

EVOLUTION OF PREY BEHAVIOR IN RESPONSE TO CHANGES IN PREDATION REGIME: DAMSELFLIES IN FISH AND DRAGONFLY LAKES

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Abstract.—In a large behavioral experiment we reconstructed the evolution of behavioral responses to predators to explore how interactions with predators have shaped the evolution of their prey's behavior. All *Enallagma* damselfly species reduced both movement and feeding in the presence of coexisting predators. Some *Enallagma* species inhabit water bodies with both fish and dragonflies, and these species responded to the presence of both predators, whereas other *Enallagma* species inhabit water bodies that have only large dragonflies as predators, and these species only responded to the presence of dragonflies. Lineages that shifted to live with large dragonflies showed no evolution in behaviors expressed in the presence of dragonflies, but they evolved greater movement in the absence of predators and greater movement and feeding in the presence of fish. These results suggest that *Enallagma* species have evolutionarily lost the ability to recognize fish as a predator. Because species coexisting with only dragonfly predators have also evolved the ability to escape attacking dragonfly predators by swimming, the decreased predation risk associated with foraging appears to have shifted the balance of the foraging/predation risk trade-off to allow increased activity in the absence of mortality threats to evolve in these lineages. Our results suggest that evolution in response to changes in predation regime may have greater consequences for characters expressed in the absence of mortality threats because of how the balance between the conflicting demands of growth and predation risk are altered.

Key words.—Antipredator behavior, damselfly, evolutionary loss, foraging, predator-prey interactions, trade-off.

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Prey have developed a bewildering array of defenses to avoid, deter, or escape predators (reviewed in Edmunds 1974; Endler 1986). However, many of these defenses come at costs to other fitness components such as growth and reproduction (Werner and Gilliam 1984; Sih 1987, Lima 1998). One aspect of the phenotype in which such a trade-off is readily apparent is in behavioral responses to predators. Theory predicts that prey should generally reduce their activity in environments where the risk of predation is greater, and should reduce activity even further when a predator threat is imminent (e.g., when a prey is within the detection field of a predator) (Endler 1986; Abrams 1991; Werner and Anholt 1993). Activity of the prey modulates this trade-off, because greater activity results in greater foraging returns to the prey but also results in more exposure to and a greater likelihood of detection by the predator.

Many animal taxa respond behaviorally to predators. Consistent with theoretical predictions, prey generally reduce their activity (e.g., spending more time in refuges [Sih 1992; Eklov and Persson 1996], moving less frequently or more slowly [Kohler and McPeck 1989; McPeck 1990a; Peckarsky 1996; Anholt et al. 2000; Relyea 2000, 2001; Richardson 2001a]) and consequently reduce their foraging efforts when presented with predation threats. This reduced foraging often leads to reduced growth and development (Skelly and Werner 1990; Skelly 1992; Peckarsky et al. 1993; Relyea and Werner 1999; Relyea 2000; Johansson et al. 2001) and may affect the dynamics of ecological communities (McPeck 1990b, 1998; Werner and Anholt 1996; Peacor and Werner 1997,

2000; Diehl et al. 2000; Relyea 2000; Schmitz and Suttle 2001).

We know that predators impose selection on the antipredator behaviors of their prey (Brodie 1992; Watkins 1996; Cousyn et al. 2001; Juliano and Gravel 2002; but see Van Buskirk et al. 1997), and that many of these behaviors possess heritable variation (Breden et al. 1987; Riechert and Hedrick 1990; Brodie 1992, 1993; Magurran et al. 1992; De Meester 1996; Storfer and Sih 1998; Cousyn et al. 2001, O'Steen et al. 2002; but see Watkins 2001). Thus, the potential for adaptive evolution is present in many populations and species. Our understanding of the role that predation plays in the evolution of prey phenotypes is incomplete, however, because it is based on studies scattered across disparate taxa and phenotypes. Only rarely has the evolution of antipredator traits been examined in a group of prey species for which predator-prey relations are well characterized and phylogenetic relationships are well established so that the direction of character change can be inferred. One notable exception is the work of Richardson (2001a,b), who studied 14 frog species from three families. Richardson found no consistent differences in activity among species that coexist with different predators. However, the change in activity in response to different predators appeared to be evolutionarily constrained. The evolution of activity level in the presence of various predators was positively correlated across species, suggesting that the behavioral response to one predator may not evolve independently of the behavioral response to another (Richardson 2001a). Using evolutionary contrasts analyses (Felsenstein 1985, 1988) at an intraspecific level, Downes and Adams (2001) also found that the presence or absence of snakes had driven the evolution of antipredator behavior in a gecko. Sih et al. (2000) concluded that the ineffective antipredator behavior of a salamander species that

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TABLE 1. Summary of source populations for larvae of the ten *Enallagma* species included in this study. *N* identifies the total sample size for each species; equal numbers of replicates were performed for the three treatments for each species. The habitat affinities of species are identified by F, fish lakes; and D, Dragonfly lakes. The last two columns identify which author observed replicates on a given species and the year in which those replicates were performed.

Species	<i>N</i>	Source population	Habitat	Observer	Year
<i>E. annexum</i>	24	Sylvester Pond, Norwich, VT	D	RS	2001
<i>E. aspersum</i>	24	KBS Pond Laboratory, Hickory Corners, MI	D	MM	1988
<i>E. boreale</i>	24	Ferson Road Marsh, Hanover Center, NH	D	JM	1994
<i>E. geminatum</i>	15	Palmatier Lake, Hastings, MI	F	MM	1988
<i>E. hageni</i>	24	Little Salem Pond, Derby, VT	F	RS	2001
<i>E. ebrium</i>	24	McDaniel's Marsh, Enfield, NH	F	RS	2001
<i>E. vernale</i>	24	McDaniel's Marsh, Enfield, NH	F	JM	1994
<i>E. divagans</i>	24	Lee's Pond, Moultonborough, NH	F	RS	2001
<i>E. signatum</i>	24	Little Salem Pond, Derby, VT	F	RS	2001
<i>E. vesperum</i>	24	Palmatier Lake, Hastings, MI	F	MM	1988

coexists with fish could partly be explained by its recent divergence from a sister species that does not live with fish.

