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ELENI KALOGIANNI, NICHOLAS KOUTSIKOS,  
LEONIDAS VARDAKAS, SOFIA GIAKOUMI, YORGOS  
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## Impacts of the alien mosquitofish on the abundance and condition of two Mediterranean native fish

Eleni KALOGIANNI<sup>1</sup>, Nicholas KOUTSIKOS<sup>1</sup>, Leonidas VARDAKAS<sup>1</sup>, Sofia GIAKOUMI<sup>1</sup>, Yorgos CHATZINIKOLAOU<sup>2</sup> and Anthi OIKONOMOU<sup>1</sup>

<sup>1</sup>Hellenic Centre for Marine Research, Institute of Marine Biological Resources and Inland Waters, Anavissos, 19013, Attica, Greece

<sup>2</sup>Freelancer PhD Environmentalist Ecologist, 54634, Ioulianou 19 Thessaloniki, Greece

Corresponding author: [ekalog@hcmr.gr](mailto:ekalog@hcmr.gr)

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### Abstract

The rapid decline of the Greek native *Valencia letourneuxi*, and its recently described sister species *Valencia robertae*, has been often attributed to habitat degradation, as well as aggression from or resource competition with the alien mosquitofish *Gambusia holbrooki*. The mosquitofish is extremely wide-spread in Mediterranean freshwater ecosystems, however, documentation on its presumed negative impacts on native freshwater fishes is limited. In this study, we compared abundance, condition and gonad weight of *Valencia* spp. in no co-occurrence versus co-occurrence to the mosquitofish, using data collected from eight aquatic systems of Greece, over a five year period (2005 to 2009). Our results show that *Valencia* abundance is significantly lower in co-occurrence to the Eastern mosquitofish. Furthermore, female *Valencia* spp. individuals in co-occurrence to the mosquitofish were smaller in length and had poorer somatic condition compared to females in habitats not invaded by the mosquitofish. The mechanisms responsible for these effects of the alien mosquitofish as well as the conservation implications for native *Valencia* populations are discussed.

**Keywords:** *Valencia robertae*; *Valencia letourneuxi*; mosquitofish; condition.

### Introduction

The decline or even extinction of native freshwater fish species has been attributed to two often concurrent and potentially synergistic processes, those of anthropogenic habitat degradation and the introduction of exotic species (Arthington *et al.*, 1983; Kennard *et al.*, 2005; Olden *et al.*, 2010). Exotic species are often superior competitors in relation to native species, due to their high reproductive potential, more successful feeding strategies, wider environmental tolerances, as well as being free from predators, parasites, etc. that regulate their population size within their native range (Townsend, 1996; Mills *et al.*, 2004; Kennard *et al.*, 2005).

Among the most successful invaders worldwide is the Eastern mosquitofish *Gambusia holbrooki* Girard, 1859, native to the United States and Mexico, which has been introduced to Europe in the 1920s to control mosquito populations, through predation on mosquito larvae (Courtenay & Meffe, 1989; García-Berthou

*et al.*, 2005; Pyke, 2005). *Gambusia holbrooki* is now very abundant in many Mediterranean countries (Vidal *et al.*, 2010; Ribeiro & Leunda, 2012; Piria *et al.*, 2018), including Greece. Its first documented introductions in Greek aquatic systems, through Italy and France, were between 1927 and 1937 (Livadas & Sphangos 1941, see also Economou *et al.*, 2007; Barbieri *et al.*, 2015). The Eastern mosquitofish is an extremely successful invader, due to its early maturation, viviparity, high reproductive rate, high behavioural plasticity and adaptability to a wide range of, often degraded, environmental conditions (Vargas & de Sostoa, 1996; Pen *et al.*, 1993). Many studies have associated the mosquitofish with the decline and extinction of several small native fish species worldwide, with predation on egg and larvae and interference and/or exploitation competition being proposed as the main mechanisms underlying these processes (Meffe, 1985; Arthington & Lloyd, 1989; Mills *et al.*, 2004; Alcaraz *et al.*, 2008; Keller & Brown, 2008; for the Mediterranean, see Ribeiro & Leunda, 2012).

