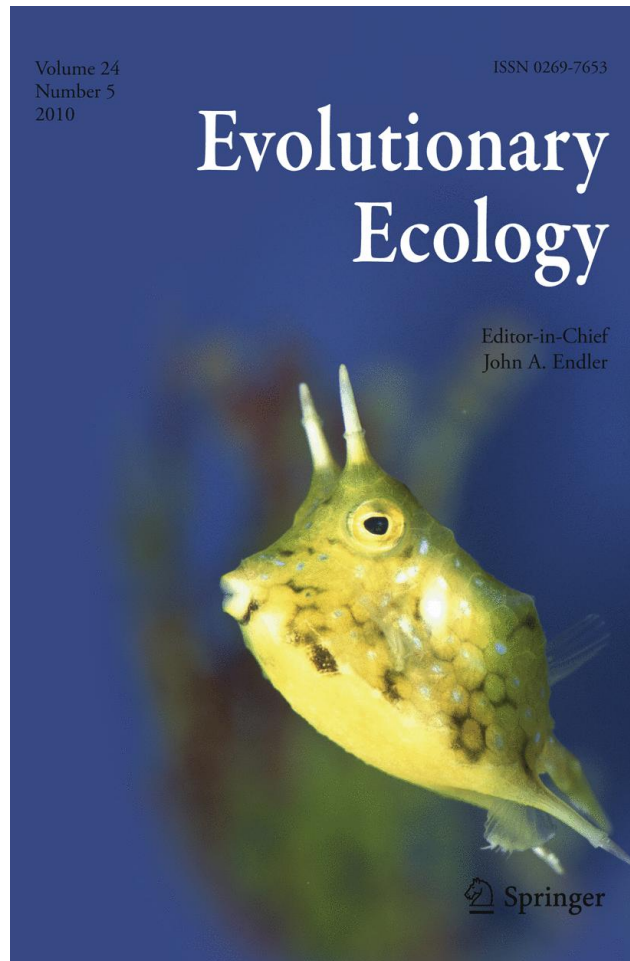


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## Predators shape distribution and promote diversification of morphological defenses in *Leucorrhinia*, Odonata

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**Abstract** Predators strongly influence species assemblages and shape morphological defenses of prey. Interestingly, adaptations that constitute effective defenses against one type of predator may render the prey susceptible to other types of predators. Hence, prey may evolve different strategies to escape predation, which may facilitate adaptive radiation of prey organisms. Larvae of different species in the dragonfly genus *Leucorrhinia* have various morphological defenses. We studied the distribution of these larvae in relation to the presence of predatory fish. In addition, we examined the variation in morphological defenses within species with respect to the occurrence of fish. We found that well-defended species, those with more and longer spines, were more closely associated with habitats inhabited by predatory fish and that species with weakly developed morphological defenses were more abundant in habitats without fish. The species predominantly connected to lakes with or without fish, respectively, were not restricted to a single clade in the phylogeny of the genus. Our data is suggestive of phenotypic plasticity in morphological defense in three of the studied species since these species showed longer spines in lakes with fish. We suggest that adaptive phenotypic plasticity may have broadened the range of habitats

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accessible to *Leucorrhinia*. It may have facilitated colonization of new habitats with different types of predators, and ultimately, speciation through adaptive radiation.

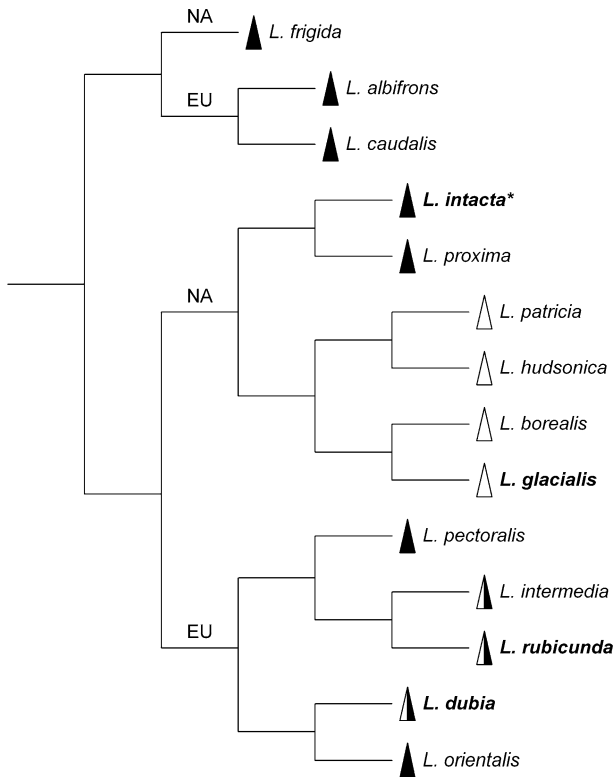
**Keywords** Adaptive phenotypic plasticity · Aquatic insects · Fish predation · Odonates · Polyphenism · Permutation test

