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COMPARATIVE EFFICACY OF *PIMEPHALES PROMELAS*, *FUNDULUS DIAPHANUS*, AND *GAMBUSIA AFFINIS* AND INFLUENCE OF PREY DENSITY FOR BIOLOGICAL CONTROL OF *CULEX PIPIENS MOLESTUS* LARVAE

MATTHEW W. BICKERTON,[1] JOSEPH CORLETO,[2] THOMAS N. VERNA,[3] ERIC WILLIGES[4] AND DEEPAK MATADHA[5]

ABSTRACT. Larval survival times and density-dependent feeding behavior were evaluated with the use of 2 species of fish native to the northeastern USA (*Pimephales promelas* and *Fundulus diaphanus*), and the potentially invasive *Gambusia affinis*. Each species was provided 10, 20, 30, 45, and 60 4th-stage larvae of *Culex pipiens molestus*/fish in the laboratory and digital images were recorded to quantify the number of surviving larvae at various intervals. Daily feeding rates were greatest at the highest larval density. These were 49.69 ± 4.07 larvae for *P. promelas*, 60 larvae for *F. diaphanus*, and 36.44 ± 6.6 larvae for *G. affinis*. Survival analysis was used to compare efficacy of each fish species over time. All fish species consumed larvae at similar rates at lower densities, but significant differences occurred at densities of 30–60 larvae/fish. Survival times of larvae at the highest density were 44 ± 7.9 h for *P. promelas*, 15 ± 3.4 h for *F. diaphanus*, and 70.6 ±13 h for *G. affinis*. In order to evaluate feeding rate as a function of prey density, we compared consumption rates 1.5 h after feeding with the use of a 4-parameter logistic model. *Fundulus diaphanus* and *G. affinis* feeding aligned with the 4-parameter model, indicating that initial feeding rates for these species increased with prey density to an upper limit (satiation). *Pimephales promelas* feeding within 1.5 h did not align with this model, suggesting that early feeding rates for this species are not heavily influenced by prey density.

KEY WORDS Biological control, *Fundulus diaphanus*, *Gambusia affinis*, *Pimephales promelas*, survival analysis

INTRODUCTION

Integrated mosquito control often involves the use of biological control agents to manage nuisance mosquito species and those involved in disease transmission. In spite of widespread use of commercial insecticides, the practice of stocking fish continues to be a major component of many regional mosquito control programs. When used in the appropriate environments, there are major advantages to using fish for mosquito control. Larvivorous fish can consume all stages of the mosquito larvae and can provide quick knockdown and long-term control of larval mosquito populations (Homski et al. 1994). Mass production can be easily achieved (Hoy 1985, Meisch 1985). When used as a supplement to conventional insecticide programs, fish can help prevent insecticide resistance by reducing the need for repeated insecticide applications. This is especially important as the number of active ingredients available in commercial insecticides has been declining for some time (Walton 2007).

Many different species of predatory fish are used throughout the world. Mosquito control agencies in New Jersey utilize 3 species: the western mosquitofish *Gambusia affinis* Baird and Girard, the fathead minnow *Pimephales promelas* Rafinesque, and the banded killifish *Fundulus diaphanus* Lesueur. All are generalist predators that feed readily on mosquito larvae and other invertebrates (McIvor and Odum 1988, Stewart and Watkinson 2004, Pyke 2005). When larvivorous fish are present in sufficient numbers, mosquito production in a body of water can be prevented from exceeding public health thresholds (Ghosh et al. 2005).

Gambusia affinis is native to the Gulf Coast drainages of the southeastern United States and has been widely introduced outside of this range (Rauchenberger 1989). These introductions have led to competitive displacement and elimination of native fish, amphibians, and invertebrates (Meffe and Snelson 1989, Rupp 1996, Leyse et al. 2004, Haas 2005, Alcaraz and Garciaberthou 2007, Walton 2007). Although stocking any species of predatory fish can disrupt vulnerable aquatic ecosystems when used indiscriminately, the judicious use of native species can prevent many of the detrimental effects caused by nonnatives (Cech and Linden 1987, Ahmed et al. 1988). For this reason, the New Jersey Department of Environmental Protection restricts the use of *G. affinis* to isolated mosquito habitats that are not occupied by vulnerable aquatic species, and that do not drain into a larger body of water. Effective native species are, therefore, necessary for use in all

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[1] Bergen County Department of Health, Division of Environmental Health, 220 East Ridgewood Avenue, Paramus, NJ 07652.