Mediterranean Cyprinodontiformes especially, such as the Iberian endemic Spanish toothcarp *Aphanius iberus* (Valenciennes, 1846) and the *Valencia* toothcarp *Valencia hispanica* (Valenciennes, 1846), appear to have been displaced by the mosquitofish from their native habitats (Elvira, 1995; Leunda, 2010). Similarly, in Greece, the Eastern mosquitofish, now the most widespread alien fish species, has been implicated in the decline of many native fishes (Economidis, 1995; Economou *et al.*, 2007). These include the Corfu *Valencia* *V. letourneuxi* (Sauvage 1880) and its recently described sister species the Peloponnese *Valencia* *V. robertae* Freyhof, Kärst & Geiger, 2014, that encompasses the most southern populations of the genus in Greece, previously known as *V. letourneuxi*. *Valencia letourneuxi*, a small ecomorphologically unique cyprinodont (Oikonomou *et al.*, 2018) is listed in Annex II of the Bern Convention and in Annex II of the European Union Habitats Directive 92/43/EEC. It has been also classified as critically endangered by IUCN in 2006, based on restricted and fragmented distribution, suspected population decline, projected decline in the next 10 years and impacts of introduced alien species (Crivelli, 2006). *Valencia robertae*, though not yet assessed by IUCN, is a potentially critically endangered species due to the decline and extirpation of its local populations (Barbieri *et al.*, 2015). The highly fragmented geographical range of both species in Western Greece has been reduced in relation to the past, with some populations gone extinct and others in a vulnerable state (Economidis, 1991; Bianco *et al.*, 1996; Barbieri *et al.*, 2000, 2002; Kalogianni *et al.*, 2010a). Furthermore, in a recent study, *V. letourneuxi* was ranked as the second most susceptible European freshwater fish species to climate change according to its life history trait diversity and climatic niche (Jarić *et al.*, 2018).

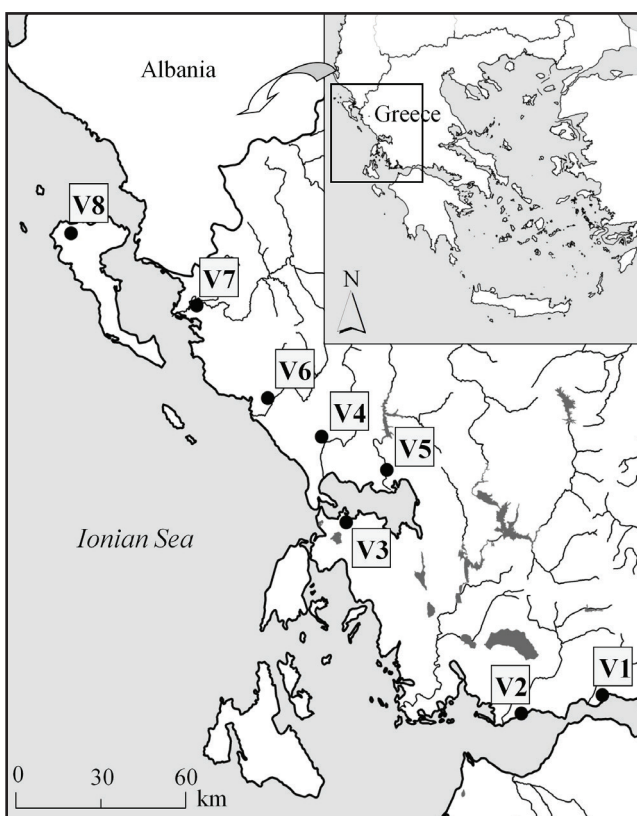
The decline of the *V. letourneuxi* and *V. robertae* has been mostly attributed to habitat degradation and fragmentation, as well as aggression from or resource competition with the mosquitofish (Bianco & Miller, 1989; Barbieri *et al.*, 2000; Kottelat & Freyhof, 2007). However, there are few studies assessing the presumed negative impacts of the mosquitofish on the two Greek *Valencia* species or the mechanisms underlying them. In a recent comparative dietary study, evidence has been presented that the underlying mechanism of the putative negative impacts of the mosquitofish on the declining Corfu *Valencia* is mainly trophic competition, regulated by resource variability, though there is also evidence of larvae predation by the mosquitofish (Kalogianni *et al.*, 2014). More experimental as well as *in situ* studies are, however, required in order to determine the effects of the mosquitofish/native species' agonistic interactions and/or trophic competition on the abundance, condition and reproduction of native fishes. The aim of this study was to assess differences in abundance, somatic condition and reproduction of the two Greek *Valencia* species living in co-occurrence and no co-occurrence with the mosquitofish. The estimation of these attributes is related to the population status of the two *Valencia* species and to their risk of extinction and can contribute to prospective conservation actions targeting these species.