## Introduction

Predation is a key factor affecting morphological defenses in prey (Edmunds 1974) and has been suggested to facilitate the adaptive radiation of prey taxa (Vamosi 2005; Nosil and Crespi 2006; Meyer and Kassen 2007). Adaptive radiation might arise because morphological defenses that are effective against one type of predator may constitute a disadvantage against another type of predator (Reimchen 1980; Reimchen and Nosil 2002; Mikolajewski et al. 2006; Marchinko 2009). A broad approach is necessary to understand how morphological defenses relate to species diversity: we need to examine the relationship between morphology and species distributions, and we need to study how morphological variation among species is reflected in the phylogenetic relationships. However, few studies have considered both at the same time (McPeck 1995; Hovmöller and Johansson 2004). Hence, knowing the abundances of well-defended and poorly defended prey in habitats with and without certain types of predators, and knowing the phylogeny of the prey allows assessing how often the invasion of systems with certain types of predators resulted in the evolution of new, efficiently defended prey species (cf. McPeck 1995).

Morphological defenses may be developed constitutively, meaning they are fixed, or they may phenotypically vary depending on environmental conditions, even within the same genotype (Tollrian and Harvell 1999). Such phenotypic plasticity often is adaptive, for instance when the development of defenses is induced by environmental cues that signify the presence of a predator (Ghalambor et al. 2007). In an ecological context, phenotypic plasticity may thus allow the use of a wider range of different habitats, those with and without a particular type of predator (Moran 1992; Agrawal 2001). In an evolutionary context, plasticity may facilitate speciation by easing shifts between peaks across the adaptive landscape. This may happen when plasticity provides more time, i.e., by allowing a population to survive in the new environment long enough for local selection to act on standing genetic variation generated by recombination and mutation (Agrawal 2001; Price et al. 2003). In fact, genetic studies suggest that plasticity may be a prerequisite for speciation (Stern and Orgogozo 2009).

The dragonfly genus *Leucorrhinia* is an ideal model taxon for studying the evolutionary ecology of morphological defenses against predators for several reasons: (1) *Leucorrhinia* exhibits large variation in the development of defensive traits among species and differences in preferred habitat types (reviewed in Johansson and Mikolajewski 2008). (2) The phylogenetic relationships among the *Leucorrhinia* species have recently been resolved (Hovmöller and Johansson 2004). (3) *Leucorrhinia* larvae develop abdominal spines that provide protection against fish predators, but are a disadvantage when evading invertebrate predators (Mikolajewski and Johansson 2004; Mikolajewski and Rolff 2004; Mikolajewski et al. 2006). (4) *Leucorrhinia* larvae can be classified according to the number of their dorsal spines: fully spined, partially spined, and non-spined (Fig. 1). The non-spined species lack dorsal spines; however, they develop lateral abdominal spines. (5) Predator-induced phenotypic plasticity in spine length has been suggested for two species: *L. dubia*



**Fig. 1** Phylogenetic topology of the genus *Leucorrhinia* [redrawn and modified from Hovmöller and Johansson (2004) with kind permission from Elsevier]. Note that branch lengths are not available. The clades are labeled with NA (*North America*) and EU (*Eurasia*) to denote the geographical distribution of the taxa. *Filled triangles* denote species that are fully spined, *half-filled triangles* species that are partially spined, and *open triangles* species lacking dorsal spines. The names of the species for which we inferred phenotypic plasticity in the present study are plotted in *bold face*. Note that *L. intacta* has been suggested to be plastic in another study and that it has therefore been additionally marked with an *asterisk* (McCauley et al. 2008)

(Johansson and Samuelsson 1994; Arnqvist and Johansson 1998; Johansson 2002; Johansson and Wahlström 2002) and *L. intacta* (McCauley et al. 2008). The remaining species have not been examined with regard to this plasticity. Our understanding of morphological defenses in *Leucorrhinia* together with our knowledge of the phylogenetic relationships among *Leucorrhinia* species allowed us to develop hypotheses on the importance of morphological defenses for speciation in *Leucorrhinia*.

Our first goal was to study whether the presence of predatory fish affected the composition of larval *Leucorrhinia* species assemblages. We expected species with many and long abdominal spines to be more abundant in lakes with fish and those with fewer and shorter spines to occur mainly in lakes without fish, which are dominated by invertebrate predators. Our second goal was to study the prevalence of phenotypic variation in abdominal spines within the larvae of *Leucorrhinia* species in the presence and absence of predatory fish. We will argue that such phenotypic variation is due to phenotypic plasticity, which might facilitate the invasion of new habitats with a different type of predator.

## Methods

We studied the distribution, abundance, and abdominal spine lengths in nine of the 14–15 described species (depending on applied classification) in the dragonfly genus *Leucorrhinia* (Odonata, Libellulidae) (Davies and Tobin 1985) using three data sets [summarized in Appendix (Electronic supplementary material)].

### *Leucorrhinia* species assemblages in lakes with and without fish

We used two of the three data sets to study the effect of the presence of fish on *Leucorrhinia* species assemblages, one data set from Sweden and another one from Maine. The third data set, from Ontario, did not comprise sufficiently high numbers of individuals to be included in the analysis of species assemblages.