[2] New Jersey Department of Environmental Protection, Office of Mosquito Control Coordination, 501 East State Street, PO Box 420, Trenton, NJ 08625.

[3] Burlington County Department of Mosquito Control, PO Box 6000, Mount Holly, NJ 08060.

[4] Essex County Department of Mosquito Control, 900 Bloomfield Avenue, Verona, NJ 07040.

[5] Middlesex County Mosquito Commission, 200 Parsonage Road, Edison, NJ 08837.

other bodies of water, e.g., ditches, storm water facilities, ponds, etc.

Gambusia spp. are among the most popular species for mosquito control worldwide because of their surface-feeding characteristics, high fecundity rates, and their tolerances to low oxygen and high water temperatures (Bay 1985, Walton 2007). However, there have been numerous attempts to supplement or replace *Gambusia* sp. with endemic species. Cech and Linden (1987) released *Gambusia holbrooki* Girard with Sacramento blackfish, *Orthodon microlepidotus* Ayres, in rice paddies, but determined that low fecundity and host-switching prevented *O. microlepidotus* from improving control. Kramer et al. (1987) compared efficacy of *G. affinis* with *Menidia beryllina* Cope in wild rice systems and determined that neither species reduced larval densities. Offil and Walton (1999) supplemented *Gambusia* releases with *Gasterosteus aculeatus* L. and determined that control was not improved by the addition of *G. aculeatus*. Few studies have demonstrated equivalent or improved control with the use of alternative species. Among them, Nelson and Keenan (1992) showed that *Fundulus zebrinus* Jordan and Gilbert provided similar efficacy to *Gambusia* sp. Homski et al. (1994) showed that the *Aphanius dispar* Rüppell was a more effective predator on late-stage *Culex* larvae than *G. affinis*.

The use of *P. promelas* and *F. diaphanus* for mosquito control in New Jersey is considered to pose less environmental risk because each is endemic to the northeastern US. In contrast to *G. affinis*, there has been comparatively little scientific work using these species as biological control agents. Additional data regarding efficacy, stocking rates, ideal habitat types, and other ecological parameters are needed to substantiate continued use of *P. promelas* and *F. diaphanus*. Irwin and Paskewitz (2009) examined the feeding rates of *P. promelas* on mosquito larvae and determined that this species may be an effective alternative to insecticides, and that the feeding rate of individual fish increases with larval density. The maximum feeding rate of *P. promelas* fed on *Culex pipiens* was determined to be 76.4 larvae/24 h when 100 larvae were provided.

Much more is known about the capabilities of *Gambusia* sp. as biological control agents. Kramer et al. (1988) found the composition of mosquito larvae in guts of *G. affinis* corresponded with larval densities in the field, indicating that *Gambusia* sp. may preferentially feed on mosquitoes compared to other invertebrates. However, Pandian and Reddy (1971) found that *Gambusia* sp. did not preferentially feed on *Culex* sp. over *Chironomus* sp. larvae. Estimated feeding rates for *G. affinis* vary dramatically; the same authors recorded 29.6 larvae/24 h, whereas Chatterjee and Chandra (1997) recorded between 31 and 51 larvae/24 h, and Verma et al. (2016) recorded 292.2/24 h. These studies differed in standardization of individual fish, as well as the quantity and species of larvae provided. Thus, a comparative examination of feeding rates between 3 different species with a controlled set of parameters needed to be performed.

The primary goal of this work was to determine whether the native species *F. diaphanus* and *P. promelas* can provide similar or superior control to that of *G. affinis* by investigating survival times of larvae. The secondary goal was to determine how each species responded to increasing larval densities.

