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ORIGINAL PAPER

Experimental evidence for the use of artificial refugia to mitigate the impacts of invasive *Gambusia holbrooki* on an endangered fish

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Abstract Habitat degradation has a major impact on freshwater ecosystems and also facilitates biological invasions thus intensifying the problem. The survival of native species under threat from invaders can be improved either by eradicating the invading species or by providing resources or conditions that benefit the native species. One such resource is shelter and in degraded habitats artificial refugia may be a viable option. However, the use of artificial refugia to promote coexistence between native and invasive species remains poorly understood. We assessed the potential for artificial refugia to ameliorate the disruption of social interactions in an endangered native Iberian toothcarp, *Aphanius iberius*, by the invasive mosquitofish, *Gambusia holbrooki*. We found that mosquitofish do not compete for access to refugia despite their higher level of aggression compared to native fish. Native fish use refugia more overall, particularly in the presence of mosquitofish. Despite this, the benefits of refugia are not clear cut: increases in refuge use by male toothcarp induced by mosquitofish aggression correspond to decreases in attention to conspecifics. However, changes in refugia use over time together with constant attention to conspecifics indicates that it is not refugia use itself that disrupts social interactions but the interrelated effects of mosquitofish aggression. Provision of artificial refugia in degraded freshwater ecosystems may thus be a viable management tool to protect native populations under imminent threat of invasion.

Keywords Artificial refugia · Social interactions · *Gambusia holbrooki* · *Aphanius iberus* · Habitat degradation · Aggression

Introduction

Freshwater ecosystems are of fundamental importance yet are among the most endangered in the world (Dudgeon et al. 2006; Vörösmarty et al. 2010). Anthropogenic influences have led to modifications of freshwater characteristics and impacted on the biodiversity of freshwater ecosystems (Mack et al. 2000; Dudgeon et al. 2006; Vörösmarty et al. 2010). Habitat degradation also facilitates biological invasions (Mack et al. 2000; Matsuzaki et al. 2012; Dudgeon et al. 2006) thus intensifying the problem; ecosystem degradation and biological invasions are two of the five greatest threats to freshwater ecosystems (Dudgeon et al. 2006).

The survival of native species under threat from invaders can be improved either by eradicating the invading species or by providing resources to support the native species (Yokomizo et al. 2007; Simberloff

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2014). One such resource is shelter (Schlaepfer et al. 2005; Yokomizo et al. 2007; Westhoff et al. 2013), which is particularly important in degraded freshwater ecosystems as habitat alteration reduces the availability of refugia (Matsuzaki et al. 2012). The presence of invaders is likely to reduce the availability of refugia even further through excluding native species from shelter (Matsuzaki et al. 2012), which increases the risk of predation and exposes vulnerable native species to stochastic events (Hayes et al. 2009; Matsuzaki et al. 2012). Refugia include all habitats and environmental factors that temporally or spatially reduce the negative effects of biophysical disturbances compared to surrounding places or times (Magoulick and Kobza 2003). For example, habitat refuges may be created by differences in flow regime (Light 2005) or by materials such as wood (Nagayama and Nakamura 2010); or consist of a physical tolerance to an environmental stressor (Magoulick and Kobza 2003). In general, any type of habitat heterogeneity can provide a refuge for some species (Nagayama and Nakamura 2010) and in degraded habitats artificial refugia may be a viable option (Matsuzaki et al. 2012; Westhoff et al. 2013). However, despite the importance of shelter, and although several species have been shown to use artificial refugia (e.g. Matsuzaki et al. 2012; Westhoff et al. 2013), the role of refugia in aquatic systems remains poorly understood (Magoulick and Kobza 2003) and more work is needed to assess the use of artificial refugia to promote coexistence between native and invasive species (Matsuzaki et al. 2012; Westhoff et al. 2013).