In Sweden, exuviae (cast skins) of larvae were collected along the shorelines of 17 permanent lakes (14 with fish, three fishless) around Stockholm and six permanent lakes (five with fish, one fishless) close to Karlstad. The presence of fish was determined by visual inspection, through interviews with the local angling societies, and by reference to data sets compiled by local and provincial environmental authorities. The by far most common insectivorous fish in Swedish lakes is perch (*Perca fluviatilis*). Fishless lakes are rare in Sweden due to widespread fish introductions, and we were unable to identify any additional lakes in that category. It should be noted, however, that the *Leucorrhinia* species common in fishless lakes also are common in small ponds and pools lacking fish (Johansson and Brodin 2003). We did not include such small ponds and pools in our study because that would confound fish occurrence with the size and permanence of the water bodies. The sampling effort was standardized by collecting exuviae from a 25 m section of lake shoreline chosen to represent typical *Leucorrhinia* habitat characterized by emergent macrophytes (Johansson and Brodin 2003). The sites were sampled by walking slowly along the shoreline and collecting all visible exuviae for 30 min (cf. Johansson and Brodin 2003). Sites were sampled two to three times per week from the end of May until mid-July. All dragonfly exuviae were counted and identified to species. In total, the Swedish data set comprised 1,227 individual exuviae from five Eurasian species: *L. albifrons*, *L. caudalis*, *L. dubia*, *L. pectoralis*, and *L. rubicunda*.

We also assessed the effect of fish presence on North American *Leucorrhinia* species assemblages in another set of study lakes. Dragonfly larvae were quantitatively collected from the littoral zones of 26 permanent lakes (16 with fish, ten fishless) in Maine, USA, with submerged light traps and sweep nets (see Schilling et al. 2009 for complete methods). Each lake was sampled once in summer during the period 2002–2005. The presence of fish was determined with gillnets and minnowtraps. Fish in the Maine lakes included cyprinids, pumpkinseed sunfish, three-spined stickleback, and smallmouth bass among others. All dragonfly larvae were counted and identified to species, and data for common *Leucorrhinia* species (i.e., those occurring in >10% of study lakes) were analyzed: *L. glacialis*, *L. frigida*, and *L. hudsonica*. In total, 702 individuals were included in this data set.

We compared *Leucorrhinia* species assemblages between lakes with and without fish with redundancy analyses (RDA) on chord distances (Legendre and Gallagher 2001). Similar to canonical correspondence analysis (CCA), in RDA a set of species abundances is related to one or several environmental variables, which allows assessing how much of the variation in the species abundances may be explained by the environmental gradients. However, the two methods require different assumptions regarding the distribution of the

species along the environmental gradients. A preliminary analysis of our data suggested that the species response curves were linear, rather than unimodal. We therefore preferred RDA to CCA. The RDA results are presented in a similar way as CCA results. In our study, we constrained the ordinations to the presence of fish as the only explanatory variable. Consequently, variation in *Leucorrhinia* species abundances along the first axis was related to the presence or absence of fish, whereas variation along the second and all subsequent axes was related to other unspecified environmental gradients. We were thus able to assess how much of the variation in *Leucorrhinia* species abundances was accounted for by the presence of fish (cf. Woodward et al. 2002). An ANOVA-like permutation test was then applied to assess the significance of the constraint using the ratio of the constrained and unconstrained total inertia ( $F$ ) as the test statistic (Oksanen et al. 2008). We analyzed the species abundance data for Sweden and Maine separately, as sampling methods differed between the regions.

We also analyzed the abundances of each species separately, because we were also interested in the effect of fish presence on the total abundances of the different *Leucorrhinia* species independently from each other. Hence, we compared total abundances between lakes with and without fish using generalized linear models. Fish presence was treated as a fixed factor and total abundance as the dependent variable with each lake representing a replicate. Sites lacking *Leucorrhinia* were retained as they constitute real data, and as we were unable to ascertain that the absence of *Leucorrhinia* does not reflect an effect of the presence of fish. We assumed a quasi-Poisson error distribution, which accounted for the nature of the count data and allowed for modeling of the dispersion factor at the same time (Venables and Ripley 2002). We assessed the significance of the effect of fish presence on total abundances with an  $F$ -test (Venables and Ripley 2002).

#### *Leucorrhinia* spine lengths in lakes with and without fish

We used two data sets to study the effect of the presence of fish on *Leucorrhinia* spine lengths: one data set from Sweden and one from Ontario. The Swedish data set is identical to the one that we used to study the effect of the presence of fish on *Leucorrhinia* species assemblages. The larvae from Maine were unavailable for spine measurements.

In Ontario, Canada, sweep net sampling was conducted in seven permanent lakes (three with fish, four fishless) in the vicinity of Sudbury. Fish presence was determined with minnowtraps (McNicol et al. 1996). The main fish species were cyprinids, brook stickleback, and pumpkinseed. In late May, each shoreline site was sampled qualitatively with a sweep net for 15 min to collect last instar *Leucorrhinia* larvae that were close to emergence. The larvae were kept individually in plastic containers in the laboratory where they emerged within a week. The exuviae of the emerged dragonflies were then used in our study of the effect of fish presence on *Leucorrhinia* spine lengths. Forty-seven exuviae comprising a subset of the North American species, *L. glacialis*, *L. frigida*, and *L. proxima*, were analyzed in total.