MATERIALS AND METHODS

Fourth-stage larvae of *Culex pipiens* f. *molestus* Forskål reared at the Burlington County Division of Mosquito Control (Mt. Holly, NJ) were used in all trials. Experiments were conducted on paired groups of adult fish established in 19-liter white polyethylene buckets. Pairs were used instead of individual fish because the absence of conspecifics can induce foraging latency in shoaling species like *F. diaphanus* and *Pimephales* spp. (Morgan 1988, Hensor et al. 2003). Fish used in trials were standardized to a weight that was well represented by adults of each species (1.8 ± 0.5 g). Individual fish were weighed first. Then pairs were selected to a weight of 3.5 ± 0.6 g. In doing so, individuals at the lower end of the weight range were always paired with those at the higher end. In the case of the sexually dimorphic species, *G. affinis*, males were paired with females to balance the size discrepancy between sexes. In addition, this species is unique from the other species in this study in that it is ovoviviparous. Therefore, visibly gravid females were excluded from the trials in order to minimize gestational effects on feeding rates. Weights of fish compared via 1-way ANOVA were not significantly different between species ($F_{2,133} = 1.77$; $P = 0.18$). Buckets were filled with 10 liters of dechlorinated H_2O. Aeration was supplied via a central 36-way manifold connected to a single Pentair diaphragm pump (SL194A) delivering approximately 1.42 liters/min to each bucket. Fish were acclimated for 48 h without food prior to the start of each trial. Conditions were maintained at 14 h light and 10 h dark at 25.5 ± 1 °C for acclimation and trial periods. All fish species were tested simultaneously across 2 different larval density ranges, each with 4 repetitions per species–density combination. The first density range was 20, 40, and 60 larvae/2 fish, and the second was 60, 90, and 120 larvae/2 fish. Two control buckets without fish were used for each larval density. Both density ranges were replicated twice for a total of 4 separate runs. Hereafter, larval densities are denoted as 10, 20, 30, 45, and 60 larvae/individual fish. Food for larvae was not added to experimental buckets, so that fish would not have access to suspended food material, and the short trial period was expected to have little effect on larval survival. Water temperatures were measured throughout acclimation and trial periods and were held constant by adjusting ambient air temperature.

Digital imagery was used to record rates of consumption. Preliminary work (unpublished data) agreed with Pandian and Reddy (1971) that when food is provided after being withheld for 48 h, feeding rate is highest within the first 2 h. Therefore, for the first 2 h of each trial, digital photographs were taken at 20-min intervals to record initial feeding rates. Thereafter, photographs were taken at 4-h intervals between 0800 and 1600 h each day until all larvae were depleted or began to emerge as adults. This period was between 72 and 120 h for each of the trials. However, because the pupal stage for *Cx. pipiens* lasts about 48 h at 25 °C (Lang 1963, Rueda et al. 1990), the critical period for fish to consume larvae was less than 72 h to prevent adult emergence.

In order to improve resolution of images, aeration was disconnected during each photo interval. Images were processed and analyzed with the use of ImageJ version 1.49 software (Rasband 2016). The Cell Counter plugin was used to label and count surviving larvae at each interval. Dead whole larvae, as well as partially consumed or damaged larvae, usually descended to the bottom of containers and were not counted among the survivors. Evacuated food was usually present in containers several hours after the start of each trial and was readily distinguished from surviving larvae.

Statistical analysis

Survival analysis was used to determine the effects of fish species on survival times of larvae at different densities. The LIFETEST procedure (SAS Institute, Inc., SAS Campus Drive, Cary, NC) was used to calculate estimates of the survival function: $S(t) = Pr(T > t)$. This function describes the probability that larval survival time (T) is longer than a predicted time period (t). The predicted time period is any interval of interest over the course of the study. Survival time is defined as the amount of time in which all larvae were consumed by the fish or when the final observation was made when adults began to emerge. The latter were denoted as "censored" observations in the model. The nonparametric product-limit (Kaplan–Meier) method was used to generate survival curves. Evaluation of the log (–log of the estimated survivor function) plots indicated the proportional hazard ratio is higher at earlier survival times; therefore, Wilcoxon tests were used to compare the survival curves between species. Mean larval survival times were evaluated with the use of the Generalized Linear Model Procedure (PROC GLM) and Tukey's Honestly Significant Difference test.

Nonlinear regression was used to examine the feeding response of the fish to different prey densities. When the range of prey provided in a given interval is easily depleted in that period of time, the response is nearly linear as prey density increases. However, when the range of prey provided in a given interval exceeds that which can be consumed in that interval, the feeding response plotted over prey density assumes a curvilinear shape that can be indicative of density dependence. For this reason, the density response was evaluated separately for each species with feeding rates recorded from the first 1.5 h of each experiment. In a density-dependent system, the decrease in prey density over longer periods of time can result in slower feeding rates. Using this early time interval was expected to minimize this effect. A density-dependent response is expected to be sigmoidal in shape, reaching a plateau when satiation has occurred (Holling 1966, Schenk et al. 2002). The increase in feeding response Y as a function of density x is therefore defined by the logistic equation:

