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TRENDS IN AQUATIC ECOLOGY III

Invasional meltdown: an experimental test and a framework to distinguish synergistic, additive, and antagonistic effects

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Abstract The potential role of positive interactions among co-invaders is at the core of the invasional meltdown hypothesis. The interaction of non-native species could result in an exacerbation of each other's effects. Thus, the resulting effect of multiple non-native species on ecosystems can be greater than the sum of their individual effects. We designed an analytical framework and a set of mesocosm experiments to assess the potential synergistic effects of three non-native species (*Limnoperna fortunei*, *Astronotus crassipinnis*, and *Hydrilla verticillata*) in a highly invaded floodplain in southern Brazil. We analyzed ecosystem, community, and population attributes in scenarios with non-natives. Our hypothesis of a synergistic effect was not supported. Even though effects of the invasive species were detected for all ecological levels, evidence indicated that these effects were additive. In addition to adding to the statement that origin (i.e., native vs. non-native status) does matter, we provide a tool to differentiate additive, synergistic, and antagonistic effects in situations with multiple invasions, and experimentally demonstrate additive effects of non-native species at different ecological levels.

Keywords Ecological impact · Multiplicative impacts · Invasive mutualism · Interactive impact

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Introduction

The intense human-mediated transport of species around the globe is promoting unexpected negative impacts through novel interactions between the native and non-native biota, as well as among non-native species. Much attention has been given to the outcome of interactions between non-native species and the recipient community, leading to a well-documented increasing rate of negative impacts (e.g., Elton, 1958; Simberloff et al., 2013; Simberloff & Vitule, 2014; Gallardo et al., 2016; Catford et al., 2018). The potential role of positive interactions among co-invaders, which is far less studied, is at the core of the invasional meltdown hypothesis (Simberloff & Von Holle, 1999). In general, the term "Invasional Meltdown" (hereafter IM) describes "the process by which a group of non-native species facilitate one another's invasion in various ways, increasing the likelihood of survival and/or of ecological impact, and possibly the magnitude of impact" (Simberloff & Von Holle, 1999).

The IM is among the most supported hypotheses in invasion science (Jeschke & Heger, 2018), but still contains relevant biases (e.g., towards evidence form plant–insect interactions) and gaps (e.g., shortage of studies at the community and ecosystem level) (Braga et al., 2018a, b). Although there is currently a wide body of evidence concerning facilitation and mutualism among non-native species, researchers have generally failed to find cases where positive interactions have led to an increase in the magnitude of their effects upon the native biota (Meza-Lopez & Siemann, 2015; Braga et al., 2018a, b). Within the context of invasion science, it is important to differentiate additive effects (i.e., the summed effects of two or more non-native species) from synergistic effects. Synergistic effects entail the exacerbation of each other's effect when two species invade, so that the resulting impact to the native biota is higher than the sum of their individual impacts (Simberloff & Von Holle, 1999; Von Holle, 2011). For effective management and conservation actions in the current Anthropocene, it is crucial to understand how multiple invaders interact and whether their interactions can change from simple additive to more extensive synergistic effects (Simberloff, 2006; Sutherland et al., 2008; Blois et al., 2013).

The highly invaded Upper Paraná River floodplain (hereafter PRF) in southern Brazil may provide a good scenario for testing the IM hypothesis since this ecosystem has been invaded by many taxa (Júlio Júnior et al., 2009; dos Santos et al., 2018; Gubiani et al., 2018; Tonella et al., 2018). Among the non-natives recorded, three species have drawn attention by their effects on native species and by the potential for positive interactions among them (Fig. 1). First, the golden mussel (*Limnoperna fortunei* Dunker 1857) is widely distributed along the PRF and its tributaries and is known to cause severe economic and environmental impacts in South-American ecosystems (for extensive examples, see: Boltovskoy & Correa, 2015; Duchini et al., 2018). Both larvae and adults of this bivalve have been reported to serve as important food items for some fish species (García & Protogino, 2005; Paolucci et al., 2007; Cantanhêde et al., 2008). In addition, consumed adults of *L. fortunei* are able to survive passage through the gut and, thereby, increase their dispersal (de Oliveira et al., 2010). Second, *Astronotus crassipinnis* (Heckel, 1840) is a fish that has colonized the PRF and may potentially consume the golden mussel. Species of the genus *Astronotus* are territorial and voracious predators with indiscriminate feeding habits, but with a preference for smaller fish (Froese & Pauly, 2018), associated with decrease in species richness and diversity of native fish in lakes (Latini & Petrere, 2004). Additionally, shredding behavior of *A. crassipinnis* can promote propagule dispersal of the submerged macrophyte *Hydrilla verticillata* (L.F.) Royle (1977) (Ribas et al., 2017). Third, the macrophyte *H. verticillata* has drawn attention due to its severe negative ecological impacts (e.g., highly competitive and dominant over native macrophytes) and extensive colonization in the PRF (Sousa, 2011). This species also apparently facilitates invasion by the golden mussel by providing colonization sites (Michelan et al., 2014). On the other hand, the filter-feeding mussels may enhance water transparency (Duchini et al., 2018), what potentially favors submerged macrophytes and also visually oriented predators such as *A. crassipinnis*.

Present evidence of invasion by these three non-native species and the effects and interactions among

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Fig. 1 Three non-native species recorded in the Upper Paraná River floodplain that have potentially positive interactions, and for this reason were used to test the invasional meltdown hypothesis (IM). The predator fish *Astronotus crassipinnis* (Heckel, 1840) (**A**), the golden mussel *Limnoperna fortunei* (Dunker 1857) (**B**), and the submerged aquatic plant *Hydrilla verticillata* (L.F.) Royle (1977) (**C**). Photos by Raul Rennó Braga (**A**), Suelen Alves Cristina da Silva Pereto (**B**), and Roger Paulo Mormul (**C**)

them makes them strong candidates to be part of an IM scenario because of their potential for additive or synergistic effects. Here we designed an analytical framework and a set of mesocosm experiments to directly assess the potential of a synergistic IM among different combinations of the non-native species in a simplified version of the PRF. In particular, we differentiated their antagonistic, additive, or synergistic effects at different ecological levels. With this, we tested the hypothesis that the combined effect of two or three non-native species is higher than the sum of their individual effects. Therefore, as described in the IM hypothesis, we predict that a synergistic effect will occur in scenarios of multiple invasions.

