

Infestation of *Lernaea cyprinacea* (Copepoda: Lernaeidae) in two invasive fish species in Romania, *Lepomis gibbosus* and *Pseudorasbora parva*

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ABSTRACT

Key-words:
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molecular markers

In this study we analyzed comparatively the host-parasite associations between two fish host species invasive in Europe (*Lepomis gibbosus* and *Pseudorasbora parva*) and one known generalist parasite species, the copepod *Lernaea cyprinacea*. We used a fragment of the hypervariable region D1-D2 of the 28S rRNA to confirm that the copepod specimens collected on both host species in our study are indeed conspecific. The prevalence of infection was significantly different between the two host species in all three aquatic ecosystems. Two populations of *L. gibbosus* exhibited a positive correlation coefficient between the standard body length and infection intensity, while a negative correlation coefficient was observed in one population of *P. parva*. This is one of the few studies providing parasitological parameters of infections of *Lernaea cyprinacea* in *Lepomis gibbosus* and *Pseudorasbora parva*.

RÉSUMÉ

Infestation de *Lernaea cyprinacea* (Copépoïde : Lernaeidae) sur des espèces envahissantes de poissons *Lepomis gibbosus* et *Pseudorasbora parva* en Roumanie

Mots-clés :
poissons non indigènes,
coévolution hôte-parasite,
parasitologie quantitative,
ADN barcoding,
marqueurs moléculaires

Dans cette étude, nous avons analysé comparativement les associations hôte-parasite entre deux espèces hôtes de poissons envahissantes en Europe (*Lepomis gibbosus* et *Pseudorasbora parva*) et une espèce de parasite généraliste connue, le copépode *Lernaea cyprinacea*. Nous avons utilisé un fragment de la région hypervariable D1-D2 de l'ARNr 28S pour confirmer que les échantillons de copépodes prélevés sur les deux espèces hôtes dans notre étude sont en effet de la même espèce. La prévalence de l'infection était significativement différente entre les deux espèces hôtes dans les trois écosystèmes aquatiques. Deux populations de *L. gibbosus* présentaient un coefficient de corrélation positif entre la longueur standard du corps et l'intensité de l'infection, tandis qu'un coefficient de corrélation négatif a été observé dans une population de *P. parva*. C'est l'une des rares études fournissant des paramètres parasitologiques de l'infection de *Lernaea cyprinacea* sur *Lepomis gibbosus* et *Pseudorasbora parva*.

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INTRODUCTION

Host-parasite coevolution may lead to either host or parasite local adaptation in spatiotemporally varying environments, e.g. when invasive species expand their range (Lemoine *et al.*, 2012). One of the factors considered to explain the success of these species is the release of the newly founded populations from the pressure exercised by their original parasites left behind in the initial environment (Mastitsky *et al.*, 2014; Torchin *et al.*, 2003). This theory relies on the idea that co-evolution leads to extensive local adaptations, and as such the parasites in a particular area infect hosts from the same area more efficiently than they infect hosts from another geographically distinct population (Morand *et al.*, 1996; Roth *et al.*, 2012). On the other hand, since the newly conquered environments are not parasite-free, the new host-parasite interactions can lead to parasite maladaptation, where parasites can temporarily have higher fitness in the newly arrived (allopatric) hosts than in the sympatric hosts (Dybdahl and Storfer, 2003).

In this study we analyzed comparatively the host-parasite associations between two fish host species invasive in Europe (*Lepomis gibbosus* and *Pseudorasbora parva*) and one known generalist parasite species, the copepod *Lernaea cyprinacea*.