In this paper, we present a phenotypic and evolutionary analysis of behavioral responses to predators for ten species of *Enallagma* damselflies (Hexapoda: Odonata). These constitute two ecological groups of species that inhabit the permanent ponds and lakes of eastern North America (Johnson and Crowley 1980; McPeck 1990b, 1998). One group of *Enallagma* species occurs as larvae only in ponds and lakes with fish, the top predator in this environment; whereas the remaining *Enallagma* species occur as larvae only in ponds and lakes without fish but with large dragonflies (e.g., *Anax*, *Aeshna*, and *Tramea* species) as the top predator (hereafter, we refer to these two ecological groups as fish-lake *Enallagma* and dragonfly-lake *Enallagma*, respectively). These two ecological groups of species segregate between the two lake types because they are differentially vulnerable to fish and dragonflies (McPeck 1990a,b). Previous phenotypic analyses of behavior for two species in each of these two groups show that dragonfly-lake *Enallagma* are active in the absence of predators, respond only to the presence of dragonflies by decreasing their activity, and swim away from attacking predators, which is a very successful evasive tactic against dragonflies but not against fish (Pierce et al. 1985; McPeck 1990a). In contrast, fish-lake *Enallagma* are less active than dragonfly-lake *Enallagma* in the absence of predators, respond to the presence of both fish and dragonflies by decreasing their activity (a set of smaller and less active dragonfly species also coexist with fish [Morin 1984a,b; McPeck 1990b, 1998]), and perform no evasive actions when attacked by a predator (Pierce et al. 1985; McPeck 1990a). Fish lakes are the ancestral habitat for *Enallagma*, and the dragonfly-lake species are the result of at least two fish-lake lineages recently invading and adapting to live with these large dragonfly predators that dominate fishless ponds and lakes (McPeck and Brown 2000; Brown et al. 2000). Previous functional and comparative analyses have shown that part of this adaptation involved phenotypic changes to increase swimming speed in order to increase the likelihood of evading an attacking dragonfly (McPeck 1995a,b, 1999, 2000; McPeck et al. 1996).

The objective of the present paper is to test whether the behavioral differences between the two *Enallagma* groups can be attributed to adaptive evolution associated with these hab-

itat shifts from a predation regime with fish and small, relatively inactive dragonflies to a predation regime without fish but with large, active dragonflies. To test these hypotheses, we reconstruct the evolution of these behavioral traits on the phylogeny of the *Enallagma* using evolutionary contrasts analysis (Felsenstein 1985, 1988; McPeck 1995b). Evolutionary contrasts provide an ideal methodology for testing such hypotheses because they encapsulate both direction and rate of character evolution in one metric (Felsenstein 1985, 1988). Behavioral characters that evolved in response to this change in selective environment should show: (1) consistent directions of change in both lineages that shifted into dragonfly lakes, and (2) substantially accelerated rates of evolution along the branches of the phylogeny on which the habitat shifts are hypothesized to have occurred relative to rates of character change on the rest of the phylogeny (McPeck 1995a,b). The evolution of greater activity and feeding in situations in which these species apparently perceive no imminent danger would suggest that behavioral evolution in this group has been shaped primarily by changes in the balance of the conflicting demands of the growth/predation risk trade-off.

MATERIALS AND METHODS

Phenotypic Differences in Behavior

Study species and maintenance of larvae

Throughout this study we closely followed the methods used by McPeck (1990a). We included larvae of ten *Enallagma* species in this study: three dragonfly lake *Enallagma* species (*E. annexum*, *E. aspersum*, and *E. boreale*) and seven fish lake *Enallagma* species (*E. geminatum*, *E. hageni*, *E. ebrium*, *E. vernale*, *E. divagans*, *E. signatum*, and *E. vesperum*). Data were collected over 11 years by the three authors. Table 1 gives the source populations, sample sizes, years when trials on each species were performed, and which author observed the trials. We acknowledge that ideally all species should have been tested in a randomized sequence by the same observer. However, because each of us observed at least one dragonfly-lake *Enallagma* species and one fish-lake *Enallagma* species in a randomized sequence (Table 1) and the striking similarity of behaviors among *Enallagma* species within the same ecological group irrespective of the

observer (see results: Fig. 2), it is highly unlikely that our results are biased by an observer/year effect. Moreover, we were unable to detect an observer effect when comparing observations on *E. boreale*/*E. cyathigerum* done by M. A. McPeck in 1988 with those done by J. L. Mitchell in 1994 (for each individual behavior, all $P > 0.20$ for differences between observers).

We have excluded data for *E. boreale* and *E. cyathigerum* collected by McPeck in 1988 that were included in the previous report (McPeck 1990a). These two species are nearly impossible to distinguish as larvae, and the ones used by McPeck in 1988 were a mixture of the two species (McPeck 1990a). We also now refer to *E. cyathigerum* as *E. annexum*, because recent genetic analysis shows that the North American and Eurasian taxa previously described both as *E. cyathigerum* are actually very distantly related in the phylogeny of the *Enallagma* (Stoks et al., unpubl. ms.). We therefore will use the specific epithet *annexum* to identify the North American species (Hagen 1861). We are confident that the larvae observed by Mitchell in 1994 were all *E. boreale*, because only this species was found as adults (which are easily identified to species) ovipositing the previous summer in the pond where the larvae were obtained. The same is true for *E. annexum* observed by Stoks in 2001.

For each species, a large stock of larvae were collected from the source lake and larvae were housed individually in 20 ml vials containing pond water and wooden dowels (3 mm diameter) for perches. Each larva was fed *Daphnia pulex* (Cladocera: Daphnidae) ad libitum every two days and maintained in an incubator at 20–21°C on a 14:10 L:D photoperiod until they were used in trials. For species observed by McPeck in 1988 and Mitchell in 1994, larvae were only included in trials after they had molted into the final instar in the laboratory. For species observed by Stoks in 2001, both larvae that molted in the laboratory into the final instar and larvae that were final instars when collected in the field were included. We found no difference in behavior within a species for larvae handled in these two ways.