Methods

Study system

The PRF in southern Brazil is known as an area critical for freshwater conservation in the second largest hydrographic basin in South America (Agostinho et al., 2004). At the same time, PRF is highly invaded (e.g., of the 211 recorded fish species, 70 are possibly non-native) (Júlio Júnior et al., 2009; Vitule et al., 2012; Gubiani et al., 2018; Ota et al., 2018) as a consequence of intense human alterations such as the changes in hydrological regime promoted by cascading reservoirs located upstream from the floodplain (Agostinho et al., 2004). Indeed, there is evidence that alterations caused by the reservoirs are the main cause of the introduction of a large number of non-native species (Júlio Júnior et al., 2009; Sousa, 2011; Vitule et al., 2012; Skóra et al., 2015). The PRF has been the subject of systematic surveys of aquatic communities for nearly 30 years through the Brazilian Long-Term Ecological Studies (PELD/CNPq, http://www.peld.uem.br) and other earlier projects (see http://www.nupelia.uem.br). This allowed the detection and monitoring of non-native species over a long period of time.

Experimental design

To understand the role of interactions among non-native species and their impacts on ecological attributes, we conducted a mesocosm experiment that simulated a simplified scenario including native and non-native species found in the PRF (Fig. 2). We assessed individual and multiple effects, along with the interactive roles of non-native species. First, we constructed mesocosms representing a native community. The simplified native biota included phytoplankton and zooplankton collected from the river, two common small-sized fish species to act as secondary consumers (*Astyanax altiparanae* Garutti and Britski, 2000, and *Moenkhausia forestii* Benine, Mariguela & Oliveira, 2009), stands of one submerged macrophyte (*Egeria najas* Planchon), and a top predator fish species (*Hoplias malabaricus* (Bloch, 1794). Then, we created mesocosms adding one, two, or three non-native species to the same native community (see details below). As stated in the introduction, the non-native species were a submerged macrophyte (*H. verticillata*), a mussel (*L. fortunei*), and a predatory fish (*A. crassipinnis*). When we added the non-native *H. verticillata* and *A. crassipinnis*, we removed the same amount of the corresponding native species (*E. najas* and *H. malabaricus*, respectively) in order to maintain equal biomass among mesocosms. Since there is not a native species corresponding to the mussel *L. fortunei*, mesocosms with this species had a higher biomass (see details below). The mesocosms with pairs of non-native species were assembled with all possible combinations of the three non-native species. In the last treatment, all three non-native species were added (Fig. 2). Thus, the experimental design included seven treatments, corresponding to seven invasion scenarios (i.e., with the addition of one or more non-native species), and one scenario with only native species included (our control). Although we use the term invasion scenario, we are not referring to an increased abundance or density of the non-native

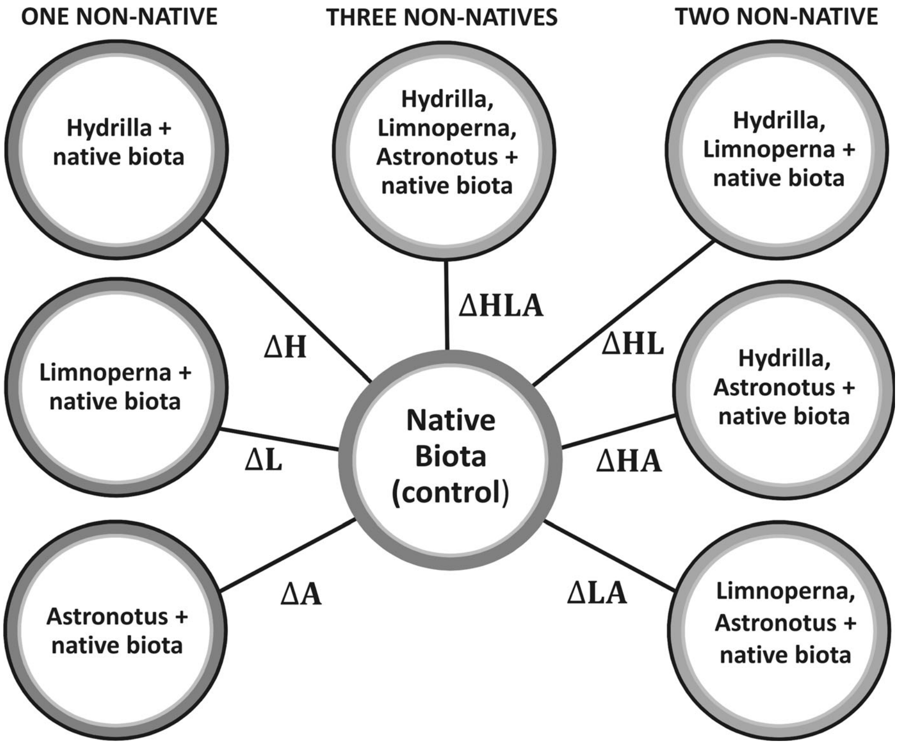

Fig. 2 Experimental design representing mesocosms with only the native biota—the control mesocosm; and mesocosms with one, two, or three non-native species (for real pictures of mesocosms, see Online Resource 1). Acronyms with Δ indicate the effects of treatments with the non-natives: the macrophyte *Hydrilla verticillata* (Δ*H*), the mussel *Limnoperna fortunei* (Δ*L*), the predator fish *Astronotus crassipinnis* (Δ*A*), *H. verticillata* + *L. fortunei* (ΔHL), *H. verticillata* + *A. crassipinnis* (ΔHA), *L. fortunei* + *A. crassipinnis* (ΔLA), *H. verticillata* + *L. fortunei* + *A. crassipinnis* (ΔHLA)

species. Instead, we are referring only to the presence of non-native species.

Each treatment was replicated five times in a block design resulting in a total of 40 mesocosms. Each mesocosm's position was sorted at random within each block. The block design was used to avoid the influence of light intensity over phytoplankton due to mesocosm position (more details in Online Resource 1). Mesocosms were 1,000 l round tanks filled with river water and continually aerated. Experiment duration of each mesocosm was 20 days. The duration of the experiment was limited by mesocosm integrity and were determined during trial runs (e.g., no prey species left or excessive macrophytes destroyed by fish activity). Abiotic factors, such as pH, water temperature, and conductivity, were measured every 2 days, always at 16:00 h (see values in Tables 2 and 3 in Online Resource 1). Water temperature and conductivity were measured using a multiparameter device (Extech model DO700), while pH was measured using an Adwa pH meter (model AD11).

