Predation and the evolution of prey behavior: an experiment with tree hole mosquitoes

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We tested for facultative changes in behavior of an aquatic insect in response to cues from predation and for evolution of prey behavior in response to experimental predation regimes. Larvae of the tree hole mosquito *Aedes triseriatus* reduced filtering, browsing, and time below the surface in response to water that had held a feeding larva of the predator *Toxorhynchites rutilus*. We subjected experimental *A. triseriatus* populations to culling of 50% of the larval population, either by *T. rutilus* predation or by random removal. After two generations of laboratory culling, behavior of the two treatment groups diverged. *Aedes triseriatus* in control-culled lines retained their facultative shift from filtering to resting, but tended to lose the response of reduced browsing below the surface in water that had held a feeding predator. Predator-culled lines lost their facultative response of reduced filtering in water that had held a feeding predator and evolved toward more time resting and less time filtering in both water that had held a feeding predator and water that had held only *A. triseriatus*. Predator-culled lines retained their facultative response of reduced browsing below the surface in water that had held a feeding predator. Two field populations and their reciprocal hybrids responded similarly to cues from predation and did not differ in their evolutionary response to experimental culling. We conclude that consistent presence or absence of predation can select rapidly for divergence in prey behavior, including facultative behavioral responses to predators. *Key words:* activity, *Aedes triseriatus*, behavioral responses, constitutive behavior, facultative antipredator behavior, feeding behavior, predation, predator avoidance behavior, *Toxorhynchites rutilus*. *[Behav Ecol 13:301–311 (2002)]*

Predation appears to cause strong selection on behavior of prey (reviewed by Lima and Dill, 1990; Sih, 1987). The result often appears to be facultative predator avoidance behaviors (e.g., reduced feeding, reduced activity, and refuge use) that are expressed when prey perceive the predator (e.g., Ball and Baker, 1995, 1996; Boersma et al., 1998; Gilliam and Fraser, 1987; Grostal and Dicke, 1999; McPeek, 1990; Sih, 1980, 1986; Stein and Magnuson, 1976; Tikkanen et al., 1996; Wisenden et al., 1999). Facultative changes in behavior are often taken as evidence for evolution of prey behavior in response to selection by predation (Blumstein et al., 2000; Gilliam and Fraser, 1987; Main, 1987; Sih, 1980, 1987; Stein and Magnuson, 1976; Watkins, 1996) because behavior patterns induced by the presence of a predator are often associated with lower vulnerability to predation (Grill and Juliano, 1996; Lawler, 1989; McPeek, 1990; Peckarsky and Wilcox, 1989; Skelly and Werner, 1990; Woodward, 1983). Investigators have rarely taken the next step of using controlled experiments to test the prediction that populations subjected to different predation regimes diverge in behavior. Such experimental tests would provide the strongest evidence that behavior evolves in response to predation. In this study, we first documented facultative changes in prey behavior in response to cues from predation and then tested for evolution of prey behavior (including modification of facultative responses to cues from predation) in response to controlled predation regimes.

Our study organisms are larvae of the tree hole mosquitoes *Aedes triseriatus* (Say) (prey) and *Toxorhynchites rutilus* (Coq.) (predator). These subjects were chosen because (1) behavior patterns of *Aedes* larvae are a major determinant of short-term probability of predation by *Toxorhynchites* larvae. *Toxorhynchites rutilus* is primarily an ambush predator (Steffan and Evenhuis, 1981), though searching behavior by larvae sometimes does occur (Linley and Darling, 1993). Prey appear to be detected primarily by mechanoreceptors (Russo, 1986; Steffan and Evenhuis, 1981). More active individuals that move and feed more frequently, and individuals below the water's surface, are more likely to be victims of predation, whereas resting at the water's surface entails a relatively low risk of predation (Grill and Juliano, 1996; Juliano and Reminger, 1992; Rubio et al., 1980; Russo, 1986). (2) There is significant interpopulation variation in behavior of *A. triseriatus* (Juliano et al., 1993; Juliano and Reminger, 1992), suggesting local adaptation. (3) Late instar *A. triseriatus* alter their behavior in the presence of *T. rutilus* larvae (Juliano and Reminger, 1992); however, these facultative behavioral responses by *A. triseriatus* to this predator are not well understood. For example, these behavioral responses are not detectable under all experimental conditions (e.g., not with caged predators; Hechtel and Juliano, 1997), and they do not appear to vary consistently between populations with different histories of exposure to this predator (Hechtel and Juliano, 1997; Juliano and Reminger, 1992).

We addressed two specific questions about the potential for predation by *T. rutilus* to cause evolution of behavior of *A. triseriatus*. First, does *A. triseriatus* adopt low-risk behaviors in response to chemical and physical traces left by the act of predation (as opposed to *T. rutilus* larvae themselves, as in past studies; Juliano and Reminger, 1992)? Based on previous studies with this predator–prey system (Juliano and Reminger, 1992; Juliano et al., 1993), we predict that cues from *T. rutilus* predation should induce decreased movement and feeding and increased time at the surface.

Second, does prey behavior change in response to controlled predation over several generations, and if so, how? Both constitutive and facultative behaviors could change in

response to selection by predation. We quantified behavior under two conditions: with no cues to the presence of *T. rutilus,* and in water that has held a feeding *T. rutilus,* which may provide cues indicating the presence of this predator. By analyzing effects of selection by predation on behavior in these two environments, we sought to determine whether consistent selection by predation favors enhanced or modified facultative behavioral responses to the predator or favors a shift to constitutive patterns of low-risk behavior.

We addressed these questions in a laboratory experiment comparing experimental lines subjected to predation to control lines raised under identical conditions but without predation. Thus, we held constant other selective forces (e.g., density, competition, resource quantity and quality) and isolated the effect of predation alone on short-term evolution of prey behavior.

