reference).

the predator (Lima and Dill 1990). Kesavaraju et al. (2007) compared the behavioral responses of *Ae. albopictus* and *Ae. triseriatus* to water-borne predation risk cues from the act of predation and to the physical presence of *C. appendiculata.* They showed that both species reduce activity in response to the physical presence of *C. appendiculata* and that the responses of the two species to the physical presence of this predator were more similar than were the responses to water-borne cues, which were much smaller for *Ae. albopictus* (Kesavaraju et al. 2007). In this study, we tested for evolution of behavioral responses of *Ae. albopictus* to the presence of water-borne cues from the act of predation. It is possible that we failed to detect changes in *Ae. albopictus* behavioral response over the generations because larvae of this species rely more on visual or tactile cues or on combinations of visual and chemical cues that are dependent on the physical presence of the predator.

Populations that regularly co-occur with predators may evolve adaptations that reduce risk of predation, and those adaptations may be limited or absent in populations in predator-free habitats (Downes and Adams 2001). *Ae. albopictus* populations from tree hole habitats with high *C. appendiculata* abundances did not show a greater degree of reduced movement in response to water-borne predator cues compared with populations collected from cemetery and tire habitats that are largely predator free (Kesavaraju et al. 2008). The base population for this study was established with *Ae. albopictus* collected from the same sites described in Kesavaraju et al. (2008). The lack of differentiation among *Ae. albopictus* populations in antipredator behavioral responses could arise because these populations are not sufficiently isolated and are experiencing considerable gene flow, which could homogenize populations. The results of our laboratory study showed that there is little response to predatory selection, even when gene flow is absent, and argues for the alternative explanation for lack of differentiation: low genetic variation for predator-related behaviors. *Ae. albopictus* showed low variability in mitochondrial sequences compared with other taxa, which may be a result of North American populations being founded by extremely small numbers of individuals (Birungi and Munstermann 2002). Similarly low nuclear genetic variance in *Ae. albopictus* for antipredator characters may limit the potential of natural selection in either the laboratory or the field to produce evolutionary change in behavior.

Another interpretation of our laboratory results is that, because *Ae. albopictus* has occurred within the range of *C. appendiculata* since the 1990s (Lounibos et al. 2001), any evolution of behavioral or life history responses to this predator has already occurred. Behavioral studies on mulitiple Florida populations of *Ae. albopictus* from locations with different abundances of *C. appendiculata* (Kesavaraju et al. 2008) do not support this hypothesis, because those populations show no significant differentiation of behavioral responses. Furthermore, even if considerable evolution of behavior or life history of the invader had occurred since its arrival in Florida, we still would expect laboratory populations exposed to the two extreme predation regimens we created (high predation in each generation versus no predation in any generation) to diverge in behavior or life history if predation was an effective agent of selection. All results are thus consistent in indicating little evolutionary response of this invader to this native predator.

Behavioral experiments and field studies have indicated that, in habitats where *C. appendiculata* are abundant, they may function as keystone predators, facilitating co-existence between *Ae. albopictus* and *Ae. triseriatus* and in particular limiting declines in the competitively inferior native species (Kesavaraju et al. 2007). Native predators may thus be one component of the biotic resistance of communities to invasion, but adaptive evolution of invaders in response to novel predators may, in principle, erode that biotic resistance (Mooney and Cleland 2001). For *Ae. albopictus,* we did not find evidence of local differentiation of

antipredatory behavior among field populations (Kesavaraju et al. 2007) or of evolution of antipredatory behavior in response to controlled selection in the laboratory (this study). If lack of response to selection that we observed indicates low genetic variation for these traits, adaptive evolution of antipredator responses of invading *Ae. albopictus* is unlikely. If, however, the observed lack of response is a result of small laboratory populations predominantly showing effects of drift and other random processes, it remains possible that antipredator behavior may evolve where populations are large and selection very strong, but these conditions would likely represent only a subset of natural field populations of this invader (Kesavaraju et al. 2007). In either case, our results suggest that the contribution of predatory *C. appendiculata* to biotic resistance to invasion and disruption of native communities may remain stable and effective in many habitats invaded by *Ae. albopictus,* at least in the immediate future. Thus, we expect that tree hole habitats will continue to be partial barriers to invasion by *Ae. albopictus* and refuges for *Ae. triseriatus.*

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