Know Your Enemy: Effects of a Predator on Native and Invasive Container Mosquitoes

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Abstract

We tested the effect of the native container-dwelling predator *Toxorhynchites rutilus* on the codominant container-dwelling mosquitoes: native *Aedes triseriatus* and invasive *Aedes japonicus*. We established two predator treatments (predator, no predator) by removing *T. rutilus* from all containers, and stocking *T. rutilus* larvae (1/3.5 liters) in the predator treatment. Removal and stocking was repeated every 3 d and established significantly different predator abundances in both large and small containers. Repeated-measures analysis of variance (ANOVA) on standard samples showed larvae+pupae/liter of *A. japonicus* was greater without versus with predation, and this difference increased across samples. In contrast, repeated-measures ANOVA showed larvae+pupae/liter of *A. triseriatus* was statistically indistinguishable for predation treatments and was greater in small versus large containers. Thus, predation reduced invasive *A. japonicus* while having no detectable effect on *A. triseriatus* larvae and pupae. A final destructive census of pupae showed that predation reduced pupae/liter of both species, but this effect was greater and more consistent across container sizes for *A. japonicus*. Predator effects on abundances were not products of the nonlethal effect of predator avoidance by ovipositing females, as *T. rutilus* presence did not lead to reduced egg inputs by either *Aedes*, nor by *Aedes* spp. as a group. Effects of predation thus are best explained by differential success of developing larvae due to the greater lethal effect of *T. rutilus* on *A. japonicus* than on *A. triseriatus*. Thus, this system is consistent with the hypothesis that native predators can limit success and potential impacts of invasive mosquitoes.

Key words: biotic resistance, *Aedes triseriatus*, *Aedes japonicus*, *Toxorhynchites rutilus*, lethal versus nonlethal predator effect

The biotic resistance hypothesis postulates that greater community diversity enhances a community's ability to resist invasion by or impacts of a nonnative species (Elton 1958, Levine et al. 2004). If this hypothesis is correct, it is species interactions such as predation, parasitism, competition, or mutualism within the community that are the source of biotic resistance (Shea and Chesson 2002, Levine et al. 2004). As originally described, the biotic resistance hypothesis focused on species (more generally, phylogenetic) diversity (Elton 1958); however, species diversity in this context is likely a surrogate for functional diversity, or the variety of ecological roles and interactions present in the community (Petchey and Gaston 2006). Species in higher trophic levels are likely to add to both species diversity and functional diversity. Native predators and other enemies contribute to biotic resistance when those predators have greater density- or trait-mediated effects on invading species than on native species (e.g., deRivera et al. 2005, Smith 2006, Juliano et al. 2010, Dumont et al. 2011, Hackerott et al. 2013, Harley et al. 2013) and thus reduce the

success or the impact of invasive species. This situation can arise when native prey are adapted to the threat of native predators, and competing invasive species, lacking history with the predator, lack these adaptations. Meta-analyses of published studies of effects of interactions on invasion success or impact have shown that the effects of enemies are consistently important for terrestrial (Levine et al. 2004, Kimbro et al. 2013), marine (Kimbrow et al. 2013), and freshwater (Alofs and Jackson 2014) communities. Typically, enemies are not absolute barriers to invasion, but instead contributors to limitation or containment of the population of the invader, which often will limit their spread and potential impact (Levine et al. 2004). There are cases in which enemies have no effect on invasion success (e.g., Hackerott et al. 2013), and some cases where enemies can enhance invasion impact on natives via apparent competition (e.g., Noonberg and Byers 2005). When enemies contribute to biotic resistance to invasions, there remains the question: What are the mechanisms by which enemies contribute to biotic resistance? Direct lethal effects of

enemies are only one possibility (Juliano et al. 2010, MacNeil et al. 2013), along with nonlethal effects such as avoidance of enemy-infested habitats (e.g., Salo et al. 2008).

The impact of enemy contributions to biotic resistance takes on greater practical importance when the invaders are vectors of disease-causing pathogens. Many of the worst vectors of human pathogens are invasive mosquitoes that have created novel disease threats by changing vector–pathogen–host interactions in the invaded area (Lounibos 2002, Juliano and Lounibos 2005, Westby et al. 2015, Lounibos and Juliano 2018). Previous field and laboratory studies of invasive *Aedes albopictus* (Skuse) (Diptera: Culicidae) in North America have implicated native predators in water-filled container habitats as important barriers to invader success and impacts on native species (Kesavaraju et al. 2008, Alto et al. 2009, Juliano et al. 2010). Theory describing community ecology of freshwater systems postulates that predator importance in aquatic systems increases with water body size and permanence (Wellborn et al. 1996). Though there is observational evidence for this pattern for mosquito systems (Bradshaw and Holzapfel 1988, Chase and Knight 2003), experiments manipulating independently container sizes across a substantial range (0.35, 3.5, 35, and 140 liters) and drying found that effects of those manipulations on community composition had no detectable relationship to abundance of *Toxorhynchites rutilus* (Coquillett) (Diptera: Culicidae), the dominant predator in those water-filled container systems (Westby and Juliano 2017). Thus, there is a need for direct manipulation of predator abundance to test for the influence of native predators on invasive species in mosquito-dominated container habitats.