We used the ocular scale of a dissecting microscope (at 310× magnification) to measure the length from base to tip (cf. Johansson and Samuelsson 1994) of all (lateral and dorsal) abdominal spines of each exuvia from Sweden and Ontario. In addition, we measured the length of the right front tibia as a proxy of body size to test whether larger individuals within species developed longer spines. Body size is correlated with tibia length in dragonflies (cf. Brodin and Johansson 2002). We measured the left tibia if the right front tibia was not preserved. Although head width may be a preferable measure of body size in *Leucorrhinia*, this metric could not be used, as the larval skin ruptures dorsally during imago emergence.

There was no relationship between body size and spine lengths within species: only the lateral spines of the ninth segment in *L. rubicunda* appeared to be longer in larger individuals (resampling: Pitman's correlation,  $P = 0.044$ ); however, given that we ran more than 60 separate tests to look for correlations between trait length and body size, this result is likely spurious (cf. Johansson 2002; Johansson and Wahlström 2002). Finally, we re-measured all traits of a subset of the exuviae ( $n = 20$ ) to assess the reproducibility of the measurements. The results from the original and repeated measurements were highly correlated (all  $r > 0.99$ ). All measurements were taken by the same rater (ZP).

For data analysis, the larvae were first classified in three categories depending on spine prevalence. We classified the larvae because the occurrence of abdominal spines determined the susceptibility of *Leucorhina* to predation by fish and invertebrates (Mikolajewski and Johansson 2004; Mikolajewski and Rolff 2004; Mikolajewski et al. 2006): fully spined species developed dorsal spines on abdominal segments 3–8, non-spined species completely lacked dorsal spines, and partially spined species grew dorsal spines only on abdominal segments 3–6. The spines of partially spined species were shorter than those of fully spined species. Note that all species additionally developed lateral spines at least on abdominal segments 9–10. We employed a linear discriminant analysis to check the reliability of our classification of *Leucorhina* species as fully spined, partially spined, or non-spined. Therefore, we treated species as the dependent variable and the different spines as independent variables. Ninety-five percent of the populations were classified to the correct species. However, the few misclassified populations all belonged to the same class. Consequently, all populations (100%) were classified correctly into fully spined, partially spined, and non-spined species.

Parametric assumptions were not met for the spine length data. Hence, we assessed whether the different abdominal spines grew longer in the presence of fish with multivariate permutation tests for each *Leucorhina* species separately, followed by a univariate test for each spine when the overall differences were significant. Our hypothesis was one-sided, and we therefore used one-sided tests when comparing the lengths of abdominal spines. In all instances, we treated the lakes, rather than individual exuviae, as replicates to avoid pseudoreplication. For similar reasons, we retained only the dorsal and right lateral spines, ignoring the left lateral spines, when running the statistical tests. The measurements for the left and right side were highly correlated (all  $r > 0.95$ ). To allow for the possibility that not all spines within a species need to exhibit a similar effect to the presence of fish, we refrained from choosing one of the classical test statistics for multivariate tests that require the above assumption, such as Hotelling's  $T^2$ . Instead, we computed the maximum of the differences in the weighted means as the test statistic (see Good 1994, p. 70f for details). For the univariate tests we used the sum of the observations for lakes with fish as the test statistic (Good 1994, p. 29f). Observations were retained when the spine length was zero. To examine if selective predation of larvae with short and long spines by fish could have caused potential intraspecific differences in spine length between lakes with and without fish, we assessed whether the distributional skew in spine length covaried with fish presence.

We also assessed whether fish presence affected spine occurrence differently on different larval segments. Therefore, we used the chi-square statistic for multivariate contingency tables. Each species was analyzed separately. For this test we enlisted the mean of the proportion of individuals at each site that developed the particular spines, but preserved the number of location labels at each lake type in each permutation when generating all possible permutations (Manly 2007, p. 312ff).

All statistical analyses were done in R 2.7.0 (R Development Core Team 2008). We used two-sided tests, except when explicitly stated otherwise.

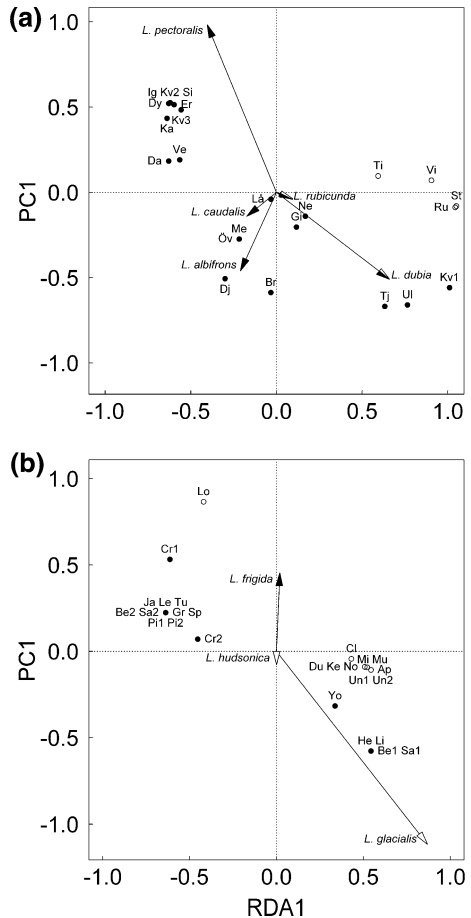
**Results**

We found an effect of the presence of fish on both *Leucorrhinia* species assemblages and spine lengths.