Experiment assembly

All species used in the experiment were collected directly from aquatic habitats in the PRF. Submerged macrophytes were collected during free dives and separated in order to get 700 g m^{-2} fresh weight (FW) in each trial (see Online Resource 1 for detailed weighing method). In treatments with the non-native *H. verticillata*, we used 350 g m^{-2} FW of each species. Densities were chosen according to a plausible representation of natural conditions (Pelicice et al., 2005). After removal of attached organisms, macrophytes were planted into four containers with sand and ground soil retrieved from river margin to allow establishment.

Submerged wooden trunks with attached individuals of *L. fortunei* were used at a density of approximately 1,500 ind m^{-2} (see Online Resource 1 for details). Because *L. fortunei* does not have any native equivalent species in the PRF, it was considered an additional component to the PRF community and its effects were measured without removing any native component of the mesocosm.

The native fish *A. altiparanae* (standard length = 60 mm, standard deviation ± 14 mm) and *M. forestii* (standard length = 31 mm, standard deviation ± 03 mm) was seined with trawls and acclimatized to laboratory conditions for at least 2 days before the experiment. During this period, fish were fed with commercial pellets to satiation. Ten adult individuals of each species were added to each treatment 2 h before the predators to allow them time to find refuge and to prevent early ambush predation. The density (10 ind m^{-2}) represented plausible values for the PRF (see Agostinho et al., 2007) and was sufficient to avoid predation of all individuals during the experiment (trial experiment information). Four six-holed bricks (9 × 14 × 19 cm) were added to each × mesocosm to provide extra refuge (see Online Resource 1).

Native *H. malabaricus* (standard length = 218 mm, standard deviation ± 22 mm) and non-native *A. crassipinnis* (standard length = 211 mm, standard error ± 14 mm) predators were seined with trawls or caught with rods. Prior to the experiment, individuals were acclimatized to experimental conditions for at least 2 days in separate aquaria (one fish per aquarium). This period was important to assign healthy and similar-sized individuals. They were fed to satiation with small prey species different from those used in the experimental trials until 24 h prior to the beginning of the experiment. We added two individuals of *H. malabaricus* in the mesocosm without non-native fish predators, and one individual of *H. malabaricus* and one of *A. crassipinnis* in treatments with the non-native predator. Predators were used at the minimum density possible due to their size in relation to the mesocosm. Additional detailed information on experimental assembly procedures is available in Online Resource 1.

Response variables

Our response variables encompassed ecosystem, community, and population attributes, which were obtained at the end of the experiment. The ecosystem attributes included dissolved oxygen (a surrogate of ecosystem metabolism; see Dodds et al., 2018), chlorophyll-*a*, phytoplankton densities (surrogates of primary productivity), and zooplankton density (a surrogate of secondary productivity).

Chlorophyll-*a* (mg l^{-1}) was sampled before the water had been disturbed by mesocosm disassembly. One liter of water was retrieved from each mesocosm and maintained at 5 °C for later laboratory analysis. Samples were protected from incident light until analyzed. In the laboratory, chlorophyll-*a* samples were filtered using glass fiber filters. Chlorophyll-*a* was extracted from the filtered material with acetone and measured in spectrophotometer (Golterman et al., 1978).

For phytoplankton sampling, we mixed the water of each mesocosm to reduce water stratification before taking samples. Samples were obtained by filtering 3 l of water through a 15-µm mesh net. Samples were stored in amber flasks and fixed with acidic Lugol's iodine. Phytoplankton density (cells ml^{-1}) was measured according to the Utermöhl (1958) technique using random fields observed with an inverted microscope (Olympus IX70). The dilution conversion was calculated from the formula:

$$\text{Phytoplankton density} = \frac{VCS \times N}{VQ \times VF},$$

where VCS refers to the volume of concentrated sample, *N* to the number of cells quantified, VQ to the volume quantified, and VF to the filtered volume.

In addition to quantify phytoplankton biovolume, phytoplanktonic taxa were also identified to the lowest taxonomic level. The number of taxa was used as a community attribute (phytoplankton richness). Zooplankton samples were obtained after mixing the water column. For each sample, 60 l of water, measured using 20-l capacity buckets, was filtered through 40-µm plankton netting (standardized sampling effort). The collected material was maintained in polyethylene bottles in formaldehyde solution (4%) buffered with calcium carbonate. In order to determine the abundance of organisms, the samples were concentrated to 75 ml and sub-assayed with a Hensen–Stempel pipette (2.5 ml), counting at least 50 individuals of each group in Sedgewick-Rafter chambers under an optical microscope. The total abundance of organisms was expressed in terms of individuals per cubic meter (ind m^{-3}). The counting of the organisms was based on the methodology of Bottrell et al. (1976), being a quantification of a minimum of three and a maximum of 10 subsamples. For adult copepods, we counted all the individuals in the samples when they had a small number of individuals. We did not use zooplankton richness as response variable (as we did for phytoplankton) because of the less detailed taxonomic resolution.

Population attributes were the survival of native prey (*A. altiparanae* and *M. forestii*), measured as the number of fish remaining at the end of the experiment (indicating predation), and macrophyte fragment abundance. Loose fragments of macrophytes remaining at the end of the experiment were divided into three fragment size classes (class 1, < 15 cm; class 2, between 15 and 30 cm; and class 3, > 30 cm) and counted to allow further evaluation of potential survival (indicated by fragment size) and dispersal of fragments (indicated by the number of fragments).

Conceptual framework and data analyses

We first estimated the *effect* of an invasion scenario by calculating the difference of the response variables between the experimental mesocosm and the control (delta values illustrated in Fig. 2). We did this for each paired mesocosm within each block. Thus, five paired effects were calculated for each of the seven invasion scenarios, and for each attribute considered (i.e., 5 pairs × 7 invasion scenarios × 10 attributes = 350 effects). We then addressed the following questions:

Question 1: is there an overall effect of non-native species on native communities?