Container mosquitoes are an ideal system for experiments testing for effects of native predators on invasion success and impact. A relatively small assemblage of specialist aquatic invertebrates, dominated by larval mosquitoes, colonizes water-filled containers. In North American forests, larvae of the native mosquito *Aedes triseriatus* (Say) (Diptera: Culicidae) often co-occur with predatory larvae of another native mosquito, *T. rutilus*. *Aedes triseriatus* appear to have some adaptations to minimize predation threat by *T. rutilus*. *Aedes triseriatus* larvae reduce predation risk by reducing movement in response to feeding *T. rutilus* (Juliano and Reminger 1992; Kesavaraju and Juliano 2004, 2010; Kesavaraju et al. 2007a,b, 2008; Alto et al. 2009), and do so in a threat-sensitive way (Kesavaraju et al. 2007b). In these forest habitats *Aedes japonicus* (Theobald) (Diptera: Culicidae) is an increasingly common invasive container mosquito (Andreadis and Wolfe 2010, Kaufman and Fonseca 2014) that competes with *A. triseriatus* (Alto 2011, Hardstone and Andreadis 2012, Freed et al. 2014) with some evidence that it may replace *A. triseriatus* (Andreadis and Wolfe 2010). Both *Aedes* species are vectors of La Crosse encephalitis virus (Westby et al. 2015) and are prey for *T. rutilus* (Freed et al. 2014). Laboratory data indicate that both *A. japonicus* and *A. triseriatus* are more responsive to cues from predation than is *A. albopictus* (Kesavaraju and Juliano 2004, Kesavaraju et al. 2011). Estimates of relative vulnerability to predation by *T. rutilus* sometimes have suggested slightly greater population impact on *A. japonicus* than for native *A. triseriatus* (Freed et al. 2014) but controlled predation experiments have found no significant difference in vulnerability (Murrell and Juliano 2013). Distributions of *A. triseriatus* and *A. japonicus* across water-filled containers are somewhat segregated by container size, with *A. japonicus* more common in larger containers and *A. triseriatus* more common in smaller containers (Westby and Juliano 2017).

Direct manipulation of predator abundance in the field is likely to provide the best direct evidence that enemies can limit the success of invaders (Juliano et al. 2010). Careful analysis of results of predator manipulation can indicate the relative importance of

density-mediated effects of the predator (e.g., differential vulnerability to predation) versus trait-mediated effects of the predator (e.g., behavioral avoidance of habitats with greater predation threat) (Juliano et al. 2010). The container habitat system and its mosquitoes are tractable for field experiments testing the role of predation as a barrier to invasion because of the small size and replicability of containers, the limited number of insect species involved and the presence of several invasive mosquitoes (Westby and Juliano 2017, Supplementary Table S1). Different sizes of containers provide an opportunity to investigate context-dependent predator effects. We tested the hypothesis that native *A. triseriatus* are less impacted by predatory larvae of *T. rutilus* than are invasive *A. japonicus*, and the hypotheses that both density-mediated lethal effects of predation, and trait-mediated nonlethal effects of behavioral oviposition choice in response to the predator contribute to this predator's effects on abundances of *A. triseriatus* versus *A. japonicus*.

Materials and Methods

Our experiment was done at Tyson Research Center of Washington University, St. Louis, near Eureka, MO in 2014. On 27 May 2014, containers were set in two transects along either side of a gravel road and 1–3 m from the road. The transects were centered approximately on 30.5° N, 90.6° W, under the canopy of second growth, mixed Oak-Hickory forest. We filled experimental containers two sizes often occupied by container mosquitoes (small = 7.6-liter buckets; large = 18.9-liter buckets; N = 24 each) with water (3.5 and 14.0 liters, respectively) and added 1 g/liter of White oak (*Quercus alba*) leaf detritus. Containers on each transect were placed in an alternating pattern (predator/small, no predator/small, predator/large, no predator/large).