Lepomis gibbosus (Linnaeus, 1758) is a freshwater fish species native to North America, belonging to the family Centrarchidae. Introductions of pumpkinseed sunfish beyond its native range have occurred around the world for many years, and for many reasons, such as potential sport, ornamental pond fish, fry and fingerlings, and prey for native wild stocks (Jordan, 2009). Introduced to Europe in the 1880s as a pond and aquarian fish, the species is quite widespread throughout this continent, being particularly abundant in Mediterranean countries (Hanel *et al.*, 2011). The second host in our study, *Pseudorasbora parva* (Temminck & Schlegel, 1846), is an Asian native freshwater cyprinid species considered a typical invasive species and occurring on all continents except for Antarctica (Margaritov and Kiritsis, 2011). The accidental transfer and release of *P. parva* with carp species translocations for aquaculture is considered the primary pathway of species introduction into new areas (Gozlan, 2012). After the first introduction into Europe in the 1960s (Nucet, Romania), the species extended its range through natural dispersal, recreational fishing and ornamental fish trade. Its high phenotypic plasticity and the ability to cope with novel pathogens have predisposed the topmouth gudgeon to being a strong invader (Hanel *et al.*, 2011). The parasite species, the anchor worm *Lernaea cyprinacea* Linnaeus, 1758, is the only cosmopolitan species in the genus *Lernaea* (Piasecki *et al.*, 2004). This copepod is non-specific, infecting many freshwater fish species (Williams and Bunkley-Williams, 1996), but also affecting marine fish hosts (Gutierrez-Galindo and Lacasa-Millan, 2005), as well as adult and larval amphibians (Kupferberg *et al.*, 2009; Nagasawa *et al.*, 2007) and larval aquatic insects (McAllister *et al.*, 2011). *L. cyprinacea* is native to Asia, but currently it is widely distributed (Oscoz, 2010).

The aim of the present study was to compare the parasitological parameters of *L. cyprinacea* infections on the two invasive fish species in several lentic systems in Romania.

MATERIALS AND METHODS

We conducted parasite surveys of fish fauna during the 2008 summer season in three Romanian lentic ecosystems: Moara Domneasca Lake, Ilfov County – 46°43'56"N, 27°44'41"E (2 surveys in June and July); Brateş Lake, Galaţi County – 45°28'59"N, 28°4'13"E (1 survey in July); Crapina Lake, Tulcea county – 45°22'24"N, 28°14'42"E (1 survey in July). The fish specimens were collected by electrofishing with a Samus 720 MP device (Samus Special Electronics, Poland) and immediately after the sampling, they were placed in 95% ethanol and transported to the laboratory. Further analyses of parasite fauna were performed only on *L. gibbosus* and *P. parva*, because both species exhibited a consistent abundance in all samples. For each fish specimen, the standardized length was measured with a calliper to the nearest 0.1 mm. Each fish specimen was examined using a Krüss Optronic dissecting microscope at the level of the branchial cavities, head, fins and skin for presence of copepod

parasites. The copepod specimens removed were placed in 95% ethanol for further analysis. The morphological identification of the *L. cyprinacea* specimens was performed according to Bauer (1987). The copepod preference for a particular attachment site on the host was recorded and 95% confidence intervals of the parasite proportions on different host regions were computed with the modified Wald method (Agresti and Coull, 1998). The infestation parameters prevalence, mean intensity, median intensity and mean abundance, as well as the corresponding 95% C.I., were calculated using the software package Quantitative Parasitology 3.0 (Rozsa et al., 2000). The Spearman rank correlation coefficient (r_s) was used to measure correlation between host fish size and the abundance of infection, while a p -value for the null hypothesis of r_s being equal to zero was computed based on 10000 Monte Carlo replications using the same software package. The comparison of prevalence between populations of the same host species and between host species in the same lake were performed by Fisher's exact test. In all cases p values lower than 0.05 were considered significant.

We tested that the copepod individuals collected on both fish hosts are conspecific (*L. cyprinacea*) by DNA sequencing of a fragment of the 28S rDNA. Total DNA was extracted from eight copepod individuals, from both host species, using the NucleoSpin[®] Tissue kit (Macherey–Nagel GmbH & Co. KG, Düren, Germany), according to the manufacturer's specifications.

The amplification of a fragment of the hypervariable region D1-D2 of the 28S rDNA fragment was performed with the primers 28SF (5'-acaactgtgatgcccttag-3') and 28SR (5'-tgggccgtgtttcaagacg-3') (Song et al., 2008). The amplification was performed in a 50 μ L reaction containing 10 mM Tris-HCl (pH 8.8 at 25 °C), 50 mM KCl, 0.08% (v/v) Nonidet P40, 2.5 mM MgCl₂, each dNTP at 0.1 mM, each primer at 0.2 μ M, 1 unit of Taq DNA polymerase (Fermentas UAB, Vilnius, Lithuania), and approximately 50 ng of DNA template. The temperature profile of the polymerase chain reaction for the 28S marker consisted of initial denaturation at 94 °C for 30s, followed by 30 cycles at 93 °C for 30s, 50 °C for 45s, 72 °C for 45s, and a final extension step performed at 72 °C for 10 min. The PCR products were separated on 1% agarose gel, and the fragments of interest purified using the innuPREP DOUBLEpure Kit (Analytik Jena AG, Jena, Germany). The DNA sequencing was performed by MacroGen Europe (1105 AZ, Amsterdam).

