

UNIVERSIDADE FEDERAL DO PARANÁ

LUIZ GUILHERME DOS SANTOS RIBAS

COMPLEMENTARY EFFECTS OF NON-NATIVE AND NATIVE
ORGANISMS ON THE ESTABLISHMENT AND PROPAGULE
PRESSURE OF THE INVASIVE MACROPHYTE *Hydrilla verticillata*

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OF THE INVASIVE MACROPHYTE *Hydrilla verticillata*

Dissertação apresentada ao Curso de Pós-Graduação em Ecologia e Conservação, Setor de Ciências Biológicas da Universidade Federal do Paraná, como requisito parcial para obtenção do grau de Mestre em Ecologia e Conservação.

Orientador: Prof. Dr. André
Andrian Padial

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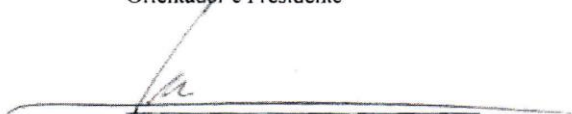
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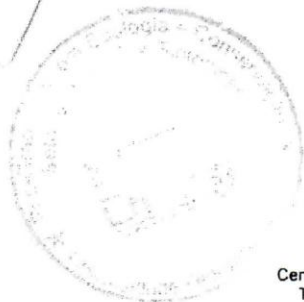
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“A mente que se abre a uma nova ideia jamais voltará ao seu tamanho original”

Albert Einstein

RESUMO

Hydrilla verticillata (L.f.) Royle é uma das espécies de macrófitas invasoras mais críticas. Diversas características abióticas são relacionadas ao seu sucesso de invasão, porém ainda é incerto como as interações biológicas com a fauna residente podem afetar este sucesso. Na ecoregião do Alto Rio Paraná, a hydrilla invadiu com sucesso o canal principal do Rio Paraná, porém não obteve sucesso invadindo ambientes de lagos da planície de inundação do rio. Além de certo filtros abióticos a fauna nativa e não-nativa pode impor resistência a invasão da hydrilla. Utilizando métodos empíricos, selecionamos três espécies de organismos aquáticos que potencialmente provem resistência ao estabelecimento da hydrilla em lagos da planície de inundação. Essas espécies foram selecionadas para representar as mais prováveis interações ecológicas que podem impactar negativamente o estabelecimento da hydrilla: um peixe herbívoro e um gastrópode herbívoro, ambos nativos, e um peixe fragmentador não nativo invasor. Ambos os efeitos isolados e em conjunto dessas espécies foram avaliados experimentalmente, utilizando tratamentos que afetam diferentes facetas da performance da hydrilla em escala local. O gastrópode nativo é o organismo que mais limita o crescimento devido a herbivoria; enquanto que o peixe fragmentador não-nativo invasor rompe as plantas do sedimento e previne o estabelecimento local. Nossos resultados sugerem que a resistência biótica e distúrbios em escala local são importantes fatores que reduzem o estabelecimento da macrófita invasora *Hydrilla verticillata* nos lagos dessa planície de inundação Neotropical. Entretanto, por romper as plantas em escala local, o peixe fragmentador não nativo invasor pode aumentar a pressão de propágulos, favorecendo o estabelecimento da planta em escalas maiores.

Palavras-chave: invasão biológica, espécies exóticas, resistência biótica, ecologia animal-plantas, interação entre espécies.

ABSTRACT

Hydrilla verticillata (L.f.) Royle is one of the most critical invasive macrophytes. Several abiotic characteristics are related to its invasion success, but it is still uncertain how biological interactions affect this success. In the Upper Paraná Freshwater ecoregion, hydrilla has invaded the main channel of Parana river but has been unable to establish in floodplain lakes. Along with abiotic filters, native and non-native species in lakes can impose resistance to hydrilla invasion. Using empirical methods, we selected three species of aquatic organisms that potentially provide resistance to hydrilla establishment in floodplain lakes. These species were chosen to represent the most probable ecological interactions that can negatively impact hydrilla establishment: an herbivore fish and an herbivore gastropod, both natives; and an invasive non-native shredder fish. Both the isolated and joint effects of these species were evaluated experimentally using treatments that affect different facets of hydrilla performance on a local scale. The native gastropod was the organism that mostly limited plant growth due to herbivory; whereas the invasive non-native shredder fish disrupted plants from the sediment and prevent their local establishment. Our results suggest that biotic resistance and small-scale disturbance play an important role in reducing the establishment of the invasive macrophyte *Hydrilla verticillata* in lakes of this Neotropical floodplain. However, by disrupting plants at a local scale, the invasive non-native fish may increase propagule pressure, favoring plant establishment at a broad landscape scale.

Keywords: biological invasion, exotic species, biotic resistance, animal-plant ecology, species interactions.

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APRESENTAÇÃO

Essa dissertação é apresentada no formato de um artigo científico que está submetido a uma revista especializada em biologia e ecologia de invasões, intitulada *Biological Invasions*, publicada pela *Springer International Publishing*. O manuscrito está editado conforme as normas da respectiva revista. O artigo tem como intuito entender como interações biológicas entre a macrófita invasora *Hydrilla verticillata* (L.f.) Royle e a fauna residente de lagos da planície de inundação do Alto Rio Paraná pode controlar a invasão desta planta em tais ambientes. Tendo em vista que as condições ambientais dos ambientes lênticos formados pelas águas do Rio Paraná não podem explicar o insucesso da invasão da hydrilla, realizamos experimentos com três espécies de ecologia distintas e tipicamente dos ambientes lênticos do Rio Paraná para tentar entender como a resistência biótica pode controlar o estabelecimento desta macrófita invasora.

Tal experimento foi delineado a fim de representar as condições de ambientes lênticos do Rio Paraná, no qual a variável avaliada é referente ao sucesso e a performance vegetativa da hydrilla mediante diferentes tratamentos, dentre quais é testado o efeito de cada espécie sobre a planta e da combinação das três espécies sobre a planta. Tais tratamentos são representados por uma espécie de peixe herbívoro nativo de pequeno porte, um peixe fragmentador invasor, um gastrópode herbívoro nativo e, por fim, um tratamento com a combinação das três espécies. Mais detalhes são apresentados nas seções seguintes e no decorrer do manuscrito.