Sampling Eggs

On 12 July 2014, 3 d before the start of the predator manipulation, we affixed a standard sized piece of seed germination paper (11 × 10 cm) to each container's wall with tape, with the bottom 7.5 cm submerged and a standard sample area (2.5 × 10 cm) above the water level and not covered by the tape. These papers were collected 3 d later and served as a standard sample for *Aedes* oviposition activity (Westby and Juliano 2017). On 15 July 2014, we collected the oviposition papers and removed from the surface of the water in each container all *T. rutilus* eggs and *Culex* egg rafts.

Sampling Larvae

On 15 July, after 7 wk of colonization by resident invertebrates, we measured the depth of the water in each container to the nearest mm. We sampled the assemblage of aquatic macroinvertebrates in each container using rectangular aquarium nets of two net sizes corresponding to small and large buckets, respectively (small = 7.1 × 5.5 cm or 39.05 cm² opening; large = 9.7 × 7.0 cm or 67.90 cm²) both with mesh sizes small enough to retain newly-hatched mosquitoes. The nets had their handles bent at 90° to the opening of the net. We placed the net with its opening up on the bottom of each container and waited 1 min for disturbance of larvae to subside, and then pulled the net vertically through the water column, thus capturing invertebrates in the water column in a volume of water defined by the depth of the water column and the opening of the net. This approach enabled us to estimate the volume of water sampled and to estimate what fraction of the volume of the container was sampled in each case. Across all our samples, we estimate that we sampled a mean ± SE of 1.76 ± 0.02 and 0.58 ± 0.01 liters for the large and small buckets, respectively. These values represent

means $\pm$ 95% confidence intervals of $19.5 \pm 0.4\%$ and $13.6 \pm 0.2\%$ of the water volumes of the large and the small buckets, respectively. Thus, we are confident that we have samples that capture similar proportions of the assemblages in each container. Larvae and pupae were returned to the laboratory alive for identification to species; when first- or second-instar larvae were present in the sample they were reared in the laboratory on bovine liver powder at 24°C for 1–4 d and then identified to species.

Initial Predator Manipulation

After these initial samples were taken, the manipulation of predator abundance began. We ran the contents of the containers through a 100- μ m sieve, resuspended larvae and detritus in clean water in white pans, returned all container water and detritus to the containers, then removed all *T. rutilus*, before returning other larvae to the containers. We then stocked half the containers of each size with second and early third-instar *T. rutilus* at a density of 1/3.5 liters (i.e., 1 in each small bucket, 4 in each large bucket) and left half as predator-free controls.

Sampling Eggs and Larvae Following Manipulation

We sampled containers for immature mosquito abundances and mosquito oviposition on days 12 and 24 after the start of the predator manipulation. For these samples, we used the same procedures described above under *Sampling eggs* and *Sampling larvae*. After the 12-d sampling, the water volumes in all containers were returned to their original 3.5 and 14 liters.

Predator Monitoring

We visited containers every 3 d, removing *T. rutilus* eggs and any excess *T. rutilus* larvae, determining stages of all *T. rutilus*, and replacing dead, missing and likely cannibalized, or pupated *T. rutilus* with third- or fourth-instar larvae, all using the same sieving procedure described above under *Initial predator manipulation*. We also monitored for colonization of the buckets by any other predatory aquatic invertebrates (e.g., Dytiscidae, Notonectidae, *Chaoborus*), and found none of these predators in the experimental containers. The only other predaceous colonist of the containers was *Anopheles barberi* Coquillett (Diptera: Culicidae), a facultative predator on small culicid larvae. We did not remove *A. barberi* during the experiment. As *A. barberi* were rare during our experiment (<1% of all collected larvae; see **Results**), we are confident that they had little effect on our results.

Final Day Census of Pupae

After the samples taken on day 24, we collected all larvae and pupae, and allowed all pupae to eclose for identification. We used this final number of pupae as a measure of successful production of adults of each species by the end of the experiment. Larvae present at the end of the experiment were preserved in ethanol. The only rainfall during the experiment occurred over the final 8 d, with 0.8 mm falling between 1 and 2 August, and more importantly, 55.9 mm falling from 5 to 8 August. The latter rainfall likely induced hatching of many larvae that appeared in the final census as first instars. We were not confident of identifying preserved first instars to species, and therefore confine our analysis of the final day census to the pupae.

Egg Hatching

Egg samples collected on papers from each container on days 0, 12, and 24 of the experiment (each sample of eggs laid over 3 d) were brought to the laboratory where we counted eggs in the 2.5 $\times$ 10 cm

sample area using a dissecting microscope. Eggs were allowed to develop for 4 d so that all eggs would reach a stage capable of hatching (25°C, 14 h photophase), then hatched in nutrient broth (0.4 g/liter). Larvae were reared to third or fourth instar before identifying larvae from each container. Some eggs were laid below the standard sample area (as container water levels fell) so that numbers of hatched larvae commonly exceeded numbers of eggs in the standard area.

