Degree of high phenotypic plasticity in wild populations of *Daphnia* in early spring

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ABSTRACT

Inducible defenses have been empirically known as defensive phenotypes that are triggered by predator kairomones. We hypothesized that morphological defense of wild *Daphnia* varies not only with predator density, but also with the predator regime in the field. We observed how the morphological defenses of two *Daphnia* species (*D. ambigua* and *D. pulex*) changed according to the population density in the water column at daytime of predatory insect larvae *Chaoborus flavicans* in Lake Fukami-ike, Japan, from February to July. In both *Daphnia* species, the inducible defense morphology was highly expressed in March and April. Its degree of expression decreased, and did not change with increases in predator density from May to July. These seasons are generally considered as when the number of the larvae and fish increases, and predation becomes more active due to their growth and breeding. We suggest that the degree of inducible defense of *Daphnia* is higher at the end of the overwintering season, when *Chaoborus* larvae began to inhabit the water column during the daytime and is constant in other seasons regardless of predators' regime changes. Field observation of wild populations is important for understanding the seasonal changes in the morphology, and to provide more realistic explanations of phenomena in inducible defense.

Key words: Inducible defense; morphological plasticity; phenotypic plasticity; predator-prey interaction; principal component analysis.

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INTRODUCTION

Phenotypic plasticity is the ability of an organism to change its phenotype in response to heterogeneous environments (DeWitt and Scheiner, 2004). The ability of freshwater organisms to bring about adaptations in reaction to environmental shifts under several ecological contexts has been shown in laboratory experiments (Relyea, 2002; Kopp and Tollrian, 2003; Decaestecker *et al.*, 2002). The organisms using experiment and breeding environments of the laboratory used are relatively simple and unrealistic, and are different from those found in natural environments. In order to understand this ability, experiments with sufficient field observations and controls are necessary. However, research in this field is dominated by simplified laboratory studies. In situations where environmental complexity is reduced and relatively predictable, we considered that it may be worthwhile to measure the phenotypic plasticity of wild populations of plankton.

The genus *Daphnia* (Cladocera) occurs in variety of freshwater habitats and has been described as exhibiting cyclic or seasonal changes in morphology (cyclomorphosis) since the 1900s (Wesenburg-Lund, 1926; Havel, 1985). The cyclomorphosis of *Daphnia* is interpreted as predator-induced plasticity, *i.e.*, an inducible defense (Tollrian and Harvell, 1999). *Daphnia* seasonally changes its morphology in response to the presence of fish and invertebrate predators (Dodson, 1989). *Daphnia* develops defensive traits only when it detects predators or chemical compounds, *i.e.*, kairomones, released by predators (Harvell, 1990; Lass and Spaak, 2003). *Daphnia* has been shown to exhibit inducible defenses in numerous experimental studies by making changes in several morphological features: total body size, head spine (*i.e.*, helmet) size, and tail spine length can change, and neckteeth can be developed (Hebert and Grewe, 1985; Dodson, 1989; Laforsch and Tollrian, 2004). Several morphological defenses occur simultaneously (Boersma *et al.*, 1998; Boeing *et al.*, 2006), and have been revealed using multi variable analysis to vary within species (Dennis *et al.*, 2010). Although multivariate analysis is useful tool for analysis of complex morphological defenses, few recent studies have assessed how morphological defenses vary with the seasons in wild *Daphnia* population (except of Pijanowska, 1990; Sell, 2000).

The level of inducible defense varies in relation to predator characteristics, such as species, age-structure, density, and behavior (Lass and Spaak, 2003). In a lake ecosystem, the larvae of invertebrate insect *Chaoborus* are a major predator of *Daphnia*. The feeding habits of larval *Chaoborus* change by age: only older instars of larvae can eat *Daphnia*, depending on their gape size (Swift, 1992). Typical the yearly cycle of *Chaoborus*

larvae in temperate lakes is recognized due to the high density of all instars in summer. Before summer, *Chaoborus* larvae are observed to be present only as fourth instar larvae, since they had remained in the diapause stage in the sediment over the winter (Stahl, 1966; Xie *et al*., 1998; Voss and Mumm, 1999). In early summer, the year's first generation emerges. The larvae that coexist with fish reside deeper in the water column or sediments during the daytime and migrate to upper layer at night, *i.e*., diel vertical migration (DVM, Teraguchi and Northcote, 1966; Dawidowicz *et al*., 1990; Tjossem, 1990). The migration amplitudes of the larvae vary by season; large migration amplitude is observed in summer while short or no migration occurs in winter and at low temperatures (Goldspink and Scott, 1971; Nagano *et al*., 2015). Given the impact of *Chaoborus* larvae on *Daphnia*, the larvae change distribution areas from sediment to water column in the daytime with simple age structure before summer. Although fishes are a predominant predator of *Daphnia*, fishes inhabited the lake throughout year, and rapidly increase their populations from spring to summer. Therefore, we considered that wild *Daphnia* from winter to early spring would show a high degree of inducible defense in response to the overwintering larvae altering distribution area from sediment to water column in daytime.