Most studies of refugia with invasive species measure the outcome of refuge presence in terms of species displacement (Becker et al. 2005). For example, many studies evaluate the effectiveness of refugia via differences in the survival of native species (e.g. Segev et al. 2009; Westhoff et al. 2013), and those which examine a less lethal endpoint generally focus on some measure of relative abundance (e.g. Pander and Geist 2010; Hanshew and Garcia 2012). These studies, while important, do not address the individual level mechanisms which result in these population level effects. Although individual behaviors, such as competition, are more difficult to study than mortality or abundance (Light 2005), the behavior of individuals of both the invading and native species are influential (Light 2005; Hayes et al. 2009) and incorporation of behavioral processes into management strategies would be beneficial (Schlaepfer et al. 2005). Individual behaviors that have received some attention include time in refugia (e.g. Light 2005; Hayes et al. 2009), aggression (e.g. Hayes et al. 2009), and escape behavior (e.g. Light 2005). Effects on growth rate (Matsuzaki et al. 2012; Segev et al. 2009), which are probably an indirect effect of refugia presence reducing the metabolic stress of predator avoidance activities (Matsuzaki et al. 2012), have also been recorded. However, although both reductions (Lawler et al. 1999) and increases (Light 2005) in activity levels are sometimes recorded, social interactions between native individuals in the presence of invasive species and refugia have been neglected. In this study we therefore address the individual level mechanisms of social interactions, along with aggression and shelter use, by which refuge availability ameliorates (or not) the impacts of an invasive on a native species.

The eastern mosquitofish, *Gambusia holbrooki*, and the closely related western mosquitofish, *G. affinis*, are well known, highly successful invaders. They have been introduced into more than 50 countries as mosquito control agents (Lever 1996; Pyke 2008) and have adversely affected many freshwater species (e.g. Lawler et al. 1999; Segev et al. 2009; Westhoff et al. 2013) Indeed, they are considered to be among the 100 worst invasive species worldwide (Lowe et al. 2000). *Gambusia holbrooki* has colonized much of southern Europe including the Iberian Peninsula (García-Berthou et al. 2005), where it has replaced the endemic Iberian toothcarp, *Aphanius iberus*, and other Mediterranean cyprinodontiforms in much of its former habitat (Doadrio 2002). The Mediterranean region is heavily affected by anthropogenic habitat degradation which has aided the spread of mosquitofish (Ruiz-Navarro et al. 2013). Although physical parameters such as salinity provide partial refugia for toothcarp from mosquitofish (Alcaraz et al. 2008; Ruiz-Navarro et al. 2013), toothcarp are becoming marginalized, and are now considered Endangered (EN A2ce) under the IUCN Red List, and protected by the Bern Convention on the Conservation of European Wildlife and Natural Habitats (Freyhof and Brooks 2011). Management tools are therefore needed to enhance the survival of existing toothcarp populations that inhabit mosquitofish suitable habitats.

In this study, we simulate conditions at the 'invasion front' of the mosquitofish invasion in a laboratory experiment to assess whether artificial refugia can mitigate the individual level impacts on toothcarp populations in imminent danger of being invaded by mosquitofish. As any beneficial effects of refugia would be negated if mosquitofish exclude toothcarp, we first investigated the differential use of refugia by each species and assessed the hypothesis that toothcarp are able to use refugia even in the presence of mosquitofish. Then, as several previous studies have shown that a major cause of mosquitofish impact is their high levels of aggression to toothcarp (e.g. Alcaraz et al. 2008; Carmona-Catot et al. 2013) and other species (e.g. Laha and Mattingly 2007), we hypothesized that mosquitofish would be more aggressive than toothcarp. Finally, we predicted that toothcarp would be able to use refugia to escape mosquitofish aggression, and that refugia presence would ameliorate any deleterious effects of mosquitofish on toothcarp social interactions. Both toothcarp (Ruiz Navarro and Oliva Paterna 2012) and mosquitofish (Evans et al. 2011) show clear sexual dimorphism and have distinctive roles in reproduction. Males and females may thus exhibit distinct aggression, vulnerability to aggression (Magellan and García-Berthou 2015) and interest in conspecifics, and have different shelter requirements. We therefore included individual sex in our analyses. Moreover, as we were interested in the immediate influence of mosquitofish presence, but behavior may change even in the short term as toothcarp and mosquitofish become accustomed to each other, we also incorporated the effects of time into each of our hypotheses. Based on our results, we discuss whether the provision of artificial refugia is likely to be beneficial for toothcarp populations under threat of mosquitofish invasion.