Effectiveness of *T. rutilus* Manipulation

During the experiment, the containers were open to colonization by *T. rutilus*. To assess the effectiveness of our manipulation, we quantified the number of *T. rutilus* found at 3-d intervals when we checked predator abundance. For each container, if a *T. rutilus* individual was present in a given day's monitoring, we counted three predator-days since the last day's monitoring. We summed the number of predator-days across the 24 d of the experiment, and used that sum as an indication of the effectiveness of our manipulation, analyzing cumulative predator-days by analysis of variance (ANOVA) (SAS Software, PROC MIXED, SAS Institute Inc. 2014), with predation treatment, container size, and interaction as fixed effects, and transect as a random block effect.

Statistical Analysis of *Aedes* Abundances

For samples of larvae and pupae, we converted counts of each species to number/liter of volume. Numbers of larvae and pupae of *A. triseriatus* and *A. japonicus*/liter, numbers of all *Aedes* eggs laid on standard papers, and numbers of *A. triseriatus* and *A. japonicus* identified from eggs were all analyzed using generalized linear models for repeated measures (SAS Software, PROC GLIMMIX, SAS Institute Inc. 2014). Container size, predator treatment, and interaction were fixed effects, transect a random block effect, and samples (days 0, 12, and 24) repeated measures. We used a zero inflated Poisson error term to account for the non-normal distribution of abundances and the excess of 0's in abundance data (SAS Software, PROC GLIMMIX). For the final census of pupae on day 24, we converted numbers of pupae of *A. triseriatus*, *A. japonicus*, and total *Aedes* to number/liter, and analyzed using generalized linear models (SAS Software, PROC GLIMMIX) with container size, predator treatment, and interaction as fixed effects, and transect as a random block effect. We used a zero inflated Poisson error term (SAS Software, PROC GLIMMIX).

Results

Effectiveness of Predator Manipulation

The manipulation was very successful in establishing two levels of predation. Some *T. rutilus* were found in the no-predator containers, representing hatching of eggs during the 3-d interval between visits, but these were mostly small and removed during the 3-d visits. Mean predator-days of exposure per container was significantly affected by predation treatment ($F_{1,43} = 191.95$, $P < 0.0001$), container size ($F_{1,43} = 65.90$, $P < 0.0001$), and interaction ($F_{1,43} = 78.25$, $P < 0.0001$). Mean $\pm$ SE predator-days for the four treatment combinations of container size and predator manipulation are shown in Fig. 1. For comparison, perfect manipulation, with no *T. rutilus* colonization and no-predator deaths, would have yielded mean predator-days corresponding to the arrows in Fig. 1. For predator/large containers, the actual mean was lower than expected, suggesting some cannibalism of *T. rutilus* larvae. In contrast, for all other combinations the actual mean was greater than expected, suggesting effects of oviposition and hatching of *T. rutilus* in the containers. Differences among these observed treatment means underestimate

differences in predation pressure, because the cases where a predator occurred in a no-predator container were overwhelmingly first- or second-instar larvae, which would exert relatively little predation pressure on mature larvae and pupae prior to their removal relative to the larger larvae that we stocked in the predator treatments.

Abundances of Mosquito Larvae and Pupae

Over the three samples, >96% of all larvae and pupae were *Aedes*. *Aedes triseriatus*, and *A. japonicus* were by far the dominant species, constituting 48 and 47%, respectively, of all larvae and pupae. No other species accounted for >2% of larvae and pupae. Rare taxa included: *Aedes albopictus* (Skuse) (Diptera: Culicidae) (0.7%), *Culex restuans* Theobald (Diptera: Culicidae) (0.2%), *Culex territans* Walker (Diptera: Culicidae) (1.6%), *Orthopodomyia signifera* (Coquillett) (Diptera: Culicidae) (0.4%), *Anopheles barberi* Coquillett (Diptera: Culicidae) (0.4%), *Culicoides* spp. (Diptera: Ceratopogonidae) (0.3%), and Chironomidae (0.5%).

The manipulations affected the total number of all mosquito larvae and pupae/liter and the effect of manipulation increased as time went on (Table 1). Mean density was significantly greater without *T. rutilus* compared to containers with *T. rutilus* in the last sample (Fig. 2A). Although there were effects on overall mosquito abundance, the more important question was whether the same predator effects were detected for both

Figure 1. Mean $\pm$ SE for cumulative predator-days for *Toxorhynchites rutilus* larvae for treatment combinations of predator-container size. ANOVA statistics reported in the text. Arrows to the left of the vertical axis indicate expected means for predator-days if additions and removals had been 100% effective, with no *T. rutilus* oviposition or deaths. Means associated with the same letter are not significantly different at experimentwise $\alpha = 0.05$ by Tukey's test.