## Materials and Methods

### Study area and sampling methods

*V. letourneuxi* from Northern-western and Central-western Greece and *V. robertae* from South-western Greece henceforth referred to as *Valencia* were used in this study. Fish were obtained from eight sampling sites during the dry season (from June to mid-October, which coincides with the reproductive period of the species, Kalogianni *et al.*, 2010a) with several locations sampled periodically over a five-year period (2005-2009, Fig.1; Table 1). Periodic sampling was opted for in order to avoid depleting populations in some localities where local densities are very low.

Sampling was conducted during daylight hours always by the same individual, using a D-shaped net (0.65 m x 0.35 m, 2 mm mesh size) with an extensible handle (area covered, per each net sweep, 0.45 m x 1.5 m). Samples were consistently collected from a 20-60 m long and 2m wide stretch of the stream, from its banks. At each sampling site, a minimum of eight net sweeps (trials) were conducted at eight consecutive vegetated spots, spaced at least 2m apart. When a site was sampled periodically at multiple sampling dates, samples were always collected from the same stretch of the site. During sampling, fish were identified to species level (nomenclature follows Barbieri *et al.*, 2015) and counted. Prior to preservation in 10% formalin for subsequent laboratory analysis, *Valencia* specimens were anesthetized with quinaldine (in accordance with Directive 2010/63/EU). Temperature, conductivity, pH and salinity were also measured *in situ* with a Consort C535 multi-parameter analyser.



**Fig. 1:** Study area with the eight sampling sites (from south to north, V1-V8). V1 and V2 are currently identified as *Valencia robertae* and V3-V8 as *Valencia letourneuxi*.

**Table 1.** Sampling dates, mosquitofish and *Valencia* relative abundances and main features of the *Valencia* populations studied, i.e. number (N) and standard length range (SL, mm). Fish samples not used for laboratory analysis are marked with an asterisk. Populations from V1 and V2 belong to *Valencia robertae*, and those from V3-V8 to *Valencia letourneuxi*.

Site	Basin	Date	<i>Gambusia</i>		<i>Valencia</i>	
			% Ab	% Ab	N	SL range
V1	Mornos	22/06/05	0	20.57	32	8.8 – 35.3
V1	Mornos	03/07/06	0	35.04	41	5.6 – 38.2
V1	Mornos	26/09/06	0	31.08	23	16.5 – 56
V1	Mornos	27/06/09	0	26.83	33	9.5 – 30
V2	Evinos	07/06/08*	0	53.49	23	6 – 43
V3	Vlychos	14/10/05	85.57	3.61	21	8.8 – 24.8
V3	Vlychos	04/07/06	0.46	1.37	7	27.5 – 39.5
V3	Vlychos	27/09/06	14.78	1.74	12	11.1 – 43
V3	Vlychos	01/07/09*	23.68	1.32	1	–
V4	Louros	16/10/05	29.55	9.09	17	9.7 – 24.8
V4	Louros	06/07/06	52.31	16.92	15	10 – 30
V4	Louros	27/09/06	58.44	5.19	7	12 – 20.5
V4	Louros	29/06/09	21.8	13.53	25	9 – 38
V5	Arachthos	17/10/05	4.55	22.73	11	10 – 24.5
V5	Arachthos	05/07/06	1.89	18.87	10	11.6 – 47.4
V5	Arachthos	26/09/06	1.47	13.24	9	11 – 25.2
V6	Acheron	25/09/09	4.88	17.07	9	7 – 29.2
V7	Kalamas	19/10/05	78.89	2.78	5	9.5 – 11.5
V8	Sidari	23/09/09	0	65.75	32	8.2 – 52