Here, we report how the inducible defenses of two *Daphnia* species in a lake vary seasonally with changes in the predatory *Chaoborus* population. We focused on two *Daphnia* species, *D. ambigua*, which has a smaller body size, and *D. pulex*, which has a larger body size, in Lake Fukami-ike, Japan from February to July 2004. During this observation season, the larvae inhabiting the lake were present only as fourth instar of overwintering individuals, but the habitat of the larvae gradually changed from sediments to the water column in early summer. A multivariate analysis was performed on all morphological values of inducible defense of an individual on each observed day, because multiple morphological changes of *Daphnia* can occur simultaneously in the presence of *Chaoborus* larvae. We predicted that the degree of inducible defense of *Daphnia* would be high when the larvae begin to emerge from sediment to the water column at daytime.

METHODS

Field observations

We sampled two *Daphnia* populations and *Chaoborus flavicans* from Lake Fukami-ike, Nagano Prefecture, Japan (35° 32' N, 137° 81' E; maximum water depth is 7.75 m), eight times from February to July, 2004. The lake is a small monomictic eutrophic lake (Nakagawa *et al*., 2012; Suda *et al*., 2016). The circulation and stagnation periods were from November to March and April to October, respectively. Two *Daphnia* species (*D. ambigua* and *D. pulex*), *C. flavicans* (Nagano *et al*., 2015), and planktivorous fish (bluegill sunfish *Lepomis macrochirus* and largemouth bass *Micropterus salmoides;* Kawanobe and Hosoe, 2010) inhabited the lake. *Chaoborus* larvae and daphnids were captured on the same observation date by vertically towing three times with a plankton net (NXX7, φ30 cm) above 7.5-m depth, with sampling carried out at approximately 10:00 hrs. Samples were preserved in 10% formalin in the field, and larvae were counted and daphnids measured in the laboratory.

Morphological measurements for *Daphnia* species

We measured several body parts for the two *Daphnia* species from at least 10 individuals at each sampling date (n=330 in total). Body length (µm), head length (µm), and tail length (µm) (Fig. 1), and the presence or absence of head spines of *Daphnia* were measured using a 400× microscope.

Quantification and analysis of phenotypic plasticity

We used a principal component analysis (PCA) to compare the morphological variability of daphnids for six traits: body size, tail and head length, relative spine length (head length/body length, RHL relative head length; tail length/body length, RTL, relative tail length), and morphological type (type 1, *Daphnia* with tail spine; type 2, *Daphnia* without any spine; and type 3, *Daphnia* with tail spine and head spine (*D. ambigua* only), or tail spine and neckteeth (*D. pulex* only)). These were converted to Z distributions prior to analysis (mean, 0; standard deviation, 1).

The degree of inducible defense present at each sampling date was calculated based on the difference
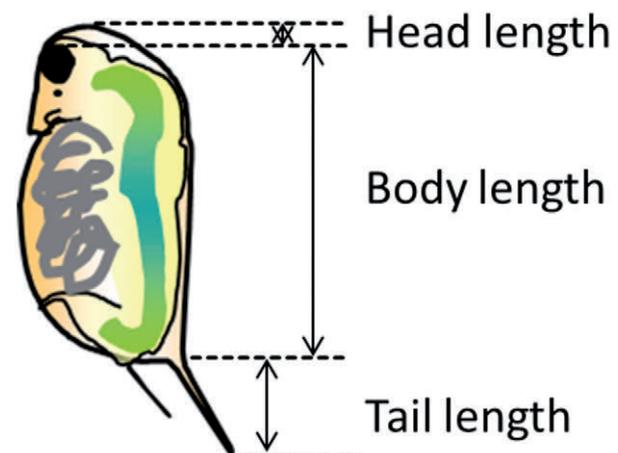

Fig. 1. Standard measurements of *Daphnia* individuals.