A. japonicus and *A. triseriatus*. *Aedes japonicus* abundance was significantly affected by predator treatment, sample, and predator-sample interaction (Table 1). There was no significant difference due to predator treatment in sample 1, but *A. japonicus* were significantly more abundant in the no-predator treatment than in the predator treatment in samples 2 and 3 (Fig. 2B). In contrast, abundance/liter of *A. triseriatus* was not significantly affected by any effect involving the predator (Table 1, Fig. 2C), but was significantly affected by container size and size-sample interaction (Table 1). *Aedes triseriatus* abundance/liter was initially greater in small containers (Fig. 2D) and decreased regardless of predation treatment over the three samples taken during the experiment (Fig. 2C). None of the other mosquitoes (*A. albopictus*, other rare mosquitoes), nor the nonmosquitoes, yielded significant effects of treatments (data not shown). Thus, predation by *T. rutilus* shaped this community by reducing *A. japonicus* and simultaneously having no significant effects on *A. triseriatus* combined larval and pupal abundance.

Oviposition Into Containers

The observed effect of *T. rutilus* manipulation on *Aedes* larval abundances was not a product of habitat choice by ovipositing females, because although analysis of egg inputs yielded a significant three-way interaction of container size-predator manipulation-sample interaction for egg input by *Aedes* (Table 2), *Aedes* egg inputs tended to be greater where *T. rutilus* was present (Fig. 3A and B). The differences varied with both sample and container size (Fig. 3A and B) with the greatest and least differences occurring in the second sample for large and small containers, respectively. The effects of predation treatment-sample interaction, and sample were significant for *A. japonicus* (Table 2); however, the trend was opposite that observed for *A. japonicus* larvae (compare Fig. 3C and Fig. 2B). In the first sample representing oviposition prior to beginning the manipulation of predators, more *A. japonicus* eggs were laid in the no *T. rutilus* containers, but this difference declined across the three samples so that in the last sample there was a nonsignificant tendency for more *A. japonicus* eggs in the containers with *T. rutilus* (Fig. 3B). The effect of predation treatment and its interactions on eggs laid were not significant for *A. triseriatus* (Table 2), and the only significant effects involved container size and sample (Table 2, Fig. 3D). There were no significant effects on the small number of *A. albopictus* in the samples (data not shown). The best explanation for the observed negative effects of *T. rutilus* on larval abundances seems to be differential success of developing larvae due to the greater lethal effect of this predator on *A. japonicus* than on *A. triseriatus*.

Final Abundance of Pupae

There were significant effects of container size, predation treatment, and interaction on pupae/liter in the final census (Table 3, Fig. 4).

Table 1. Repeated-measures analysis for abundances of larvae and pupae/liter of total mosquitoes, *Aedes japonicus*, and *Aedes triseriatus*

Effect	df	All mosquitoes		<i>Aedes japonicus</i>		<i>Aedes triseriatus</i>	
		F	P > F	F	P > F	F	P > F
Container Size	1, 44	2.33	0.1344	0.89	0.3494	1.65	0.2060
Predator	1, 44	4.96	0.0311	12.34	0.0010	0.45	0.5069
Size $\times$ Predator	1, 44	2.21	0.1442	2.09	0.1554	0.00	0.9739
Sample	2, 88	35.81	<0.0001	25.08	<0.0001	19.19	<0.0001
Size $\times$ Sample	2, 88	1.65	0.1977	1.63	0.2015	3.21	0.0450
Predator $\times$ Sample	2, 88	3.22	0.0447	3.46	0.0358	1.76	0.1775
Size $\times$ Predator $\times$ Sample	2, 88	0.32	0.7272	0.25	0.7827	0.71	0.4956

Effects significant at $\alpha = 0.05$ are emphasized in bold type.

Figure 2. Least squares means ($\pm$ SE) for effects of treatments on abundances of larvae and pupae/liter over the course of three samples during the field experiment. Repeated-measures ANOVA results are reported in Table 1. (A) Effect of predator-sample date interaction on all mosquitoes. (B) Effect of predator-sample date interaction on *Aedes japonicus*. (C) Effect of predator-sample date interaction on *Aedes triseriatus*. For panels A, B, and C, least squares means for predator versus no predator on the same sample day were compared using a Bonferroni approach at experimentwise $\alpha = 0.05$, and pairs significantly different are marked with an Asterisk (*). (D) Effect of container size-sample date interaction on *Aedes triseriatus*. For panel D, least squares means were compared using a Bonferroni approach within a container size class at experimentwise $\alpha = 0.05$ and means marked with the same letter are not significantly different.