*Leucorrhinia* species assemblages in lakes with and without fish

The *Leucorrhinia* species assemblages differed between lakes with and without fish (Fig. 2), and this association appeared related to the prevalence of abdominal spines. One of the European partially spined species, *L. dubia*, loaded highly on the abscissa, as did the fishless lakes (Fig. 2a). In contrast, the lakes inhabited by fish had on average lower scores. At the same time the three European species that are fully spined, *L. albifrons*, *L. caudalis*, and *L. pectoralis* had lower loadings on the abscissa, and hence were associated with lakes inhabited by fish (Fig. 2a). Similarly, the non-spined *L. glacialis*, a North American species lacking dorsal spines, had a high loading on the abscissa and was therefore associated with lakes lacking fish (Fig. 2b). The proportion of the variation explained by fish presence was relatively small, but the effect was nevertheless significant (Fig. 2;  $F_{\text{Sweden}} = 0.9$ ,

**Fig. 2** Redundancy analysis on species-abundance data for *Leucorrhinia* from **a** Sweden and **b** Maine. The first axes (*RDA1*) are constrained to represent the presence of fish. All further axes are unconstrained. **a** *RDA1* accounts for 15.7% of the variance, the first principal component (*PC1*), i.e. the second axis, accounts for 18.5% of the variance. **b** *RDA1* accounts for 23.8% of the variance, *PC1* accounts for 21.3% of the variance. *Filled dots*, sites inhabited by fish; *open dots*, sites lacking fish; *filled arrowheads*, species that are fully spined; *half-filled arrowheads*, species that are partially spined; *open arrowheads*, species lacking dorsal spines





**Table 1** Effect of fish presence on *Leucorhina* species' abundances and results from the generalized linear models for comparisons in total abundances (*F*-tests)

Species	<i>L. albifrons</i> (▲)	<i>L. caudalis</i> (▲)	<i>L. frigida</i> (▲)	<i>L. pectoralis</i> (▲)	<i>L. dubia</i> (▲)	<i>L. rubicunda</i> (▲)	<i>L. glacialis</i> (△)	<i>L. hudsonica</i> (△)
Mean total abundance (and standard deviation) in lakes with fish and fishless lakes								
Fish	9.5 (19.0)	0.7 (1.4)	0.3 (0.7)	14.0 (25.0)	5.8 (18.2)	3.3 (5.4)	1.1 (1.7)	1.3 (4.7)
Fishless	1.5 (3.0)	0	0.7 (1.9)	1.8 (3.5)	116.8 (93.0)	28.3 (50.5)	63.1 (128.4)	2.2 (4.0)
Effect of fish presence on total abundance								
<i>F</i>	1.1	2.0	0.9	1.6	18.7	9.3	10.7	0.2
<i>P</i>	0.304	0.174	0.346	0.227	<b>&lt;0.001</b>	<b>0.006</b>	<b>0.003</b>	0.649

Bold *P*-values denote significant differences ( $P < 0.05$ )

*Fish* lakes with fish, *Fishless* lakes lacking fish, *F* *F*-value, *P* *P*-value; Filled triangles (▲) denote species that are fully spined, half-filled triangles (▲) species that are partially spined, and open triangles (△) species lacking dorsal spines

$P_{\text{Sweden}} = 0.005$ ;  $F_{\text{Maine}} = 0.9$ ,  $P_{\text{Maine}} = 0.005$ ). The European *L. rubicunda* and the North American *L. hudsonica* that are partially spined and the North American *L. frigida* that is fully spined did not show any clear association with either lake type, but note that *L. hudsonica* and *L. frigida* occurred in very small numbers in the sampled lakes (Table 1).

When the total abundances were compared separately, the partially spined species *L. dubia* and *L. rubicunda* and the non-spined *L. glacialis* occurred more often in lakes lacking fish than in those inhabited by fish (Table 1). None of the other species exhibited any significant difference in total abundances. When the mean total abundances were calculated ignoring lakes lacking the respective *Leucorhina* species, and when the percentages of lakes occupied by *Leucorhina* were calculated, the pattern remained the same (not shown). *L. caudalis* never occurred in lakes without fish, but its abundances were generally small, and the variation in abundances was comparatively large. In summary, the partially and non-spined species *L. dubia*, *L. rubicunda*, and *L. glacialis* were more common in lakes lacking fish. The fully spined species *L. albifrons*, *L. caudalis*, and *L. pectoralis* were more closely associated with lakes with fish, although their total abundances did not differ between lakes with and without fish.