All sites are characterized by the typical *Valencia* habitat conditions described elsewhere (Kalogianni *et al.*, 2010a), i.e. they are small spring fed streams or canals with a narrow depth and width range, stable hydrological conditions, silty substrate, stable thermal conditions, a close to zero altitude and a high aquatic vegetation surface coverage, that provides to the target species food, spawning substrate and protection from predators (Barbieri *et al.*, 2000; Kalogianni *et al.*, 2010a). The target species were usually found together with two native species, the Western Greece goby *Economidichthys pygmaeus* (Holly, 1929) and the Stymphalia minnow *Pelagus stymphalicus* (Valenciennes, 1844) or its sister species, the Thesprotian minnow *Pelagus thespoticus* (Stephanidis, 1939). More specifically, in V1 and V2 (Fig. 1), *V. robertae* was found with the natives *P. stymphalicus* and *E. pygmaeus*. In V3, *V. letourneuxi* was found with *P. stymphalicus* and *E. pygmaeus*, and in one sampling occasion, also with *Telestes pleurobipunctatus* (Stephanidis, 1939). In V4, *V. letourneuxi* was found with *P. thespoticus* and *E. pygmaeus*, in one occasion also with *Cobitis hellenica* Economidis & Nalbant, 1997, and in one occasion with *Gasterosteus gymnuris* Cuvier, 1829. In V5, *V. letourneuxi* was found with *P. thespoticus* and *E. pygmaeus*, in one occasion also with *T. pleurobipunctatus* and in one occasion with *Cobitis arachthosensis* Economidis & Nalbant, 1997. In V6, *V. letourneuxi* was found with *P. thespoticus* and *G. gymnuris*. In V7, *V. letourneuxi* was found with *P. thespoticus* and *E. pygmaeus*, while in V8, it was found only with *P. thespoticus*. Overall, only three *Valencia* spp. habitats are free from the mosquitofish (V1, V2 and V8).

#### Laboratory methods

After their transfer to the laboratory, *Valencia* specimens were measured (standard length SL to the nearest 0.1 mm) and weighed before and after being eviscerated (net weight NW, to the nearest 0.01 mg). Gonads were examined to determine sex and then weighed (gonad weight GW, to 0.01 mg). For size class variation, fish were assigned to three length classes, i.e. larvae (<13 mm SL) where 13 mm is the standard length of the target species when scale coverage is complete and there is an almost total loss of larval characters, juveniles (SL $\geq$ 13 and <17 mm) and adults (SL $\geq$ 17 mm) where 17 mm is the maximum SL of immature individuals (Barbieri *et al.*, 2000).