Table 2. Repeated-measures analysis for eggs laid on oviposition papers for total mosquitoes, *Aedes japonicus*, and *Aedes triseriatus*

Effect	df	All mosquitoes		<i>Aedes japonicus</i>		<i>Aedes triseriatus</i>	
		<i>F</i>	<i>P</i> > <i>F</i>	<i>F</i>	<i>P</i> > <i>F</i>	<i>F</i>	<i>P</i> > <i>F</i>
Container Size	1, 44	1.04	0.3135	1.23	0.2730	0.29	0.5910
Predator	1, 44	5.46	0.0241	0.69	0.4095	1.07	0.3061
Size $\times$ Predator	1, 44	0.27	0.6088	0.04	0.8339	0.91	0.3465
Sample	2, 88	33.72	<0.0001	20.59	<0.0001	22.04	<0.0001
Size $\times$ Sample	2, 88	5.33	0.0065	0.55	0.5784	3.39	0.0381
Predator $\times$ Sample	2, 88	0.47	0.6258	4.08	0.0203	1.49	0.2361
Size $\times$ Predator $\times$ Sample	2, 88	3.42	0.0372	0.76	0.4729	1.12	0.3318

Effects significant at $\alpha = 0.05$ are emphasized in bold type.

Pupae/liter for individual *Aedes* species showed a significant predator effect for *A. japonicus* pupation success (Table 3) with significantly more pupae in the absence of *T. rutilus* for both container

sizes (Fig. 4). For *A. triseriatus*, predator manipulation and predator-container size effects were both significant (Table 3). The interaction arose because in large containers, *T. rutilus* significantly

Figure 3. Least squares means ($\pm$ SE) for effects of treatments on eggs laid over the course of three samples during the field experiment. Repeated-measures ANOVA results are reported in Table 2. (A) Effect of predator–sample date combinations within large containers for all mosquitoes. (B) Effect of predator–sample date combination within small containers for all mosquitoes. (C) Effect of predator–sample date combination for *Aedes japonicus*. (D) Effect of container size–sample date combination for *Aedes triseriatus*. For each analysis, least squares means for predator versus no predator, or for large versus small containers were compared using a Bonferroni approach within sample dates and none of the pairs were significantly different at experimentwise $\alpha = 0.05$.

Table 3. ANOVA for pupae/liter in the final census of all containers for total mosquitoes, *Aedes japonicus*, and *Aedes triseriatus*

Source	df	All mosquitoes		<i>Aedes japonicus</i>		<i>Aedes triseriatus</i>	
		<i>F</i>	<i>P</i> > <i>F</i>	<i>F</i>	<i>P</i> > <i>F</i>	<i>F</i>	<i>P</i> > <i>F</i>
Container size	1, 43	4.93	0.0317	1.84	0.1823	0.84	0.3643
Predator	1, 43	29.30	<0.0001	15.42	0.0003	9.58	0.0035
Size*Predator	1, 43	7.74	0.0080	2.58	0.1153	5.46	0.0242

Effects significant at $\alpha = 0.05$ are emphasized in bold type.

reduced *A. triseriatus* pupae, whereas in small containers, the effect of *T. rutilus* was not significant (Fig. 4). Thus, predation by *T. rutilus* in these habitats does reduce significantly the production of adult mosquitoes, to some degree, for both of these species, but this effect is larger and more consistent across container sizes for *A. japonicus* than it is for *A. triseriatus*.

Discussion

Our manipulation of abundances of *T. rutilus* succeeded in establishing distinctly different predation environments in the experimental containers for the duration of the experiment. Inputs of *T. rutilus* eggs to our containers were steady and sometimes high, with a mean

Figure 4. Least squares means ($\pm$ SE) for pupae/liter in the final census of containers. ANOVA results are reported in Table 3. Within the separate analyses of all mosquitoes, *Aedes japonicus*, and *Aedes triseriatus*, means were compared using Tukey's test at experimentwise $\alpha = 0.05$, and means within a group marked with the same letter.