O experimento foi realizado em 10 dias e nos apresentou resultados interessantes. A resistência biótica pode ser um fator determinante no controle da invasão da hydrilla. Os tratamentos que mais afetam a planta são os tratamentos com peixe fragmentador invasor e gastrópode herbívoro nativo, porém o tratamento com as três espécies é o que apresenta efeito complementar na resistência biótica. Esse trabalho inova por apresentar a relação do insucesso de invasão da hydrilla por meio de fatores biológicos e não abióticos como fora extensivamente descrito na literatura.

COMPLEMENTARY EFFECTS OF NON-NATIVE AND NATIVE ORGANISMS ON THE ESTABLISHMENT AND PROPAGULE PRESSURE OF THE INVASIVE MACROPHYTE

Hydrilla verticillata

Luiz Guilherme dos Santos Ribas¹, Eduardo Ribeiro Cunha², Jean Ricardo Simões Vitule¹, Roger Paulo Mormul², Sidinei Magela Thomaz² and André Andrian Padial^{1,2}

¹ Pós-graduação em Ecologia e Conservação (PPGECO), Universidade Federal do Paraná (UFPR). Av. Coronel Francisco H. dos Santos, P.O. Box: 19031, CEP: 81531-990. Curitiba, PR, Brazil. email: lg_ribas@hotmail.com, Telephone: +55 41 9608-7703

² Pós-graduação em Ecologia de Ambientes Aquáticos Continentais, Núcleo de Pesquisa em Limnologia, Ictiologia e Aqüicultura (Nupélia), Universidade Estadual de Maringá (UEM). Av. Colombo, 5790, bl. H-90, CEP: 87020-900. Maringá, PR, Brazil.

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Abstract

Hydrilla verticillata (L.f.) Royle is one of the most critical invasive macrophytes. Several abiotic characteristics are related to its invasion success, but it is still uncertain how biological interactions affect this success. In the Upper Paraná Freshwater ecoregion, hydrilla has invaded the main channel of Parana River but has been unable to establish in floodplain lakes. Along with abiotic filters, native and non-native species in lakes can impose resistance to hydrilla invasion. Using empirical methods, we selected three species of aquatic organisms that potentially provide resistance to hydrilla establishment in floodplain lakes. These species were chosen to represent the most probable ecological interactions that can negatively impact

hydrilla establishment: an herbivore fish and an herbivore gastropod, both natives; and an invasive non-native shredder fish. Both the isolated and joint effects of these species were evaluated experimentally using treatments that affect different facets of hydrilla performance on a local scale. The native gastropod was the organism that mostly limited plant growth due to herbivory, whereas the small herbivore fish mostly consumed hydrilla leaves and the invasive non-native shredder fish disrupted plants from the sediment and prevent their local establishment. Our results suggest that biotic resistance and small-scale disturbance play an important role in reducing the establishment of the invasive macrophyte *Hydrilla verticillata* in lakes of this Neotropical floodplain. However, by disrupting plants at a local scale, the invasive non-native fish may increase propagule pressure, favoring plant establishment at a broad landscape scale.

Keywords: biological invasion, exotic species, biotic resistance, animal-plant ecology, species interactions.

Introduction

The original idea of biotic resistance proposed by Elton (1958) suggests that some invaders are absent because of specific ecological interactions with resident species. Probably, some resident species are most prone than others to repel or regulate potential invaders. In the particular case of plant invasions, herbivores and other drivers of physical disturbance play a significant role in regulating the establishment of non-native species (Maron and Vilà 2001; Colautti and MacIsaac 2004; Levine et al. 2004; Parker et al. 2007). The regulation of establishment includes decreased individual performance and increased mortality rates due to the consumption of structures or the entire individual (Virtanen et al. 1997; Hulme 1998; Sirotinak and Huntly 2000; Maron and Vilà 2001). Non-consumptive interactions such as breeding and foraging activities can also injure plants by physical disturbance (e.g., Anastácio et al. 2005; Landis and Lapointe 2010). Thus, resident fauna can decrease the establishment of invasive plants through small-scale disturbances and especially affect the initial establishing stage (Hobbs and Huenneke 1992; Davis et al. 2005).

Fish and aquatic invertebrate species may affect the establishment of macrophyte species (e.g., Wheeler and Center 2001; Baker et al. 2010). However, despite some notorious cases showing resistance to macrophyte invasion by resident biota (Parker and Hay 2005), the underlying mechanisms of biotic resistance are not completely understood in aquatic ecosystems. Because the effects of biotic resistance by different taxa are commonly addressed separately, most investigations provide fragmented pictures of the resistance posed by resident fauna to plant invasions. For example, few studies separately showed that fish (Wanda et al. 2011), gastropods (such as *Pomacea insularum*; Baker et al. 2010) and immature stages of aquatic insects (like

Hydrellia spp.; Wheeler and Center 2001) may injure, decrease the growth and hamper the establishment of *Hydrilla verticillata* (L.f.) Royle, an invasive submerged macrophyte of great concern. However, these studies using enemy species separately do not allow concluding on the individual or combined effects of multiple species, a situation that is expected in the field.

Hydrilla has invaded freshwater ecosystems worldwide, causing changes in species composition (e.g., Posey et al. 1993; Theel et al. 2008; Mormul et al. 2010) and having negative effects on navigation, fisheries and leisure activities (Monterroso et al. 2011). Despite the well-known abiotic filters that negatively affect the growth of hydrilla (e.g., Barko and Smart 1983; Gu 2006; Sousa et al. 2009; Wu et al. 2009; Yu et al. 2010; Silveira and Thomaz 2015), the role played by biotic resistance is not well elucidated, most likely because of complex and unknown interactions with resident biota.