#### Statistical analyses

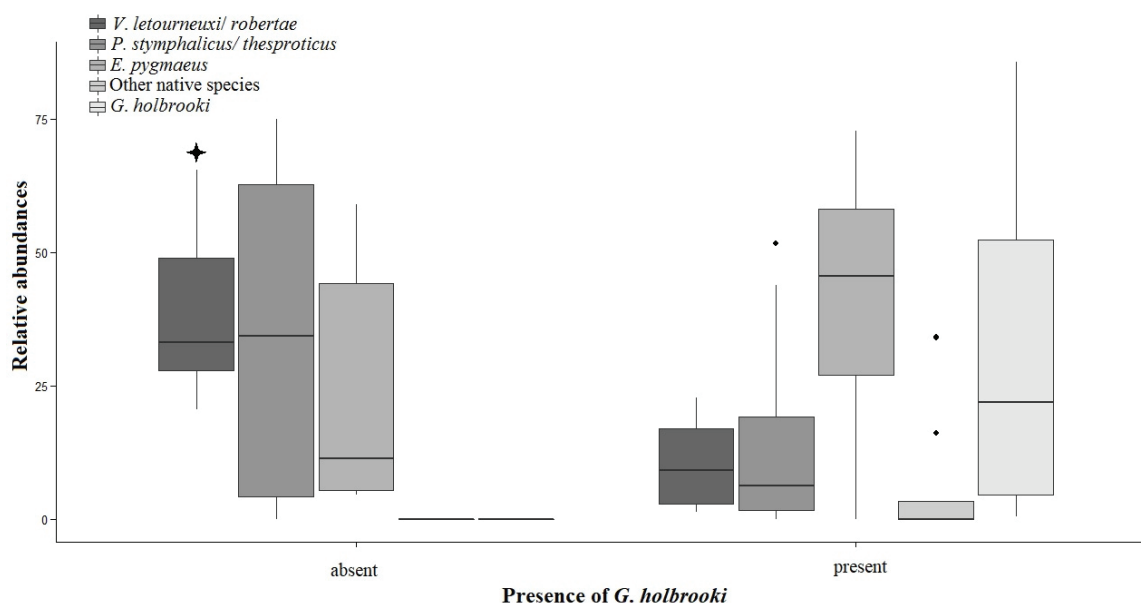
*Valencia* relative abundances and the relative abundances of the commonest native species found together with the two *Valencia* species, were compared in the presence and absence of the mosquitofish, using the Mann-Whitney Nonparametric Test of Group Differences. We then applied permutational multivariate analysis of variance (PERMANOVA) to test for differences in species relative abundances on a Bray–Curtis distance matrix among samples, using environmental variables and mosquitofish presence as explanatory factors (predictors), providing P-values by permutations (n=19999). To test for the effects of mosquitofish presence on the population structure of *Valencia*, analysis of variance (ANOVA) was performed with the percentages of larvae, juveniles, females

and males, and adult sex ratio among samples using the presence of *Gambusia* as a binary factor. SL was compared with t-tests for *Valencia* larvae, juveniles, female and male adults in co-occurrence versus no co-occurrence to the mosquitofish. In order to estimate if there is a variation in the net weight (NW) and gonadal (GW) of *Valencia* sp. in co-occurrence versus no co-occurrence with *G. holbrooki*, a generalised linear model, namely Analysis of covariance (ANCOVA) was performed. Net and gonad weight of individuals were regressed as dependent variables to the factor of the presence/ absence of *G. holbrooki*, while SL was treated as a covariate to account for the variation because of body size differences of the individuals. Prior to analyses, log-transformation was implemented to fulfil linearity between NW and GW with SL. All analyses were performed in the R statistical and programming environment (R 3.0.2., R Development Core Team, 2013) using the “vegan”, “ggplot2” and “car” packages.

## Results

### Mosquitofish effects on *Valencia* population abundance

In the current study, mosquitofish relative abundances ranged from 0 (absence of the species), to 85.57, while *Valencia* relative abundance ranged from 1.32 to 65.75 (Table 1). This value ranged between 20.57 and 53.49 for *V. robertae* (V1 and V2, Table 1) and between 1.32 and 65.75 for *V. letourneuxi* (V3-V8, Table 1). When comparing *Valencia* relative abundances, pooling data for both *Valencia* species (Fig. 2), in co-occurrence versus no co-occurrence to the mosquitofish, *Valencia* relative abundance was found significantly lower in co-occurrence to the mosquitofish (Mann Whitney test  $P < 0.001$ ).



