Experimental evidence that induced defenses promote coexistence of zooplanktonic populations

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Received April 2, 2010; accepted in principle July 15, 2010; accepted for publication September 13, 2010

Corresponding editor: Beatrix E. Beisner

Theoretical predictions suggest that adaptive phenotypic plasticity, and induced defenses in particular, exert a stabilizing effect on ecological systems and increase the likelihood of species coexistence. Nonetheless, up to now, there is little empirical support for this hypothesized mechanism of diversity preservation. We experimentally assessed the effects of induction of plastic morphological responses triggered by a predator kairomone, on patterns of co-occurrence of two herbivore populations of rotifers (Brachionus calyciflorus and B. havanaensis) sharing resources (Chlorella vulgaris) and predators (Asplanchna brightwelli). To our knowledge, this is the first experimental work conducted at the population level where non-induced and induced states of same prey species are obtained through manipulating the level of predator signal. Our objective was to assess the consequences of induced defenses on the mean population density, population variability, evenness and likelihood of persistence of competitor populations. Our results show that induced defenses promote species coexistence through increasing the likelihood of persistence and evenness of competing populations, over a gradient of resource availability.

KEYWORDS: Non-lethal effects; phenotypic plasticity; stability; rotifers; persistence

INTRODUCTION

Since the pioneering work of Gardner and Ashby (Gardner and Ashby, 1970) and May (May, 1972), ecologists have increasingly accepted that the persistence of populations embedded in natural multispecific assemblages cannot be understood without considering biological stabilizing mechanisms. Adaptive predator and prey responses to changes in the abundance or behavior of interacting species have been postulated as important stabilizing factors in food webs of different complexity (Ives and Dobson, 1987; Abrams, 2000; Kondoh, 2003, 2007).

Inducible antipredator defenses (Harvell, 1990) constitute a type of adaptive prey response that decreases predation success via shifts in behavioral, morphological or life-history traits of prey perceiving predator cues (see Tollrian and Harvell, 1999). A number of theoretical studies have explicitly addressed the effects of inducible...
defenses on the local stability and persistence probability of model systems consisting of a few interacting populations (Ives and Dobson, 1987; Ramos-Jiliberto et al., 2002; Ramos-Jiliberto, 2003; Rinaldi et al., 2004; Vos et al., 2004a,b; Yamauchi and Yamamura, 2005; Ramos-Jiliberto et al., 2008a,b), up to large complex communities (Kondoh, 2003, 2007). These theoretical results support the conclusion that the presence of induced defenses exerts a stabilizing effect on the model systems and increase the likelihood of species coexistence.

The suggested mechanism underlying the stabilizing effect of induced defenses is as follows. At low predator abundances, prey exhibits an inexpensive basic uncommitted state, which favors their growth and indirectly that of their predators. A consequent increase in predator density induces prey to exhibit modified traits that reduce their vulnerability (e.g. longer spines relative to those present in the basic morph), decreasing predators’ feeding and per capita growth. In addition, the growth of the prey decreases, due to the costs of carrying their arms. Therefore, adaptive prey defenses are expected to create negative feedback loops that prevent strong population oscillations, enhancing community stability and persistence (Abrams, 1984; Kopp and Gabriel, 2006; Dambacher and Ramos-Jiliberto, 2007). Nevertheless, when more than a single species per trophic level is considered to exhibit inducible defenses, the dynamic outcome and underlying mechanisms are little understood (van der Stap et al., 2008; Garay-Narváez and Ramos-Jiliberto, 2009).

Despite the extensive evidence on the prevalence of induced defenses across many taxa and ecosystems (Harvell and Tollian, 1999), and the soundness of modeling developments, there is still a wide gap between data and theory regarding the role played by adaptive prey responses in the puzzle of biodiversity maintenance. The empirical validation of theoretical proposals has not been fully convincing, mainly due to the inherent difficulties in manipulating the level of responses of the experimental organisms to inductor signals (Underwood and Rausher, 2002; Verschoor et al., 2004; van der Stap et al., 2006, 2007, 2008). Previous studies have found difficulties in maintaining a control consisting of a population composed of basic non-induced organisms under predation pressure, in order to contrast its properties with the population of organisms displaying an induced response.