METHODS

Source populations

We used two populations of *A. triseriatus,* both of which encounter *T. rutilus* in nature. One population was collected from tree holes at Parklands Merwin Reserve (and adjacent private lands) near Normal, Illinois, USA (88°50′ W longitude, 40°39′ N latitude), where *T. rutilus* is relatively rare (Juliano et al., 1993). *Toxorhynchites rutilus* was collected in only 4 of 10 years beginning in 1990, only in late summer and fall, and never in spring, suggesting that they do not overwinter at this latitude (Juliano, 1996). During the two summers preceding this experiment, *T. rutilus* occurred in only 1 of 23 tree holes (4%) and 7 of 18 discarded tires (39%) sampled at this site (Juliano and Gravel, unpublished data). The second population was collected from tree holes at Horseshoe Lake State Park near Olive Branch, Illinois (89°13′ W longitude, 37°6′ N latitude), where *T. rutilus* appears to be more common (Juliano et al., 1993). During the two summers preceding this experiment, *T. rutilus* occurred in 9 of 36 tree holes (25%) and in 1 of 2 tires (50%) sampled at this site (Juliano and Gravel, unpublished data). We collected *A. triseriatus* as larvae and pupae, raised them to adulthood in environmental chambers, and allowed them to breed in 0.6-m³ cages. We collected *T. rutilus* as eggs or larvae in the field at Vero Beach, Florida, USA (82°23′ W longitude, 27°31′ N latitude), the site where we conducted the culling phase of the experiment. *Toxorhynchites rutilus* from this site prey regularly on *A. triseriatus* in nature, and using this population simplified the acquisition of sufficient numbers of predators and does not affect our test of these hypotheses.

Field-collected *A. triseriatus* formed the parental (P) generation. In addition to these populations, we created reciprocal hybrid populations, which we deemed important for this design because of the possibility that genetic variation in behavior within a single population may be low due to the effect of selection on behavior (Hoffman, 2000; Mousseau and Roff, 1987). If there is interpopulation differentiation in behavior, as suggested for the Normal and Olive Branch populations (Juliano et al., 1993), and if that differentiation has some genetic basis, hybrid populations may show greater genetic variation and so may respond more rapidly to our laboratory selection regimes. Thus, the hybrid populations may give us more power to detect effects of selection by predation. The P generations for both pure populations and for two reciprocal hybrid populations consisted of the following numbers of adults: male Normal/female Normal (abbreviated NN) = 851/767; male Olive Branch/female Olive Branch (OO) = 524/602; male Normal/female Olive Branch (NO) = 845/604; and male Olive Branch/female Normal (ON) = 520/

767. We reared the progeny of the P generation (F₁) for one generation in the laboratory in environmental chambers at 25°C with a 14:10 h day : night light cycle. We used their progeny (F₂) to start the culling experiment. Thus, we began the experiment with individuals only two generations removed from field environments, minimizing the effects of unintentional laboratory selection on behavior. We reared the F₁ parents of the generation that initiated the experiment under identical laboratory conditions, which should reduce any maternal effects of field environments. Finally, any genetic variation in the hybrid populations that is associated with recessive traits (and therefore masked in the F₁ individuals) should be phenotypically expressed in the F₂ individuals, increasing the amount of genetic variation upon which selection may act. We cannot rule out the possibility that the two generations of laboratory rearing prior to initiating the experiment altered the behavior of larvae relative to that of field-collected larvae. However, because all individuals were subjected to the same conditions during this period, this preexperimental rearing cannot be responsible for any effects of our treatments on behavior. Even if the behavior of larvae was affected by the preexperimental rearing, our experiment still provides us with a test of whether controlled predation regimes result in divergence of behavior.

Culling experiment

We chose to test for evolution of behavior in response to predation using a laboratory culling experiment (Rose et al., 1990, 1996). Culling experiments differ from traditional artificial selection experiments, in which the investigator measures phenotypic values for a number of individuals and then chooses the parents for subsequent generations based on these values. In contrast, in a culling experiment, replicate populations are exposed to some potential agent of selection (in our case, sustained predation by a single predator species) in circumstances that will yield a limited number of survivors, and the survivors are used as parents for the next generation. The traits of interest (in our case, prey behavior) in these selected populations may then be compared to those in control populations without the agent of selection (or with some alternative agent of selection). Culling experiments can produce responses to selection quickly and, because they are less labor intensive than selection experiments, may facilitate investigation of larger experimental populations or greater replication (Rose et al., 1990, 1996). Most important, however, a culling experiment provides us with a means of testing the effectiveness of a particular agent of selection (in this case predation) as a cause of evolution. To our knowledge, this approach has not been used in the study of evolution in response to predation but has been used to test for evolution in response to several other factors (Chippindale et al., 1994; Goodnight and Craig, 1997; Joshi and Mueller, 1988; Mueller, 1988; Rose, 1984; Rose et al., 1990; Zwaan et al., 1995).

From each of the four populations (i.e., NN, OO, NO, ON), we created replicate lines for predator and control culling regimes. For each replicate line, we placed 300 larvae in a 1-l container with 800 ml of water. Initially, we established eight lines from each of the four populations. For the first generation of culling, when larvae were 8 days old (and culling was to begin), we equalized densities of all replicate lines by pooling within populations, resulting in a density at the start of culling of 250 larvae per line. This pooling is justifiable because at this point in the experiment, all larvae had been treated identically and had the same history. This pooling resulted in a reduction of replicate lines to four for OO and NO, six for ON, and eight for NN. For the second generation of culling, after each line had its own unique history, we could

not equalize densities at the start of the culling phase; therefore, the number of larvae in each line when culling began on day 8 varied from 57 to 280. By the start of the second generation of culling, 6 of 22 lines had gone extinct due to combinations of poor production of adults and poor production of eggs. Though we lost lines from the experiment, by the second generation we still had eight control and eight predator lines; hence extinction of lines was not related to the culling treatments.

For each generation of culling, we subjected larvae in predator lines to predation by *T. rutilus* until the population of larvae in the line was reduced by approximately 50%. For different replicate lines, this required 2–6 days of exposure to the predator. We added the predator when the *A. triseriatus* larvae were 8 days old, at approximately the third instar. To keep effects of density consistent between culled and control lines, we culled larvae in control lines randomly, every other day, to densities necessary to produce the same proportion survivorship as the mean of the corresponding predator lines. For random culling of control larvae, we poured the water and larvae onto a screen marked with a numbered grid. We used a random number table to determine from which grid sections larvae were to be removed (regardless of instar). We chose grid sections until we had removed the required number of larvae. We also collected larvae in lines culled by the predator on a screen and counted them every other day to determine the appropriate reduction for the control lines. We fed larvae in each line 15 mg of bovine liver powder at the start of the experiment, and we also provided an additional 0.05 mg/larva every other day, beginning on day 8, after each count. Thus, all replicate lines received the same amount of liver powder per larva.