$$Y(x) = \delta + \frac{\alpha - \delta}{1 + \left(\frac{x}{\gamma}\right)^{\beta}},$$

where the parameters represent the upper (δ) and lower (α) limits of quantity consumed, (β) represents the slope, and (γ) represents the x-coordinate of the point of inflexion (Gray et al. 2005, Commo and Bot 2016). The NLIN (nonlinear regression model) procedure was used to obtain least-squares estimates for these parameters with the use of the Marquardt iterative method. Hougaard's measure of skewness was evaluated and minimized with the use of expected-value parameterization for interpretation of individual parameter estimates. The coefficient of determination (R^2) was calculated as: 1 – error sum of squares/corrected sum of squares. The response value used for all analyses was the average number of larvae consumed by paired fish for each observation.

RESULTS

Between 736 and 844 images were recorded and analyzed from each trial for a total of 3,159 images. Larval survival time is defined as the period when all larvae were consumed. Survival curves of larvae released at different densities into 1 of the 3 fish treatments are given in Fig. 1 and Wilcoxon tests for homogeneity of survival curves of the 3 species are given in Table 1. Mean survival times are given in Fig. 2. Survival curves of larvae were not significantly different between the 3 predatory fish species at the 10 and 20 larval densities, but significant effects were present at the higher larval densities. Differences between fish species were most pronounced at the 30-larvae density; larvae fed to *P. promelas* or *G. affinis* were 6.7 and 7.1 times longer, respectively, than those provided to *F. diaphanus*. At the 45-larvae density, larvae fed to *P. promelas* or *G. affinis* survived 5.0 and 3.9 times longer, respectively, than those fed to *F. diaphanus*. Larvae provided to *G. affinis* began to emerge at this density and had a 46.9% chance of survival past 64 h. At the 60-larvae density, larvae fed to *P. promelas* or *G. affijnis* survived 3.0 and 4.7 times longer, respectively, than those provided to *F. diaphanus*. Larvae provided to *G. affinis* began to emerge and had a 53.3% chance of survival past 64 h. The maximum daily feeding rates