**Fig. 2:** A comparison of relative abundances of *Valencia letourneuxi/robertae*, *Economidichthys pygmaeus* and *Pelasgus stymphalicus/thesproticus* populations, in the absence and presence of *Gambusia holbrooki*. Boxes represent the 25-75% range and the midline the median. Black circles indicate the outliers and the asterisk the significant difference based on Mann-Whitney Nonparametric Test.

No statistically significant differences in the abundances of *E. pygmaeus*, *P. stymphalicus* and *P. thesproticus*, which are more commonly found together with *Valencia*, in co-occurrence versus no co-occurrence to the mosquitofish, were evident. The same pattern was evident when pooled abundances of other native species found together with *Valencia*, i.e. *T. pleurobipunctatus*, *C. hellenica*, *C. arachthosensis* and *G. gymnurus*, were compared in co-occurrence versus no co-occurrence to the mosquitofish. Finally, when comparing *Valencia* relative abundances with relative abundances of *E. pygmaeus*, and *P. stymphalicus/P. thesproticus* to test for effects of these species on *Valencia* abundances, by performing the independent 2-group Mann-Whitney U Test, no statistical significant relationship was found ( $p > 0.05$ ).

PERMANOVA results indicated that species abundances did not differ significantly with the dissimilarity matrix of the environmental characteristics of samples, with no environmental variable found significant for the differences of species relative abundances in samples. Significant differences in species' composition based on abundance were, however, evident among samples with mosquitofish presence as a factor ( $p - F = 4.129$ ;  $p = 0.028$ , Table 2).

### Mosquitofish effects on *Valencia* population, condition and reproduction variables

A comparison of *Valencia* population variables among samples in co-occurrence versus no co-occurrence to the mosquitofish, revealed no significant differences in the percentages of *Valencia* larvae, of juveniles, of female and male adults, as well as in the adult sex ratio ( $p > 0.05$ ). T-tests showed that the length (SL) of juveniles and male

**Table 2.** Results of PERMANOVA analysis to test whether the set of environmental variables or mosquitofish presence could significantly explain the observed species composition in terms of abundance. Df: degrees of freedom; SS: sum of squares; MS: mean sum of squares; pseudo-F: F value by permutation. Boldface indicates statistical significance at  $p < 0.5$  based on 19999 permutations

Tested variable	df	SS	MS	pseudo-F	R2	p (perm)
Gambusia presence	1	0.349	0.349	4.129	0.251	<b>0.028</b>
Depth	1	0.01	0.01	0.123	0.007	0.910
Width	1	0.008	0.008	0.100	0.006	0.915
Conductivity	1	0.026	0.026	0.302	0.018	0.782
DO	1	0.081	0.081	0.952	0.058	0.411
pH	1	0.004	0.004	0.043	0.003	0.946
Temperature	1	0.019	0.019	0.223	0.014	0.857
Vegetation cover	1	0.050	0.050	0.586	0.036	0.603
Residuals	10	0.895	0.081		0.608	
Total	18	1.392			1	

adult *Valencia* was significantly higher and the length of female adult *Valencia* significantly lower in co-occurrence to the mosquitofish (Table 3). *Valencia* net weight (ANCOVA-adjusted for fish length) was significantly lower in females co-occurring with the mosquitofish (Table 4). No significant differences were found in the gonadal mass of female or male *Valencia*.

## Discussion

The present study presented evidence for the negative impact of the presence of the alien mosquitofish on the abundance of two *Valencia* species, with the native *Valencia* spp. being significantly less abundant in co-occurrence to the Eastern mosquitofish. Statistical analysis also demonstrated altered community structure in habitats invaded by the mosquitofish, a pattern observed when alien species, as more effective predators, are introduced into

freshwater ecosystems (Matthews, 1998; Jackson *et al.*, 2001), in a process called biotic homogenisation (Rahel, 2002). Our study also showed that mosquitofish presence had significant negative effects on the body condition of female adult *Valencia*. No significant negative effects on the target species' reproduction were evident. The absence of an effect on *Valencia* gonad weight, may reflect an investment on reproduction, at the cost of condition. However, a possible negative effect on reproduction at the level of e.g. fecundity, not analyzed in the current study, cannot be excluded. Nevertheless, cumulative negative effects on *Valencia* condition and reproduction are expected to be more pronounced at the end of the reproductive period (autumn), however, the small size of our seasonal samples did not permit such an analysis.