We reared survivors from each predator and control line to adulthood and propagated adults in 15-l bucket cages housed in a screened porch at Florida Medical Entomology Laboratory, Vero Beach, Florida. Adults were subjected to ambient temperature and photoperiod of subtropical Florida (September–November). We provided adults in each line with sugar water and weekly blood meals from restrained chickens. We collected and synchronously hatched (Novak and Shroyer, 1978) eggs from each replicate line and subjected resulting larvae to the next generation of culling. In each generation, we raised larvae from the same hatch as those used for the culling experiment individually for behavioral observations. We subjected lines to two generations of culling and collected behavioral data for three generations: preculling (F_2), after one generation of culling (F_3), and after two generations of culling (F_4). We refer to these as preculling, first generation, and second generation, respectively.

Behavioral observations

Rearing of individuals
We placed newly hatched larvae individually into 18-ml vials with 11 ml of water. Thus, in all cases, the tested individuals from predator lines had never been exposed to *T. rutilus* or any stimuli emanating from *T. rutilus* until the time they were tested. Therefore, any differences in behavior between predator and control lines cannot be products of the experience of the test larvae. We fed these larvae a liver powder suspension (LPS) prepared by mixing 0.3 g of bovine liver powder with 1 l of water. The suspension was stirred continuously on a magnetic stir plate and transferred by Eppendorf variable-volume pipette to rearing vials. We provided each larva with 0.50 ml of LPS on days 1 and 4, 1.00 ml of LPS on days 9 and 12, and 2 ml of LPS on day 14, and every other day thereafter until pupation. We raised these larvae in an environmental chamber at 23°C and a 14:10 h day : night cycle.

Observation protocol
We videotaped behavior of one-day-old fourth instar larvae of the preculling, first, and second generations. Before all observations, we held larvae individually in a 50-ml cup with 30 ml of water with no food for 24 h to standardize hunger before transfer to the test water. We recorded activity and position of each larva every minute for 30 min in instantaneous scan censuses (Juliano and Reminger, 1992; Martin and Bateson, 1986). We classified activity into four categories: (1) browsing—the larva moved along the surfaces of the cup propelled by movements of the mouth parts, which brushed the surfaces of the cup; (2) resting—the larva was completely still and not feeding; (3) filtering—the larva drifted through the water column, propelled by movement of mouth parts, which created filtering currents; and (4) thrashing—the larva propelled itself through the water by vigorous lateral flexion of the body. We also classified position within the cup into four categories: (1) surface—the larva's spiracular siphon was in contact with the surface; (2) bottom—the larva was within 1 mm of the bottom of the cup; (3) wall—the larva was within 1 mm of the sides of the cup; and (4) middle—the larva was not in contact with the surface and was more than 1 mm from the cup's surfaces. We classified larvae simultaneously at the surface and wall as at the surface, and larvae simultaneously at the bottom and wall as at the bottom. Among activities, resting is the least likely to lead to predation, thrashing is the most likely to result in predation, and feeding behaviors are intermediate (Juliano and Reminger, 1992). Among positions, the surface is the least likely to lead to predation, the bottom is the most likely to lead to predation, and middle and wall are intermediate (Grill and Juliano, 1996; Juliano and Reminger, 1992, Juliano et al., 1993).

We tested for facultative behavioral changes in response to perception of the predator and examined whether such facultative changes are modified by selection due to predation by observing larvae under two experimental water treatments designed to provide cues to the presence of *T. rutilus*. We observed one group of larvae in water that had held a fourth instar *T. rutilus* larva feeding on *A. triseriatus* (predator + prey water). We observed the other group in water that had held only *A. triseriatus* (prey-only water). We prepared predator + prey water by placing one fourth instar *T. rutilus* in 50 ml of water with 10 third and fourth instar *A. triseriatus* larvae and replacing eaten or dead *A. triseriatus* larvae daily. We prepared prey-only water similarly, but with only 10 *A. triseriatus* larvae. We did not feed the *A. triseriatus* larvae during preparation of either water treatment. After 5–7 days of preparation, we removed all larvae from both treatments and recorded behavior patterns of test larvae as described above. We observed only one larva in each beaker of prepared water and then discarded the water.

Statistical analysis

Behavioral observations were converted to proportions of observations in each activity or position category. To reduce the number of variables and to obtain uncorrelated descriptors of behavior patterns, we summarized the activity and position of the preculling generation using principal components analysis (PCA; Hatcher and Stepanski, 1994). We estimated principal components (PCs) for activity and position combined (SAS Institute Inc., 1990; PROC FACTOR), and retained resulting principal components only if their eigen values exceeded 1.00 (Hatcher and Stepanski, 1994). To follow changes in the same measure of phenotype over the subsequent generations (first, second), we used the principal component loadings generated during the preculling generation to determine PC scores in the first and second generation. Because we used the same

Table 1

Frequencies of each activity and position (proportion of observations) during the preculling generation, all populations and water treatments pooled, means and SDs ($N = 127$ individuals)

Activity	Mean	SD	Position	Mean	SD
Resting	0.534	0.326	Surface	0.786	0.241
Browsing	0.224	0.246	Wall	0.058	0.089
Filtering	0.170	0.149	Bottom	0.130	0.164
Thrashing	0.072	0.066	Middle	0.026	0.033

functions, we can compare principal component scores across generations and determine how much these summary variables change in response to experimental culling.

We then analyzed PC scores for all three generations using MANOVA (SAS Institute Inc., 1990; PROC GLM). We interpreted MANOVA results via standardized canonical coefficients (Scheiner, 1993), which indicate by their magnitudes which variables contributed to significant MANOVA effects. When appropriate, we compared populations using multivariate contrasts (Scheiner, 1993). For each of the two postculling generations, we tested for effects of population (Normal, hybrid, Olive Branch), culling regime (predator, control), replicate line within population-culling regime, water (predator + prey, prey only), and all interactions. We considered line within population-culling regime, and all its interactions, to be random effects. For the preculling generation, the analysis was similar, but without effects involving culling regime and line within population-culling regime, because replicate lines were only established after culling had begun.

For the first and second generations, we could not reject the null hypotheses of normally distributed residuals for PC scores. Residual plots indicated no pronounced heterogeneity of variances. For the preculling generation, residual plots indicated approximately homogeneous variances, but residuals were not normally distributed ($p < .05$, Shapiro-Wilk test). We found no transformation that improved this situation, and various transformations did not alter the overall conclusions concerning statistical significance. We therefore analyzed all PC scores without transformation.