Methods

Study fish

Sixty litre stock aquaria (60 × 30 × 32 cm) were set up containing a gravel substrate, conditioned water and a filtered air supply. Aquaria were maintained at a constant photoperiod (12:12 h light:dark cycle) using 6 W bulbs and temperature was kept at 25 ± 1 °C. Mosquitofish were collected from the Ter (42.0451 N, 3.1960 E), Fluvià (42.1875 N, 3.0851 E) and Muga (42.2527 N, 3.0756 E) rivers, where they had no prior contact with toothcarp. Toothcarp came from Fra Ramon lagoon (42.0149 N, 3.1129 E), Baix Empordà salt marshes and while they may have previously encountered mosquitofish this contact was minimal. This asymmetrical pattern of contact between species was assumed to mimic the spreading process of a biological invasion. All fish were transported to the laboratory and placed in three stock aquaria per species, with mosquitofish from all three populations being housed together. Population differences in behaviour were addressed in a separate experiment in which no differences were apparent (K. Magellan: unpublished data). Fish were allowed to acclimate to laboratory conditions, and in the case of mosquitofish to their mixed populations, for at least 2 months prior to the start of experimentation. During acclimation fish were fed to satiation once per day with commercial food flakes or frozen bloodworms (*Chironomus* spp.).

Experimental set-up

For experiments, six 26 L aquaria (45 × 22 × 28 cm) containing a gravel substrate, an air supply and an aquarium heater, with an overhead light source, were filled with conditioned water to a depth of 20 cm. Refugia, consisting of clear plastic jars, 5 cm diameter, 12.5 cm tall and covered with a grey mesh, were added to each tank and situated so that the open ends faced the front of the tank. Six separate aquaria (without refugia) were set up for use as holding tanks for mosquitofish during experiments. Six trials could thus be carried out simultaneously.

The evening before a set of trials, two toothcarp were added to each of the six experimental tanks, with refugia present, fed and left overnight to acclimate. The toothcarp remained in their experimental tank throughout the 3 days for each trial (one evening with overnight acclimation, 3 × Day 1 treatments, 1 × Day 2 treatment) so that they always had prior residence and had previous experience with refugia. The following morning all refugia were carefully removed from aquaria. At the same time, pairs of mosquitofish were added to the separate holding aquaria (1 pair per tank). Each mosquitofish pair was matched with a toothcarp pair throughout their trial so avoiding effects of individual differences between treatments. All fish were randomly selected with the proviso that each individual within the group of four fish could be identified using species, sex and size. For mosquitofish, this resulted in mixed sex, all female, and all male pairs, while for toothcarp, the limited number of males meant there were no male-male pairs. All fish were provided with food and left to feed for 1 h. Food was provided in excess so that pre-observation hunger levels were standardized and to ensure fish were motivated to interact with each other rather than forage.

Observations

On Day 1, fish were observed in three different treatments in a random order: two toothcarp with a refuge (treatment R); two toothcarp and two mosquitofish without a refuge (treatment M); and two toothcarp and two mosquitofish with a refuge (treatment RM). After feeding, refugia (treatments R and RM) and mosquitofish (treatments M and RM) were added to aquaria as required. Fish were left for 1 h to settle, after which 10 min observations were conducted; all observations were videoed and scored later. After each observation, refugia and mosquitofish pairs were removed or added as necessary, fish were left for 1 h and the next 10 min observation was conducted. This protocol was repeated for a third time. At the end of Day 1 observations, the mosquitofish were removed and each pair placed in their stock aquarium, refugia were added to each of the experimental tanks if needed, and fish were again fed and left overnight.

The following morning (Day 2) fish were fed and left for 1 h as before, then mosquitofish pairs were added to each tank and the 10 min observations (treatment RMday2: as treatment RM) were videoed. After Day 2 observations were completed, the sex of each fish was recorded and total length was measured to the nearest millimetre using a ruler to facilitate individual identification in the videos. Fish were placed in separate stock tanks so that they would not be reused.

Five sets of six trials were conducted. Thirty sets of 2 toothcarp and 2 mosquitofish were thus observed in all of the four treatments.

Behavioural analysis

Each fish's behavior was individually scored from the 10 min videos recording the total time spent in refugia and the number of aggressive acts carried out towards each other individual for all fish; and for toothcarp, the time spent paying attention (attendance) to the other conspecific. Aggression included lunges (fast movements towards another individual without making contact), chases (longer movements towards another individual) and bites (as lunges but with contact). Attendance was recorded when a fish was oriented directly towards another individual. The relatively small size of the experimental tanks meant that individual fish were able to see the other fish from anywhere within the tank.