In the Upper Paraná Freshwater ecoregion, hydrilla has been recorded in prolific abundances since it started colonization (c. 2005), but this plant has been restricted to the main river and secondary channels (Sousa 2011). When found in lakes in the Upper Paraná River floodplain, contrary to what is found in the main channel, hydrilla develops small biomass and soon disappears, never outcompeting resident species (like *Egeria najas* Planchon and *Egeria densa* Planchon; Sousa et al. 2010). The presence of only young plants that soon disappear in lakes indicates that hydrilla is able to sprout new plants but not to develop successfully (establish a new population) in these lentic ecosystems. In addition, despite lakes presenting abiotic characteristics that limit hydrilla development (e.g., high levels of sediment organic matter; Silveira and Thomaz 2015), most of the variation in hydrilla biomass does not seem to be explained by abiotic variables (~65%; Sousa et al. 2010), indicating that biotic factors may play a role on unsuccessful early establishment of hydrilla in lakes. Establishment of new propagules is a critical stage to hydrilla given the lack of sexual propagation.

Accordingly, several indications suggest that biotic resistance helps suppress hydrilla in lakes of the Upper Paraná River floodplain. For example, hydrilla is frequently consumed by native fish species of the genus *Moenkhausia* (N.Carniato, R. R. Braga and L. G. S. Ribas, personal observation) and *Schizodon* (Isaac et al. 2014), and by the apple snail *Pomacea* spp., which is very abundant in floodplain lakes (C. Calvo, unpublished; see also Baker et al. 2010). In addition to these herbivores, the abundant and invasive non-native fish *Astronotus ocellatus* (Agassiz 1831) mechanically disrupts hydrilla (authors' observation) by performing a shredder activity. Although this species may decrease hydrilla establishment, it potentially increases hydrilla propagule pressure and spread by disrupting and fragmenting the plant.

To better understand the role of simultaneous biological interactions on the establishment and potential spread process of hydrilla invasion, we conducted a mesocosm experiment that assessed the individual and combined effects of three aquatic species that damage hydrilla in different ways and represent a variety of possible interactions found in lakes. According to our prior knowledge (see also Langeland 1996; Luz and Okada 1999; Fellerhoff 2002; Sousa 2011), *Moenkhausia* aff. *sanctaefilomenae* may eat hydrilla leaves and the gastropod *Pomacea* spp. is able to eat the whole plant, which could be particularly important for decreasing the performance of hydrilla and reducing the quality of propagules produced by this plant. In addition, the shredder fish *A. ocellatus* disrupt hydrilla and may also decrease the performance of the plant during the establishing stage. Thus, we first hypothesize that the presence of an invasive shredder fish and two native herbivores, which employ different strategies in their plant use, affect hydrilla's performance (i.e. vegetative growth and local establishment). If we find evidence to support our hypothesis, the presence of such organisms can help to explain the unsuccessful early establishment of hydrilla in lakes. In this sense, we expect that a scenario with the three species will have the highest effect on hydrilla's performance given that effects may not be redundant. We further hypothesize that the behavior of *A. ocellatus* may increase hydrilla propagule pressure by disrupting plants. Consequently, the local negative effect of fragmentation can be used to infer propagule pressure and invasiveness on a coarse scale (Dantzer 2015).

Methods

Experimental context

Our study scenario was based on the Brazilian Long-Term Ecological Studies (PELD/CNPq, <http://www.cnpq.br/>), conducted in the Upper Paraná Freshwater ecoregion, South America (Abell et al. 2008; and <https://www.feow.org>). Systematic surveys of several aquatic communities have been conducted for over 30 years in the Upper Paraná River floodplain (see <http://www.nupelia.uem.br>), where the first records of hydrilla date to 2005 (Sousa 2011). The hydrilla that invaded this region is a dioecious female (Sousa 2011), probably originating from a single introduction (according to molecular analysis; L.C. Lúcio, unpublished) that spread throughout the floodplain. Despite the large phenotypic plasticity of hydrilla, it is not able to succeed in stable populations in floodplain lakes (Sousa et al. 2009; Sousa 2011), which are inhabited by the resistance-promoting organisms we considered in this study (*M. aff. sanctaefilomenae*, *Pomacea* spp. and *A. ocellatus*). In secondary channels of the Upper Paraná River floodplain, where abundances of the resistance-promoting organisms are low, hydrilla develops extensive stands and keep viable populations.

Experimental design

Hydrilla was sampled in three different locations of the main channel of the Upper Paraná River (22° 46' 20" S; 53° 16' 01" W), Brazil. We collected only plants that grew in the shallows (e.g., maximum of 1.5 m) and within the edge of the stand, avoiding young or senescing plants. This was done to avoid plants in different stages of development, in which physiology, instead of ecological mechanisms, would drive the growth. Hydrilla plants were carefully washed to remove attached material prior to the experiment. We used 30 cm height apical portions (hereafter “apical branches”) without lateral branches and with minimal signs of herbivory or other damages. This length is representative of plants in early stages (1-2 months) of establishment in the floodplain lakes (see Figure 4H in Sousa et al. 2010). Fresh apical branches were evenly planted in four 250 ml plastic pots, which were filled with sediment composed of 3/4 river sand substrate and 1/4 organic matter rich sediment. Apical branches had 500 g of fresh weight, which corresponded to c. 25 g of dry weight - DW. Initial plant DW was obtained by using a regression function between dry x fresh weights generated after drying several spare plants of different fresh weights. The pots were settled into water tanks (c. 600 L) filled with river water and fixed to avoid mechanical disturbances. Hydrilla apical branches were left in tanks for rooting for two days before the experiment was initiated to mimic the early establishment stage, and each tank characterized an experimental unit. The time required for establishment was determined by removing spare plants planted at the same time as those used in the experiment, and observing their developed roots and healthy.

We prepared a total of 35 experimental units to allocate five treatments (seven replicates) simulating different types of damage on hydrilla: control (C), native herbivorous fish (NHF), invasive non-native shredder fish (ISF), native herbivorous gastropod (NHG), and a treatment with the three organisms together (ALL). The C treatment consisted of plants of experimental units growing without any possible disturbers of its performance; the NHF treatment received 10 individuals of the native *M. aff. sanctaefilomenae*, starved for 24 hours; the ISF treatment received one individual of the invader *A. ocellatus*; the NHG treatment received three individuals of the native apple snails (*Pomacea* spp.), starved for 24 hours; and finally, the ALL treatment combined all species, with 10 individuals of *M. aff. sanctaefilomenae*, three individuals of apple snails and one individual of *A. ocellatus*.