For the preculling generation, we compared phenotypic variances for PCs. We first removed effects of water and population on the means by running ANOVA on each PC score and obtaining residuals. We then tested whether phenotypic variance of these residuals within hybrid populations (ON and NO pooled) was greater than phenotypic variance of these residuals within pure populations (NN and OO pooled) using F tests.

RESULTS

Preculling generation

Across all populations and water treatments, resting was the most common activity, and surface the most common position (Table 1). There were significant positive correlations between position and activity categories, particularly for resting and surface, and for browsing and wall, bottom, and middle (Table 2). Within positions, there were significant positive correlations among bottom, wall, and middle (Table 2). Positive, but statistically nonsignificant, correlations were also present for browsing, filtering, and thrashing (Table 2). Resting and surface were significantly and negatively correlated with other activity and position variables, respectively (Table 2). Because such strong correlations complicate interpretations of any changes that may occur in activities and positions in response to the environment (i.e., prey only vs. predator + prey water) or to the culling regime (i.e., predator vs. control), our analysis focused on PCs.

Three PCs with eigen values >1 summarized >89% of the variation in activity and position (Table 3). The fourth eigen value was less than half of the third (Table 3). Rotated factor scores show that PC1 quantifies allocation of time between resting at the surface versus browsing at the wall and bottom (Table 4). Low scores on PC1 result from frequent resting at the surface (large negative coefficient for resting, surface; Table 4) and infrequent browsing of walls and bottom (large positive coefficients; Table 4). PC2 quantifies the frequency of thrashing in the middle (Table 4). High scores on PC2 result from frequent thrashing in the middle and infrequent other activities. PC3 quantifies the allocation of time to resting and filtering (Table 4). High scores on PC3 result from frequent filtering and infrequent resting, and low scores from the reverse (Table 4).

A significant water effect in MANOVA (Table 5) resulted primarily from larvae in predator + prey water having lower scores for PC1 and, to a lesser extent, PC3 (note large canonical coefficients for PC1 and PC3, relative to that for PC2; Table 5) than did larvae in prey-only water. Thus, predator + prey water induced reduced browsing below the surface and increased resting at the surface, and also some reduction in filtering (Figure 1A). In contrast to the water effect, the significant population effect resulted primarily from PC2 (note large canonical coefficient for PC2 relative to those for PC1 and PC3; Table 5), with hybrids (ON, NO) having lower PC2 scores than did pure populations (OO, NN). Thus, hybrids show a lower frequency of thrashing in the middle (Figure 1B). Pure populations did not differ, and reciprocal hybrids did not differ (Table 5). The nonsignificant interaction (Table 5) indicates that behavioral responses of these populations

Table 2

Frequencies of each activity and position (proportion of observations) during the preculling generation, all populations and water treatments pooled, correlation matrix ($N = 127$ individuals)

	Browsing	Filtering	Thrashing	Surface	Wall	Bottom	Middle
Resting	−0.864	−0.599	−0.369	+0.849	−0.705	−0.782	−0.420
Browsing		+0.166	+0.172	−0.944	+0.831	+0.867	+0.352
Filtering			+0.084	−0.161	+0.084	+0.180	+0.058
Thrashing				−0.314	+0.199	+0.225	+0.636
Surface					−0.806	−0.935	−0.488
Wall						+0.572	+0.356
Bottom							+0.322

Correlations listed in boldfaced type are statistically significant at overall $\alpha = 0.05$ (Bonferroni correction; Sokal and Rohlf 1995).

Table 3
Principal components analysis for behavior in the preculling generation

	PC1	PC2	PC3	PC4	PC5	PC6
Eigen value	**4.71**	**1.32**	**1.11**	0.44	0.34	0.07
Proportion of variance	**0.59**	**0.17**	**0.14**	0.05	0.04	0.01
Cumulative proportion variance	**0.59**	**0.76**	**0.89**	0.95	0.99	1.00

Principal components listed in boldface type are those that were retained in the analysis of behavior.

and the hybrids to cues from feeding *T. rutilus* are similar (Figure 1A).

Because resting and surface are both the safest (Juliano and Reminger, 1992) and most frequent behaviors, we also analyzed frequencies (arcsine square-root transformed) of these two behaviors alone as a test of whether our conclusions are robust relative to manipulations of the data. MANOVA on frequencies of resting and surface showed effects similar to those derived from PCs, with a highly significant effect of water (Pillai's trace = 0.507, df = 2, 118, p = .0001) and significant effects of population (Pillai's trace = 0.106, df = 6, 238, p = .0418) and the interaction of the two (Pillai's trace = 0.111, df = 6, 238, p = .0337). Proportion of time at the surface (adjusted mean ± SE: prey only = 0.62 ± 0.02; predator + prey = 0.94 ± 0.02) and time resting (adjusted mean ± SE: prey only = 0.31 ± 0.03; predator + prey = 0.75 ± 0.03) both increased significantly in predator + prey water. The difference between prey only and predator + prey water was significant within all four populations, though the magnitude of the difference was somewhat less for the NN population (data not shown). Although analysis of only these two variables cannot indicate the real nature of behavioral shifts in response to cues from the predator (i.e., what decreases as resting and surface increase?), the similarity of the significant effects supports our interpretation of PC1 and indicates that cues from predation induce *A. triseriatus* to adopt a safer pattern of behavior.

Variation of PCs within each population was similar (Figure 1). Our F tests on residuals indicated that phenotypic variance in PCs for behavior was not significantly greater for hybrid than for pure populations ($F_{60,67}$ = 1.11, 0.27, and 1.16 for PC1, PC2, and PC3, respectively; $p \gg$ .10 in all cases).

Effects of culling by predation

Because there were no differences between the reciprocal hybrid populations ON and NO (see above), we pooled the re-

ciprocal hybrid populations into a single hybrid population for analyses of the first and second generations. After one generation of controlled culling in the laboratory, there were neither significant differences among populations nor significant effects involving culling for the three PCs (Table 6). The only significant MANOVA effect present after one generation of culling was the effect of water treatment, which derived primarily from PC1 (Table 6). For all populations combined, effects of water treatment on PC scores were qualitatively similar to those observed in the preculling generation (Figure 2). In predator + prey water, larvae had lower mean scores on PC1 than did larvae in prey-only water (Figure 2).