Statistical analyses

All analyses used Generalized estimating equations (GEEs: extensions of Generalized linear models (GLMs) to account for data dependency; see e.g. Magellan and García-Berthou 2015) in IBM SPSS version 20. In each case non-significant interaction terms (and associated main effects that were also non-significant) were removed and the analysis repeated to obtain the simplest model to explain the variation in the data. Posthoc pairwise tests using GLM algorithms with sequential Sidak correction for multiple comparisons were used to elucidate significant differences in main effects in cases where three levels of the independent variable were included in the analysis.

First, refuge use was examined. To assess differences in refugia use between species and over time, data from treatments RM and RMday2 (the two treatments that contained both species with refugia) were used in a GEE with fish group as the between subjects factor and species using the refuge, fish number within a species pair (i.e. toothcarp 1 or 2, mosquitofish 1 or 2), and time (day 1 or day 2) as within subject factors. The model included species, time and fish sex as independent factors. Sex was included as this is likely to affect intra- (and maybe inter-) specific interactions. The dependent variable, refuge use, had Poisson distribution assumed with a log-link function.

Second, to assess the effects of mosquitofish presence on toothcarp refuge use another GEE was carried out using data from treatments R (toothcarp with refugia) and RM (toothcarp with refugia and mosquitofish).The between subjects factor was again fish group and the within subjects factors were toothcarp number and mosquitofish presence. The dependent variable of toothcarp refuge use (Poisson distribution, log-link function) was tested against the independent variables of mosquitofish presence and toothcarp sex. Again, sex was included as male and female toothcarp may exhibit differential refuge use.

Third, differences in the frequency of attacks between species and treatments was examined using data from treatments M, RM and RMday2 (all treatments that included mosquitofish) in a GEE with fish group as the between subjects factor, and fish number, attacking species, and treatment as within subjects factors. The independent factors were species, treatment and sex. Sex was included as males and females of both species are likely to show different levels of aggression (e.g. Magellan and García-Berthou 2015). The dependent variable, attacks carried out, assumed a Poisson distribution and log-link function.

Finally, the effects of refugia for toothcarp were assessed. Two measures of potential effects were examined: attacks received by toothcarp from mosquitofish and social interactions between toothcarp pairs, the latter using attendance (described above) as a proxy for interactions. The effects of refugia were assessed indirectly by examining the influence of refugia presence on the two measures of potential effects using two separate GEEs with the dependent variables of attacks received (Poisson distribution, log-link function) and attendance (normal distribution, identity-link function) using data from treatments M, RM and RMday2. The between subjects factor was fish group and the within subjects factors were fish number and treatment. The final model included treatment and toothcarp sex as independent variables.

The influence of refugia use on toothcarp social interactions was further assessed directly. As there were no effects of treatment in the previous analysis (see "Results", Table 3; Fig. 4b) the means of toothcarp refuge use and attendance for treatments RM and RMday2 were taken for each toothcarp, which served to provide a more general measure of refuge use by removing any effects of time. Attendance was then used as the dependent variable (normal distribution, identity-link function) in a GEE with fish group as the between subjects factor, fish number as the within subjects factor, sex as the independent variable and toothcarp refuge use as the covariate.

Results

Refuge use was overall highly variable. In some trials none of the fish used refugia in any of the treatments, while in others all four fish used refugia at least once during the four treatments. Refuge use varied even within individuals with some fish using refugia only once and others entering refugia during every treatment. Occasionally at least one fish of each species were together in refugia but there were no trials in which all four fish were in refugia at the same time. There was a significant interaction between species and time for refugia use (Table 1a). However, when non-significant interaction terms were removed this significant interaction disappeared (Table 1b). Toothcarp made significantly more use of refugia than mosquitofish and refugia use increased on the second day (Fig. 1).