The reduced condition of female *Valencia* adults can be attributed to a variety of possibly synergistic forms of negative interactions, based on the findings of previous laboratory studies on the interactions between the

**Table 3.** A comparison of standard length (SL) between *Valencia* individuals in the presence (P) and absence (A) of *Gambusia holbrooki*. All data were log10 transformed prior to analyses. \*  $P < 0.05$ .

		n	mean	t	df	P
<i>Larvae</i>						
SL	P	39	1.026	-1.842	79.035	0.069
	A	48	1.050			
<i>Juveniles</i>						
SL	P	36	1.171	2.651	65.363	<b>0.010*</b>
	A	32	1.152			
♀ <i>adults</i>						
SL	P	30	1.366	-2.195	68.331	<b>0.032*</b>
	A	42	1.431			
♂ <i>adults</i>						
SL	P	33	1.428	2.018	54.369	<b>0.048*</b>
	A	48	1.379			

**Table 4.** ANCOVAs of net (NW) and gonad weight (GW) of *Valencia* with presence/absence of *G. holbrooki* (factor) and SL (covariate). All quantitative variables were log10 transformed. Signif. codes: p< \*\*\*0.001, \*\*0.01, \*0.05

	Net weight			Gonad weight		
	Sum Sq	df	F value	Sum Sq	df	F value
<i>Juveniles</i>						
Intercept	0.254	1	17.223***			
SL	0.795	1	53.870***			
Presence/Absence	0.001	1	0.081			
Residuals	0.979	66				
♀ <i>adults</i>						
Intercept	2039	1	323.026***	11885	1	109.142***
SL	10685	1	1692.021***	16069	1	147.573***
Presence/Absence	<b>0.036</b>	1	<b>5.787*</b>	0.008	1	0.071
Residuals	0.436	69		7514	69	
♂ <i>adults</i>						
Intercept	1118	1	205.366***	14942	1	138.404***
SL	7478	1	1373.301***	18116	1	167.799***
Presence/Absence	0.001	1	0.241	0.001	1	0.011
Residuals	0.425	78		8421	78	

mosquitofish and other native fish species. These studies have shown that Eastern mosquitofish is a much more efficient forager, with much higher levels of satiety (maximum prey number) and voracity (number of prey consumed per unit of time) than the related native Iberian cyprinodonts *A. iberus* and *V. hispanica* in experimental conditions (Caiola & de Sostoa, 2005) and that it caused a decrease or absence of feeding activity to the native species in the laboratory (Rincon *et al.*, 2002; Caiola & de Sostoa, 2005). This led to the conclusion that habitat exclusion combined with a slower feeding rate and presumably increased costs of foraging (Gill & Hart, 1996), disfavors the native species. Based on the above, the poorer *Valencia* condition reported in this study could also be a result of competition for food, through exclusion interference. However, exploitation competition as yet another mechanism of negative interaction in nature where resources are limited is also possible, since the *Valencia* diet (mostly aquatic macroinvertebrates) is generalist and reflects food resource diversity and availability, much the same way as the Eastern mosquitofish diet (Kalogianni *et al.*, 2010b; García-Berthou, 1999). Furthermore, it has recently been shown that there is high dietary overlap between adult *Valencia* and mosquitofish regulated by resource availability (Kalogianni *et al.*, 2014).