Behavioral assays

We observed the behavior of solitary larvae of each species under three experimental treatments: in the presence of (1) no predators, (2) dragonfly larvae, or (3) fish. Each treatment was performed in one of three identical rectangular Plexiglas containers (34.5 cm long × 24.0 cm wide × 12.5 cm deep). Each container was divided into two compartments. A 10.0 cm × 10.5 cm inner compartment was enclosed against the middle of one wall with three Plexiglas sides. Damselfly larvae and their *Daphnia* prey were placed in this compartment during replicates (hereafter referred to as the damselfly compartment). Predators were placed in the larger outer compartment (predator compartment) surrounding the damselfly compartment on three sides. A 2 cm × 2 cm hole, covered with 0.5 mm Nitex screen, in one of the dividing walls allowed movement of water between these two compartments. This container design allowed the damselfly larvae to detect the presence of predators in the outer compartment by sight, olfaction, and mechanoreception, but prevented the predators from consuming the damselfly larvae or the *Daphnia* prey

during replicates. Fiberglass window screening covered the bottoms of both compartments to provide footing for odonates, but no other structure was included in either compartment. In laboratory feeding trials, predator feeding biases among species under these conditions are indistinguishable from those when natural macrophyte densities are added to containers for structure (McPeck 1990b). Also, larvae held in the laboratory under similar conditions display growth rates that are nearly identical to those in natural populations (McPeck et al. 2001).

All replicates were performed in three identical containers. Before performing replicates for a species, each container was randomly assigned to a predator treatment (i.e., all replicates of a predator treatment for a species were done in the same container). We intentionally confounded any potential container effects with predator treatment effects to minimize the potential carryover of any chemical residues left by the predators in a container. Also, water was replaced each day. For all behavioral assays, each container (irrespective of the predator treatment) was placed in the same position in the laboratory to minimize any potential laboratory positioning effects among containers (hence treatments). Because the containers were identical in construction and all were placed in the same position in the laboratory when used, confounding effects that would systematically bias larval behavior should be negligible.

The three treatments were constructed by placing either (1) no predators, (2) three final-instar *Anax junius* larvae (Odonata: Aeshnidae), or (3) one *Lepomis* sunfish in the predator compartment. Bluegill sunfish (*L. macrochirus*) were used in the 1988 trials and pumpkinseed sunfish (*L. gibbosus*) were used in the 1994 and 2001 trials. *Anax* dragonflies are a common predator of damselflies in fishless ponds and lakes (McPeck 1990b, 1998; Werner and McPeck 1994). Both bluegill and pumpkinseed sunfishes are major littoral predators in lakes with fish (Brown and Ball 1942; Cooper et al. 1971; Osenberg et al. 1988; Werner and McPeck 1994), and they impose similar levels of mortality and show similar feeding biases among these damselflies (McPeck and Stoks, unpubl. data). Predators were fed natural prey every two days after the last replicate on that day was finished. The same set of 1–2 fish and 3–5 *Anax* were used as predators in all relevant replicates of all *Enallagma* species tested by an observer. That way we minimized the possibility that differences in antipredator behavior among *Enallagma* species were due to differences in predator behavior. Again, the striking similarity in behavior among *Enallagma* species of the same ecological group tested by different observers (and different sets of fish and dragonfly predators; see results, Fig. 2) strongly argues against any biases caused by differences among individual predators.

All replicates consisted of continuous 30-min behavioral observations of the damselfly larvae. Replicates were done between 1000 and 1730 h with water at room temperature (20–23°C), which is well within the range of temperatures that larvae experience in nature over much of their larval period (M. A. McPeck, pers. obs.). Containers were filled to a depth of 8.5 cm two hours before the start of the first assay on that day. Then one final-instar larva *Anax* or one *Lepomis* sunfish was placed in each of the damselfly and predator

compartment of the respective predator treatment containers. The predators were left in the damselfly compartments until the replicates for that day began, and were then removed. The predator treatments were then established in the respective containers. Each replicate began with 25 *Daphnia pulex* in the damselfly compartment. Generally, five to seven *Daphnia* were killed during a replicate, and they were not replaced as eaten because of the disturbance involved. The 30-min observation period began when a single damselfly larva was introduced and reached the bottom of the damselfly compartment. Larvae usually became responsive to prey within a few seconds of reaching the bottom of the container. Larvae were randomly assigned to treatments.

A laptop computer was used to record the behaviors of damselfly larvae. The complete set of behavioral variables recorded in this study are described in McPeck (1990a). Walking was defined as a change in position on the substrate when a larva moved its legs. Swimming was defined as a larva leaving the bottom substrate and moving through the chamber by swinging its abdomen and caudal lamellae. In the abdominal bend display, a larva bends its abdomen through an arc from side to side while standing on the bottom (Baker 1981). Orienting toward prey was defined as a larva turning its head or body toward a *Daphnia* without changing its position on the substrate. Advancing toward prey was defined as a walk to chase a *Daphnia*. We separately scored unsuccessful strikes and successful strikes (called captures) at prey. It is important to note that there is no necessary sequence to any of the behaviors we recorded, example, larvae routinely struck at prey without first orienting towards them. In other words, the occurrence of one behavior was not conditional upon another behavior. In this paper we analyzed only a subset of these variables. Our choice of variables to include in this analysis was based on their importance to discriminating species and responses to predators in the previous analysis (McPeck 1990a).

Statistical analyses

In a previous analysis of a subset of these data (McPeck 1990a), principal components were first extracted from the original data to reduce the dimensionality of the data set to a few summary variables. The resulting principal component scores were then analyzed to determine species differences and treatment responses. When this same analysis was applied to the present dataset containing ten species, the resulting principal components had no simple interpretations (even after rotations), as in the first study. We, therefore, have taken a different approach to the analysis.

We first reduced the number of variables in the analysis by including only those variables that loaded heavily on principal components in the first study and that were important to the interpretation of the results (McPeck 1990a). We retained eight variables that naturally group into three sets. The first variable set contains three variables describing movement and activity (Movement Set): *WALKNUM*—the number of walks performed during a trial; *WALKMEAN*—the mean duration of individual walks (in seconds); and *INACTIVE*—the duration of the longest motionless period (in minutes). The second set contains four variables describing feeding:

ORIENT—the number of orientations of the head or body towards prey; *ADVNUM*—the number of walking advances towards prey; *STRIKEMISS*—the number of unsuccessful strikes at prey; and *CAPTURE*—the number of prey captures. The third variable set contains one variable (*ABDNUM*) quantifying the number of abdominal bends, a conspicuous behavioral display for which the function is currently questionable (Baker 1981; Richardson and Anholt 1996). In the previous analysis, each set focused most heavily on a different principal component, indicating that these sets describe largely independent features of behavior in these species. We then applied the same analyses to each set of variables. This approach to the data analysis resulted in easily interpretable results that are consistent with the previous analysis.