In this work, we advance our understanding of the relationship between phenotypic plasticity and community stability. This is done by performing a controlled experiment to evaluate the effects of induction of morphological responses triggered by predator kairomones, on the coexistence of two competing zooplankton species (each as a single clone) in populations that share predators and resources. We developed a trirophic experimental model system with four species: two herbivorous rotifers (Brachionus calyciflorus and B. havanensis), a single resource (Chlorella vulgaris) and a common predator (Asplanchna brightwelli) that is able to induce morphological defenses in the two herbivores. To isolate the impact of the induced defenses, we were able to separate the lethal effect of predators from the net effect, by manipulating the level of inducing signal (kairomone) in the simple experimental communities. Our results present experimental evidence that induced defenses promote species coexistence, increasing the evenness and likelihood of persistence of animal populations.

**METHOD**

**Experimental organisms**

Predator Asplanchna brightwelli and the herbivores B. calyciflorus and B. havanensis were isolated from two temperate lakes from central Chile: Pitama (33°27’S–70°49’W) and Rapel (34°09’S–71°26’W). Clonal cultures were initiated from a single amictic female and kept under laboratory conditions for 2 months prior to experiments. To maintain the cultures and carry out the experiments, we used artificial freshwater (U.S. EPA) enriched with vitamins: thiamine (100 mg L⁻¹), biotin (0.5 mg L⁻¹) and cyanocobalamine (0.5 mg L⁻¹).

Prey rotifers in the stock cultures were fed daily with the green alga Chlorella vulgaris at a density of 0.5 × 10⁶ cells mL⁻¹, which was grown in axenic 2 L glass bottles with Bold basal medium (Borowitzka and Borowitzka, 1988). The algae were harvested in the exponential growth phase, centrifuged at 3000 rpm for 5 min and re-suspended in EPA water prior to presenting it as food. Algal density was estimated by cell counting in a Neubauer chamber.

The predator A. brightwelli was cultured in 500 mL glasses and fed daily ad libitum with a Brachionus mixture (B. calyciflorus, B. caudatus, B. havanensis and B. patulus). The culture media were replaced daily.

Standard culture and experimental conditions were: pH 7.5 ± 0.1, temperature 25 ± 0.5°C and diffuse fluorescent lighting with 12:12 (light:dark) photoperiod.

**Plastic response to predator kairomones**

An independent assay was run in order to measure the morphological response of prey to low and high kairomone concentrations. Signal concentrations of 5 and
100 Asplanchna per liter were chosen as the low and high conditions, respectively, based on previous laboratory measurements and reported field abundances (Nandini, 1999). In this test, B. calyciflorus and B. havanaensis, at an initial density of 1 ind. mL⁻¹ in 150 mL, were exposed to low and high kairomone levels, in addition to a control (without signal) level. Control and treatment media were prepared daily as follows. The control medium consisted of EPA freshwater pre-conditioned with the mixture of rotifers used as prey for Asplanchna. Kairomone treatments were carried out with predator-conditioned medium, which was prepared daily with EPA water, containing the corresponding Asplanchna densities. Asplanchna was fed with a mixture of brachionids at a density of 4 ind. mL⁻¹. Every 24 h, all rotifers were removed, the media were filtered through a 0.45 µm membrane and the pH adjusted to 7.5 ± 0.1 through addition of 1N HCl when required. Once the control and treatment media had been set up, C. vulgaris at a density of 10⁶ cells mL⁻¹ was added as food. The media were distributed into 18 experimental jars, consisting of six treatments (2 rotifer species × 3 kairomone levels) × 3 replicates. Rotifers were transferred daily to freshly prepared medium. After 6 days, 50 egg-bearing prey individuals were sampled from each experimental jar and fixed in 5% formalin. Digital images of each animal were obtained under a dissecting microscope, from which the lengths of the anterior and posterior spines were measured.

Differences between morphological measures among treatments were evaluated, for each species, by means of t-test after verification of parametric assumptions.