To answer this question, we calculated an overall effect of invasion scenarios for each response variable by combining effects from the seven different invasion scenarios using Hedges' *g* effect sizes and a random model meta-analytic tool (Borenstein et al., 2009). Hedges' *g* effect sizes were estimated for each invasion scenario by calculating the mean effect across paired replicates divided by their standard deviation (standardized mean difference) (see Borenstein et al., 2009). We interpreted the mean effect size for invasion to be significant if the Bootstrap confidence intervals around mean effect size did not cross the zero line. We used MetaWin for summary meta-analyses (Rosenberg et al., 1997).

Question 2: is the effect of each individual invasion scenario significant? Do the effects differ among invasion scenarios?

We compared effects among invasion scenarios by using the 95% confidence intervals of Hedges' *g* effect size. Confidence intervals were also used to infer if the effects of each invasion scenario differ from 0, i.e., if there is an effect different from the control. As well as for the overall mean effect size in meta-analyses, the effects of treatments were considered significant if the 95% confidence intervals around mean effect size did not cross the zero line.

Question 3: how comparable are effects with only one non-native species and with a combination of two or three non-native species? Are the effects of interactive invasive species antagonistic, additive, or synergistic?

By calculating paired effects for each treatment, we could also make inferences about the differences in invasion scenarios considering that effects of invaders on ecosystem, community, and population attributes can be antagonistic, additive, or synergistic (Simberloff & Von Holle, 1999; Braga et al., 2018a, b; Fig. 3A). When making these determinations, we followed a conceptual framework illustrated in Fig. 3 B. Our inferences are based on a rationale that takes into account three facets: (i) a comparison of treatment effects against the control (i.e., significant effects), (ii) a comparison of the direction and magnitude of treatment effects among each other, and (iii) a comparison of the sum of effects derived from treatments with only one non-native species with the effects derived from treatments with the combination of non-native species.

We first considered that antagonistic, additive, or synergistic effects could only be identified if at least one effect differs from the control. If effects of treatments with only one non-native species or the effects from treatments with the combination of non-native species are not significant (did not differ from the control mesocosm), then we were not able to generate evidence of either antagonistic, additive, or synergistic effects, given that effects were non-existent (even with a significant mean effect size in meta-analysis).

However, if at least one (any) treatment effect was significant, we then evaluated the significance, magnitude, and direction of the effects (Fig. 3B). We first considered whether individual effects (i.e., effects of treatments with only one species) are in the same direction, i.e., whether the mean effects of the treatments are all positive or negative. If true, we then compared the sum of individual effects with the joint effect (i.e., the effect of treatment with the combination of non-native species; Fig. 3A). Evidence for synergistic effects was generated if the joint effect is greater than the sum of the individual effects (Fig. 3A, B). For example, when considering the individual effects of the non-native species *H. verticillata*, *L. fortunei*, and *A. crassipinnis* (H, L, and A, respectively; see Fig. 2), the synergistic effect HLA would be in evidence if the ΔHLA is greater than $\Delta H + \Delta L + \Delta A$. Following the same rationale, additive effect would be in evidence if the ΔHLA does not differ from $\Delta H + \Delta L + \Delta A$, while an antagonistic effect would be found if the ΔHLA is smaller than $\Delta H + \Delta L + \Delta A$. The standard variation of the summed effects was considered as the pooled standard variation of individual effects (Borenstein et al., 2009). We calculated 95% confidence intervals and inferred significant differences if the confidence interval of one effect did not cross the confidence interval of the other effect.

If the individual effects were not at the same direction, we inferred the nature of joint effect as follows: if the joint effect had the most extreme mean effect, and was significantly different from the control, we compared the sum of individual effects with the joint effect as above (Fig. 3). However, if the joint effect was not significantly different from the control, but at least one individual effect was, we interpreted this to be evidence for an antagonistic effect: the effect of one non-native species was canceled by the presence of another non-native (Fig. 3). Antagonistic effects were also in evidence if the joint effect was significant, although significantly lower than at least one of the individual effects in the same direction as the joint effect (Fig. 3). In this case, the rationale is similar to the above-mentioned: the effect of one non-native species was (partially) canceled by the presence of the other non-native. Graphs and statistical analyses were performed using the STATISTICA software v. 7.1 (StatSoft, 2005).

Results

The treatments had similar environmental conditions during the course of the experiment. There were no differences in means of pH (7.0; ± SD 0.12) ($F_{7;32} = 1.961$; $P = 0.091$), temperature (27.0 °C; ± SD 0.5) ($F_{7;32} = 0.304$; $P = 0.946$), and conductivity (54.7; ± SD 7.62) ($F_{7;32} = 0.272$; $P = 0.961$) among treatments. Prey survival in control mesocosms was on