Table 1 Results from the generalized estimating equations examining differences in refugia use between species, sex and time (treatments RM and RMday2): (a) fully factorial analysis, (b) with non-significant interaction terms removed

Variables	Wald χ^2	*df*	*p*
(a)			
Species (S)	35.820	1	**<0.001**
Time (T)	5.325	1	**0.021**
Sex (X)	0.434	1	0.510
S × T	5.268	1	**0.022**
S × X	0.002	1	0.966
T × X	3.114	1	0.078
S × T × X	0.014	1	0.907
(b)			
Species (S)	31.153	1	**<0.001**
Time (T)	9.652	1	**0.002**
S × T	2.792	1	0.095

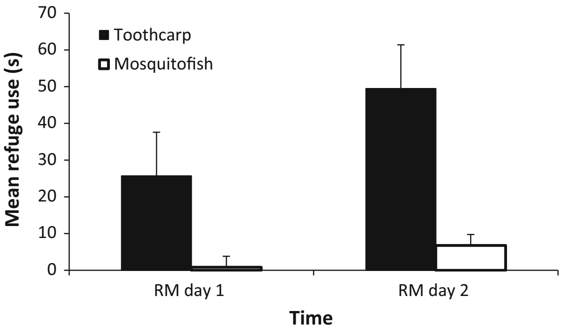

Fig. 1 Differences in refugia use between species on day 1 (*RMday1*) and day 2 (*RMday2*). *Error bars* represent standard error

When refuge use by toothcarp alone was considered, although there was no difference between males and females (Wald $\chi^2 = 0.05$; $df = 1$; $p = 0.818$) and the interaction term was non-significant (Wald $\chi^2 = 2.63$; $df = 1$; $p = 0.105$), refugia use increased in the presence of mosquitofish (Wald $\chi^2 = 6.38$; $df = 1$; $p = 0.012$) (Fig. 2).

Aggression was likewise variable with some individuals consistently carrying out multiple attacks during every treatment while others exhibited no aggression at all. The factorial analysis of frequency of attacks showed a significant difference only with treatment (Table 2a). Removal of the non-significant interaction terms revealed that mosquitofish carried out significantly more attacks than toothcarp, with the highest aggression being shown when no refugia were present (Table 2b; Fig. 3). Posthoc tests indicated a significant increase in attacks when no refugia were present on day 1 ($p = 0.013$). However, attacks increased significantly on day 2 ($p = 0.010$) such that there was no significant difference between attacks with refugia on day 2 and without refugia on day 1 ($p = 0.154$).

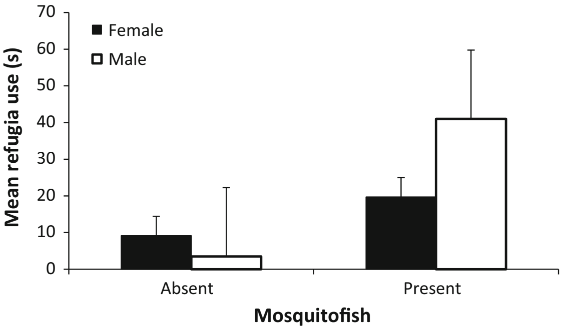

Fig. 2 Differences in refugia use between toothcarp sexes with and without mosquitofish. *Error bars* represent standard error

Table 2 Results from the generalized estimating equations examining differences in aggression between species, treatment and sex: (a) fully factorial analysis, (b) with non-significant interaction terms removed

Variables	Wald χ^2	*df*	*p*
(a)			
Species (S)	2.578	1	0.108
Treatment (T)	16.918	2	**<0.001**
Sex (X)	1.543	1	0.214
S × T	4.179	2	0.124
S × X	0.720	1	0.396
T × X	1.716	2	0.424
S × T × X	0.894	2	0.640
(b)			
Species (S)	7.288	1	**0.007**
Treatment (T)	23.967	2	**<0.001**
Sex (X)	1.934	1	0.164

Treatment includes treatment M (without refugia), treatment RM (with refugia on day 1) and treatment RMday2 (with refugia on day 1): see main text for details. Significant results are highlighted in bold

Although some mosquitofish did show intra-specific aggression, most attacks were directed at heterospecifics. All toothcarp directed some attention to at least one other fish, usually the conspecific though often also one or both of the mosquitofish, in every treatment of each trial. In the indirect assessment of the effects of refuge use, sex affected the attacks received by toothcarp from mosquitofish with males being the target of more attacks when there were no refugia and females being the main target when refugia were present (Table 3; Fig. 4a). Male toothcarp also spent significantly more time attending conspecifics than females in all treatments while treatment itself had no effect on attendance (Table 3; Fig. 4b).