We applied multivariate analyses of variance to the movement and feeding sets and analogous univariate methods to the number of abdominal bends to test a priori hypotheses about the differences among the two ecological groups (i.e., dragonfly-lake *Enallagma*, fish-lake *Enallagma*), and responses to the presence of predators within each group. One general model cannot be constructed to simultaneously test hypotheses about group differences and predator treatment differences. Consequently, we have taken a two-step approach to the analysis. The first step was to test for group differences without regard to predator treatments in a multivariate nested analysis of variance (MN-ANOVA), with group and species nested within group as independent variables in the model. Species nested within group was used as the error variance for group. To test for group differences between dragonfly-lake versus fish-lake *Enallagma*, we constructed a linear orthogonal contrast with the species nested within group term as the error variance for each (Sokal and Rohlf 1995). We examined the characteristic equations from the multivariate analyses of variance and performed univariate analyses employing the same model to examine which variables were responsible for significant results (Morrison 1990).

In the second step of the analysis, we examined responses to the presence of predators for each species group separately, because we had a priori expectations that each would group respond differently (McPeck 1990a). As above, the same model was applied to each set of variables. We constructed a multivariate analysis of variance model (MANOVA) with species, predator treatment, and the species \times predator interaction term as independent factors. We consider species a random effect and predator a fixed effect, and so the species \times predator interaction was used as the error variance estimate in all significance tests of the predator treatment main effect and contrasts among predator treatments (Sokal and Rohlf 1995). Because we had a priori expectations that the two species groups would differ in their responses to the predator treatments, the set of linear, orthogonal contrasts used to test for differences among predator treatments differed between the groups. In the analysis reported by McPeck (1990a), dragonfly-lake *Enallagma* responded significantly only to the presence of dragonfly larvae, so we tested the contrast set for (1) dragonflies present versus (predators absent + fish present), and (2) predators absent versus fish present. In contrast, fish lake *Enallagma* responded to the presence of both fish and dragonflies, and therefore we tested the contrast set

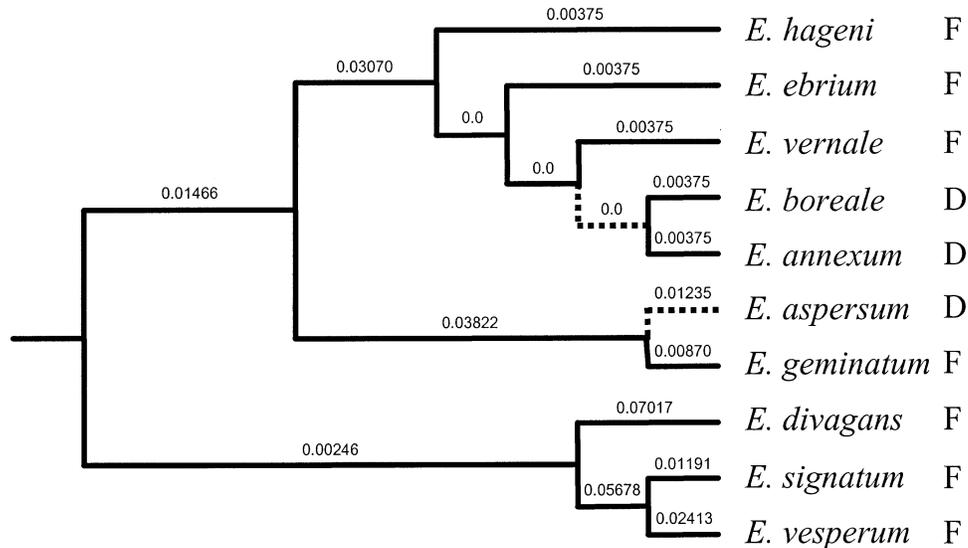


FIG. 1. The phylogenetic hypothesis for the 11 species included in this study. This phylogenetic hypothesis is derived from the analysis of Brown et al. (2000) based on 815 bp of mtDNA sequence and 51 morphological characters. The two dashed branches identify branches along which habitat shifts of lineages from fish lakes to dragonfly lakes are hypothesized to have occurred. The habitat affinities of species are identified by F, fish lakes; D, dragonfly lakes. Numbers on the branches are branch lengths derived using maximum likelihood methods assuming the HKY model of nucleotide substitutions (see Brown et al. 2000).

for (1) predator absent versus (dragonflies present + fish present), and (2) dragonflies present versus fish present. Again, we examined the characteristic equations from the multivariate analyses of variance and performed univariate analyses employing the same model to examine which variables were responsible for significant results (Morrison 1990).

Evolution of Antipredator Behavior

We employed evolutionary contrasts analysis (Felsenstein 1985, 1988; Martins and Garland 1991; McPeck 1995b) to test whether the present behavioral differences between the dragonfly-lake and fish-lake *Enallagma* were the result of adaptive evolution associated with recent habitat shifts of formerly fish-lake lineages into the dragonfly lake habitat (for similar analyses of other characters, see also McPeck 1995a,b, 1997, 1999, 2000; McPeck et al. 1996; McPeck and Brown 2000; Brown et al. 2000). Evolutionary contrasts orthogonally partition the evolutionary change in a character to estimate the direction and rate of character change across a phylogeny (Felsenstein 1985, 1988; McPeck 1995b). Although most analyses construct all evolutionary contrasts using pairs of branches, alternative orthogonal partitions that isolate character change on single branches can be made (McPeck 1995b). We employed this alternative partitioning technique to estimate character change along the two branches of the *Enallagma* phylogeny on which the shift in habitat from fish lakes to dragonfly lakes are hypothesized to have occurred. By comparing evolutionary contrasts values for these two single branches to the rest of the evolutionary contrasts, we are testing whether the direction of character change associated with these two habitat shifts is consistent and whether the rate of evolution is significantly accelerated over the rate of character change that occurs within habitats.

To perform these analyses we used the phylogenetic hy-

pothesis derived by Brown et al. (2000) for the North American *Enallagma*. Branch lengths were derived using maximum-likelihood methods assuming the HKY model (Hasegawa et al. 1985) of nucleotide substitutions on the nucleotide sequence data for 815 bp of sequence from the mitochondrial cytochrome oxidase I and II genes and the intervening tRNA (Brown et al. 2000). Species were then pruned from the tree until only those included in this study remained (Fig. 1). The systematics study could not resolve the clade containing *E. boreale*, *E. annexum*, *E. ebrium*, *E. hageni*, and *E. vernale*. This clade explosively radiated very recently (Brown et al. 2000; McPeck and Brown 2000; Turgeon and McPeck 2002), and so we consider this a "hard" polytomy (Purvis and Garland 1993). We resolved this clade for analysis by first placing *E. boreale* and *E. annexum* together so that only one habitat shift is assumed in this clade. We then arbitrarily resolved the remaining splits among species included in this study, and assigned branch lengths of 0.0 to all these arbitrarily resolved branches (Purvis and Garland 1993). We tried many resolutions within this clade and all gave qualitatively identical results.