Coexistence experiments

Levels of morphological induction were set from the results of the experiment described in the previous section (5 and 100 Asplanchna per liter), following the same procedures for the preparation and renewal of treatment media. At the “low kairomone concentration” level, predators were presented at a fixed low density (5 Asplanchna per liter). The concentration of kairomones resulting from such a predator density did not induce morphological defenses in the clones tested, but lethal effects occurred (see results). At the “high kairomone concentration” level, predators are present at the same low density (5 Asplanchna per liter), but the medium was conditioned with a predator density of 100 Asplanchna per liter. This allowed us to evaluate lethal effects of predation together with the induction of morphological defenses. The experimental design consisted of 2 (kairomone levels) × 4 (food densities: 0.5, 1, 2 and 4 × 10⁵ cells mL⁻¹ of C. vulgaris) × 4 (replicates), resulting in 32 experimental units. Every beaker containing 200 mL of medium was initiated with 200 individuals of each prey species and on individual Asplanchna. The initial groups of brachionids used in the induction treatments were grown in the predator-conditioned medium for a period of 7 days prior to beginning the experiment. This ensured that founder individuals exhibited defenses from the first experimental day.

Predator density was maintained constant, through replacing dead or inactive individuals and removing each newborn, after an exhaustive search of each beaker performed each 12 h. Maintaining a constant predator density during the experiment allows a clearer separation of the net effect of predation between lethal and non-lethal effects.

Herbivorous rotifers from each experimental beaker were counted every 24 h, using three to four 10 mL aliquots. After this, all the rotifers were transferred to new medium using a flame-narrowed pipette. Coexistence experiments were terminated after 16 days, when brachionid populations in most replicates began to stabilize.

Population density of brachionids was analyzed based on their mean values and population variability of de-trended density over time (excluding an initial transient phase of 6 days) by means of one-way ANOVA and Bonferroni post hoc tests. Raw data were log-transformed in order to satisfy parametric assumptions.

Pielou’s evenness index \( J' \) was estimated daily, as \( J' = H' / \ln S \), where \( H' \) is Shannon’s diversity index and \( S \) the number of species (Pielou, 1966). Pielou’s index was estimated based on the densities of both brachionid species in order to obtain a measure of the homogeneity of herbivore densities. This index was considered adequate due to its symmetry, sensitivity and range of variation, and because it is comparable with many other evenness indices (Biesele et al., 2003). Finally, a population viability analysis (PVA) was performed following Dennis et al. (Dennis et al., 1991) for the estimation of mean and variance of the growth rate. This analysis was developed in order to roughly assess the probability of population persistence from the observed variation of rotifers’ abundances, and not for calculating the viability of endangered populations. Under this approach, the extinction probability \( P(\epsilon) \) of each replicate was estimated on a 60-day time horizon. A population was considered extinct when its density dropped below 0.002 ind. mL⁻¹, corresponding to <0.5% of initial densities. The calculated persistence probabilities \( 1 - P(\epsilon) \) were analyzed using two-way ANOVA, after arcsine transformation, with resource density and defense induction as factors.
RESULTS

Spine elongation in both prey species was effectively induced by the high kairomone level (100 *Asplanchna* per liter), reaching 40% of elongation of anterior spines relative to the control (without kairomone) in both species, and 100 and 60% of elongation of posterior spines in *B. calyciflorus* and *B. havanaensis*, respectively. On the other hand, anterior and posterior spines of *B. calyciflorus* exposed to a low kairomone level (5 *Asplanchna* per liter) showed a slight increase of less than 20%. In the case of *B. havanaensis*, spines were marginally lower (anterior spines) or of similar length as those measured in control individuals (Fig. 1). We considered induction of morphological defenses as a significant elongation of both spines relative to measures obtained from control individuals, and not a novo formation of spines. From these results, it was considered that at the low kairomone level, the organisms were not induced to exhibit morphological defenses, and at the high kairomone level the organisms were effectively induced. Although body sizes were measured in both species, no significant differences were observed between treatments and control.

Without induction of morphological defenses at the low kairomone level, mean population density of *B. calyciflorus* increased with resource density, while *B. havanaensis* reached its highest mean density at a low resource level (Fig. 2 and Supplementary data, Fig. S1). Also, population densities of *B. calyciflorus* showed larger variations at highest resource levels, unlike *B. havanaensis* whose densities were more variable at lowest resource densities. With induced defenses at the high kairomone level, mean density values of both species were homogeneous over the entire resource gradient (at about 0.5 ind. mL$^{-1}$, see Supplementary data, Fig. S1), and interspecific differences were only significant at the highest level of resource. In addition, where defenses were induced, variability of density trajectories decreased consistently in both species and did not differ between species over the resource gradient (Supplementary data, Fig. S1).