However, refuge use directly affected male and female attendance (Wald $\chi^2 = 13.13$; $df = 1$; $p < 0.001$). Attendance overall decreased with increasing refuge use (Wald $\chi^2 = 10.89$; $df = 1$; $p = 0.001$) while the effects of toothcarp sex were highly significant (Wald $\chi^2 = 15.62$; $df = 1$; $p < 0.001$), with females slightly reducing attendance with refugia use while males showing a much more

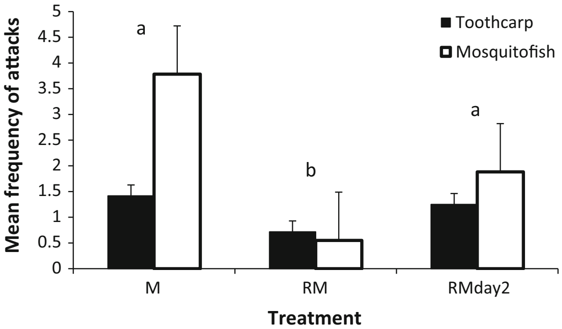

Fig. 3 Differences in attacks between species without refugia (*M*) and with refugia on day 1 (*RM*) and day 2 (*RMday2*). *Different letters* above *bars* indicate significant differences in posthoc tests. *Error bars* represent standard error

Table 3 Results from the generalized estimating equations examining the effects of the presence of refugia (treatment) and toothcarp sex on attacks received by toothcarp from mosquitofish and social interactions between toothcarp (attendance)

Variables	Attacks received			Attendance		
	Wald χ^2	*df*	*p*	Wald χ^2	*df*	*p*
Treatment (T)	0.930	2	0.628	0.534	2	0.766
Sex (X)	0.322	1	0.570	15.346	1	**<0.001**
T × X	7.056	2	**0.029**	0.073	2	0.964

Treatment includes treatments M (without refugia), RM (with refugia on day 1) and RMday2 (with refugia on day 2): see main text for details. Significant results are highlighted in bold

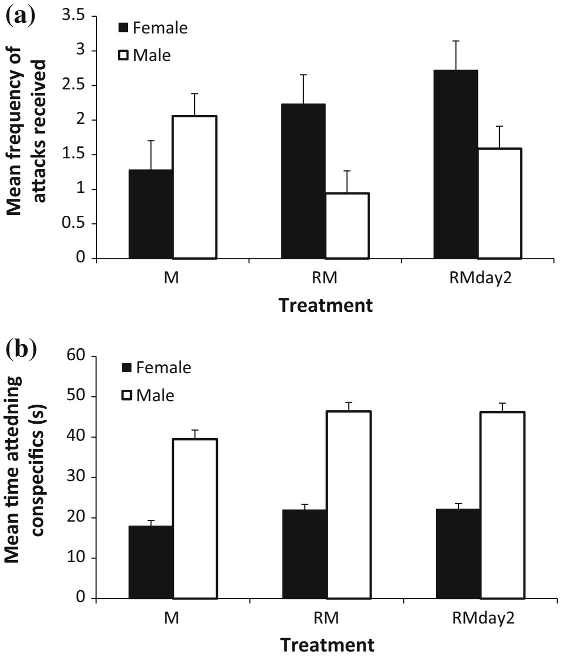

Fig. 4 Mean **a** frequency of attacks received from mosquitofish and **b** time spent attending conspecifics by toothcarp females and males with (*RM* and *RMday2*) and without (*M*) refugia. *Error bars* represent standard error

dramatic decrease in attendance with increased refugia use (Fig. 5).