The experimental design resembled natural conditions as much as possible. The *A. ocellatus* is typical of slow water habitats (Soares et al. 2006) and together with *M. aff. sanctaefilomenae* is caught higher densities in lakes than in the Paraná River channel (da Luz et al. 2004; A. A. Agostinho, personal communication). Similarly, *Pomacea* spp. is also rare in the habitats of the River Paraná main channel, but abounds in its

floodplain lakes (R. P. Mormul, unpublished). Thus, all species are more abundant in lakes, where hydrilla does not succeed, than in the river channel, where it succeeds. The densities of individuals were based on natural densities found in lakes and by earlier pilot experiments. We used 10 indv/m² of *M. aff. sanctaefilomenae* based on Agostinho et al. (2007), who reported 4-49 indv/ m² in lakes. We tried to standardize individual fish size (5 cm) and age (only adults) to reduce individual variations in the response variables. We used 3 indv/m² of *Pomacea* spp. (5 cm shell width), based on natural densities (5-15 adult snails per 100 m²; Donnay and Beissinger 1993, but in specific lakes we found densities of 1 to 3 indv/m², R. P. Mormul, personal observation). The non-native invader *A. ocellatus* was used in a minimum density (1 individual) due its size, experimental logistics and territorial behavior. *M. aff. sanctaefilomenae* was chosen to this experiment not only by their capacity to eat hydrilla leaves but also by the capacity to avoid *A. ocellatus* predator behavior in presence of submerged plants. We have also chosen large snails to avoid predation by *A. ocellatus*. Hydrilla densities were chosen based on the values found in the Paraná lateral channels (mean of 235 g DW/m² and a maximum of 2,608 g DW/m²; see Sousa et al. 2010). Finally, we also confirmed the viability of manipulations and organisms densities after pilot experiments.

The experiment lasted 10 days, given that previous studies have shown that plants respond to manipulation after a few days (see Steward and Van 1987; Hofstra et al. 1999) and hydrilla can double its biomass in twenty days (Bianchini et al. 2010). Our own experiment also showed that this was enough time for plants establishment, to produce lateral branches in the control (where they were not disturbed) and show differences between treatments (see Results). At the beginning and throughout the experiment, we monitored electrical conductivity ($\mu\text{S}/\text{cm}$), pH, temperature (C^o) and light incidence (lux). Measurements were taken at five different times throughout the day in all experimental units: 6:30 (sunrise), 09:30 (middle of morning), 12:30 (middle of day - period with the highest light incidence), 15:30 (middle of afternoon) and 18:30 (sunset). Abiotic measurements were performed only to monitor the environmental variation within and among the treatments. The experiment was set up in a block design structure, considering the possibility of differences in the amount of sun radiation among the experimental units.

Data processing and analysis

After finishing the experiment, we removed all rooted plants and floating fragments, which were considered vegetative propagules (hereafter “propagules”) from each tank. To estimate hydrilla performance, we obtained several quantitative and qualitative measurements from both rooted plants and propagules using

photographs and dry weight. Photographs were taken of all rooted plants and propagules on a graduated table in centimeters. All photographs were taken at an angle of 90° and at a 50 cm distance from the sample to the lens for accurate measurement. The dry weight was obtained after drying the plant structures (40° C) to a constant weight. Regarding quantitative measurements, the performance of rooted plants was obtained from measuring the following parameters: the number of rooted plants; total plant dry weight (g); total plant length (cm); and mean plant length (cm). Low values of these parameters indicate a negative effect of the species on plant growth. With regards to the performance of propagules, we considered the following parameters: the number of lateral branches in planted hydrilla (interpreted as a dispersion structure in plant with clonal reproduction such as hydrilla; see Grace 1993); the number of floating propagules; total propagule dry weight (g); total propagule length (cm); and mean propagule length (cm). Numerous floating propagules indicate high plant fragmentation, what is a negative effect for established plants at a local scale. However, numerous floating propagules also indicate increasing propagule pressure, what may contribute to spread of hydrilla via water flow on a coarse scale (landscape). Propagule pressure of disrupted plants is particularly relevant for large propagules, given that there is a positive relation between hydrilla propagules size and the viability of dispersal structures (Netherland 1997; Rybicki et al. 2001).

Two qualitative aspects of both rooted plants and propagules were also measured as indicators of plant health and quality, using the pictures we took. The first aspect is the presence or absence of senescence in a plant or propagule. We consider senescent plants or propagules that have one or more of the following characteristics on more than 5% of their length: dark color, loss of healthy leaf structure, chlorosis and apparent decomposition of the leaf and rooted plants. The second aspect, which is highly relevant as a metric of biotic effects, was leaf herbivory; the presence of leaf herbivory was recorded when each plant or propagule had at least 10% consumed leaves, in addition to the initial herbivory or injury condition. All imaging variables were obtained using the image analysis program ImageJ (Rasband 2014).

We firstly tested if there was difference among blocks considering our experimental design using an ANOVA. There was no effect of blocks. Therefore, we interpreted effect of treatments by using the Kruskal-Wallis test, because all response variables did not achieve the ANOVA assumptions. In addition, we used a standard comparison of multiple independent samples (see Pohlert 2014) represented in the Fig. 1 and 2 as letters upside of means bars to demonstrate the effects of treatments on each response variable. We performed a log transformation on data to minimize problems with heteroscedasticity. Although the overall performance could be evaluated using a multivariate ANOVA (i.e., PERMANOVA, see Anderson et al. 2008), we decided to analyze

variables separately because our objective was to explain each one of the effects individually. Thus, only separated univariate analyses were carried out individually for each predictor. We then interpreted the effect of treatments considering each of the metrics of hydrilla performance. If the effect size of treatment ALL is higher than other treatments considering all performance metrics, then effects are not completely redundant. In this case, combined effects are observed. Redundant or non-existent effects would be observed if a certain treatment always have the same effect of the treatment ALL or if the treatment ALL never differ from the control. We presented results in graphs considering the effects on i) plant growth (the number of rooted plants, total plant dry weight, total plant length, mean plant length, presence of senescence in rooted plants, presence of herbivory in rooted plants), and ii) plant dispersion structures (the number of lateral branches, the number of floating propagules, total propagule dry weight, total propagule length, mean propagule length, presence of senescence in propagules and presence of herbivory in propagules). Significant values were considered when type I error was lower than 5%. Analyses and graphs were carried out in STATISTICA software v. 7.1 (StatSoft 2008).