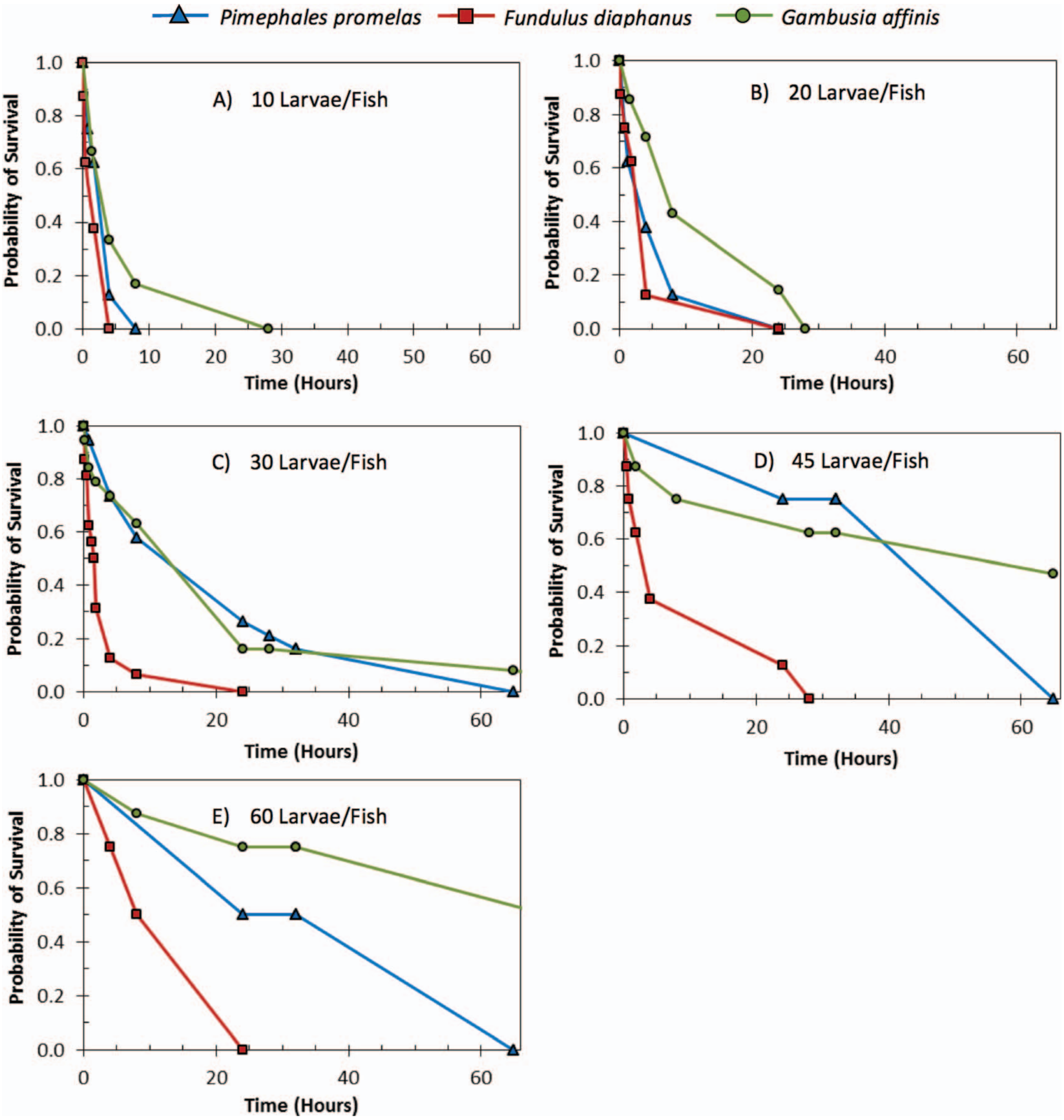

Fig. 1. Survival curves for 4th-stage larvae of *Culex pipiens molestus* provided to 3 fish species at different densities.

Table 1. Wilcoxon tests for homogeneity between survival curves of 4th-stage larvae of *Culex pipiens molestus* provided to fish at different densities.

Larval density	χ^2	df	*P*
10	3.1	2	0.21
20	4.08	2	0.13
30	23.4	2	<0.0001
45	13.5	2	0.001
60	12.5	2	0.0019

by all species in these trials occurred at the 60-larvae density. These were 60 larvae for *F. diaphanus*, 49.69 ± 4.07 larvae for *P. promelas*, and 36.44 ± 6.6 larvae for *G. affinis*.

Nonlinear models constructed for feeding rates at the 1.5-h interval produced significant near-linear estimates for *F. diaphanus* ($F_{3,44} = 72.18$, $P < 0.0001$, $R^2 = 0.83$) and *G. affinis* ($F_{2,45} = 5.61$, $P = 0.007$, $R^2 = 0.2$), but not for *P. promelas* ($F_{2,45} = 1.31$, $P = 0.38$, $R^2 = 0.05$ [Fig. 3]). This indicates that *F. diaphanus* and *G. affinis* fed in a density-dependent manner during the first 1.5 h of feeding. For *F. diaphanus*, the observed mean at the lowest density (8.75 ± 0.53 larvae) was within range of the

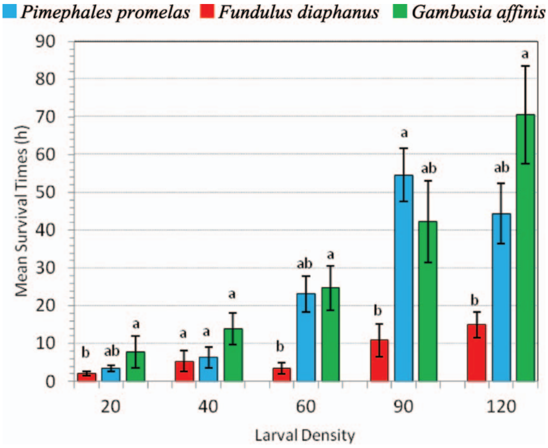

Fig. 2. Mean survival times ± SE of 4th-stage larvae of *Culex pipiens molestus* provided to fish at different densities. The survival time is the amount of time for fish to consume 100% of larvae. Means with the same letter are not significantly different ($P < 0.05$) by Tukey's Honestly Significant Difference test.

predicted lower limit ($\alpha = 7.91 \pm 2.57$ larvae) of the model. Higher densities resulted in progressively higher proportions of larvae consumed (relative to density provided) up to an inflexion point of 25.19 larvae at the predicted density of (γ) = 26.72 ±1.86 larvae. The proportion of larvae consumed began to plateau at densities above this point. The observed mean at the highest larval density (40.5 ± 3.44) was within range of the upper limit (δ) of 42.46 ± 3.01 larvae.