Discussion

Native Iberian toothcarp readily entered the artificial refugia provided. Moreover, toothcarp used refugia more than mosquitofish. As the fish were forced into close contact by the experimental situation, they were likely to have to compete for resources (Becker et al. 2005), suggesting that toothcarp dominated refugia use. Although a previous study also found that mosquitofish could not outcompete a small fish, *Galaxias maculatus*, for refugia, this was thought to be due to the larger size of the native species (Becker et al. 2005). Toothcarp have no such size advantage though their prior residency may confer a competitive advantage (Stammler and Corkum 2005; Heavener et al. 2014). However, the higher level of aggression shown by mosquitofish suggests that they would win any direct competitive interactions so it is more likely that mosquitofish were not interested in the refugia. Mosquitofish aggression during this experiment was therefore not a consequence of competition for shelter. Competition for food is also improbable. While there may have been a small amount of food left from toothcarp feeding in the experimental aquaria, mosquitofish were fed in excess before each trial so were unlikely to be motivated to forage and therefore fight for food resources. Mosquitofish aggression must have an alternative explanation. Toothcarp not only utilized refugia more, they also increased refuge use in the presence of mosquitofish. The increased density of fish with the addition of mosquitofish, which mimics the increased local density when mosquitofish initially migrate into an area, may have induced toothcarp to seek shelter. Although even this increased density was relatively low compared to natural habitats: toothcarp density estimates range around 0.41 individuals per liter and 86–131 individuals per square meter (Ruiz Navarro and Oliva Paterna 2012) while mosquitofish density is highly variable (Evans et al. 2011); a density effect cannot be entirely ruled out. However, the

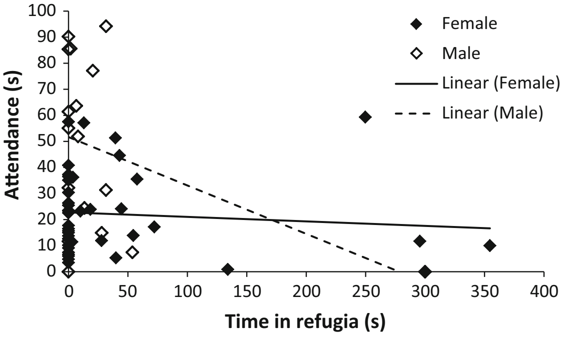

Fig. 5 Time spent attending conspecifics by toothcarp females ($R^2 = 0.01$) and males ($R^2 = 0.16$) as a function of time spent in refugia

aggression shown by mosquitofish, together with toothcarps' increased refugia use with mosquitofish, suggests that mosquitofish were attacking toothcarp, and toothcarp were using refugia to escape mosquitofish aggression.

Toothcarp thus appear to benefit from refugia presence, a perception reinforced by the observation that mosquitofish showed more aggression and toothcarp overall received more aggression when no refugia were present. However, while for male toothcarp the increased attacks from mosquitofish without refugia are mirrored by the decreased attacks received when refugia were present, for toothcarp females the opposite is apparent. Toothcarp females used refugia less than males overall, and their increase in refugia use with mosquitofish was more moderate than that of males. In addition, females received more attacks when refugia were present. Taken together, these results suggest that when male toothcarp were not available, because they were hiding in refugia, mosquitofish switched their aggression towards female toothcarp, which did not use refugia to escape attacks to the same extent as males. This may be due to larger females being less vulnerable to damage from attacking mosquitofish. The results for attendance as a proxy for social interactions are more conclusive. Males paid much more attention to conspecifics than females regardless of refugia presence, which presumably reflects their reproductive interest in females. The limited female interest in conspecifics decreased slightly with increasing refugia use, while for males the decrease in attendance with refugia use was dramatic. This suggests that aggression from mosquitofish indirectly interferes with toothcarp social interactions by prompting toothcarp males to use refugia, which reduces the time they spend interacting with females.

However, over the short-term period of 1 day used in this experiment some changes can be seen in the levels of recorded variables. Although there was a significant increase over time in mosquitofish attacks when refugia were present, mosquitofish aggression decreased overall between day 1 and day 2 and the attacks received by toothcarp increased slightly (though non-significantly) indicating a change in intra-, rather than interspecific aggression by mosquitofish. At the same time, toothcarp refuge use increased by the second day and it is clear that it was males that predominantly used refugia. These changes likely reflect increased experience of heterospecifics for both species (Hayes et al. 2009; Heavener et al. 2014). However, the overall level of toothcarp attendance of conspecifics did not change over time, which taken together with the increase in refugia use indicates that any effects that refugia may have on toothcarp social interactions is overshadowed by the interrelated influence of mosquitofish aggression. Overall then, mosquitofish aggression, which contributes to its invasive success (e.g. Segev et al. 2009; Carmona-Catot et al. 2013) and is likely to have immediate impacts, has longer-term impacts on toothcarp in terms of disruption of social interactions, which probably results in decreased reproductive success and a reduction in population survival potential. Although the benefits of refugia presence are not clear cut, it is apparent that some of these impacts may be at least partially ameliorated by provision of refugia.