A second form of negative interaction, i.e. agonistic behaviors, is an additional mechanism that could be responsible for the effects observed in the current study on *Valencia* somatic condition when in co-occurrence with the mosquitofish. Previous studies have shown mosquitofish aggression towards native species in the laboratory (Barrier & Hicks, 1994; Keller & Brown, 2008; for *V.*

*hispanica* see Rincon *et al.*, 2002) that can result to increased stress (Ejike & Schreck, 1980) and increased vulnerability to predation. Furthermore, mosquitofish presence can also elicit an aggression response by the native species themselves, such as that reported in the case of *V. hispanica* (Rincon *et al.*, 2002), which also translates to lost feeding opportunities, and consequently decreased feeding and poorer condition. Such aggressive behavior of the Corfu and the Peloponnese *Valencia* towards the Eastern mosquitofish has been also observed in the field in Greece during the course of the current study.

Finally, among the potential negative interactions between *Valencia* and the Eastern mosquitofish, predation by adult mosquitofish on *Valencia* larvae and juvenile has been also suggested as a factor contributing to *Valencia* decline (Bianco & Miller, 1989). Previous studies have indeed presented evidence of mosquitofish predation on larvae of other native fish (Barrier & Hicks, 1994; Belk & Lydeard, 1994; Howe *et al.*, 1997). Our study has shown that neither the percentage of larvae nor that of juveniles was negatively affected by the presence of the mosquitofish, confirming the findings of those studies that have reported either no predation or very infrequent predation of the mosquitofish on other fish species (Pen & Potter, 1991; Stoffels & Humphries, 2003). The same result was also shown in a recent study focusing on *V. letourneuxi* that provided evidence of opportunistic mosquitofish piscivory (Kalogianni *et al.*, 2014); thus, size-selective predation by mosquitofish as a major interactive mechanism between the two species in natural conditions is not supported by our data.

In summary, the results of this study represent the

first *in situ* evidence of the negative effects of the Eastern mosquitofish on two *Valencia* species, i.e. namely a decrease in somatic condition of female *Valencia* adults, with non-predatory (i.e. dietary competition and/or agonistic) interactions, possibly implicated as the underlying mechanisms, as suggested elsewhere (Kalogianni *et al.*, 2014). These effects were evident using data of the dry season, which coincides with the extended reproductive period of the Greek *Valencia* species (Kalogianni *et al.*, 2010a; Giakoumi *et al.*, 2010). These effects cannot be attributed to abiotic factors, since environmental conditions were quite stable during the dry season in the habitats sampled. Rather, the reproductive season is a period of increased energy demands for breeding *Valencia* adults, therefore this could lead to increased encounters between adults of the two species. *Valencia letourneuxi*, and its sister species *V. robertae*, are habitat specialists exhibiting a high affinity towards spring-fed wetlands with rich aquatic vegetation (Kalogianni *et al.*, 2010a) that, as relatively stable environments, constitute refugia for the species. The results of the current study indicate that, apart from habitat degradation and loss, previously identified as the major threats to *Valencia* survival, the presence of the alien mosquitofish at high densities is possibly responsible for *Valencia* population decline. Thus, any conservation actions targeting the Greek *Valencia* species should also incorporate actions to control the range and population densities of the Eastern mosquitofish. In the light of the results of the current study, the prevention of mosquitofish invasion to the non-invaded *Valencia* habitats, through e.g. construction of barriers to prevent mosquitofish invasion of the spring areas from the lower section of the respective stream or prevention of the construction of irrigation canals connecting invaded and non-invaded habitats, arises as a main conservation priority. The eradication or reduction of syntopic mosquitofish populations in those locations hosting the Greek *Valencia* emerges as an alternative conservation option, despite its various methodological limitations (for a review see Donaldson & Cooke, 2016).

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### Author contributions

E.K. Conception, design and supervision of the study, Acquisition of sampling data, Drafting and revising the article, Obtained funding, N.K. Drafting the article, Critical revision of the manuscript for important intellectual content, L.V. Analysis and interpretation of data, Revising the article, S.G. Acquisition of sampling and laboratory data, Revising the article, Y.C. Design of the study, preliminary statistical analysis, A.O. Revising the article, Statistical analysis and interpretation of results.

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