For each species we calculated the mean of each behavioral variable in each predator treatment (e.g., each species has three values for WALKNUM, one for each predator treatment). We take these means to represent the behavior of the species in the presence of each predator. We then applied a modification of Felsenstein's (1985, 1988) method of evolutionary contrasts to isolate evolutionary changes on the single branches of the phylogeny on which habitat shifts from fish to dragonfly lakes are hypothesized to have occurred (McPeck 1995b): the external branch leading to *E. aspersum* and the branch leading to the immediate ancestor of *E. boreale* and *E. annexum* (Fig. 1).

We applied this methodology to calculate a set of standardized evolutionary contrasts for each behavior in each

predator treatment. As stated in the introduction, the test of character change consistent with adaptive evolution involves both directional and rate components. For the two single branches along which habitat shifts are hypothesized to have occurred, we subtracted the phenotypic value for the fish-lake ancestral taxon from the derived dragonfly-lake taxon, so that an increase in the character as a result of the habitat shift would result in a positive contrast value. We then compared these two single branch contrast values to the remaining evolutionary contrasts that quantify the rate of character change within the two habitats. The direction of subtraction was arbitrary for contrasts describing character change within habitats. This comparison simultaneously tests whether the direction of change in the two branches with habitat shift is consistent (i.e., if their direction of change is not consistent, their average will be near zero) and whether the rate of evolutionary change in a character is significantly higher when habitat shifts are hypothesized to have occurred as compared to the rate of change within the habitats (i.e., are the two habitat shift values outliers when compared to character change within habitats). We performed multivariate ANOVAs (equivalent to Hotelling's multivariate T^2 test [Morrison 1990]) on the movement and feeding variable set within each predator treatment, and a univariate t -test (Sokal and Rohlf 1995) on the number of abdominal bends for each predator treatment.

All statistical analyses were performed in SAS version 8.0 (SAS Institute 1990).

RESULTS

Phenotypic Differences in Behavior

Overall group differences in behavior

Behaviors characterizing movement differed greatly among the two *Enallagma* groups (Fig. 2a–c: MN-ANOVA for overall group effect $F_{3,6} = 8.80$, $P < 0.02$). The two *Enallagma* groups did not differ overall in the frequency of movements (WALKNUM $F_{1,8} = 4.33$, $P > 0.071$), but the fish-lake *Enallagma* had a much longer mean duration of walks than the dragonfly-lake *Enallagma* (WALKMEAN $F_{1,8} = 8.16$, $P < 0.03$). Our observations indicate that this difference in mean duration of walks reflects the fact that fish-lake *Enallagma* move much more slowly than dragonfly-lake *Enallagma*. Although we did not measure distances, damselflies in this study typically moved about 1–3 cm with each walk, and mean duration of walks reflects the average time each species takes to move this distance. The two groups did not differ in the longest inactive period during trials (INACTIVE $F_{1,8} = 1.46$, $P > 0.26$).

The groups also differed overall in their performance of the abdominal bend behavior (Fig. 2d: $F_{1,8} = 15.53$, $P < 0.01$). Dragonfly-lake *Enallagma* routinely performed this behavior, sometimes for extended periods of time, whereas fish-lake *Enallagma* species performed the behavior much less frequently on average, and three fish-lake *Enallagma* never performed this behavior (Fig. 2d).

The results of the MN-ANOVA indicated that the two groups did not differ overall in their feeding behavior (Fig. 2e–h: $F_{4,3} = 1.67$, $P > 0.29$).

Behavioral responses toward predators

The two ecological groups of damselflies showed fundamentally different patterns of behavioral response to the presence of dragonfly and fish predators.

Overall MANOVAs showed that dragonfly-lake *Enallagma* responded behaviorally only to the presence of dragonflies (Fig. 2). Dragonfly-lake *Enallagma* walked less frequently and were inactive for a much longer period of time in the presence of dragonflies than when no predators or fish were present (contrast comparing dragonfly treatment to the no predator and fish present treatments: overall MANOVA $F_{3,2} = 49.80$, $P < 0.02$; WALKNUM $F_{1,4} = 9.82$, $P < 0.04$; INACTIVE $F_{1,4} = 26.43$, $P < 0.01$), but their movement did not differ between the no predator and fish treatments (contrasts for overall MANOVA and the three individual movement variables with $P > 0.30$). Their performance of the abdominal bend behavior did not differ among the predator treatments ($F_{2,4} = 0.35$, $P > 0.72$). Dragonfly-lake *Enallagma* also oriented toward prey less, and struck at and captured fewer prey in the presence of dragonflies as compared to the other two predator treatments (contrasts for all feeding variables with $P < 0.05$, but the overall MANOVA test for this contrast was not significant, $F_{4,1} = 10.09$, $P > 0.23$, because of the small number of denominator degrees of freedom), but their feeding behavior in the no predator and fish treatments did not differ (contrasts for all feeding variables with $P > 0.25$).

Fish-lake *Enallagma* responded to the presence of both dragonflies and fish by decreasing their movement and feeding (Fig. 2: MANOVAs for overall predator treatment differences in movement $F_{6,20} = 4.10$, $P < 0.01$; feeding $F_{8,18} = 9.80$, $P < 0.001$). Fish-lake *Enallagma* walked less frequently and were inactive for longer in the presence of the predators as compared to the no predator treatment (contrast comparing no predator treatment to the two predator treatments: WALKNUM $F_{1,12} = 23.93$, $P < 0.001$; INACTIVE $F_{1,12} = 22.89$, $P < 0.001$), but they did not alter the duration of their movements (all these contrasts with $P > 0.15$). Fish-lake *Enallagma* did not alter their performance of abdominal bends across the predator treatments ($F_{2,12} = 0.35$, $P > 0.70$). Fish-lake *Enallagma* also greatly reduced their feeding and responsiveness to prey in the presence of both dragonflies and fish (contrasts comparing the no predator treatment to the two predator treatments all with $P < 0.001$). None of these variables differed between the dragonfly and fish treatments for the fish-lake *Enallagma* (all contrasts $P > 0.05$).