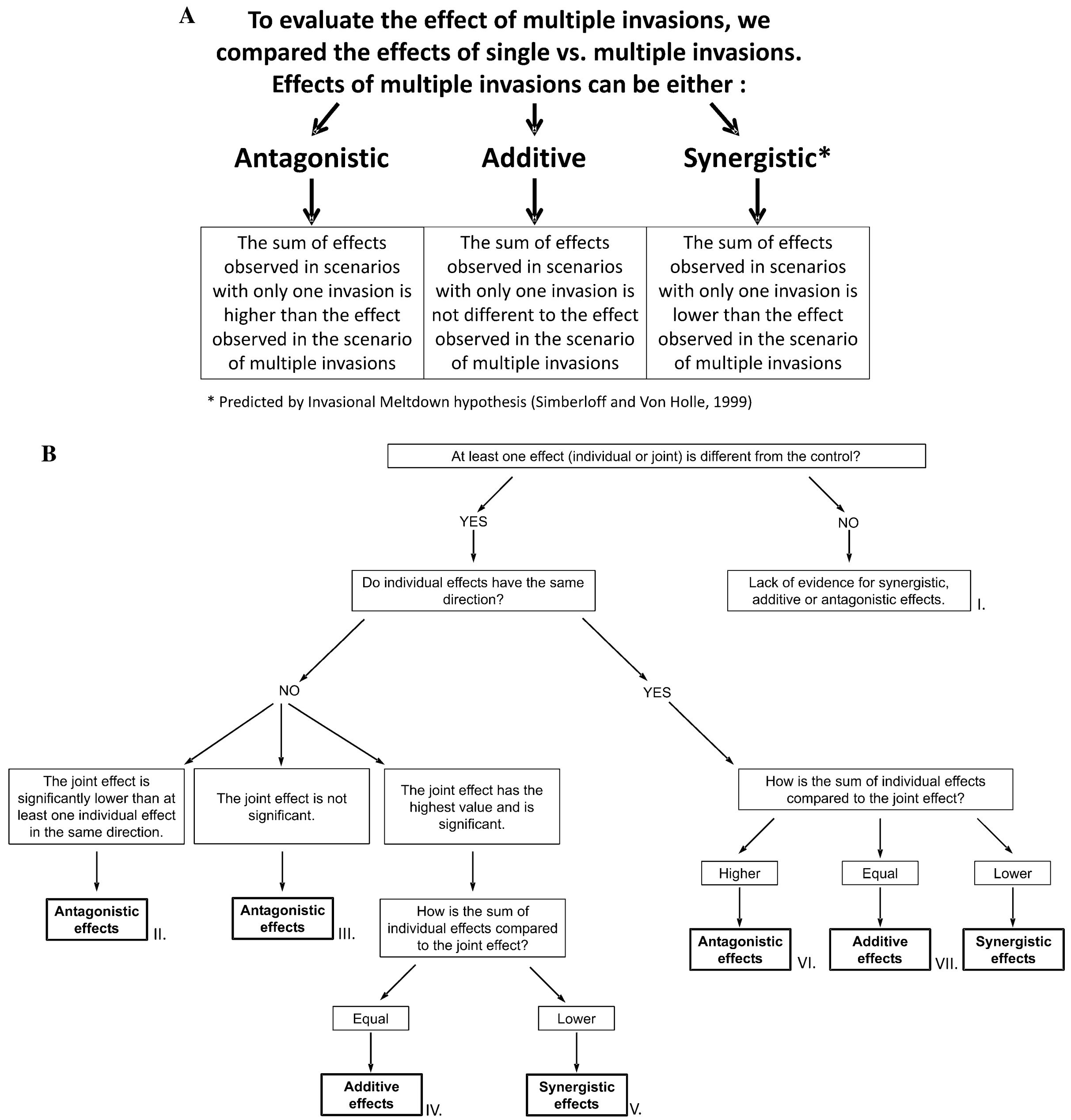

Fig. 3 Definition of antagonistic, additive, and synergistic effect of multiple invasions (**A**). Conceptual framework showing how one can identify antagonistic, additive, and synergistic effects in scenarios of invasion (**B**). See Online Resource 2 for exemplified graphs on each outcome. Roman numerals indicate which graphs of Fig. 1 in Online Resource 2 (also indicated by the same numerals) are used as an example

average 5.3 (± SD 1.89) individuals of *A. altiparanae* and 9.3 (± SD 0.96) individuals of *M. forestii*. No *L. fortunei* mortality (not related to predation) was observed at the end of the experiment therefore no mussel decomposition affected analyzed parameters.

After 20 days, effects of invasions were detected for every ecological level by summarizing the mean effect of all invasion scenarios (see Figs. 4, 5, 6). For ecosystem attributes, the addition of non-native species decreased dissolved oxygen concentration and zooplankton densities (Fig. 4). For the community

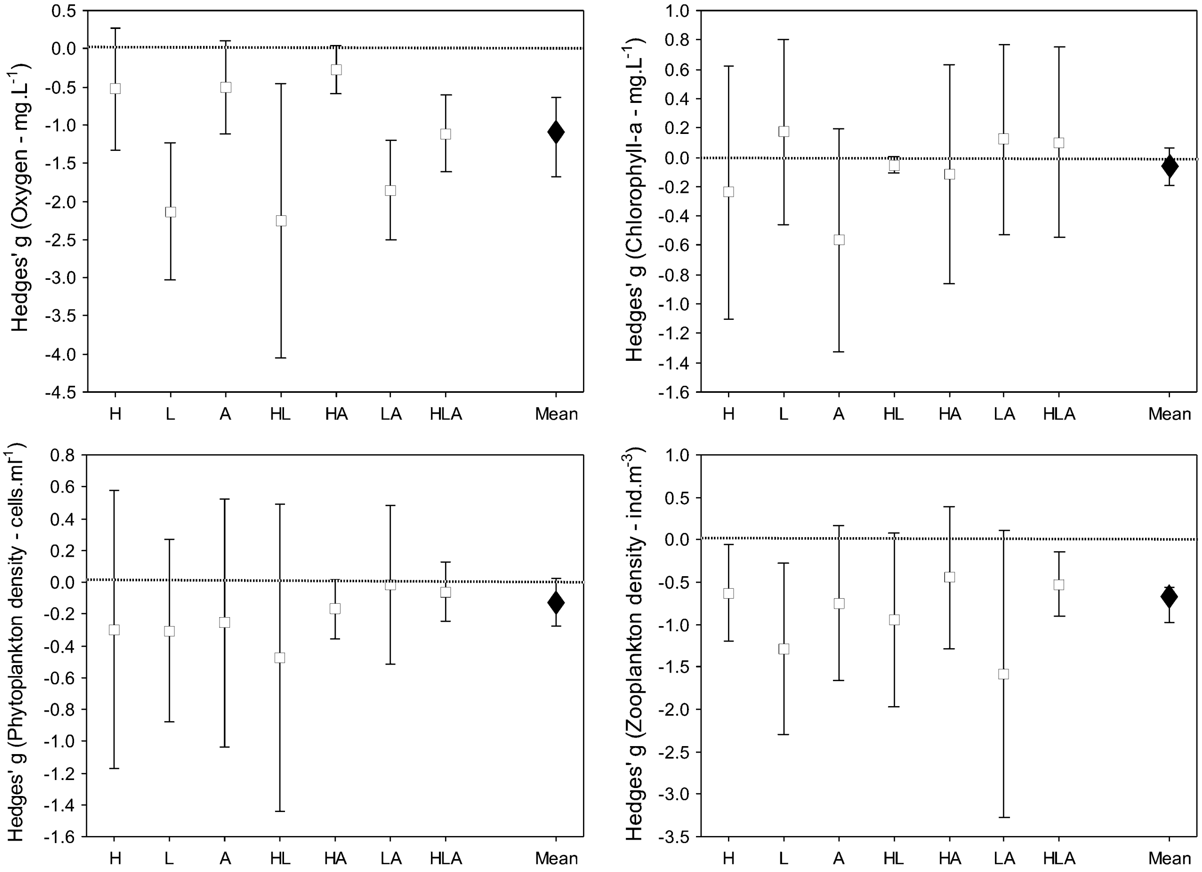

Fig. 4 Changes between the control and treatments simulating scenarios of non-native species invasion for the four ecosystem measured attributes. Means (squares) and ± 95% confidence intervals (bars), as well as Hedges' *g* mean effect size (diamonds), bootstrap confidence intervals (lines) are shown in graphs. Invasion scenarios not crossing the 0 dashed line are significantly different from the control. *H Hydrilla verticillata*, *L Limnoperna fortunei*, *A Astronotus crassipinnis*

attribute there was a decrease in phytoplankton richness (Fig. 5), and for population attributes, a decrease of prey (*M. forestii*) survival and an increase of plant propagules production (Fig. 6) were observed.