RESULTS

During the 2008 summer surveys 312 specimens of *Lepomis gibbosus* and 393 specimens of *Pseudorasbora parva* were collected. A total of 224 *Lernaea cyprinacea* adult females were collected from both *Lepomis gibbosus* (199) and *Pseudorasbora parva* (25) fish hosts, with a range of 1 to 11 parasite specimens per host. The sample sizes, mean body measurements, copepod infestation parameters and correlation coefficients between the host standard body length and abundance of infection are displayed in Table I, while the comparison of prevalence between populations/species is presented in Table II. The attaching preference of *L. cyprinacea* for a particular host body area in *L. gibbosus* is recorded in Table III. In our study two populations of *L. gibbosus* exhibited a positive correlation coefficient between the standard body length and infection intensity, while a negative correlation coefficient was observed in one population of *P. parva*. The comparison of prevalence (Table II) revealed significant differences between the *L. gibbosus* sample collected in Moara Domneasca in June 2008 and all the other *L. gibbosus* samples. On the other hand, all three *L. gibbosus* vs. *P. parva* comparisons revealed significant differences concerning the prevalence, while no significant differences were observed among the *P. parva* samples. The parasite preference for host attachment in *L. gibbosus* shows that in the Moara Domneasca samples, the skin and head were the preferred attachment areas over the fins, while the opposite situation could be observed in the remaining two fish samples (Table III).

A 417bp fragment of the hypervariable D1-D2 region of the rRNA 28S was successfully sequenced from 8 specimens of *Lernaea* sp. from both host species. Only one haplotype was identified in all cases (GenBank accession numbers KF751648.2- KF751649.2). A BLAST

Table 1
 The sample sizes, mean body measurements and copepod infestation parameters. SBL – standard body length; a – Clopper-Pearson confidence interval; b – confidence interval computed with 2000 bootstrap replications; c – p-value based on 10000 Monte Carlo replications; gray cells indicate r_s values significantly different from zero.

Sampling site	Host	Sample size	Mean SBL (min-max)	Prevalence (95% C.I.) ^a	Mean intensity (95% C.I.) ^b	Median intensity (95% C.I.) ^b	Mean abundance (95% C.I.) ^b	r_s (p-value) ^c
Moara Domneasca Lake June 2008	<i>L. gibbosus</i>	80	74.7 (45–101)	0.588 (0.472–0.696)	1.66 (1.43–2.02)	1 (1–2)	0.975 (0.725–1.25)	0.13 (0.2573)
								0.38 (0.0289)
Moara Domneasca Lake July 2008	<i>L. gibbosus</i>	34	65.6 (47–85)	0.265 (0.129–0.444)	1.89 (1.22–3)	1 (1–3)	0.5 (0.206–1)	–0.15 (0.0193)
								0.066 (0.035–0.097)
Brateş Lake July 2008	<i>P. parva</i>	228	36.7 (20–61)	0.066 (0.037–0.106)	1	1	0.066 (0.020–0.135)	0.07 (0.3972)
								0.283 (0.160–0.435)
	<i>L. gibbosus</i>	46	60.7 (17–80)	0.283 (0.160–0.435)	1.15 (1–1.31)	1	0.326 (0.174–0.478)	–0.08 (0.6110)
								0.047 (0.019–0.095)
Crapina Lake July 2008	<i>L. gibbosus</i>	152	57.6 (40–97)	0.342 (0.267–0.423)	1.71 (1.38–2.46)	1	0.586 (0.428–0.842)	0.25 (0.0025)
								0.059 (0.001–0.287)
	<i>P. parva</i>	17	49.2 (47–76)	0.059 (0.001–0.287)	1	–	0.059 (0–0.176)	–0.21 (0.5201)

Table II

Two-sided *p*-values for comparison of prevalence by Fisher's exact test; gray cells indicate significant differences.