between the February average and measurements from a *Daphnia* for each month sampled. In the field, morphological changes in *Daphnia* species are caused not only by predation, but also by environmental stresses such as food quality (Whittington and Walsh, 2015), water temperature (Miehles, 2013), and light intensity (Tollrian and Heibl, 2004; Oexle *et al*., 2016). Thus, these changes are more precisely called "degree of phenotypic plasticity," but we decided to use the term "degree of inducible defense" because we were measuring defensive morphology in particular. The degree of inducible defense was quantified by Euclidean distance. The method of estimation used PC scores from the first to the third main component:

$$\overline{|i-c|} = \sqrt{(PC1_{fm} - PC1_F)^2 + (PC2_{fm} - PC2_F)^2 + (PC3_{fm} - PC3_F)^2}$$

where: i, individual; c, control (mean value in F); fm, focal month; F, February.

Thus, if degree of inducible defense was nearly zero, *Daphnia* had no changes in morphology in February. In February, the predation activities of the larvae and fishes may be low in temperate lakes, especially as the larva often inhabit the sediment. Therefore, the morphology of *Daphnia* shown in February was used as a reference value, *i.e*., control. A two-way analysis of variance (ANOVA) was performed to examine the effects of *Daphnia* species, observation dates, and the degree of inducible defense observed. To compare the mean degree of inducible defense among observation dates, we performed a Tukey-Kramer

multiple comparison test. All analyses were conducted using R ver. 3.3.1 (R Core Team, 2016).

RESULTS

Chaoborus larvae were not observed in the water column in February (Fig. 2), probably because the larvae wintered in the diapause stage in the sediments over the winter. The larvae emerged into the water column, and were observed from March, when the larval population density remained very low. In July, the larval density was the highest observed during the season. The larval density in July was over 200 times more than that observed in March.

Daphnia ambigua was only observed in the water column from February to May 29, whereas *D. pulex* was noted as being present in the lake on all observation dates (Fig. 3). Examining the PCA scores obtained from analyzing six morphological features (Fig. 4), the variation in morphology explained by the first principal component (PC1) was 41.5% and was positively associated with relative tail length (RTL) and tail length. The second principle component (PC2) explained 25.2% of the variance, and was positively associated with relative head length (RHL). The third principle component (PC3) explained 22.0% of the variance, and was negatively associated with body length. The morphology ordinate from individuals collected in
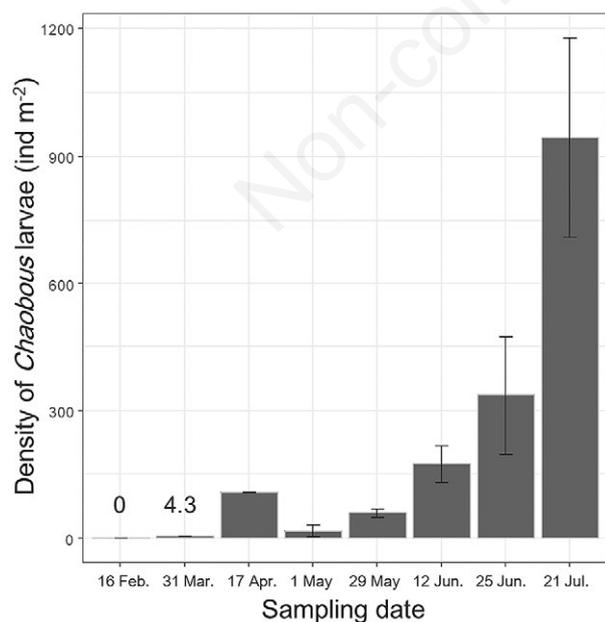
Fig. 2. Seasonal density of *Chaoborus flavicans* in Lake Fukami-ike, Japan. The error bar shows standard deviation (mean ± SD). The numbers for Feb. and Mar. shows the mean values. Ind, number of individuals.
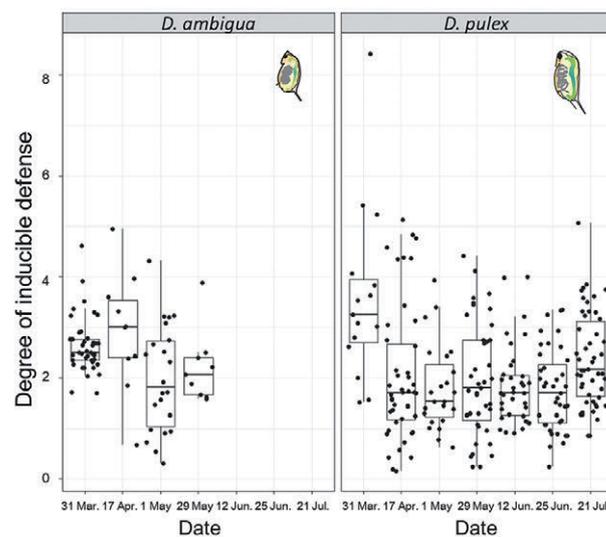