Analyzing the effect of individual and combined non-native species treatments (i.e., how they differ from the control), *L. fortunei* treatments were the ones where oxygen concentrations were most significantly reduced (15% reduction on average) (Fig. 4). Zooplankton density was reduced in treatments of *H. verticillata* (44% reduction) and *L. fortunei* alone (73% reduction) and when the three non-native species were present (76% reduction). Phytoplankton richness was reduced when *A. crassipinnis* was in combination with one other non-native species (*A. crassipinnis* with *H. verticillata* reduced 15%, and *A. crassipinnis* with *L. fortunei* reduced 39%) and in the presence of *L. fortunei* alone (30% reduction) (Fig. 5). The combination of *L. fortunei* and *A. crassipinnis*, as well as when the three non-native species were present lead to an increase in macrophyte propagule numbers increase (45% and 61%, respectively) (Fig. 6). These effects were detected for combined non-native species, which make a synergism possible to be evaluated. When the sum of individual effects were compared to their joint effects, they were not significantly different (although always with higher values), indicating additive effects only (Table 1).

Discussion

The analytical framework developed here can provide a key tool to differentiate additive, synergistic, and

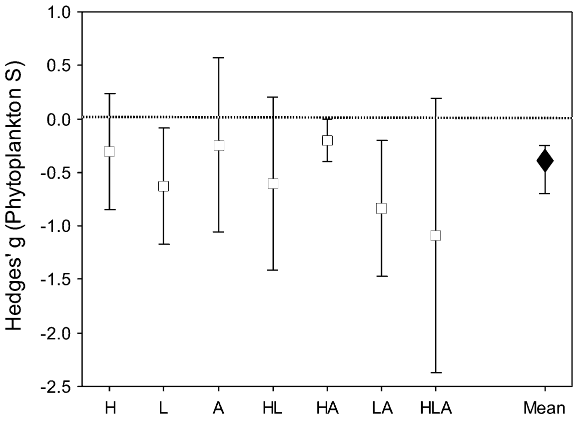

Fig. 5 Changes between the control and treatments simulating scenarios of non-native species invasion for the community measured attribute (phytoplankton richness). Means (squares) and ± 95% confidence intervals (bars), as well as Hedges' *g* mean effect size (diamonds), bootstrap confidence intervals (lines) are shown in graph. Invasion scenarios not crossing the 0 dashed line are significantly different from the control. *H Hydrilla verticillata*, *L Limnoperna fortunei*, *A Astronotus crassipinnis*

antagonistic effects caused by combinations of non-native species in an invaded ecosystem. Our experiment showed that the investigated non-native species have additive effects on different attributes at the population, community, and ecosystem levels. Thus, our results do not support the IM, which predicts synergistic effects (Simberloff & Von Holle, 1999; Simberloff, 2006). Despite not supporting the IM, our experiment provides evidence of at least two important outcomes resulting from the presence of non-native species and interactions among them. First, we did not find any sign of antagonistic effects between the non-native species added to our mesocosms. Thus, it seems that one non-native species does not reduce the negative effects caused by other non-natives in an ecosystem. Second, the additive effects indicate that ecosystems will be impacted more intensively if the number of non-native species increases. These two outcomes are a matter of concern, since the number of non-native species is still increasing globally (e.g., Seebens et al., 2017).

The observed results are valid for species with similar biological and ecological characteristics as those used in this study. For example, considering the large number of non-native fish species present in the PRF (ca. 70 species Skóra et al., 2015; Ota et al., 2018), different outcomes are possible. Our analytical framework offers the possibility to test the hypothesis (that the combined effect of two or three non-native species is higher than the sum of their individual effects) for any combination of different non-native species.

Overall effect of non-natives: does species origin matter?

For the scenarios investigated, there was an overall negative effect of non-native species on variables at different ecological levels. Despite finding only addictive effects, the population, community, and ecosystem attributes were affected by the substitution of a native species for a non-native or by the introduction of a non-native organism, regardless of the very divergent ecological or taxonomical identities of the non-native species evaluated here. More interestingly, such ecological attributes were significantly affected by the introduction of non-native species and by all possible combinations of different non-natives. This conclusion is robust because the substitution of native by equivalent non-native species (i.e., same archetype or trophic level) always resulted in significant changes in the attributes compared to the control, where only native species were present (i.e., species with evolutionary experience and biogeographically restricted). In addition, we did not simulate invasion in the sense of increased abundance or biomass of the non-native species. For example, *H. verticillata* grows rapidly, attains higher biomass, and is a better competitor than the native *E. najas* (Sousa, 2011). Therefore, our results can be an underestimation of their actual effect in the PRF, especially over long periods of time.

The experimental evidence of effects caused by non-native species when comparing response variables among mesocosm treatments adds to the statement that origin does matter and non-native species should continue to be an important ecological and conservation concern (Lambertini et al., 2011; Paolucci et al., 2013; Simberloff & Vitule, 2014). Our experimental framework allowed the substitution of native species for non-native species and therefore tested if non-native species would simply replace native species function (functional redundancy) or, despite being in the same trophic position, the non-natives would change population, community, and ecosystem attributes. The framework also allowed us to introduce a new component (the mussel *L. fortunei*) and study its interactions with other non-native species. We showed that indeed non-natives can be different from natives regarding the outcome of interactions. Our results are in accordance with the analysis of more than 1,000 interactions involving non-native plants showed that negative effects on natives are far more common than effects of non-natives on other non-natives (Kuebbing & Nuñez, 2016).