A simplified analysis of only frequencies of resting and surface after one generation of culling yielded the same conclusions as analysis of PCs, with only the effect of water significant (Pillai's trace = 0.859, df = 2,9, p = .0001). As in the preculling generation, frequencies of both resting (adjusted mean ± SE: prey only = 0.47 ± 0.02; predator + prey = 0.78 ± 0.02) and surface (adjusted mean ± SE: prey only = 0.74 ± 0.03; predator + prey = 0.95 ± 0.03) increased significantly in predator + prey water.

In contrast, after two generations of laboratory culling by the predator, there were significant effects of water and culling (Table 7). The culling-by-water interaction yielded p = .0521 (Table 7), which we interpret as a significant interaction, indicating that the behavioral response to water treatment differed between predator versus control culling regimes. Canonical coefficients indicated that PC1 and PC3 both contributed to the water and culling effects and that the culling-by-water interaction resulted primarily from PC3 (Table 7). In prey-only water, larvae in the predator-culling treatment browsed at the bottom and wall more, and filtered less, than did larvae in the control-culling treatment (Figure 2). In contrast, in predator + prey water, larvae in these groups differed little in frequencies of these activities and positions (Figure 2). The culling-by-water interaction and the trajectory of

Table 4
Principal components analysis for behavior in the preculling generation: factor loadings

Original variable	Varimax rotated factor pattern		
	PC1	PC2	PC3
Resting	**−0.79**	−0.26	**−0.54**
Filtering	+0.08	+0.02	**+0.99**
Browsing	**+0.98**	+0.09	+0.09
Thrashing	+0.08	**+0.91**	+0.09
Surface	**−0.95**	−0.26	−0.08
Wall	**+0.85**	+0.14	−0.02
Middle	+0.27	**+0.85**	−0.01
Bottom	**+0.89**	+0.13	+0.13
Interpretation	**Browsing, wall, bottom vs. resting, surface**	**Thrashing, middle vs. other behaviors**	**Filtering vs. resting**

Factor loadings listed in boldface type indicate those original variable that load strongly on each principal component (loadings > 0.4).

Table 5
MANOVA for behavior pattern in the preculling generation

			Pillai's		Standardized canonical coefficients		
Source	df	df error	trace	p	PC1	PC2	PC3
Population	9	357	0.246	.0005	+0.093	+1.104	−0.330
Water	3	117	0.511	.0001	+1.235	+0.264	+0.679
Population × water	9	357	0.085	.3239			
Multivariate contrast							
Pure vs. nybrid	3	117	0.153	.0002	+0.099	+1.016	−0.323
NN vs. OO	3	117	0.035	.2387			
NO vs. ON	3	117	0.050	.1066			

Standardized canonical coefficients for the first canonical variate are reported only for effects that were significant ($p < .05$) in MANOVA. The magnitudes of standardized canonical coefficients indicate the magnitude of the contribution of that variable to the significant MANOVA effect (see Scheiner, 1993, for details).

change in individual variables is most clearly seen when PC3 or PC1 scores are plotted individually versus by generation (Figure 3). For PC3, the variable most responsible for the significant culling-by-water interaction across the three generations of the experiment, there was an increase in allocation of time to resting at the expense of filtering in all groups (Figure 3A). For the control-culling regime, the difference in PC3 between water treatments remained similar across all three generations (Figure 3A). In contrast, for the predator-culling regime, the difference in PC3 between water treatments declined steadily across the three generations, so that by the second postculling generation, the values for PC3 in the two water treatments were virtually identical and low (Figure 3A). Culling by *T. rutilus,* then, appears to shift behavior of *A. triseriatus* to a pattern of reduced filtering even in water that contains no cues to the presence of the predator.

The behavioral response of second-generation larvae from lines culled by the predator remains evident in PC1, representing a shift from browsing below the surface to resting at the surface in predator + prey water (Figure 3B). In contrast, for PC1, second-generation larvae from control culled lines showed a reduced behavioral response to predator + prey water (Figure 3B). Effects of culling on PC1 (browsing below the surface vs. resting at the surface) are not as pronounced as those involving PC3 (filtering vs. resting; Table 7, Figure 3). However, when behavioral responses to predator + prey water for both PC1 and PC3 are considered together, second-generation predator-culled lines show uniformly low filtering (low PC1) and a strong reduction of browsing below the surface in predator + prey water (Figure 2). In contrast, second-generation control culled lines show a strong reduction of filtering, with little difference in browsing below the surface, in predator + prey water (Figure 2).

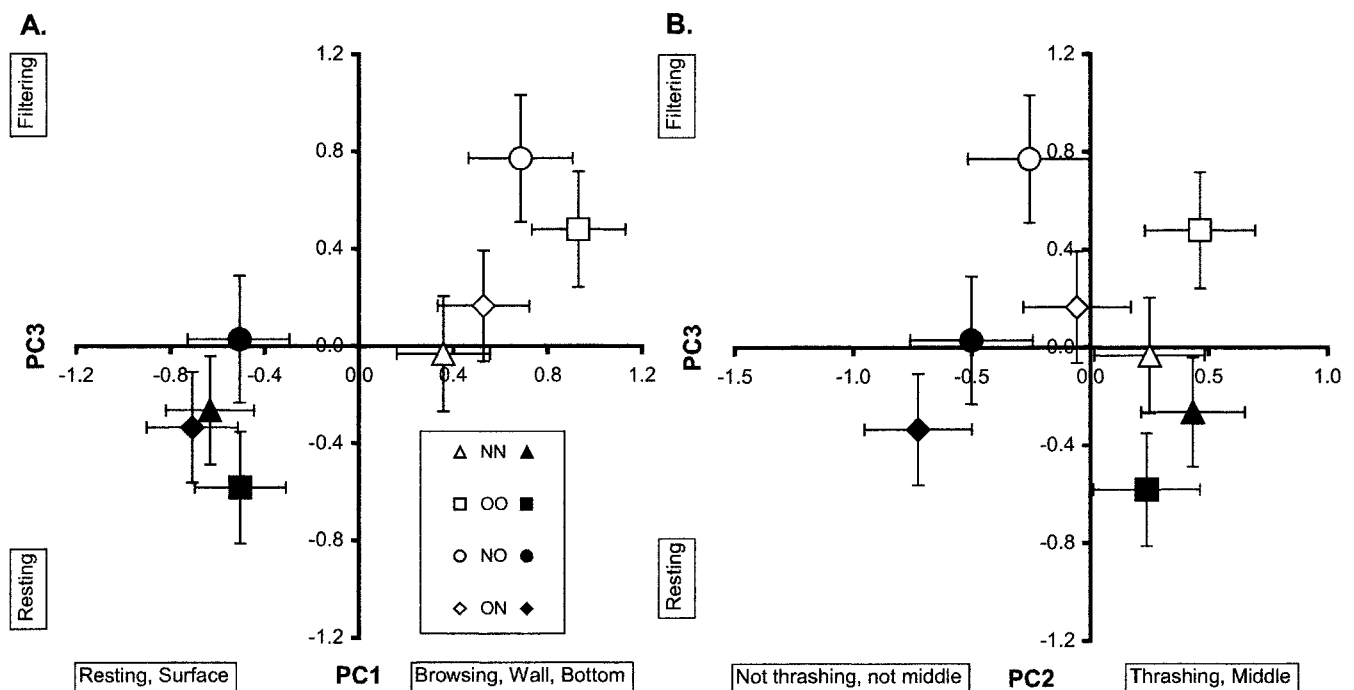A simplified analysis of only frequencies of resting and sur-