For *G. affinis*, observed means at 10- and 20-larvae densities (7.42 ± 1.41 and 9.21 ± 2.55, respectively) were in range of the predicted lower limit ($\alpha = 8.03 \pm 3.34$ larvae) of the model. The proportion of larvae consumed increased to an inflexion point of 15.39 larvae at a density (γ) of 29.93 ± 3.67 larvae. Mean larval consumption at the 45- and 60-larvae densities (22.88 ± 5.29 and 22.13 ± 6.29, respectively) was within range of the predicted upper limit (δ) of 22.76 ± 3.02 larvae.

For *P. promelas*, rates of larval consumption did not increase asymptotically towards an upper limit as in the 4-parameter logistic model. The mean number of larvae consumed after 1.5 h was lowest at the 10-larvae density (7.63 ± 0.96 larvae). Larval consumption increased at the 20-larvae density to 15.63 ± 1.74 larvae; however, it decreased at higher densities. Curves fitted to *P. promelas* consumption at other time points produced singular hessian matrices, indicating that this type of model was not appropriate.

DISCUSSION

In our study, mosquitoes that survived exposure to fish began to emerge as adults after 64 h (2.7 days). Control was considered effective when larvae did not survive beyond 64 h. Survival analysis showed that all fish species provided similar control at the lower densities. However, differences occurred at densities of 30 larvae and above. The amount of time required to achieve 100% mortality never exceeded 28 h for *F. diaphanus*. For *P. promelas* and *G. affinis*, survival times increased considerably at densities of 30 larvae and above. *Pimephales promelas* consumed 100% of larvae at all densities in less than 64 h, and adult emergence did not occur. However, there was >50% chance of survival beyond 24 h at the 45- and 60-larvae densities. Although higher densities were not investigated in this study, it is likely that larvae would have survived beyond 64 h and emerged as adults at densities above 60 larvae. For *G. affinis*, larvae survived beyond 64 h and adult emergence was recorded in 20.8% of higher-density treatments. Emergence of adult mosquitoes after 64 h implies that *G. affinis* was not able to eliminate all mosquito larvae at densities >45 larvae per fish. Comparing the survival times of larvae revealed that the most effective predator in this study was *F. diaphanus*, followed by *P. promelas* and then *G. affinis*.

The parameter estimates for the logistic equation have an important practical function. The predicted lower limit represents the lowest number of larvae consumed, which occurred at the lowest density for all species. At increasing densities, *F. diaphanus* and *G. affinis* consumption aligned with the 4-parameter model, such that the feeding rates increased with density at an accelerated rate through an inflexion point. Thereafter, feeding rates approached an upper limit, which represents the satiation point at higher larval densities. Density-dependent feeding rates were apparent for *F. diaphanus* and *G. affinis*. *Gambusia affinis* exhibited greater variance than *F. diaphanus*, as illustrated by the lower R^2 value and broader confidence bands. At the 1.5-h time period, *P. promelas* did not feed in a density-dependent manner like *F. diaphanus* and *G. affinis*. However, in the survival analysis, larval consumption at later intervals was shown to be greater than that of *G. affinis*. The negligible effect of larval density at earlier times may be related to the natural diet of this species and a possible need for a prey learning period. Detritus is a major food source for *P. promelas* and may comprise up to 79% of the adult diet. *Fundulus diaphanus* and *G. affinis* possess upturned facial appendages to facilitate surface feeding. The lack of this adaptation in *P. promelas* may be indicative of their more generalist nature or dependence on detritus rather than pelagic macroinvertebrates (Herwig and Zimmer 2007). Many generalist fish predators require a period of exposure in order to learn how to recognize and successfully capture unfamiliar prey (Reid et al. 2009). Because they were hatchery-reared and not previously exposed to mosquito larvae, these fish may have required such a period in order learn the target prey.