Evolution of Antipredator Behavior

Enallagma lineages that recently invaded the dragonfly-lake habitat from fish lakes evolved large changes in all aspects of their behavior that were quantified in this study (Fig. 3). The evolutionary contrasts analyses indicated that behavioral aspects of movement in both the absence of predators and the presence of fish evolved in association with the habitat shifts (MANOVA for No Predators $F_{3,6} = 4.98$, $P < 0.05$; Fish $F_{3,6} = 5.29$, $P < 0.05$), but movement in the presence of dragonflies did not change significantly ($F_{3,6} = 4.08$, $P > 0.05$). These responses were primarily caused by the evolution of an increased number of walks and decreased

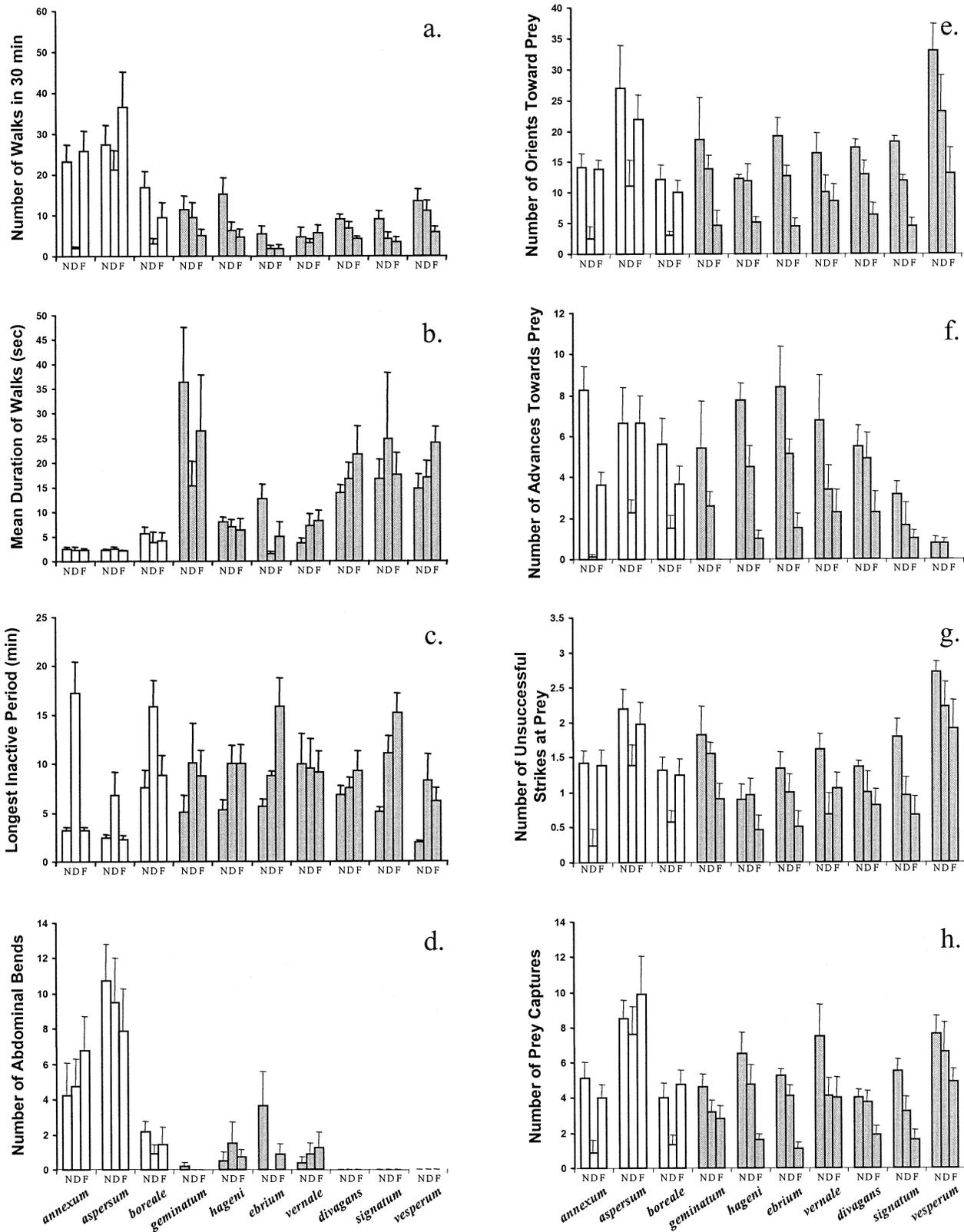


FIG. 2. The eight behavioral variables for the 10 species included in this study. *Enallagma* species found as larvae only in dragonfly lakes are identified by white bars. *Enallagma* species found as larvae only in fish lakes are identified by gray bars. Means + 1 SE are shown for each species in the No Predator (N), Dragonflies Present (D) and Fish Present (F) treatments. Dragonfly-lake species are more active in the absence of predators than the fish-lake species, and respond only to the presence of dragonflies. Fish-lake species respond to the presence of both dragonflies and fish.