Figure 1
Bivariate means (± SE) for each population-water treatment combination in the preculling generation for (A) principal component (PC) 3 and PC1 and (B) PC2 and PC1. Open symbols = prey-only water treatment. Filled symbols = Predator + prey water treatment. Boxed labels on the axes indicate interpretations of PCs derived from factor loadings (Table 4). Significant MANOVA effects are described in Table 5.

Table 6
MANOVA for behavior pattern after one generation of laboratory culling

Source	df	df error [error term]	Pillai's trace	p	Standardized CC		
					PC1	PC2	PC3
Population	6	18 [Rep(population × culling)]	0.536	.4004			
Culling	3	8 [Rep(population × culling)]	0.049	.9357			
Population × culling	6	18 [Rep(population × culling)]	0.279	.8095			
Water	3	8 [Error]	0.881	.0005	+1.790	+0.231	+0.481
Population × water	6	18 [Error]	0.751	.1550			
Culling × water	3	8 [Error]	0.287	.4137			
Population × culling × water	6	18 [Error]	0.243	.8594			

Standardized canonical coefficients (CC) are reported for the first canonical variate only for effects that were significant ($p < .05$) in MANOVA. The magnitudes of standardized canonical coefficients indicate the magnitude of the contribution of that variable to the significant MANOVA effect (Scheiner, 1993).

face after two generations of culling yielded similar conclusions to analysis of PCs, with only the effects of water (Pillai's trace = 0.704, df = 2,7, p = .0141) and culling-by-water interaction (Pillai's trace = 0.604, df = 2,7, p = .0390) significant. Larvae in the predator-culled treatment had similar frequencies of resting in prey-only and predator + prey water (adjusted mean ± SE: prey only = 0.36 ± 0.05; predator + prey = 0.44 ± 0.05), but frequencies of time at the surface (adjusted mean ± SE: prey only = 0.46 ± 0.03; predator + prey = 0.68 ± 0.03) that increased significantly in predator + prey water. In contrast, larvae in the control-culled treatment had greater frequencies of resting in predator + prey water (adjusted mean ± SE: prey only = 0.36 ± 0.04; predator + prey = 0.53 ± 0.04), but frequencies of time at the surface (adjusted mean ± SE: prey only = 0.69 ± 0.03; predator + prey = 0.71 ± 0.03) that did not differ between prey only and predator + prey water.

Figure 2
Behavior principal components: effects of laboratory culling. Mean (± SE) for principal component (PC) 3 and PC1 for four culling–water combinations across three generations. Large circles designate the preculling generation, medium symbols designate the first generation, and small symbols designate the second generation. Arrows indicate the trend in behavior over generations (preculling, first, second generations). The distance between different symbols of similar size and fill (e.g., open circles and open triangles) illustrates the effect of culling regime. The distance between open symbols and filled symbols of similar size and shape illustrates the effect of water treatment (i.e., facultative response). The significant culling-water effect in the second generation results primarily from PC3.

DISCUSSION

Our results show that *A. triseriatus* adopts low-risk behaviors in response to chemical and physical traces of predation, and support the hypothesis that predation by *T. rutilus* can cause evolution of *A. triseriatus* behavior. The behavioral response of larvae to water that has held a feeding predator diverges in lines subjected to selection by predation versus control lines, with control-selected lines responding by reduced filtering and increased resting, but predator-selected lines showing constitutively low filtering. We reached these same conclusions regardless of whether we analyzed the PC scores derived from all behavioral variables or focused only on the safest and most common behaviors (resting, surface). Hence, our results are robust with respect to data manipulation, and we focus our discussion on the more complete analysis of PC scores. We now return to the questions posed concerning selection by predation in this system.

Behavioral change in response to the predator

Larvae of *A. triseriatus* dramatically increase the frequency of low-risk behaviors in response to water that had held a feeding *T. rutilus*. As predicted, movement in general and feeding (browsing, filtering) in particular were significantly less common in predator + prey water than in prey-only water (Figure 1A). In addition, as predicted, larvae spent more time at the surface and less time at the bottom in predator + prey water compared to prey-only water (Figure 1A). These results suggest that *A. triseriatus* can perceive cues from predation and modify behavior to reduce risk of predation. As previously reported (Juliano and Reminger, 1992), there appears to be little differentiation among populations in behavioral responses to *T. rutilus*. Both populations, and their reciprocal hybrid populations, showed similar responses (Figure 1A). The major behavioral difference among populations in the preculling generation was that the hybrid populations showed much lower values of PC2, indicating infrequent thrashing in the middle, relative to the pure populations (Figure 1B). Why hybrid populations should be less active than the associated parental populations is not obvious.