The maximum feeding rates recorded in our study contrasted with findings by other authors. This is a

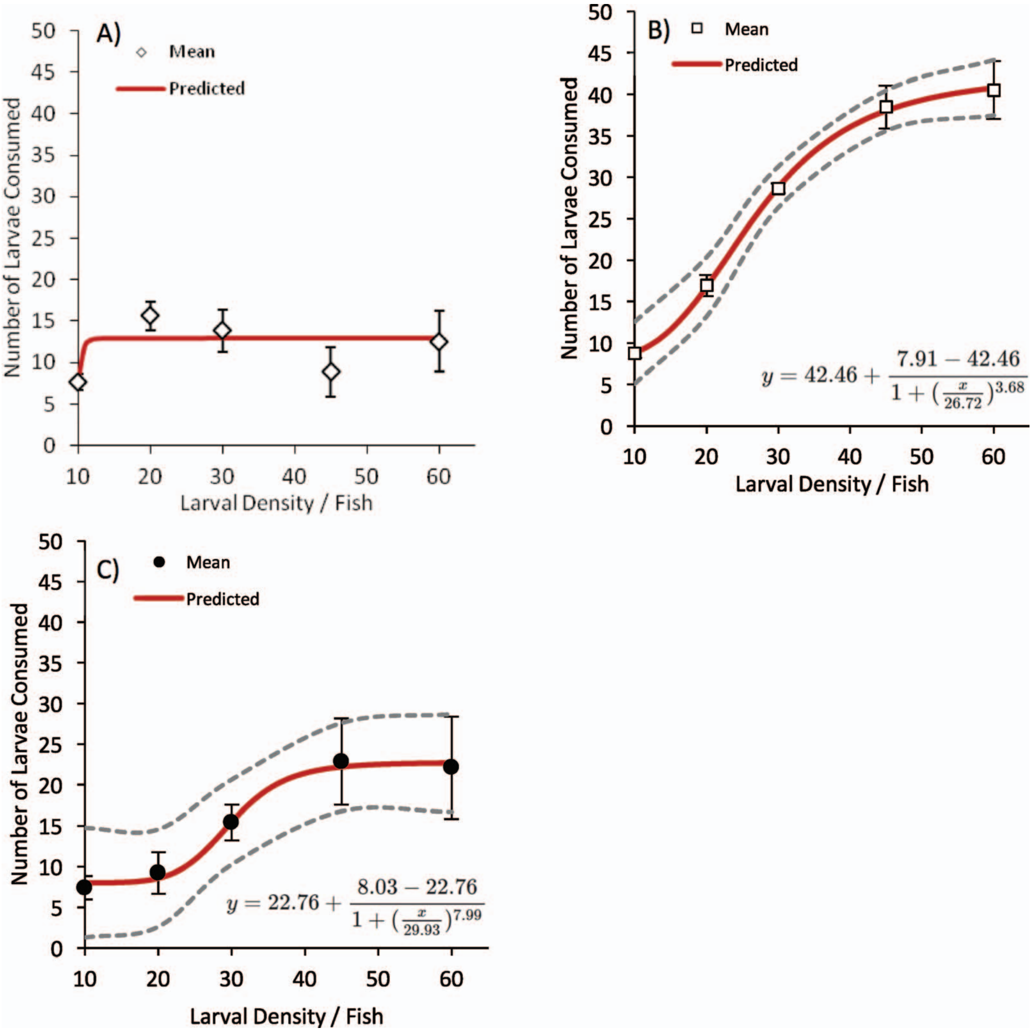

Fig. 3. Four-parameter logistic models constructed for feeding rates of fish species after 1.5 h of feeding on 4th-stage larvae of *Culex pipiens molestus*. (A) *Pimephales promelas*, (B) *Fundulus diaphanus*, (C) *Gambusia affinis*. Dashed lines show upper and lower 95% confidence intervals from the means. Four-parameter logistic equations for predicted values are given for *F. diaphanus* and *G. affinis*.

result of differences in experimental design, standardization practices, and the ranges of larvae provided. Although this study utilized only late instars, further work is needed to determine if these findings can be extrapolated for feeding on early instars. Although *F. diaphanus* and *P. promelas* were effective predators in the laboratory study, other factors present in field situations could affect season-long performance of these species. Factors such as temperature, day length, water composition (e.g., salinity, alkalinity, organic matter, dissolved oxygen), habitat complexity, the presence of alternative foods, and fecundity rates are likely to affect their efficacy.

When used in appropriate environments, native species of fish are preferable to nonnatives for mosquito control because the ecological drawbacks may be substantially reduced (Chandra et al. 2008). However, inferior control often demonstrated by native species may result in increased public health risks. Our findings demonstrate that *F. diaphanus* and *P. promelas* are superior predators in the laboratory when compared to *G. affinis*. These species should be considered in future investigations as potentially valuable biological control agents.

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