$\pm$ SE of 1.4 ± 0.2 (range: 0–29) eggs per container, per 3-d interval. Continuous removal of eggs and young larvae of *T. rutilus* necessitated some disruption of the containers, which were emptied and

sorted every 3 d. This disruption may have contributed to the decline in numbers of larvae and pupae/liter observed in the second and third samples, after manipulations began (Fig. 2). The disruption was the same for all treatment combinations, because all containers were treated the same except for removal or addition of *T. rutilus* larvae. The measured predator abundance (mean predator-days) across the experiment came very close to the targets shown in Fig. 1. In the large containers, where four *T. rutilus* larvae per container was the goal, actual mean of predator-days was less than expected, due to cannibalism or other deaths of larvae. In the small containers, where one *T. rutilus* was the goal, actual mean of predator-days was very close to the goal. For no-predator containers of both sizes, where predator removal was the goal, there were *T. rutilus* larvae that colonized the containers (Fig. 1), though means of predator-days were significantly less than that for either of the treatment combinations with predators. By removing *T. rutilus* eggs and larvae every 3 d, we kept predator abundance well below that in the yes-predator containers. Based on this experiment, it seems that removals every day would be necessary to approach complete removal more closely.

Despite any variation caused by predator colonization or death, we detected significant effects of the predator manipulation on total abundance of mosquitoes/liter and *A. japonicus*/liter, though not on *A. triseriatus* (Fig. 2). As expected, the effect of the predator manipulation increased over the three samples taken (Fig. 2), and one likely contributor to this was the growth of the *T. rutilus* larvae from their start as second or early third-instar larvae, and associated increased consumption. The first *T. rutilus* pupae were replaced at 9 d, and peak pupal replacement occurred at 12 d (34 replaced across all containers). Thereafter, a steady rate of *T. rutilus* pupation, requiring replacement of 7–12 pupae per sample date, was maintained until day 24. The tendency of *T. rutilus* fourth-instar larvae to increase consumption is well known (Steffan and Evenhuis 1981) and it appears that as the *T. rutilus* larvae matured, their impact increased.

The effect of *T. rutilus* predation was not uniform for the two dominant species in this system, *A. japonicus* and *A. triseriatus*. Invasive *A. japonicus* larval and pupal abundance was strongly and significantly affected by *T. rutilus* predation, whereas *A. triseriatus* larval and pupal abundance was not (Table 1, Fig. 2). This pattern certainly does not indicate that *A. triseriatus* are invulnerable to *T. rutilus*, as numerous studies have documented predation on *A. triseriatus* by this predator (e.g., Juliano 1989, Grill and Juliano 1996, Lounibos et al. 1997, Griswold and Lounibos 2006). Rather, the differential effect on these species shows that native *A. triseriatus* are less vulnerable than are invasive *A. japonicus*, which is a pattern that has been predicted from some (e.g., Freed et al. 2014) single-species experiments, but not others (Murrell and Juliano 2013). Such differential vulnerability may only be evident in relatively long (i.e., 24 d) experiments with both species of potential victims exposed to predator, so that slight differences in vulnerability become evident. Native *A. triseriatus* show strong behavioral responses to cues from predation by *T. rutilus* (e.g., Grill and Juliano 1996; Kesavaraju and Juliano 2004, 2010; Kesavaraju et al. 2007b) and to other predators (e.g., Kesavaraju et al. 2007b, Kesavaraju and Juliano 2010). These behavioral responses may contribute to *A. triseriatus*' better performance in a multispecies assemblage subject to predation. One other field study of the impact of container-dwelling predators similarly showed that predation by *Corethrella appendiculata* Grabham (Diptera: Corethrellidae) had a lesser impact on *A. triseriatus* abundance than on *A. albopictus* abundance (Juliano et al. 2010). These results all suggest that consistent co-occurrence with native predators has resulted in *A. triseriatus* being adapted to habitats with these predators, perhaps to a greater degree than are invasive species

like *A. japonicus*. Unfortunately, there have been no comparable published studies comparing directly behavioral responses to predators of *A. japonicus* and *A. triseriatus*. Igrassia (2006) compared the foraging behavior of *A. triseriatus* and *A. japonicus* in the absence of predation and noted that *A. japonicus* spent more time browsing below the surface whereas *A. triseriatus* spent more time filtering at the surface. This difference in foraging would be expected to render *A. japonicus* more vulnerable to *T. rutilus* predation than is *A. triseriatus* (Juliano and Reminger 1992, Grill and Juliano 1996), though firm conclusions require direct comparison of behavior in the presence and absence of the predator. In its native Asia, *A. japonicus* likely co-occurs with other species of *Toxorhynchites* (Sota 1996), as well as other predators (Sunahara et al. 2002), so that this species would not be expected to be globally predator naïve. One interesting possibility is that greater vulnerability of the invader compared to the native is that the process of invasion and establishment of *A. japonicus* in North America has led to relaxation of selection by predation. This relaxation may have occurred because *A. japonicus* invasion of North America has been concentrated in northern portions of the country (Andreadis and Wolfe 2010, Kaufman and Fonseca 2014) and predation by *T. rutilus* and *Corethrella* is considerably less common further north where colder climates limit the distribution of these subtropical/tropical groups. It is also true that invasion has substantially involved colonizing man-made containers in relatively disturbed habitats, where predation pressure may be weaker. The continued spread of *A. japonicus* into the Midwest and some parts of the south presents an opportunity to investigate how this species will fare in regions where container-dwelling predators are more common.