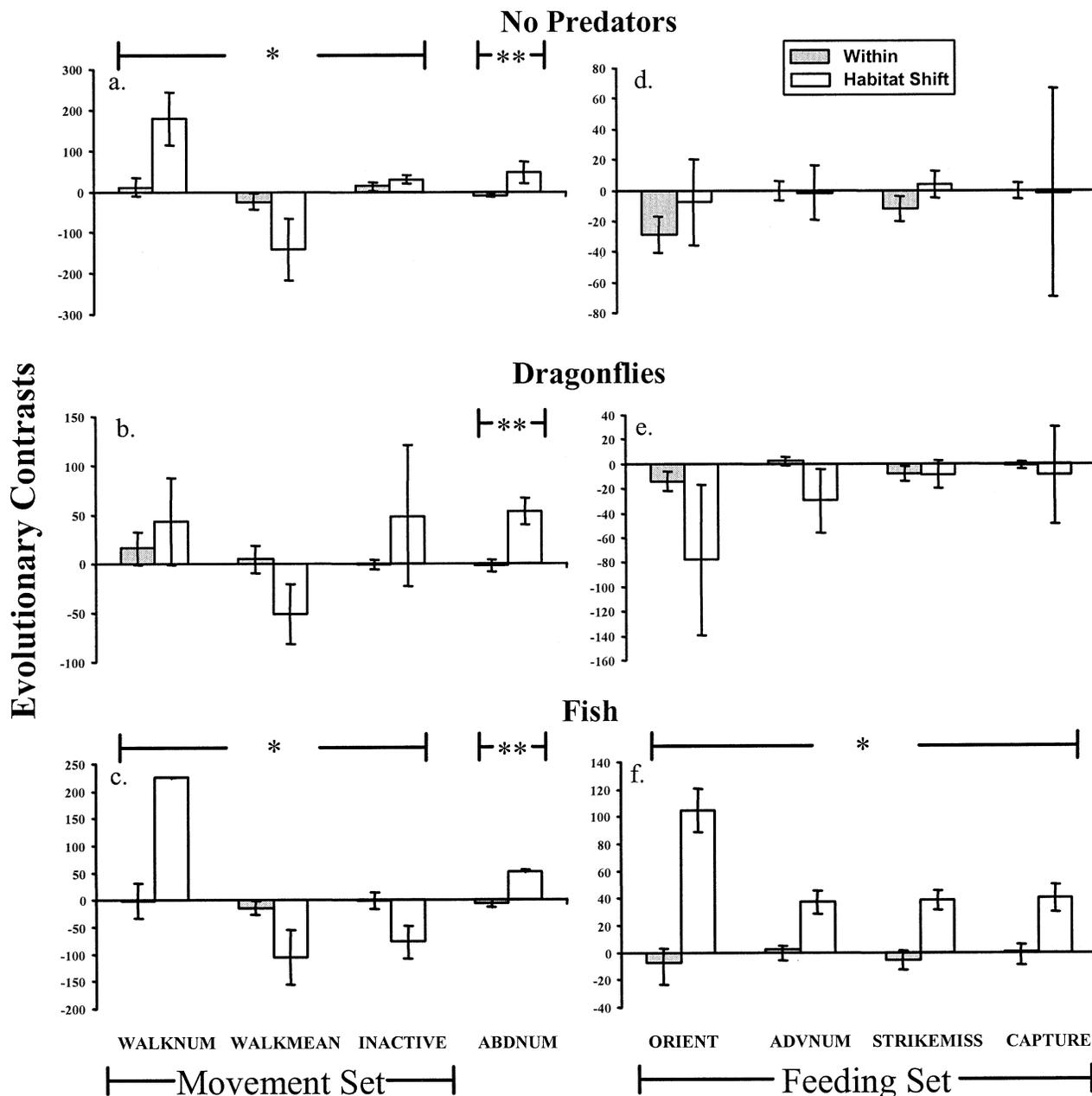


FIG. 3. Evolutionary contrasts for the eight behavioral variables measured in the three predator treatments. Each bar shows the mean (± 1 SE) of the evolutionary contrasts for either evolutionary change within habitats (gray bars) or evolutionary change associated with habitat shifts (white bars) in one of the predator treatments. Panels (a) and (d) show results for the No Predator treatment, panels (b) and (e) for the Dragonflies Present treatment, and panels (c) and (f) for the Fish Present treatment. WALKNUM and WALKMKN are the number and duration of walks, respectively. INACTIVE is the longest inactive period. ABDNUM is the number of abdominal bends. ORIENT and ADVNUM are the number of orientations and advances toward prey, respectively. STIKEMISS and CAPTURE are the number of unsuccessful and successful strikes at prey, respectively. The statistical significance of MANOVA tests for the variable sets are identified in each panel (* $P < 0.05$; ** $P < 0.01$). Evolutionary contrasts associated with habitat shifts are significantly larger than contrasts for change within habitats for the movement set in the absence of predators, the movement set in the presence of fish, and the feeding set in the presence of fish. These results indicate that dragonfly-like lineages evolved to be more active when no predators or fish are nearby.

duration of walks in the absence of predators and in the presence of fish, and less inactivity in the presence of fish (Fig. 3a,c).

Significant evolutionary increases were also apparent for abdominal bends in all three predator treatments (Fig. 3a-c: all t -tests with $P < 0.01$).

For the feeding variable set, significant evolution associated with habitat shifts was only apparent in the presence of fish (MANOVA in the fish treatment $F_{4,5} = 6.59$, $P < 0.04$), with all four variables showing significant evolutionary increases in the presence of fish (Fig. 3f).

Very few correlations among the evolutionary contrasts

for the behavioral variables in different treatments were significant when considered separately and none were significant when significance levels were corrected for multiple tests (e.g., Bonferroni or Dunn-Šidák corrections). Thus, behavioral responses to the different predators and different behavioral components appeared to have evolved largely independently in this clade.

DISCUSSION

Among natural ponds and lakes in eastern North America, *Enallagma* species segregate as larvae among ponds dominated by fish predators and ponds dominated by large dragonfly predators (Johnson and Crowley 1980; McPeck 1990b, 1998). These differences in habitat distribution are caused primarily by differences in species' abilities to avoid fish and dragonfly predators (McPeck 1998). The results of the present analysis confirm the differences among fish-lake and dragonfly-lake *Enallagma* groups seen in previous analyses, which included many fewer species (cf. Pierce et al. 1985; McPeck 1990a). All ten *Enallagma* species responded to the presence of predator types with which they coexist by decreasing their frequency of walking (Fig. 2a), and consequently increasing the length of their longest continuous inactive period (Fig. 2c). These results are consistent with previous studies of species in this genus (Pierce et al. 1985; Jeffries 1990; McPeck 1990a; Steiner et al. 2000). All ten *Enallagma* species also responded to predators by reducing their feeding and responsiveness to prey (Fig. 2e–h; see also Pierce et al. 1985; McPeck 1990a; Steiner et al. 2000). These responses to the presence of predators may be interpreted as adaptive predator avoidance behavior. Predators are much more likely to strike at damselfly larvae when they are moving than when they remain motionless (Baker et al. 1999; Elkin and Baker 2000); movement attracts the attention of predators and thus increases the rate at which prey are detected by predators (e.g., Jakobsson et al. 1995). Also, damselfly larvae with higher foraging rates have higher mortality rates from fish predation (Stoks and Johansson 2000).