Fig. 3. Seasonal variation in degree of inducible defenses of *Daphnia ambigua* and *D. pulex*. The bar in the box, upper and lower box edges, and error bar represent the median, ±25% quantile, and 1.5 × ±25% quantile, respectively. Average of the PC score in February was set to zero point. Full values for all comparisons, by post-hoc testing, can be found in Tab. 1.

February could be assumed to be the non-defensive morphology (degree of inducible defense is zero, Fig. 3), because *Chaoborus* larvae were absent from the water column at daytime and migrate at night time (Nagano *et al.*, 2015). We calculated the degree of inducible defense of the species on each sampling date (Fig. 3). Both *Daphnia* species exhibited their highest degree of inducible defense around March. In the case of *D. ambigua*, no significant relationships were found for the observation period (Tab. 1). In contrast, *D. pulex* showed a significant difference between the degree of inducible defense observed in March and that observed on other dates (Tab. 1, Tukey-Kramer multiple comparison test). A comparison of seasonal changes in the degree of inducible defense exhibited showed no differences between *D. ambigua* and *D. pulex* (Species $F_{1,318}$=0.001, P=0.97; Seasons $F_{6,318}$=7.52, P<0.001; Species × Seasons $F_{3,318}$=5.64, P<0.001, two-way ANOVA, Fig. 2).

DISCUSSION

Despite many studies on inducible defense in laboratory experiments, research on inducible defense of wild daphnids has been limited. In this study, we found that inducible defense tends to increase during the season as
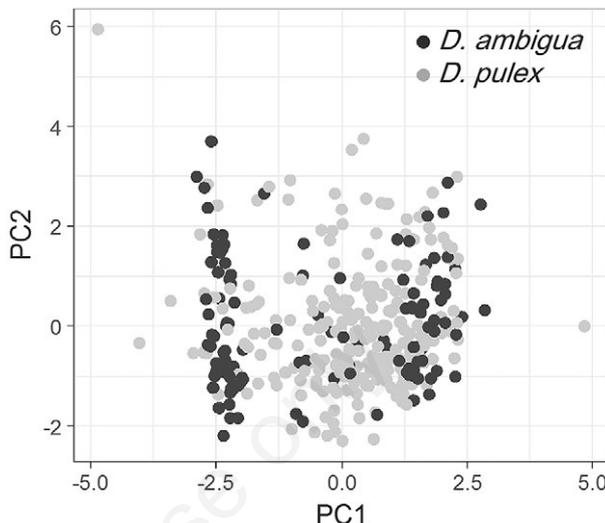
Fig. 4. Ordination of morphological values of *Daphnia ambigua* and *D. pulex* by principal components analysis (PCA).

Tab. 1. Results of Tukey-Kramer multiple comparison tests, comparing single pairs of the degree of inducible defense among *Daphnia* species and among seasons for each *Daphnia* species (99% confidence interval: Conf. interval lower/upper). P-values in bold indicate significant difference (P<0.05).