A higher evenness among population densities of the herbivores was observed in the induction treatments, as revealed by the dynamics of the Pielou’s index $J'$ (Fig. 3). In the absence of induction at the low kairomone level, evenness declined over time and more abruptly at lower resource densities. With induced defenses at the highest kairomone level, $J'$ remained virtually constant over time for all resource densities.

Finally, the results of the PVA (Fig. 4) show that induced defenses promoted a significant increase in the persistence probability of herbivores. This applies to *B. calyciflorus* across the entire resource gradient and to *B. havanaensis* at all but the lowest resource level, where the persistence probability remained quite high (Fig. 4). Homogenization of abundances between species, on the gradient of resources and along trajectories of each species (Figs 2 and 3) was translated into a homogenization of the values of $r$, which render the results of the PVA.

DISCUSSION

Induced defenses of *B. calyciflorus* and *B. havanaensis*, in response to the kairomone of *A. brightwelli*, produced a homogenization between herbivore population densities and the decreased variability of their dynamics: high densities decreased, low densities increased, while temporal oscillations were dampened, so that both species were nearly equally abundant and less variable over time. This competitive equivalence promoted by phenotypic plasticity led to either maintaining or increasing the persistence probability of herbivores over all tested...
Fig. 2. Population trajectories (mean density ± 95% CI) of *B. calyciflorus* and *B. havanaensis* in media with the low (left column) and high (right column) kairomone concentration. Prey was subjected to predation by *Asplanchna* and was competing for a single resource (*C. vulgaris*). Resource densities are given within the plots.
resource levels. Furthermore, the induction of antipredator defenses suppressed the resource-dependence that, in the absence of antipredator response effects, exhibited the mean abundance, stability and persistence of herbivore populations.

Rotifers show large interspecific and even interclonal variability in the development of spines in response to the presence of predatory zooplankton. Postero-lateral spines of non-induced \textit{B. calyciflorus} have been reported to be absent (Halbach, 1971), of 20 μm long (Stemberger, 1990), or 40 μm long (present work). Although the clones used in our experiments exhibit posterior or postero-lateral spines even without being exposed to an inducing signal, the incorporation of a high kairomone concentration caused a significant increase in length of the structures, which can be considered an effective induction. Other experiments performed with the same isolated herbivores (Aránguiz-Acuña et al., 2010) indicated that induced morphs of both \textit{B. calyciflorus} as \textit{B. havanaensis} were less attacked by \textit{Asplanchna}, and more time was required by predators to handle induced prey. This supports the fact that the morphological changes observed in both clones exposed to the high level of kairomones are effective defenses against predation by \textit{Asplanchna}.

Although uncovering the precise mechanisms by which induced defenses promote biodiversity maintenance requires dedicated experiments, our results could be explained by means of current ecological knowledge about differential abilities in resource use (Leibold, 1996), interacting with differential abilities and costs of predator avoidance tactics (Chesson and Kuang, 2008).

At a low kairomone density, where only consumption effects took place, \textit{B. havanaensis} was able to reach higher densities at low resource levels, as compared with its congeneric \textit{B. calyciflorus}. Conversely, higher resource levels favored the growth of \textit{B. calyciflorus}. Previous experiments conducted with non-induced clones of both competing species showed that this trend held also in the absence of predators (Supplementary data, Fig. S2). Thus, the incorporation of predation decreases the mean abundances of the two prey over the resource gradient due to consumption, without changing the pattern of dominance. On the other hand, when defenses were induced by the addition of the kairomone to the predation treatments (Fig 2, right column), dominant prey decreased their mean abundance, while the subordinate competitor increases or maintains its abundance.

We propose that at the low resource level, the induction of defenses drove the density increase in \textit{B. calyciflorus} due to diminished mortality as a consequence of effective antipredator responses (e.g. Gilbert, 1966, 1999). This can be seen by comparing the top left and right plots in Fig. 2. The density increase in \textit{B. calyciflorus} led to a decrease in \textit{B. havanaensis} likely due

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**Fig. 3.** Pielou's evenness index $J'$ of prey rotifers over time (mean ± 95% CI). Prey was exposed to predation with the low or high kairomone level. Resource densities are given within the plots.