Based on a phylogenetic hypothesis derived from mtDNA sequences and morphological data, the fish-lake environment appears to have been the ancestral lake type for the *Enallagma*, and the extant dragonfly-lake species arose from at least two very recent shifts of lineages into the dragonfly-lake habitat (Brown et al. 2000; McPeck and Brown 2000). Functional and comparative phenotypic studies have shown that morphological and biochemical evolution to increase swimming speed and thus increase the likelihood of evading an attacking dragonfly was a major component of the adaptation associated with these habitat shifts (McPeck 1995a,b, 1997, 1999, 2000; McPeck et al. 1996). The present analysis identifies evolutionary changes in behavior that were associated with these habitat shifts. However, unlike the behavioral shift to using swimming as an evasive tactic against attacking predators and the associated morphological and biochemical changes to increase swimming speed, the evolutionary changes identified in this study would not directly affect larval survival under dragonfly predation. When the threat of predation is imminent but before an attack has begun, prey should decrease their activity as much as possible

to minimize the likelihood of being detected by the predator. If these lineages had evolved to decrease their susceptibility to the large dragonfly predators found in fishless lakes, we would expect the evolutionary contrasts analyses to show the evolution of decreases in movement and/or feeding in the dragonfly treatment of the behavior experiment. However, no behavioral variable in the dragonfly treatment showed significant change associated with habitat shifts (Fig. 3b,e). Because dragonflies are potential mortality threats in both fish and dragonfly lakes, it is not surprising that species from both lake types would decrease their movement and responsiveness to prey to similar degrees in the presence of threatening dragonflies.

The dragonfly-lake *Enallagma* lineages appear to have lost the ability to identify fish as potential predators. The movement and responsiveness to prey by dragonfly-lake *Enallagma* were not different between the no predator and fish treatments (Fig. 2), and all variables showed significant evolutionary changes associated with habitat shifts in the fish treatment (Fig. 3c,f). Abdominal bends also increased in these lineages, but the functional significance of abdominal bending remains unclear, although it may be related to more efficient processing of ingested food (Richardson and Anholt 1995; Richardson and Baker 1996). Odonate larvae appear to use specific cues that allow them to discriminate among different types of predators (e.g., fish vs. dragonflies: Hooper 2001), but dragonfly-lake *Enallagma* did not respond to the available cues emitted by fish in this experiment.

Loss of response to cues from fish could be either the evolutionary loss of an innate response or the lack of sufficient conditioning to fish as predators in the fishless, dragonfly-lake environment. *Enallagma boreale* larvae from western North American populations have been shown to decrease movement and feeding in response to chemical cues from fish predators (northern pike, *Esox lucius*) that have been fed damselflies, and to learn to associate cues from pike with these chemical cues from injured damselflies (Chivers et al. 1996; Wisenden et al. 1997). The dragonfly-lake larvae we used in these experiments were completely naive of fish when included in experiments, and thus they would not have developed any of these learned responses to fish. However, our field experiences in eastern North America also suggest that although damselflies may be able to learn that fish are potential predators, these learning responses are not adequate to prevent their extirpation from water bodies when fish are introduced. For example, we have witnessed the rapid extirpation of all three dragonfly-lake *Enallagma* species included in this study (including *E. boreale*) from numerous water bodies after the introduction of *Lepomis* species (M. A. McPeck, pers. obs.; D. M. Johnson, pers. comm.), and fish introductions in general are known to decimate the diversity of many vertebrate and invertebrate taxa that are adapted to living in fishless waters (Knapp et al. 2001). These species may be capable of learning to alter their behavior to some degree in response to the threats that novel predators pose, but whatever learning does occur is not adequate to permit them to coexist with the fish found in eastern North America. Similar erosion of antipredator recognition in populations or species after the relaxation of a selective force associated with the change in the predation regime are documented in

several animals (Berger 1999; Berger et al. 2001; O'Steen et al. 2002; reviewed in Coss 1999, including also counterexamples where prey retained predator recognition abilities for thousands of years after predation as a selective force has been relaxed). This evolutionary loss now may prevent these naive prey species from coexisting with the predators that were syntopic with the prey's ancestors.

Not only did *Enallagma* lineages that shifted into dragonfly-lakes lose the ability to recognize fish as potential predators, they also evolved to move more frequently and more quickly in the absence of predators (Fig. 2a,b). We hypothesize that this change in movement in the absence of predators was coupled with the evolutionary shift from remaining cryptic to using swimming to avoid attacking predators. The response of most fish-lake *Enallagma* larvae to an attacking predator is to remain motionless until actually captured, whereas dragonfly-lake *Enallagma* swim away from attacking predators (Pierce et al. 1985; McPeck 1990a). Damselfly larvae are not capable of evading a fish by swimming (Stoks and De Block 2000), and thus remaining motionless until captured is a successful strategy for fish-lake *Enallagma*. This strategy places a premium on reducing detection by fish, and therefore slow and infrequent movements would seem to be the behavioral repertoire favored by phenotypic selection imposed by fish predation. In contrast, because swimming away from an attacking dragonfly is a successful evasive tactic, being detected by dragonfly predators is less important to ultimately evading predation (McPeck 1990b; Stoks and De Block 2000). Thus, selection imposed by dragonfly predation on activity when the prey perceive no dragonfly in their immediate vicinity would be relaxed, and selection imposed by other activities, namely foraging, may favor increased activity in the absence of immediate dragonfly threats (Werner and Anholt 1993). In other words, the evolution of swimming as an evasive tactic against dragonfly predators may have tipped the balance in the growth/predation risk trade-off to allow greater activity in the absence of immediate predator threats to evolve. This interpretation is consistent with the evolved behavioral changes we see, but further experiments are obviously needed to test this conjecture.

Several ecological studies, mostly at an intrapopulation level, have shown predictable responses of prey under changing levels of predation risk (reviewed in Lima and Dill 1990; Lima 1998, see also Werner and Anholt 1993). Surprisingly, the primary behavioral changes we have identified in the *Enallagma* are changes in activity in the absence of predators, and not evolution in the presence of predators. The behavioral evolution identified in this study resulted in greater activity in the absence of perceived predators (i.e., changes that would shift the balance between predator susceptibility and foraging in favor of feeding when larvae perceive no mortality threats). This result presents evolutionary evidence illustrating the importance of the foraging/predation risk trade-off to the fitness of prey. Phylogenetic comparison of species that have evolved under different selection regimes provide a powerful tool to evaluate the evolution of a shift in the optimal balance between foraging effort and predation risk. Comparisons with other species groups with known relationship that underwent similar changes in predation regime are needed to evaluate the generality of the predictive power of this trade-off frame-

work for the long-term evolution of predator avoidance and food acquisition.

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