Treatment	Diff.	Conf. interval lower	Conf. interval upper	P-value	Diff.	Conf. interval lower	Conf. interval upper	P-value
Among species								
D. ambigua-D. pulex	-0.003	-0.335	0.329	0.979				
	within D. ambigua				*within D. pulex*			
Mar.31-Apr.17	-0.282	-1.656	1.091	1	1.559	0.39	2.729	**<0.001**
May.1-Apr.17	-0.928	-2.42	0.563	0.48	-0.234	-1.24	0.773	1
May.29-Apr.17	-0.721	-2.518	1.076	0.957	-0.067	-0.928	0.793	1
May.1-Mar.31	-0.646	-1.672	0.379	0.458	-1.793	-3.091	-0.495	**<0.001**
May.29-Mar.31	-0.439	-1.872	0.995	0.996	-1.627	-2.815	-0.438	**<0.001**
May.29-May.1	0.208	-1.34	1.755	1	0.1664	-0.862	1.195	1
Jul. 21-Apr. 17	-				0.327	-0.482	1.135	0.955
Jun. 12-Apr. 17	-				-0.189	-1.075	0.697	1
Jun. 25-Apr. 17	-				-0.281	-1.141	0.579	0.993
Jun. 12-Jul. 21	-				-0.515	-1.378	0.347	0.55
Jun. 25-Jul. 21	-				-0.608	-1.443	0.228	0.226
Mar. 31-Jul. 21	-				1.233	0.081	2.384	**0.004**
May 1-Jul. 21	-				-0.56	-1.545	0.425	0.633
May 29-Jul. 21	-				-0.394	-1.23	0.442	0.865
Jun. 25-Jun. 13	-				-0.092	-1.003	0.819	1
Mar. 30-Jun. 13	-				1.748	0.541	2.955	**<0.001**
May 1-Jun. 13	-				-0.045	-1.095	1.005	1
May 29-Jun. 13	-				0.121	-0.789	1.032	1
Mar. 30-Jun. 25	-				1.84	0.652	3.029	**<0.001**
May 1-Jun. 25	-				0.047	-0.981	1.076	1
May 29-Jun. 25	-				0.214	-0.672	1.099	1

Diff., difference between the average values among the compared groups.

predatory larvae emerge from sediment. *Chaoborus* population in our study lake emerged into water column and gradually gained height, and probably migrated vertically, but limited in this observation periods (Nagano *et al*., 2015). When the lower population of larvae changed their habitat to the water column from February to March, their degree of inducible defense showed highly. After February and March, the degree of inducible defense observed decreased, and remained constant even in the presence of a high population density of *Chaoborus* (Figs. 2 and 4). Although the degree of inducible defense of *Daphnia* has been shown to be associated with predator density, *i.e*., high kairomone concentration, in several empirical studies (Tollrian, 1993; Dennis *et al*., 2010; Miyakawa *et al*., 2015), wild daphnia may not be as straightforward as implied by these empirical studies. In another field study, Luecke and Litt (1987) suggested that the rate of neck-teeth production of *D. pulicaria* did not correlate with the density of *Chaoborus* larvae. Another study showed that the head length of *D. cristata* and *D. cuculata* correlated with predator density, but that of *D. hyalina* did not, based on a two years of field observation (Pijanowska, 1990). These results may suggest that the degree of inducible defense was not related to predator density, but related predators moving from sediment to the water column. *Daphnia* fundamentally expresses different sets of inducible defense against different predators (Boersma *et al*., 1998; Riessen, 1999; Boeing *et al*. 2006). Hence, the body lengths of *Daphnia* become smaller to defend against fish predation, because fishes are visual predators, and prefer larger-sized prey. Additionally, the tail length of *Daphnia* becomes longer to escape ingestion by fish (Dodson, 1989). On the other hand, fourth-instar *Chaoborus* larvae are gape-limited predators that are unable to ingest large-sized *Daphnia* (Swift and Fedrenko, 1975; Swift, 1992), thus, the neck spine of *D. pulex* and the head spine of *D. ambigua* are induced and elongated against *Chaoborus* larvae. Although bluegill and largemouth bass inhabited in the lake, the peak egg production of largemouth bass was before the beginning of May (Kawanobe and Hosoe, 2010). The morphological changes in *Daphnia* in March and April and their effects on fish predation could not be assessed, because lower water temperature may suppress the predation activity (Beitinger and Fitzpatrick, 1979). There were no coldwater fish, such as the pond smelt (*Hypomesus nipponensis*), in the lake (Tanaka, 1992), and February is overwintering season for fishes in the lake (Kawanobe and Hosoe, 2010). The body length of both *Daphnia* species in the lake increased in March. The head length of *D. ambigua* in April was longer than that in February. Although we investigated less type 3 morphologies (set of morphological defense against *Chaoborus* larvae), type 3 is exhibited in February (*D. ambigua*) or March (*D. pulex*). Then, we concluded that the

morphological changes observed in early spring tended to be more dependent on *Chaoborus* predation than fish predation. After early May, there is little change in the both of degree of inducible defense and univariate analysis, except for in the tail length. During this period, the density of the both predators will gradually increase. The inducible defense in natural *Daphnia* is not an effect of the predator density, or may offset the plasticity for both predators. Such field observations would allow us to clarify the relationship between the degree of prey plasticity and the predation regime, such as overwintering and hatching season.