#### *Leucorhina* spine lengths in lakes with and without fish

Exuviae of *L. dubia*, *L. glacialis*, and *L. rubicunda* collected at lakes with fish had longer spines than exuviae collected at fishless lakes (Table 2; Fig. 3). The percentage increase in the length of the spines in the presence of fish was considerable in the partially spined species *L. dubia* and *L. rubicunda*, but much smaller in the non-spined species *L. glacialis* (Table 2). All spines on *L. dubia*, except for the rarely observed spine on the second segment, were longer in lakes with fish (Table 2). Only the lateral spine on the ninth segment on *L. glacialis* was longer in exuviae from lakes inhabited by fish (Table 2). In *L. rubicunda* only the dorsal spines on the fourth and sixth segment were longer in lakes with fish (Table 2). In addition, the effect of fish on the spines of *L. dubia* and *L. rubicunda* differed among spines: when fish were present, a larger proportion of larvae developed posterior dorsal spines (Fig. 3,  $P_{L. dubia} = 0.024$ ,  $P_{L. rubicunda} = 0.018$ ,  $P_{\text{other}}$

**Table 2** Effect of fish presence on *Leucorrhinia* abdominal spine length (see Fig. 3 for means and ranges)

Species	<i>L. albifrons</i> (▲)	<i>L. pectoralis</i> (▲)	<i>L. proxima</i> (▲)	<i>L. dubia</i> (▲)	<i>L. rubicunda</i> (▲)	<i>L. glacialis</i> (△)
Effect of fish presence on overall spine length						
<i>P</i>	0.375	0.583	0.250	<b>0.005</b>	<b>0.041</b>	<b>0.029</b>
<i>P</i> <sub>min</sub>	0.125	0.083	0.250	0.005	0.005	0.029
Effect of fish presence on single spine length						
<i>P</i> <sub>D2</sub>	NT	NT	NT	0.867	NA	NA
<i>P</i> <sub>D3</sub>	NT	NT	NT	<b>0.005</b> (42.0)	0.191	NA
<i>P</i> <sub>D4</sub>	NT	NT	NT	<b>0.005</b> (37.8)	<b>0.018</b> (132.0)	NA
<i>P</i> <sub>D5</sub>	NT	NT	NT	<b>0.005</b> (118.5)	0.145	NA
<i>P</i> <sub>D6</sub>	NT	NT	NT	<b>0.005</b> (251.5)	<b>0.045</b> (15,531.2)	NA
<i>P</i> <sub>D7</sub>	NT	NT	NT	<b>0.024</b> <sup>a</sup>	NA	NA
<i>P</i> <sub>R8</sub>	NT	NT	NT	<b>0.005</b> (30.3)	0.195	0.114
<i>P</i> <sub>R9</sub>	NT	NT	NT	<b>0.005</b> (32.8)	0.182	<b>0.029</b> (11.7)

First, we examined the effect on the overall length of the abdominal spines. Therefore, we used multivariate permutation tests to compute the *P*-values. For species with a significant overall effect of fish presence on spine length, we analyzed each spine separately, i.e., we computed *P*-values using multiple univariate permutation tests. For spines of significantly different length between lake types, the percentage increase of the length of the spines in the presence of fish is presented in parenthesis below the corresponding *P*-value. Bold *P*-values denote significant differences ( $P < 0.05$ ).

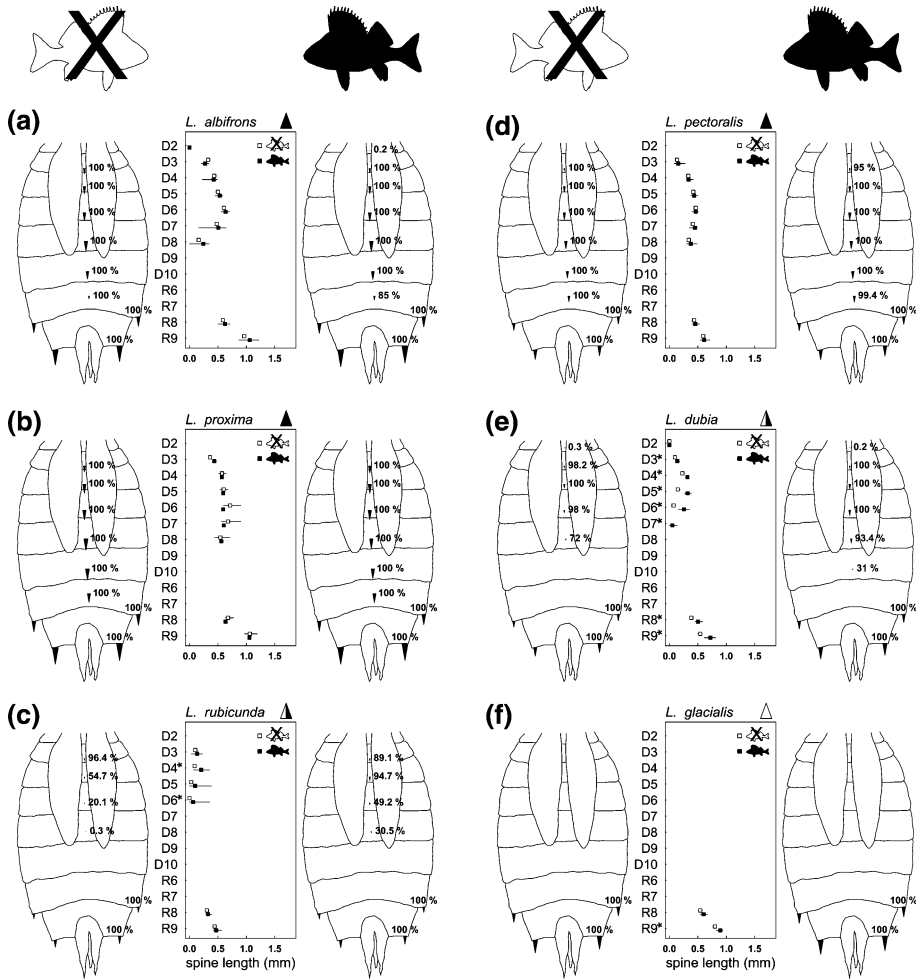
*P* overall *P*-value, *p*<sub>min</sub> lowest attainable *P*-value calculated from the number of possible permutations, the other subscripted *P*-values refer to the dorsal (D) and right lateral (R) abdominal spines of the segments 2–9, NA not available as species did not develop that particular spine, NT not tested as multivariate test indicated insignificant difference. Filled triangles (▲) denote species that are fully spined, half-filled triangles (▲) species that are partially spined, and open triangles (△) species lacking dorsal spines