Several mechanisms responsible for the observed effects are possible, especially given the multivariate nature of species interactions (Brown et al., 2001). However, we can make some inferences based on evidence shown by others. For example, the significantly greater decrease in oxygen concentration found in mesocosms colonized by non-native species may be attributed first to consumption by *L. fortunei* (not found in the control mesocosms). At the same time, mussels may have indirectly affected the zooplankton densities through a bottom-up mechanism (Sinistro, 2010). The presence of the non-native submersed macrophyte *H. verticillata* may also have contributed to decreases in phytoplankton density through release of allelopathic compounds (Gao et al., 2015), which potentially contributed indirectly to decreases of the zooplankton densities. The mechanisms associated with the presence of *L. fortunei* and *H. verticillata* are possible explanations for the overall effect observed in phytoplankton richness, i.e., by affecting certain species of algae more than others, causing changes in the plankton assemblage species richness. Finally, effects at the population level shown by an increase in

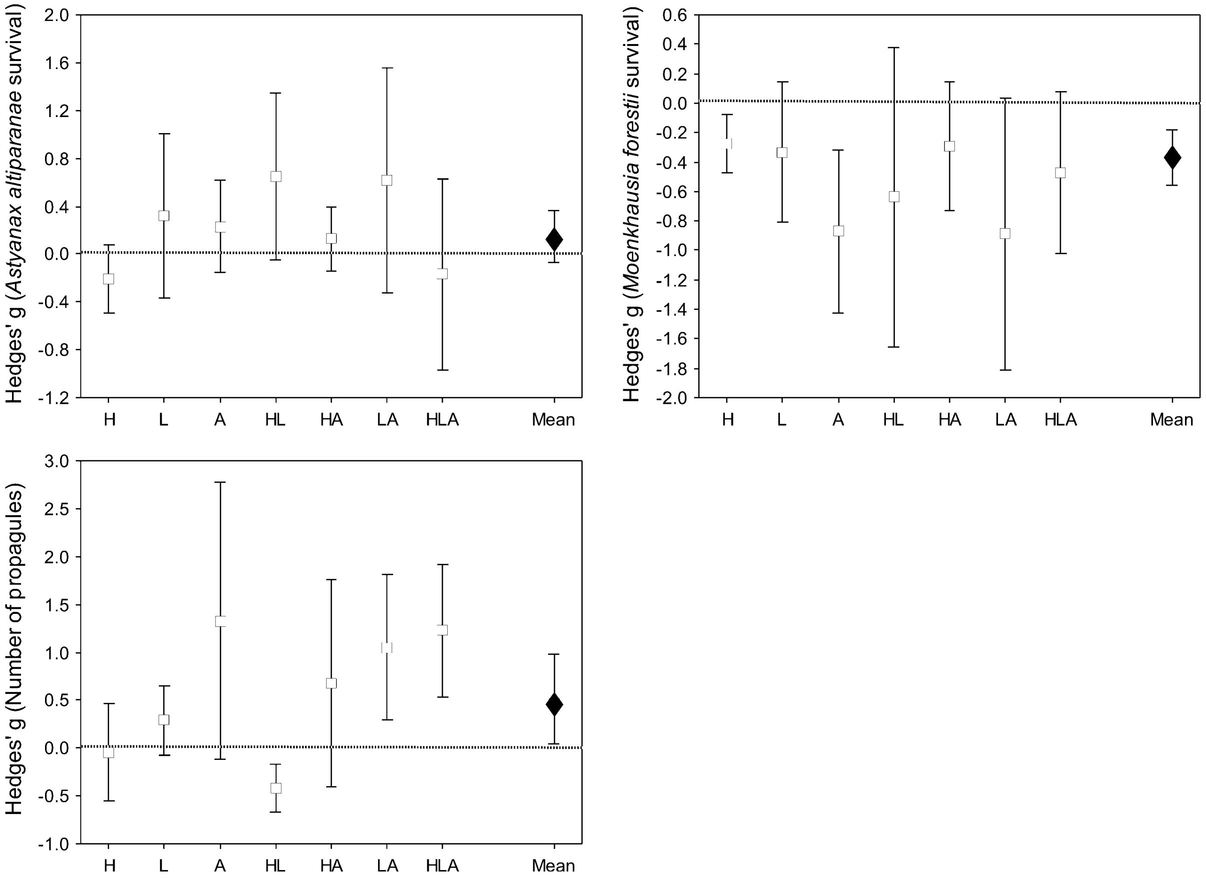

Fig. 6 Changes between the control and treatments simulating scenarios of non-native species invasion for the three population measured attributes. Means (squares) and ± 95% confidence intervals (bars), as well as Hedges' *g* mean effect size (diamonds), bootstrap confidence intervals (lines) are shown in graphs. Invasion scenarios not crossing the 0 dashed line are significantly different from the control. *H Hydrilla verticillata*, *L Limnoperna fortunei*, *A Astronotus crassipinnis*

Table 1 Comparison of summed individual with joint effects following the proposed framework (see Fig. 2)