Finally, we used meta-analyses to calculate a mean effect size of each treatment to evaluate if there was a significant overall effect on hydrilla performance considering the different metrics. In this case we summarized those effects related to plant growth and to plant dispersion structures (see above) in separate meta-analyses. We used random meta-analyses categorized by treatments to summarize effects for each treatment, assuming that real effect sizes of the different metrics may not always be the same (Borenstein et al. 2009). However, we did not compare summary effects among treatments given that our goal was to evaluate the effects for each metric of hydrilla performance. We used Cohen's d to calculate effect sizes without Hedge's correction given that the number of replicates were always the same (Borenstein et al. 2009). Also we use bootstrapping to calculate the confidence intervals. We took care on the signal of effect, we used always positive signal in case of negative effects in hydrilla performance in relation to control.

Results

The values of electrical conductivity and pH in the mesocosms remained relatively constant over the experiment (Supplementary Material 1). The other measurements indicated that plants grew in mild temperatures and abundant light (Supplementary Material 2). The constancy of environmental factors indicates that the effects we observed were not caused by water chemistry, but by the effects of the herbivorous we added in the mesocosms.

The mean plant length in C treatment was 31.25 cm (SD = 1.27), showing that plants grew only few centimeters in the mesocosms conditions. The mean total dry weight was 9.08 g (SD = 0.9847) in the C treatment, showing a reduction over time given that the initial plants dry weigh was c. 25 g in each mesocosm. Even so, roots and lateral branches were produced in all treatments.

All hydrilla plant growth measurements were significantly affected by the treatments (Fig. 1). Given that the results were the same for total and mean plant length, we showed the results of only the mean plant length. Considering individual effects, the NHG was the only treatment that differed from the control allowing for mean rooted plant length (Fig. 1A) and total rooted dry weight (Fig. 1B). Neither the NHF nor the ISF decreased these plant attributes in relation to the C treatment (Fig. 1A and Fig. 1B). The number of rooted plants was not affected by any individual treatment, only by the treatment ALL, where rooted plants decreased significantly compared to the C treatment (Fig. 1C). The treatment ALL had the highest effect size and was the only one that differed from the C treatment with regard to the senescence of rooted plants (Fig. 1D), although statistically similar to the ISF and NHG treatments. The herbivory of the rooted plant leaves was higher in the NHF and ALL treatments than in the C treatment, but did not differ among each other (Fig. 1E).

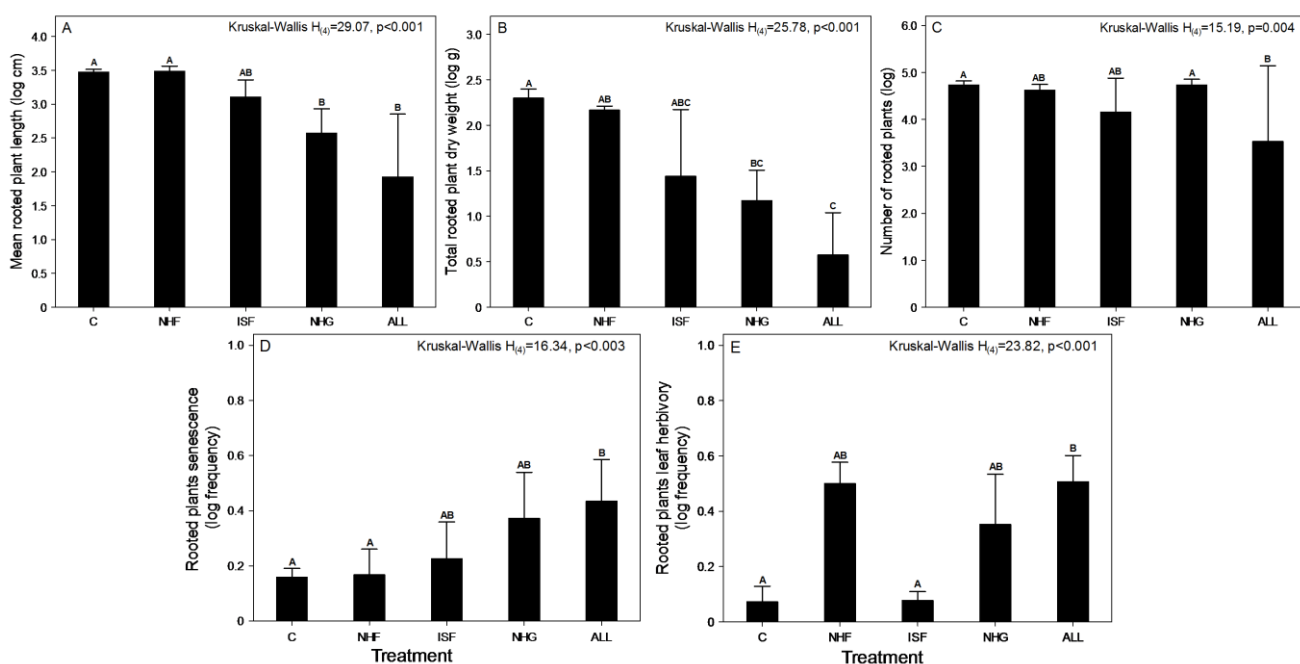


Fig. 1 Mean values (\pm standard deviation) of the mean root plant length for the five treatments (A), total rooted plant dry weight (B) and number of rooted plants per tank (C), rooted plants senescence (D) and rooted plants leaf herbivory (E). Results of a Kruskal-Wallis test are shown in graphs. Equal letters indicate statistically similar values according to a standard comparison of multiple independent samples. C = control treatment; NHF = native herbivorous fish treatment; ISF = invasive non-native shredder fish treatment; NHG = native herbivorous gastropod treatment; ALL = treatment with all species

As observed for the rooted plants, all hydrilla plant dispersion structures measurements were significantly affected by the treatments (Fig. 2). The results of the total and mean propagule lengths followed a similar pattern, and we show only the mean propagule length. The ISF treatment had significantly more propagules (Fig. 2A), which were heavier and longer (Fig. 2B and 2C respectively) than in the C treatment, but the shredder fish did not affect the number of lateral plant branches (Fig. 2D). The NHG treatment significantly increased propagule dry weight (Fig. 2B). The NHF treatment had no significant effect on any metric related to propagules or lateral branch formation (Fig. 2). The treatment ALL decreased the number of lateral branches (Fig. 2D) and increased other propagule metrics significantly in relation to the C treatment (except for mean propagule length) but not in relation to the ISF or NHG treatments. Propagule senescence (Fig. 2E) and propagule leaf herbivory (Fig. 2F) were also higher only in the treatment ALL than in the C treatment.