The observed changes in behavior of preculling generations of *A. triseriatus* when exposed to predator + prey water may be interpreted as adaptive predator avoidance behavior. However, an alternative explanation is that the change in behavior occurs in response to organic enrichment of the water, via predator feces and victim body parts, which increases microbial food for *A. triseriatus* and so modifies foraging behavior. This hypothesis suggests that the change in behavior in predator + prey water is not specifically related to predation.
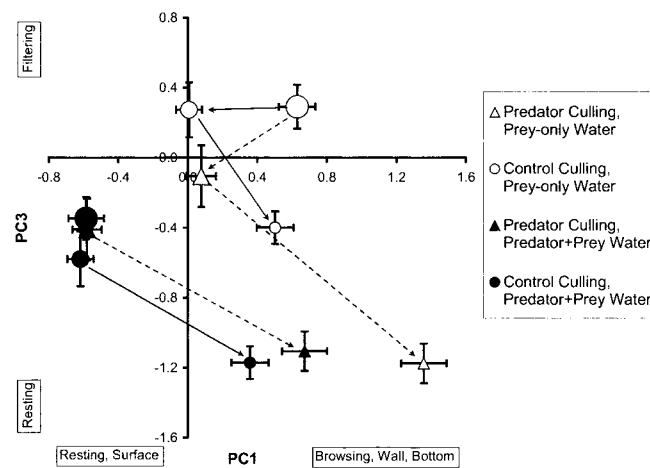
Table 7
MANOVA for behavior pattern after two generations of laboratory culling

Source	df	df error [error term]	Pillai's trace	p	Standardized CC		
					PC1	PC2	PC3
Population	6	14 [Rep(population × culling)]	0.373	.3905			
Culling	3	6 [Rep(population × culling)]	0.750	.0307	−0.826	−0.148	+1.545
Population × culling	6	14 [Rep(population × culling)]	0.419	.7344			
Water	3	6 [Error]	0.836	.0090	+2.261	+0.523	+1.658
Population × water	6	14 [Error]	0.605	.4560			
Culling × water	3	6 [Error]	0.700	.0521	+0.353	−0.498	−1.677
Population × culling × water	6	14 [Error]	0.467	.8210			

Standardized canonical coefficients (CC) are reported only for effects that were significant ($p \cong .05$) in MANOVA. The magnitudes of standardized canonical coefficients indicate the magnitude of the contribution of that variable to the significant MANOVA effect (Scheiner, 1993).

This alternative hypothesis is not supported by the apparent evolution of this facultative behavioral response under different regimes of predation, which suggests that the behavioral changes in response to predator + prey water are indeed adaptations specifically related to predation by *T. rutilus.*

Behavioral responses to cues from *T. rutilus* were similar for all populations despite apparent differences in the regularity with which individuals from Normal or Olive Branch would encounter *T. rutilus* (Juliano et al., 1993; Juliano, 1996; see Methods). Rare occurrence of this predator at the Normal site is difficult to study quantitatively, but it appears that in most containers for most of the active season (April–October), developing *A. triseriatus* from Normal do not encounter *T. rutilus.* Despite this, they show a clear and strong behavioral response to cues from this predator. Though *T. rutilus* is collected more frequently at the Olive Branch site, a substantial number of containers at this site also lack this predator. Thus, at both sites, *T. rutilus* predation varies in space and time, as it does at other sites in North America (e.g., Lounibos et al., 1997). Such variable occurrence of a predator is expected to favor facultative, rather than constitutive, behavioral responses to predation (Sih, 1987), as observed in the populations of *A. triseriatus* we have studied.

Past tests for behavioral responses of *A. triseriatus* to *T. rutilus* used different methods and obtained different results. Changes in behavior in response to the current presence of *T. rutilus* (Juliano and Reminger, 1992) were similar to, but more subtle than, those observed in the preculling generation in the present study. Juliano and Reminger (1992) placed a *T. rutilus* larva in the test water immediately before the trial, so that there were cues from the predator itself but no cues from the act of predation (e.g., victim body parts, predator feces). The likely source of quantitative differences in results is the set of cues available from the predator. The difference in results suggests that *A. triseriatus* larvae respond more strongly to chemical or physical traces left by the act of predation and less strongly to chemical or tactile cues to the current presence of the predator itself. Such responses seem ideal for detecting an ambush predator like *T. rutilus,* which may be difficult to detect if it is motionless in a dark tree hole environment. Chemical and tactile traces of predation, in contrast, are likely to be more reliably detectable and better indicators of danger of predation (Sih, 1986).

Aedes triseriatus shows no behavioral response to caged, feeding *T. rutilus* (Hechtel and Juliano, 1997). This lack of response to a caged predator, combined with our results showing responses to water that has held a feeding predator, suggests that solid residues from predation (e.g., prey body parts, predator feces), which would have been retained inside cages used by Hechtel and Juliano (1997), may be the primary cues

that induce behavioral changes in *A. triseriatus.* Similar specificity of predator-derived cues that elicit behavioral responses in prey is evident in diverse systems (e.g., Blumstein et al., 2000; Grostal and Dicke, 1999; Sih, 1986, 1987; Tikkanen et al., 1996; Wisenden et al. 1999).

Which traits evolve in response to predation?

After only two generations of culling, individuals in the predator-culled lines showed significantly reduced changes in behavior, as summarized by PC3, in response to cues from predation by *T. rutilus.* Predator-culled lines lost behavioral plasticity and showed increased baseline frequencies of the safest behaviors, regardless of cues from *T. rutilus* predation (Figures 2, 3A). In contrast, control-culled lines retained behavioral plasticity, as summarized by PC3, in response to cues from predation (Figures 2, 3A). Thus, our data clearly indicate that consistent selection by predation results in reduced facultative behavioral responses and a shift in constitutive behavior patterns. Despite the effect of culling by predation on the facultative switch from filtering to resting, both predator-culled and control-culled lines appear to retain some form of facultative response to predator-derived cues, but those responses are not the same. Individuals in the preculling generation shifted from browsing the walls and bottom to resting at the surface in the presence of predator-derived cues (Figure 1), and this behavioral change remains strong in the predator-culled lines after two generations (Figures 2, 3B). This shift from browsing walls and bottom to resting at the surface tended to be reduced, however, in the control-culled lines (Figures 2, 3B).