<sup>a</sup> The length of the spines of individuals from fishless lakes was zero. The percentage increase in spine length in the presence of fish could thus not be calculated

species  $> 0.50$ ). The skew in spine lengths did not differ between lakes with and without fish (all  $P > 0.20$ ). Thus, three of the species, all with few and short or no dorsal spines in fishless lakes, showed indication of predator-induced plasticity in spine length in the presence of fish.

## Discussion

In accordance to our predictions, *Leucorrhinia* species that were fully spined and had long abdominal spines were more closely associated with lakes inhabited by fish, whereas species with fewer and short or no spines occurred more often and in larger numbers in lakes lacking fish in both Sweden and North America. This pattern indicates that fish had a large effect on the distribution and partly also the abundance of *Leucorrhinia* species.



**Fig. 3** Length of abdominal spines of **a** *L. albifrons*, **b** *L. proxima*, **c** *L. rubicunda*, **d** *L. pectoralis*, **e** *L. dubia*, **f** *L. glacialis* in lakes with and without fish. Sketches depict mean lengths of spines and show mean percentages of individuals expressing the respective spine in each lake. Plots depict means and ranges of spine lengths, with significant differences between lakes with and without fish denoted by asterisks next to the spine names (see Table 2 for details). The larvae were classified with respect to spine length and prevalence as follows: fully spined species (filled triangles) had dorsal spines on segments 3–8, non-spined species (open triangles) lacked dorsal spines, and partially spined species (half-filled triangles) developed dorsal spines on segments 3–6, which were shorter than the corresponding spines of fully spined larvae. Note that all species developed lateral spines at least on the abdominal segments 9–10

Furthermore, our data suggest that the presence of fish predators induced longer spines and thus morphological defenses in *Leucorrhinia*: three of six *Leucorrhinia* species for which we could compare spine lengths between lakes with and without fish had longer spines when fish were present. Although laboratory experiments are needed to demonstrate and unequivocally conclude that the longer spines in lakes with fish are actually induced by the presence of fish, we note the following: Firstly, we did not find any skew in the distribution of spine lengths with respect to the presence of fish and thus no evidence for

selective consumption of individuals with short spines by fish. Secondly, differences in spine length between lakes with and without fish are unlikely to reflect fixed polymorphism alone, as adult *Leucorrhinia* disperse widely (Pajunen 1962). Thirdly, phenotypic plasticity has been shown for two *Leucorrhinia* species, *L. dubia* and *L. intacta* (Johansson 2002; McCauley et al. 2008), which are not closely related as suggested by the phylogeny (Hovmöller and Johansson 2004). Hence, possibly in addition to a more or less important fixed component, the observed pattern is suggestive of adaptive phenotypic plasticity. Again, laboratory experiments will have to follow to conclusively demonstrate plasticity. Nevertheless, together with the results from previous behavioral studies (Mikolajewski and Johansson 2004; Mikolajewski and Rolff 2004), observational studies (Johansson and Samuelsson 1994; Arnqvist and Johansson 1998; Johansson 2002), and field experiments (Johansson and Wahlström 2002), our data suggest that spines provide protection against predatory fish allowing fully spined *Leucorrhinia* larvae and *Leucorrhinia* species with predator-induced defenses to survive in lakes with fish.