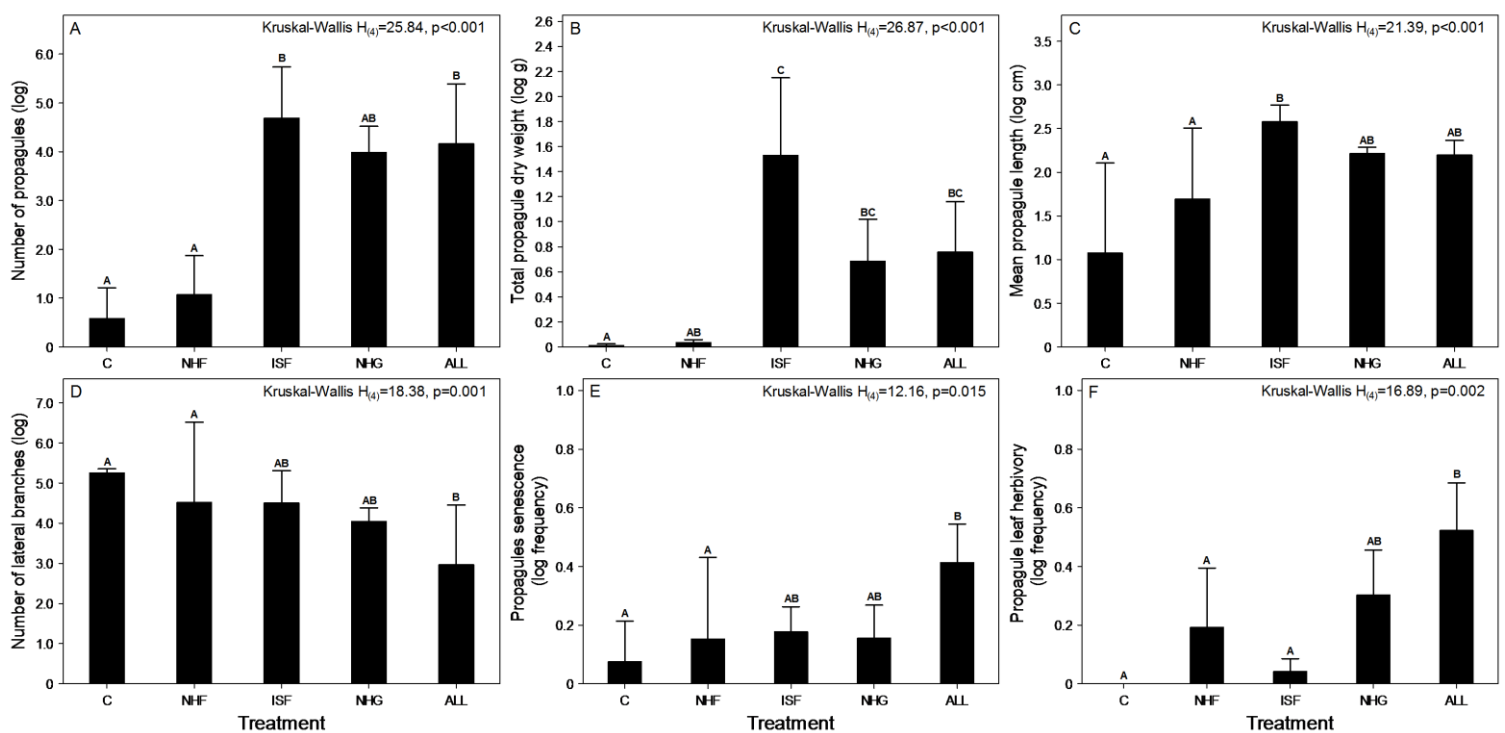


Fig. 2 Mean values (\pm standard deviation) of the number of propagules per tank (A), total propagule dry weight (B), mean propagule length (C) and number of lateral branches per tank (D), propagules senescence (E) and propagule leaf herbivory (F). Results of a Kruskal-Wallis test are shown in graphs. Equal letters indicate statistically similar values according to a standard comparison of multiple independent samples. C = control treatment; NHF = native herbivorous fish treatment; ISF = invasive non-native shredder fish treatment; NHG = native herbivorous gastropod treatment; ALL = treatment with all species

In summary, the ALL treatment was significantly different from the control in 10 out of the 11 metrics, while the ISF and NHG treatments differed from the control in only 3 metrics. The NHF treatment did not differ from the control in any measurement. Even so, the meta-analyses indicated that all treatments had significant mean effect sizes (Fig. 3). Therefore, the presence of species tested does affect overall hydrilla performance considering both plant growth and dispersion.