Indicator	Comparison	Sum of individual effects (± 95% CI)	Joint effect (± 95% CI)	Effect's nature
Oxygen (mg l^{-1})	$H + L$ versus HL	− 1.36 (− 1.96; − 0.077)	− 0.93 (− 1.28; − 0.57)	Additive
	$L + A$ versus LA	− 1.48 (− 2.04; − 0.92)	− 1.24 (− 1.48; − 0.99)	Additive
	$H + L + A$ versus HLA	− 1.76 (2.42; − 1.10)	− 1.44 (− 1.94; − 0.94)	Additive
Phytoplankton density (cells ml^{-1})	$H + A$ versus HA	− 84.28 (− 426.43; 257.87)	− 61.26 (− 154.03; 31.52)	Additive
	$H + L + A$ versus HLA	− 92.40 (− 409.03; 224.23)	− 64.33 (− 247.40; 118.74)	Additive
Zooplankton density (ind m^{-3})	$H + L$ versus HL	$- 1.09 * 10^5$ ($- 1.57 * 10^5$; $- 0.60 * 10^5$)	$- 0.44 * 10^5$ ($- 0.92 * 10^5$; $0.02 * 10^5$)	Additive
	$H + A$ versus HA	$- 0.75 * 10^5$ ($- 1.30 * 10^5$; $- 0.21 * 10^5$)	$- 0.23 * 10^5$ ($- 0.61 * 10^5$; $0.15 * 10^5$)	Additive
	$L + A$ versus LA	$- 1.03 * 10^5$ ($- 1.60 * 10^5$; $- 0.46 * 10^5$)	$- 0.64 * 10^5$ ($- 0.99 * 10^5$; $- 0.29 * 10^5$)	Additive
	$H + L + A$ versus HLA	$- 1.44 * 10^5$ ($- 1.97 * 10^5$; $- 0.90 * 10^5$)	$- 0.52 * 10^5$ ($- 1.03 * 10^5$; $- 0.01 * 10^5$)	Additive
Phytoplankton S	$H + L$ versus HL	− 6.60 (− 15.40; 3.64)	− 3.90 (− 10.34; 2.54)	Additive
	$L + A$ versus LA	− 6.00 (− 15.64; 3.64)	− 5.90 (− 9.47; − 2.33)	Additive
M. forestii survival	$H + A$ versus HA	− 3.00 (− 7.74; 1.84)	− 0.9 (− 3.02; 1.32)	Additive
Plant propagule (n)	$H + L$ versus HL	32.60 (− 180.63; 245.83)	− 56.80 (− 22.21; 106.22)	Additive
	$L + A$ versus LA	209.80 (− 14.89; 434.49)	132.40 (− 378.29; 277.41)	Additive
	$H + L +$ A versus HLA	203.80 (− 11.72; − 419.32)	174.40 (− 439.40; 293.52)	Additive

Inferences can be evaluated using the 95% confidence interval (CI). Significant differences are present if the confidence interval of one effect did not cross the confidence interval of the other effect. H (*Hydrilla verticillata*), L (*Limnoperna fortunei*), A (*Astronotus crassipinnis*), HL (*H. verticillata* + *L. fortunei*), HA (*H. verticillata* + *A. crassipinnis*), LA (*L. fortunei* + *A. crassipinnis*), and HLA (*H. verticillata* + *L. fortunei* + *A. crassipinnis*) are the invasion treatments (see Fig. 1) the mortality of *M. forestii* may be related to direct predation by the non-native *A. crassipinnis*. Since these two species of fish do not share an evolutionary history, the prey may be naïve to the new predator (Cox & Lima, 2006; Kuehne & Olden, 2012).

Non-natives combined effect: evidence for additive effects

Disentangling specific mechanisms by which ecological interactions between non-natives lead to the observed effects is difficult given that multiple direct and indirect interactions are at play in a community and that indirect interactions are especially difficult to detect (e.g., White et al., 2006; Kuebbing & Nuñez, 2016). Evidence in support of the IM hypotheses traditionally encompasses direct positive interactions, but indirect facilitation should be looked at more carefully (Kuebbing & Nuñez, 2016; Braga et al., 2018b). For example, if competitive interactions between non-natives are weaker than between a native and a non-native, then the presence of one non-native is indirectly beneficial to the second non-native (Kuebbing & Nuñez, 2016). The invasion by stillgrass (*Microstegium vimineum*), for example, facilitates a secondary invasion by garlic mustard (*Alliaria petiolata*) through suppression of native species (Flory & Bauer, 2014). This facilitation resulted in three times more biomass of the secondary invasive species.

Our hypothesis of a synergistic effect was not supported. Instead, we found evidence that additive effects resulted from the combination of detected individual effects. Similar additive effects were found for the interaction between an exotic snail (*Pomacea maculata*) and the exotic plant (*Alternanthera philoxeroides*) on native plant mass and diversity (Meza-Lopez & Siemann, 2015). Our experiment adds to this example by showing that additive effect may also reach ecosystem attributes.

So far, there is evidence for synergistic effects resulting from neutral or even negative interactions between non-native species. For instance, a previous study has shown that two non-native marine predators consume different sizes of the same prey species (resource competition) and likely have a synergistic effect upon a soft-sediment invertebrate assemblage at a large spatial scale (Ross et al., 2004). In another study, the combined effect of a non-native crayfish and a non-native snail lead a native snail's population nearly to extinction due to predatory and competitive relations. However, the non-native snail was also negatively affected by the crayfish species although to a lesser extent (Johnson et al., 2009). Jackson et al. (2014) conducted a mesocosm experiment and showed additive and synergistic impact of crayfish on the ecosystem, although with no clear benefit for each other.

Evidence of positive interactions leading to synergistic effects is scarce. Perhaps the strongest evidence comes from a combination of different studies on Christmas Island, where a network of positive interactions among terrestrial non-native species (the yellow crazy ant, *Anoplolepis gracilipes*, honeydew-secreting scale insects, *Tachardina aurantiaca*, and the giant African land snail, *Achatina fulica*) led to a severe synergistic effects on the native population, community, and ecosystem (O'Dowd et al., 2003; Abbott & Green, 2007; Green et al., 2011; O'Loughlin & Green, 2015). In any case, whether in analyzing the outcomes found in the literature or in our investigation, evidence of additive or synergistic effects on communities may be the result of the high degree of contingency found in community ecology and invasion science (Simberloff, 2004).

We propose that advances in this field could be achieved by following two lines of investigation. First, one could investigate whether antagonistic, additive, or synergistic effects are associated with particular combinations of species traits (e.g., predominance of non-native herbivores vs. a combination of herbivores and top predators) or are a characteristic of particular types of ecosystems (e.g., riverine vs. wetlands vs. lake; tropical vs. temperate) or even if invasion time matter (e.g., recently invaded vs. ecosystems invaded for a long time). Second, one could search for mechanisms behind each type of identified effect (e.g., direct vs. indirect facilitation). These investigations should necessarily involve a combination of observational studies along with experiments conducted at different spatial and temporal scales. Here, we were able to disentangle the outcome of multiple species introductions in several response variables.

In summary, our experiment corroborated the idea that additive effects predominate in mesocosms where we simulated different combinations of native and non-native aquatic species typically found in a Neotropical floodplain. The current increase of non-native species invasions worldwide (e.g., Seebens et al., 2017) means that co-occurrence and interactions among them will increase and, as shown here, intensify the negative effects on native biota more than on one another. Thus, if our results are typical of other tropical highly diverse aquatic ecosystems, we predict that these ecosystems are under an even greater threat resulting from further introductions of non-native species.

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