The difficulty of studying competition and social interactions in situ, especially in aquatic habitats (Light 2005), means that experimental studies are necessary to estimate the effects of any given factor or species (Lawler et al. 1999). However, complementary field studies in natural environments are also needed (Lawler et al. 1999; Becker et al. 2005; Matsuzaki et al. 2012). Several studies have reported broadly similar results in laboratory and mesocosm or field studies (e.g. Hayes et al. 2009; Hanshew and Garcia 2012; Westhoff et al. 2013) but this is not a universal finding (e.g. Lawler et al. 1999) and the multitude of potential influences on interactions between native and invasive species means field testing is necessary to assess the true benefits of refugia availability. For example, the propagule pressure of the invasive species (e.g. Becker et al. 2005) and the density of shelters (e.g. Hanshew and Garcia 2012) are both likely to play a role. Moreover longer-term effects such as growth rate are likely to be important (e.g. Matsuzaki et al. 2012) and effects on different life stages, especially juveniles, may have consequences for recruitment (e.g. Laha and Mattingly 2007; Westhoff et al. 2013). Finally, mosquitofish undoubtedly disrupt other aspects of toothcarp life. For example, although in this experiment fish were fed to apparent satiation so any effects on foraging would be artificially reduced, both aggression from mosquitofish and increased refugia use are likely to reduce foraging time with subsequent effects on health and growth (Segev et al. 2009; Matsuzaki et al. 2012).

The jars used in this study seem to constitute suitable refugia for toothcarp under threat from invasive mosquitofish. Different refugia may be suitable for different species (Becker et al. 2005; Pander and Geist 2010) and although different types of refugia may be preferable for toothcarp, they may equally be preferable for mosquitofish making them counterproductive. For example, natural or artificial vegetation is more similar to toothcarp's natural habitat, but vegetation facilitates mosquitofish establishment (Ruiz-Navarro et al. 2013) at least partly by protecting mosquitofish juveniles from cannibalistic adults so increasing recruitment (Segev et al. 2009). In addition, toothcarp, but not mosquitofish, were observed 'hiding' outside refugia in the angle where the refugia met the substrate which was not recorded as refugia use, and the closed design of the refugia may also help protect toothcarp from predators such as aerial or larger piscine predators, so these refugia are likely to be more effective than shown here. Thus an artificial refuge that is suitable for a specific species and excludes associated invasive species should be an effective management tool (Becker et al. 2005).

Biological invasions can be managed by eradicating the invading species or by providing resources to support the native species, both of which are costly (Yokomizo et al. 2007; Simberloff 2014). In fluctuating environments, if native population density decreases, it is more cost effective to increase resource enhancement effort than increase extermination effort (Yokomizo et al. 2007). Provision of refugia is one method of enhancing habitats (Schlaepfer et al. 2005; Matsuzaki et al. 2012) and may create conditions to protect the native species until they can adapt to the invasion (Schlaepfer et al. 2005; Westhoff et al. 2013) or promote long term coexistence of native and alien species when eradication is not a feasible option (Schlaepfer et al. 2005; Hayes et al. 2009). In the Mediterranean region mosquitofish success is linked to habitat degradation (Ruiz-Navarro et al. 2013) and both degradation and mosquitofish are implicated in the decline of toothcarp populations (Oliva-Paterna et al. 2006). Although refugia have been shown to be ineffective at promoting coexistence between western mosquitofish, *G. affinis* and topminnows, *Fundulus julisia* (Westhoff et al. 2013) and fire salamanders, *Salamandra infraimmaculata* (Segev et al. 2009), the current study using eastern mosquitofish, *G. holbrooki*, indicates that artificial refugia may be successful in aiding the survival of Iberian toothcarp populations in imminent danger of invasion. Ultimately it is less costly to prevent the spread of invasions than control invaders in a native habitat (Mack et al. 2000; Vörösmarty et al. 2010). Moreover, artificial refugia can be a viable management tool, especially in degraded aquatic habitats.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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