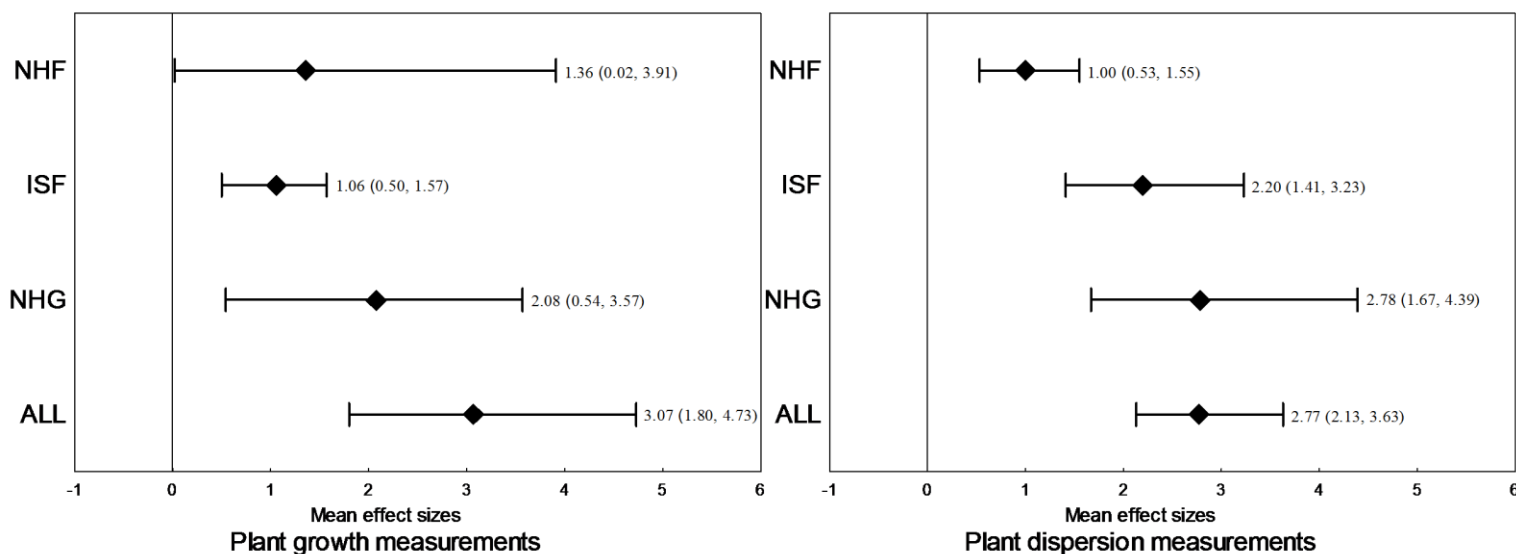


Fig. 3 Mean effect sizes, lower limit of bootstrap confidence interval, and upper limit of bootstrap confidence interval according to random-effect meta-analyses summarizing effects of treatments on plant growth and dispersion measurements. Values above zero indicate significant mean effect size of treatments. NHF = native herbivorous fish treatment; ISF = invasive non-native shredder fish treatment; NHG = native herbivorous gastropod treatment; ALL = treatment with all species

Discussion

Although we do recognize that any experiment have to be replicated to improve reliability (we used seven replicates in our experiment), our results obtained in mesocosms demonstrated that biotic resistance associated with the three organisms used in our experiment was the one that mostly affected metrics associated to the establishment of the invasive macrophyte hydrilla. Indeed, to reduce the biomass and length of establishing plants, the combined effects of biotic resistance due to herbivory and small-scale disturbance are necessary. The increased frequency of senescence and herbivory may decrease the success of invasion by hydrilla in the establishing phase. These findings reinforce the importance of specificity and the multifaceted aspects of biotic interactions in determining resistance to species invasion, at least on small spatial scales, where

interspecific interactions occur. In addition to the effective reduction in the establishment of hydrilla, our findings also demonstrate that the combination of small-scale disturbance and herbivory may play ambiguous roles in other stages of invasion processes because there was an increase in the release of vegetative propagules (plant fragments).

In general, we evidenced that the effects of hydrilla disturbers were indeed combined: the effect sizes in the treatment with all species were usually the highest. Depending on the performance metrics, the treatment ALL was similar to one other treatment, but always with high values compared to control (see Fig. 1 and Fig. 2). We highlight that the interaction among the three species caused the highest damage, indicating combined negative effects of herbivores and of the invasive shredder fish on hydrilla establishment on a local, fine scale, as shown in almost all metrics used in this study. Only mean propagule length was not most affected in the combined treatment, which can be explained by the large standard deviation. Therefore, we are confident that the biological resistance imposed by the treatment with the most diverse resident lake community on hydrilla is possibly explained by more than one interaction between hydrilla and a disturber, and not only as a sampling effect (Crawley et al. 1999; Cardinale et al. 2007). This outcome reinforces the important role of species rich communities in providing biotic resistance (Abell et al. 2008; Skóra et al. 2015). Although our experiment was not designed to elucidate the causes for the positive association between the diversity of native communities and biotic resistance, we do generate evidence that complementarity may explain biotic resistance (Alofs and Jackson 2014).

On one hand, the identity of the disturber matters: the NHG had a greater effect in hydrilla growth, whereas the NHF treatment was the one with the highest leaf herbivory and the ISF generated numerous propagules. As a consequence, the treatment with the three organisms had the highest effect for all metrics, suggesting combined effects on hydrilla performance in a broad sense. Indeed, our treatments represent a variety of possible interactions that hydrilla face in natural ecosystems in the Upper Paraná River floodplain. In addition to the known effects of abiotic factors on hydrilla performance (Barko and Smart 1986; Steward 1991; Rybicki and Landwehr 2007; Wu et al. 2009; Sousa et al. 2010; Sousa et al. 2011; Silveira and Thomaz 2015), we found that the establishment and early development of this invasive macrophyte is indeed affected by ecological interactions with vertebrates and invertebrates (see also Strayer 2010). The complexity of biological interactions, including different tactics of herbivory and biological disturbances, provided by the high diversity and organism density of floodplain lakes may help control the establishment and early development of hydrilla in these lentic habitats. However, in addition to complementarity, we cannot discard the mass effect (higher densities of

organisms) represented by our ALL treatment. However, we emphasize that this treatment is representative of real conditions because there are higher densities of animals in littoral areas of lakes compared to littoral areas of river channels (see Agostinho et al. 2000), where hydrilla grows.