**Fig. 4.** Persistence probability (mean ± 95% CI), estimated in a community experiment, of \textit{B. calyciflorus} (filled bars) and \textit{B. havanaensis} (open bars) as a function of the resource level. Prey was exposed to predation with the low (left) or high (right) kairomone level.
to stronger food limitation. This process promoted the
equalization of the abundances of both herbivores (see
Fig. 2, top right, and Supplementary data, Fig. S1, top
right). On the other hand, at the highest food level, B.
havanaensis did not change its mean abundance with the
addition of kairomones (Supplementary data, Fig. S1,
top plots). This suggests that the costs of defenses
observed in Supplementary data, Fig. S2 (bottom plots)
were not overwhelmed by the net benefits of decreased
mortality. Therefore, the defense effectiveness of B. hav-
anaensis appears to be relatively weak, especially at the
highest food level. Since there was no significant change
in the abundance of B. havanaensis at the highest food
level, the effects of the treatments on the dynamics of its
competitor allow us to discard changes in resource
availability. The species B. calyciflorus showed evidence
of high costs at intermediate and high food levels (compare bottom left and right plots of Supplementary
data, Fig. S2). Therefore, the addition of the kairomone
promoted the homogenization of trajectories at the
initial phase of population growth (left column of Fig. 2)
and sustained higher abundances in later phases as a
product of their defenses. Similar to B. havanaensis, the
effectiveness of defenses of B. calyciflorus decreases at the
highest resource level. This could be explained because
higher resources sustain higher herbivore density, which
offers a protection by numbers given the saturating (i.e.
type II) functional response of Asplanchna (Nandini and
Sarma, 1999; Sarma and Nandini, 2007). Costs of
defenses in isolated populations of rotifer prey can be
explained by reduced fertility or delayed reproduction
(Gilbert, 1980, 2009; Epp and Lewis, 1984; Stemberger,
1988; Zagarese and Marinone, 1992; Relyea, 2002). In
interacting populations, ecological costs (such as reduced
competitive ability) could also take place (Strauss et al.,
2002; Garay-Narváez and Ramos-Jiliberto, 2009). The
results of this study suggest that induced defenses of
herbivores in competition, promote evenness of their
abundances over a gradient of resource availability. This
pattern is arguably driven by diminished mortality
acquired from antipredator protection, together with the
release of resources and the effects of exhibiting defenses.
This results in the suppression of numerical dominance
between the herbivores, at all resource levels, which
increases the persistence probability of both rotifer
populations.

Prior experimental work suggested that induced
defenses in primary producers enhance a herbivore’s
density dependence (Underwood and Rausher, 2002),
preventing population fluctuations in herbivores
(Verschoor et al., 2004; van der Stap et al., 2006), and
could increase the persistence probability of competing
herbivores and their predators (van der Stap et al.,
2008). Nonetheless, those studies did not compare the
dynamics among systems including the same species/
strain of plant in induced versus non-induced states.
Overcoming this drawback, our work introduces a
methodological improvement based on an appropriate
manipulation of Asplanchna densities, adjusted for dis-
tribution between induction (non-lethal) effects and
net effects of the predator. To our knowledge, this is the
first experimental work where the population dynamics
consequences of induced defenses are assessed after
manipulating the level of phenotypic response in the
same animal populations. As with any experimental
system, which is necessarily a simplification of nature,
our results cannot be directly extended to natural con-
ditions. In the wild, rotifer species are composed of
many clones, and density of predators fluctuates, linked
with kairomone dynamics. Nevertheless, our interest in
this study was not focused on explaining species-specific
reactions, but rather on identifying the potential rel-
ance of plastic defensive responses as a driving mech-
anism of coexistence between competitors.

Our results support the hypothesis that induced
antipredator defenses promote stability of populations
and thus facilitate biodiversity maintenance. These
findings help explain the prevalence of complex
ecological models as useful tools for understanding
nature.

SUPPLEMENTARY DATA
Supplementary data can be found online at http://
plankt.oxfordjournals.org.

ACKNOWLEDGEMENTS
We thank M.I. Olmedo from the Biodiversity
Laboratory at CENMA for her support throughout this
investigation, and S.A. Navarrete for his helpful sugges-
tions on an earlier version of this work.

FUNDING
A.A.-A. acknowledges the support of a CONICYT do-
toral scholarship, grant ICM-P05-002 to Institute of
Ecology and Biodiversity, technical support in
laboratory from Universidad Andrés Bello and a grant for foreign research stay from the Vicerrectoría de Asuntos Académicos, Universidad de Chile. R.R.-J. received partial support from grant FONDECYT 1090132.

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