Although we detected some apparent effects of predator treatments on oviposition (Table 2), the actual effects (Fig. 3) are inconsistent with the hypothesis that trait-mediated avoidance of oviposition in habitats with predators contributes to the reduced abundances of larvae and pupae observed in this experiment. Egg inputs to containers were more often greater in the containers with the predator as opposed to the no-predator containers, though the differences were sometimes small (Fig. 3). This result combined with the data on abundances of larvae and pupae during the experiment imply that the effect of *T. rutilus* on populations and communities of its main victims are largely driven by the lethal effect of predation, rather than prey behavioral avoidance of oviposition in habitats with predators. We cannot rule out other, nonlethal effects of *T. rutilus* such as predator-induced reductions in growth due to behavioral changes (e.g., Lounibos et al. 1993) or changes in physiology (e.g., Stoks et al. 2006). A meta-analysis of oviposition responses of mosquitoes to predators found that *Aedes* species in general are unlikely to respond to predators by avoiding oviposition (Vonesh and Blaustein 2010), in contrast to *Culex* species, which often show strong behavioral avoidance of oviposition in habitats with a variety of predators (Blaustein et al. 2004, Silberbush and Blaustein 2008, Vonesh and Blaustein 2010). Some *Aedes* species show an oviposition preference for containers in which *Toxorhynchites* are preying on *Aedes* larvae, which seems to arise from a trophic cascade due to predation reducing the larval population and releasing bacteria from grazing pressure and adding feces and body parts of victims to the water, which would increase detritus substrates for bacteria (Albeny-Simões et al. 2014).

Our final census of experimental containers showed that *T. rutilus* had stronger and more consistent effects on pupal production of *A. japonicus* than on that of *A. triseriatus* (Fig. 4). In the case of pupal production (and by inference adult production), the presence of *T. rutilus* significantly reduced pupal abundance of

A. japonicus in both small and large containers (Fig. 4), whereas *T. rutilus* only significantly reduced pupal production of *A. triseriatus* in the large containers. Generally, our experiment showed a lack of interactive effects of container size and predation on larval and pupal abundance in samples and egg inputs, which is not surprising as our two sizes of containers held similar numbers of predators/liter. The interactions of container size and predation observed for censused pupal production may suggest that the presence of multiple, similarly sized *T. rutilus* larvae in some way creates synergistic effects on per capita predation of pupae of *A. triseriatus*. Alternatively, it is possible that predation pressure on pupae in containers does not scale with volume of water, but instead with surface area or bottom area. Because our two sizes of containers were similar in shape, they are more similar to one another in surface or bottom area than in volume. Thus, our constant of one *T. rutilus* larva/3.5 liters would produce a greater density of *T. rutilus* per unit surface or bottom area in the large, as opposed to the small, containers. It seems likely that because pupae do not feed, they will spend more of their time at the surface than do larvae, which may mean that predators per unit area at the surface is the important determinant of pupal survival. Larval *T. rutilus* are considered ambush predators, often capturing moving prey at the bottom but sometimes at the surface, where both prey and predator must obtain oxygen. The impacts of *T. rutilus* on pupae and, likely, ultimate adult production are consistent with past observations that *Toxorhynchites* in general are effective predators of pupae, sometimes preferring them to, or having a greater impact on them compared to prey larvae (e.g., Lounibos 1979, Lounibos et al. 1997). Thus, *T. rutilus* may have important effects reducing adult mosquito production from containers in forested areas. Previous attempts to use *Toxorhynchites* for biological control of mosquitoes have a variable record of success (Schreiber 2007), likely owing to the difficulty and expense of rearing and releasing sufficient numbers of these predators. Nevertheless, this species may be important as an agent of conservation biological control, which seeks to foster abundance and diversity of natural enemies already present (Tscharrntke et al. 2007). Our results suggest further that this role for *T. rutilus* may selectively impact invasive species like *A. japonicus*, and though we do not expect this predator to eliminate *A. japonicus* locally or regionally, it is likely to inhibit the spread and increase of this invader in predator-rich habitats.

The presence of a higher trophic level in a community is one component of both functional and taxonomic diversity. Our results indicate that natural enemies of mosquito larvae, like *T. rutilus*, are likely to contribute to biotic resistance of container communities to invasion, even if they do not constitute an absolute barrier to invasion. Conserving native predators in mosquito larval habitats seems like a prudent step toward limiting invasions by nonnative mosquitoes.

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