For the rooted plant length, our results suggest that the apple snail causes the highest individual damage to hydrilla. Severe herbivory is also observed where hydrilla is native and *P. canaliculata* Lamarck 1819 is invasive (Wang and Pei 2012), which in combination with our results, reinforces the snail's classification as a voracious macrophytophagous herbivore (Estebenet 1995; Estebenet and Martin 2002). In contrast, *M. aff. sanctaefilomenae* do not represent a potential control of growth and development of hydrilla in the short term but can potentially decrease hydrilla's performance in the long-term by damaging the total photosynthetic tissues due to leaf consumption from rooted plants. The biological interaction that *M. aff. sanctaefilomenae* represents – herbivory by small fish – may occur in rivers, but the negative effect is probably amplified in lakes, where the abundance of small herbivorous fish is very high (Rozas and Odum 1988; Agostinho et al. 2007; Dibble and Pelicice 2010). We cannot rule out that the effects of small fish can be even greater, given that we used only one genus, so we probably underestimated the effects of small fish herbivory on hydrilla leaves. Our results also suggest a combined effect of herbivory. Although *M. aff. sanctaefilomenae* do not represent potential controllers individually, a combined effect of leaf herbivory by this native fish and rooted plant herbivory by the gastropod may explain the lowest performance in the treatment with all species. The lack of effect of NHF in decreasing biomass may be explained by the fact that leaves represent only a small portion of the total hydrilla biomass. However, the effects of small fishes are probably enhanced over longer periods of time than the one tested in our experiment because continuous herbivory on leaves certainly affects plant performance.

Different from herbivores, *A. ocellatus* is an invasive non-native piscivore, so the negative effects came from disturbances caused by a shredder activity of this fish, which dislodges the plants. Although occasional herbivore behavior has been recorded for this fish (Santos et al. 2006), herbivory was minimal and not different from the control in our study. Instead, *A. ocellatus* represents another disturbance for hydrilla establishment in lakes because this fish had the strongest effect on plant disruption. So, at a local scale, the effect of the shredder fish is negative given that it prevents hydrilla establishment, i.e., the higher density of *A. ocellatus* in lakes is probably reflected on lower hydrilla colonization. However, the disturbance of this fish released propagules with higher quality and larger sizes than those found in the other treatments (i.e., larger mean and total length, lower herbivory and lower degree of senescence). Hydrilla propagules are usually highly viable (e.g., Umetsu et al. 2012), especially the larger ones (Netherland 1997; Rybicki et al. 2001). Thus, by generating numerous and high

quality propagules, *A. ocellatus* may increase propagule pressure at the landscape scale. As a consequence, *A. ocellatus* activity may promote the spread of hydrilla in the Upper Paraná River floodplain and facilitate the colonization of suitable sites (like the Paraná River main channel). Even so, viability of propagules still deserves further investigation. For instance, we are not completely aware how viability relates to propagule size in our ecosystem. It is important to note that *A. ocellatus* is a non-native fish in the Upper Paraná River floodplain, and a positive interaction with hydrilla at a landscape level emphasizes the unpredictability of biological interactions of invasive species (Simberloff and Von Holle 1999). Finally, we highlight the high variation in the metrics used to represent hydrilla performance in the treatment with *A. ocellatus*. This variation may be explained by the individual variability, particularly because we used only one fish per tank (Magnhagen et al. 2012).

In summary, our results support the idea that the combination of biotic resistance provided by herbivory and fine scale disturbances decrease the performance of hydrilla, suggesting that biotic interactions may reduce the performance of the invasive plant. Thus, together with already described effects of environmental filters (e.g., elevated sediment organic matter and low water transparency; Sousa 2011; Silveira and Thomaz 2015), biotic resistance helps to explain why hydrilla fails to colonize lakes in the Upper Paraná River floodplain. Such results can also help to explain patterns of species establishment found in large scale studies (e.g., Skóra et al. 2015). In addition, the resulting interaction between biotic resistance and small-scale disturbance increasing the release of high quality propagules and decreasing the rooted plant health and quality, suggest two possible short-term outcomes: (i) improvement of the spread of the invasive species by increasing propagule pressure and (ii) suppression of the establishment strategies used by hydrilla to invade habitats (Fig.3). However, the real importance of each of these outcomes for hydrilla invasion deserves further investigation. Our main conclusion is that several biological interactions with herbivores and fragmenting organisms most likely limits hydrilla invasion in natural lakes of a Neotropical floodplain.

Biological Interaction of hydrilla with:	Main Ecological Consequence	Interaction Output: local/landscape
<i>Moenkhausia</i> aff. <i>sanctaeofilomenae</i> +	Leaf consumption, but poor growth limitation, low formation of propagules	-/-
<i>Astronotus ocellatus</i> *	Plant disruption with high formation of large propagules	- -/+ + + +
<i>Pomacea</i> spp. +	Leaf and stem consumption, with strong growth limitation and low formation of poor propagules	- - -/- -
<i>Moenkhausia</i> aff. <i>sanctaeofilomenae</i> + <i>Pomacea</i> spp. + <i>Astronotus ocellatus</i> *	Strong growth limitation, plant disruptions and moderate formation of poor propagules	- - -/+

Fig. 4 Summary of main consequences of interactions between *Hydrilla verticillata* and the species used in this study as biological disturbers for the establishment of hydrilla in floodplain lakes. Positive and negative interactions are indicated for either local or landscape scale, with signal quantity indicating the intensity of each interaction (+ both species are native and * species is also non-native to the floodplain studied here)

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Supplementary Material 1

Table 1 Mean values (\pm standard deviation) of electrical conductivity ($\mu\text{S}/\text{cm}$) and pH in mesocosms at the beginning and at the end of the experiment

Abiotic conditions	Period	Mean	Standard Deviation
Electrical conductivity ($\mu\text{S}/\text{cm}$)	Initial	65.36	3.28
	Final	65.39	3.69
pH	Initial	8.07	0.14
	Final	8.24	0.13

Supplementary Material 2

Table 2 Mean (\pm standard deviation) of light incidence (lux) and temperature (C $^{\circ}$) in mesocosms from each one of the chosen periods throughout the experiment

Abiotic conditions	Period	Mean	Standard Deviation
Light Incidence (lux)	06:30	1021.73	796.56
	09:30	21270.32	14011.23
	12:30	42137.50	20295.18
	15:30	22247.25	14841.41
	18:30	290.13	151.82
Temperature (C $^{\circ}$)	06:30	25.73	1.19
	09:30	24.80	1.79
	12:30	28.25	1.66
	15:30	29.64	2.50
	18:30	28.65	0.50