Perhaps the most interesting aspect of our results is that lines subjected to different culling regimes clearly diverged in their behavioral responses to this predator in only two generations of intense selection. This result implies that there may be rapid differentiation of predator avoidance behaviors if exposure to predators changes, as suggested for other predator–prey systems (e.g., Blumstein et al., 2000). Conditions in this laboratory experiment were, of course, different from those encountered in the field by *A. triseriatus,* hence we are unwilling to predict that field populations subjected to different levels of predation would evolve the same behavior patterns as those in our experiment. However, we do expect that behavior patterns, particularly facultative behavior patterns, would diverge in some way in populations subjected to consistently different predation regimes. We would have liked to continue this culling experiment for additional generations to see if divergence of behavior patterns continued, but losses of experimental lines rendered continuation of this kind of controlled experiment with these spe-
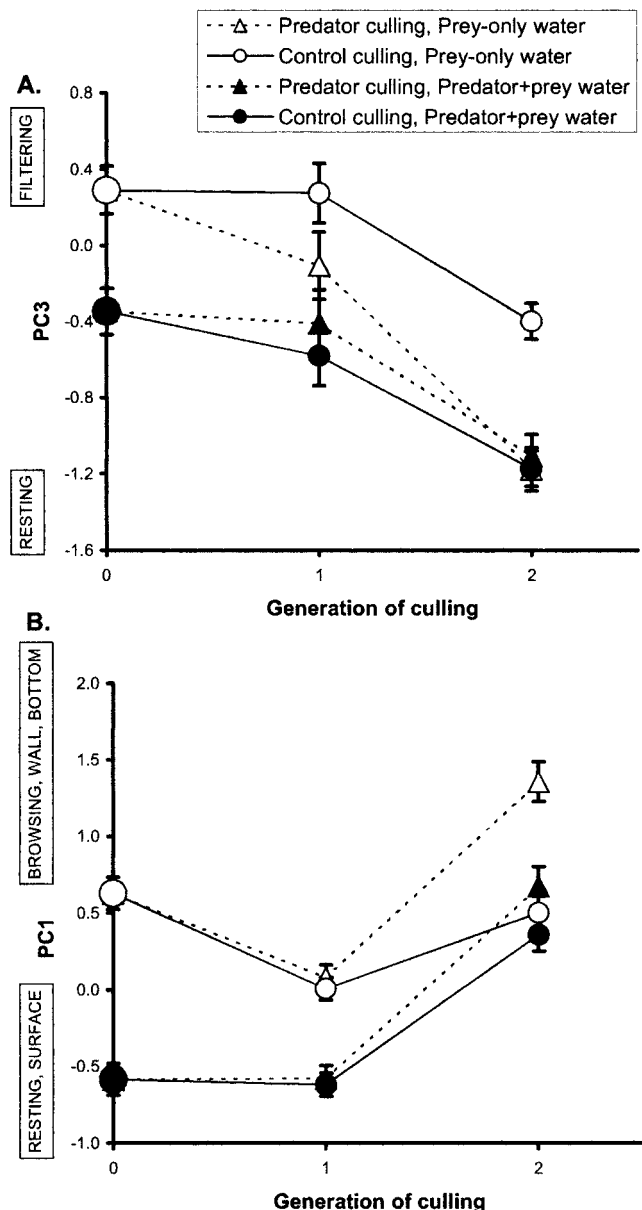
Figure 3
Means (± SE) for (A) principal component (PC) 3 and (B) PC1 across three generations. Large circles designate the preculling generation. The distance between different symbols with similar fill (e.g., open circles and open triangles) illustrates the effect of culling regime. The distance between open symbols and filled symbols illustrates the effect of water treatment (i.e., facultative response). The significant culling-water effect in the second generation results primarily from PC3 (see Table 7).

cies difficult, particularly as this experimental protocol requires dramatic reduction in population size every generation. However, even this relatively brief experiment showed that divergence of behavior did occur. Further, the brief duration of the experiment is realistic. For most of the range of these species, seasonal environments dictate that predation by *T. rutilus* is unlikely to continue at a high level for more than a few generations because of diapause in *A. triseriatus* (Sims, 1985) and winter reductions of populations of *T. rutilus* either due to freezing in the north or drought in the south (Bradshaw and Holzapfel, 1985). Spring generations of *A. triseriatus* may therefore escape predation almost

completely, and two generations of intense predation may be a reasonable representation of what occurs over much of the range of these species.

Although our results might suggest that there should be strong interpopulation differentiation in behavioral responses to this predator, the role of predation by *T. rutilus* in causing differentiation of *A. triseriatus* behavior in nature is not yet clear. At sites where *T. rutilus* occurs, container habitats form a temporal and spatial mosaic of low versus high probability of predation (see Methods; see also Bradshaw and Holzapfel, 1983, 1985; Lounibos et al., 1997), so that even where *T. rutilus* is common, the entire population of *A. triseriatus* may not be subject to consistent directional selection. Such variation, coupled with the rapid response of behavior to selection by predation, may in fact enhance intrapopulation variation in behavioral responses by causing small-scale intrapopulation differentiation. Subgroups within the population (e.g., within one tree hole) may experience directional selection on behavior, but because different subgroups are affected differently, and because adults are mobile, evolution of behavior within the entire population may show no directional trend.

Evolution of these behavioral differences in only a few generations of laboratory husbandry raises questions about studying behavioral responses to predation and other environmental cues of mosquitoes (and other organisms) maintained in laboratory colonies for many generations. Laboratory-reared lines may evolve modified facultative responses when removed from environments with appropriate agents of selection. Facultative responses are particularly problematic for laboratory study, as it seems likely that what selects for facultative responses in nature is uncertainty (e.g., of predator presence; Sih, 1987), and most laboratory-rearing programs do not incorporate uncertainty.

Differences in behavior patterns between hybrids versus parental populations were evident in the preculling individuals (Table 1) but were apparently lost in both the culled and control lines in the first generation after culling (Table 2). This apparent loss of interpopulation behavioral differences should be interpreted with caution, however, as the design of the postculling experiment, with replicate lines, results in reduced denominator degrees of freedom for MANOVA statistics (from 357 to 18, see Tables 2–4) for tests for population effects and thus reduced power for F tests to detect those population effects.

The lack of interaction of population and culling indicates that pure populations and the hybrids responded to culling regimes in similar ways. It thus appears that all populations harbored similar genetic variation for behavior (including facultative behavior) related to predation. This conclusion is consistent with the absence of differences in phenotypic variances for PC scores for pure versus hybrid populations in the preculling generation.

We have shown that populations that regularly encounter predation or the absence of predation may diverge in their behavioral responses to predation and that a constitutive pattern of increased low-risk behavior (increased resting and reduced filtering in this case), regardless of the presence of cues from the predator, may evolve in populations that regularly encounter a predator. Our experiment isolates the effect of predation as a selective agent acting on behavior, but behavior pattern is likely to affect feeding, growth, and possibly competitive ability (Grill and Juliano, 1996). Such competing selective forces make prediction of evolution of behavior in nature more difficult, even if there is consistent presence or absence of predation. Evolution of behavior in response to predation regime may only be obvious when other factors are controlled and selection by predation is ap-

plied uniformly and consistently, as was the case in our laboratory experiment.

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