Phylogenetic analyses suggest that the presence of abdominal spines in *Leucorrhinia* represents the primitive state (Hovmöller and Johansson 2004). Abdominal spines constitute an efficient defense against predatory fish by increasing rejection rate of attacked larvae (Mikolajewski and Johansson 2004; Mikolajewski and Rolff 2004). In contrast, long abdominal spines facilitate grasping by predatory invertebrates and thus are a disadvantage against such predators suggesting antagonistic selection by these two predator types (Mikolajewski et al. 2006). Such antagonistic selection may explain why species with fewer and short spines were less abundant in lakes with fish. It may also explain why species with more and long spines were less closely associated with lakes lacking fish: long spines are selected against by predatory invertebrates, which are the dominant predators in fishless lakes (Crowder and Cooper 1982; Mallory et al. 1994). We propose that the dorsal abdominal spines were likely reduced, or completely lost, in some species because they were selected against by predatory invertebrates when *Leucorrhinia* invaded habitats lacking fish in the past (Mikolajewski et al. 2006). Hence, a loss of certain defense traits made the prey more successful invaders in lakes with other kinds of predators. A phylogenetic approach demonstrated a similar loss of defensive traits against predatory fish in *Enallagma* damselflies that invaded habitats with predatory invertebrates. In such habitats with abundant invertebrate predators, the *Enallagma* damselflies developed larger caudal lamellae facilitating higher evasion speed (McPeck 1995). The opposite pattern was observed in *Lestes* damselflies where some species colonized habitats with predatory fish (Stoks and McPeck 2006). While these two studies exemplify loss as well as gain of anti-predator traits in a phylogenetic context, our study has a different focus in the same context by addressing morphological defense with a post attack effect (for further examples on morphological pre- and post-attack defenses see e.g. Grant and Bayly 1981; Harvell 1986; Lively 1986).

Predators constitute an important selective force in animal communities (Kerfoot and Sih 1987). The fish predators of *Leucorrhinia* mostly attack their prey from behind suggesting that posterior spines are most important for protecting *Leucorrhinia* against fish predation (Mikolajewski and Johansson 2004; Mikolajewski and Rolff 2004), while the same spines simultaneously render the larvae more susceptible to invertebrate predators (Mikolajewski et al. 2006). Therefore, we would expect selection to act primarily on the posterior spines. Consequently, posterior spines should be reduced first in response to predation by invertebrates. In contrast, the relatively small anterior spines, which are hidden below the wing pads in final instar larvae, would be expected to minimally affect survival and thus should not be markedly selected for or against. Among the studied

species only those with short and few spines and those lacking dorsal spines showed evidence indicative of plasticity. In both partially spined species, *L. dubia* and *L. rubicunda*, the posterior dorsal spines tended to vary more in prevalence and size than the other spines, suggesting that the posterior dorsal spines may have been most plastic and hence supporting our prediction. Indeed, evidence from other studies suggests that the traits undergoing the strongest selection are most plastic (cf. Van Buskirk and Relyea 1998). The third possibly plastic species, *L. glacialis*, did not develop dorsal spines.

We acknowledge that experiments are needed to unequivocally demonstrate that phenotypic plasticity is the mechanism that caused spine polymorphism. Nevertheless, we take the opportunity to speculate on the details of speciation in the genus *Leucorrhinia* as it might help in identifying relevant questions and developing hypotheses for future research. We have good evidence for phenotypic plasticity for two species (*L. dubia* and *L. intacta*), and we have tentative evidence for phenotypic plasticity for a further two species (*L. rubicunda* and *L. glacialis*). We did not perform a formal phylogenetic analysis with respect to the evolution of fixed and plastic traits because we lack information for more than half of the species. In addition, absence of evidence for plasticity in some of the species may reflect limitations in statistical power. Considering the phylogeny, however, three scenarios are conceivable. In the first scenario, if we assume plasticity to be ancestral to the two major European and North American clades, then this would suggest that phenotypically plastic *Leucorrhinia* species with long spines inhabiting lakes with fish invaded lakes lacking fish. Subsequently, in some cases the spines, whether short or long, or the spines' absence may have become fixed. This scenario would be supported by genetic evidence that suggests plasticity is a condition for speciation, whereas the microevolutionary fixation of a trait may hamper speciation (Stern and Orgogozo 2009). In the second scenario, assuming that the development of spines was originally fixed would suggest that plasticity independently arose at least four times in *Leucorrhinia*, because the four species belong to different clades separated by species for which we found no evidence of plasticity. A third scenario would be that all species actually are plastic, but the effect sizes tend to covary with spine length. The percentage increase in spine length in the presence of fish was overall smaller in species that either have no dorsal spines, 11.7% in *L. glacialis* (this study), or long spines, 28.9–46.8% in *L. intacta* (McCauley et al. 2008). Note that we were unable to detect any increase in spine length in fully spined species in the presence of fish in this study. However, that result may change given larger sample sizes, and thus increased power, for fully spined species. The latter scenario would suggest a greater degree of plasticity in species living under heterogeneous predator regimes, i.e., species that frequently need to adapt to different predator types (Vamosi 2005).

We speculate that predation by fish versus invertebrates is likely to have contributed to speciation in *Leucorrhinia* and to have spurred diversification (Vamosi 2005). Adaptive phenotypic plasticity may have facilitated the colonization of fishless lakes by *Leucorrhinia*, suggesting that plasticity and additional ecological mechanisms may have operated concurrently in *Leucorrhinia* speciation (Agrawal 2001; Price et al. 2003). Testing the scenarios and understanding the details of diversification in *Leucorrhinia*, however, requires further research.

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