It is important to simultaneously analyze multiple phenotypes, *i.e*., quantitative and qualitative traits of an individual. In our study, *Daphnia* individuals without any spines, *i.e*., type 2 morphology, were observed despite the presence of *Chaoborus* larvae (*D. ambigua* shows 11.1% on May 29th and *D. pulex* shows 9.5% in July). This type of *Daphnia* individuals may depend on other traits, *i.e*., behavior or life history, for their defense. Alternatively, *Daphnia* individuals that had spines were less prevalent during our observation period but were observed at a higher density of larvae (*D. ambigua* shows 4.2% in May 1st and *D. pulex* shows 12.7% in July). Hammill *et al*. (2008) scored two morphological traits of *D. pulex* and showed that the score increased as predator density increased. Dennis *et al*. (2010) quantified the levels of inducible defense of *D. pulex* using 12 iso-female lineages with 11 traits, and reported the levels converged at higher levels of predation risk, even for different populations. *Daphnia* is known to exhibit clonal succession in the field (Weider, 1985; Stibor and Lampert, 2000), so it is necessary to clarify the relationship between the gene analysis of clones and their traits using multivariate analysis by long-term field study and laboratory experiment in the future.

Previous studies have discussed the effectiveness of morphological changes. From a predation experiment, *D. pulex* with neck teeth reduced *Chaoborus*'s predation on them and were more likely to survive than other *Daphnia* without neck teeth (Tollrian, 1993). However, *Daphnia* individuals developed several types of inducible defenses at the same time (Boersma *et al*., 1998; Boeing *et al*., 2006). Hence, we supposed that the adaptation of *Daphnia* was to incorporate the integrative phenotypic plasticity. Then, we first proposed to estimate the "degree of inducible defense" as an index of integrative phenotypic plasticity using Euclidean distance, and we observed that the degree of the natural *Daphnia* population changed along with *Chaoborus* density in early spring. We only focused on the morphological traits for the Euclidean-distance based approach, while this method can also be used for the other traits, *i.e*., life history (Weider and Pijanowska, 1993) and behavioral traits (Spaak and Ringelberg, 1997). *Daphnia* responds to

chemical cues from predators with multiple traits simultaneously. *Daphnia* temporarily take refuge against *Chaoborus* larvae and fish in deep water columns or anoxic strata (Hanazato *et al*., 1989; Salonen and Lehtovaara, 1992; Spaak and Ringelberg, 1997; Vanderploeg *et al*., 2009; Larsson and Lampert, 2011). In fact, *Daphnia* in the study lake might also show this defensive behavior with diel vertical migration (DVM), since an anoxic layer was observed in this lake (Nakagawa *et al*., 2012; Suda *et al*., 2016). *Chaoborus* larvae also showed DVM in the lake against fish predation (Nagano *et al*., 2015). In future studies, it is important to integrate these multiple defensive traits into the analysis, as well as consider how *Daphnia* adapts to their multiple traits against predation pressures.

Even though inducible defense studies originally started with descriptive observations, in recent years most such studies have been laboratory experiments. Evidence of inducible defense observed in natural environment is, however, of increasing importance in predicting anthropogenic effects on environmental changes. For example, the response of freshwater plankton to environmental shifts has been evaluated by studies on the effects of climate change (Chevin *et al*., 2013; Merilä *et al*., 2014) and of urbanization (Alberti *et al*., 2017). A zooplankton species, *D. retrocurva*, does not produce defensive traits or show defensive behavior, due to the use of artificial lights at night (Moore *et al*., 2000). In experimental settings, *Daphnia* has generally been made accustomed to high predator density over a period (usually three generations) (Hammill *et al*., 2008). In contrast, *Daphnia* in the field do not become accustomed to an environment with a constant predator density, but one that has seasonal dynamics. We recognize these differences between natural and experimental environments and suggest that this could explain the degree of inducible defense of *Daphnia* in wild populations.

CONCLUSIONS

Inducible defenses in wild *Daphnia* shown highly in March, late winter. After March, the degree of inducible defense decreased and became stable until July, early summer. The trends were similar among two *Daphnia* species, *D. ambigua* and *D. pulex*. We observed that in the distribution of *Chaoborus* changed only from sediments to the water column in the daytime. Additionally, we considered that the predation activity of the larvae and fish were still limited. The degree of inducible defenses was increased at high predator density and/or predatory kairomone concentrations by numerous laboratory experiments. For wild *Daphnia* in the lake, however, the changing predator population levels after overwintering seems important for the degree of inducible defense. We

emphasize that it is even now important to investigate wild individuals and to conduct surveys in the field to further our understanding of inducible defense mechanisms.

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