		<i>L. gibbosus</i>			<i>P. parva</i>		
		MD_Jul	Brateş	Crapina	MD_Jul	Brateş	Crapina
<i>L. gibbosus</i>	MD_Jun	0.0020	0.0015	0.0005			
	MD_Jul		1.0000	0.4261	0.0012		
	Brateş			0.4800		<0.0001	
	Crapina						0.0241
<i>P. parva</i>	MD_Jul					0.5081	1
	Brateş						0.5893

query run against the Genbank Nucleotide collection (nr/nt) returned a species match with *Lernaea cyprinacea* at similarity levels between 100% and 99.76% (3 matches). The next best match scored a 91% similarity with *Lamproglena orientalis* Markevich, 1936.

DISCUSSIONS

> *L. CYPRINACEA SPECIES CONFIRMATION*

The origin and taxonomic position of *Lernaea cyprinacea* is open to discussion. According to (Margaritov and Kiritsis, 2011), *L. cyprinacea* is a European parasite, whereas *L. elegans* is considered to be an Asian species. However, the majority of experts regard both species as synonyms (Nagasawa *et al.*, 2007). Given this controversy, we used a fragment of the hypervariable region D1-D2 of the 28S rRNA to confirm that the copepod specimens collected on both host species in our study are indeed conspecific. The hypervariable regions of 28S rRNA have been used by other authors as species diagnostic markers in arthropods, annelids and mollusks (Markmann and Tautz, 2005; Raupach *et al.*, 2010; Song *et al.*, 2008). All eight examined sequences (from two host species) were identical. Moreover, the BLAST query run against the Genbank Nucleotide collection (nr/nt) returned a species match with *L. cyprinacea*. These results corroborated with the morphological identification performed according to Bauer (1987) confirmed that in our study we analyzed *L. cyprinacea* specimens on two different host species.

> *PREVIOUS RECORDS OF THE PARASITE AND/OR THE HOST SPECIES*

Recorded in Romania for the first time in 1929 by Busnita, the pumpkinseed sunfish *L. gibbosus* is currently present in almost all limnic ecosystems in the country (Gavriloaie *et al.*, 2006). Banarescu (1946) found for the first time the anchor worm *L. cyprinacea* parasitizing *L. gibbosus* in Romania and the same author provided a detailed list of the pumpkinseed sunfish parasites in 1964 (Banarescu, 1964). More recently, Stavrescu-Bedivan *et al.* (2011) reported the anchor worm from *L. gibbosus*. Radulescu and Georgescu (1969) studied for the first time the parasites of *P. parva* in Romanian waters, identifying one protozoan and one nematode species, while Banarescu (1999) cited three protozoan species. Stavrescu-Bedivan *et al.* (2012) reported for the first time in Romania the presence of *L. cyprinacea* as a parasite on *P. parva*. *Lernaea cyprinacea* was also reported in Romania from the fish species *Carasius auratus*, *Cyprinus carpio*, *Rhodeus sericeus amarus* and *Rutilus rutilus* (Cojocar, 2003; Patriche *et al.*, 2009).

> *DIFFERENCES IN PARASITOLOGICAL PARAMETERS*

In our study, the prevalence of infection was significantly different between the two host species in all three aquatic ecosystems. This finding could be interpreted in different ways,

Table III
Parasite attachment preference on *L. gibbosus*. (n) – number of copepod specimens; P – pectoral fins; V – ventral fins; D – dorsal fin; A – anal fin; C – caudal fin; MD – Moara Domneasca; light gray cells-fins; dark gray cells-head.

	Skin			P			V			D			A			C			Gills			Opercular area			Oral cavity			Eyes			Nasal cavity		
	n	%	95% C.I.	n	%	95% C.I.	n	%	95% C.I.	n	%	95% C.I.	n	%	95% C.I.	n	%	95% C.I.	n	%	95% C.I.	n	%	95% C.I.	n	%	95% C.I.	n	%	95% C.I.			
<i>L. gibbosus</i>	18			5			9			7			0			4			20			9			1			0					
MD	23.1			6.4			11.5			9.0			0.0			5.1			25.6			11.5			1.3			0.0					
Jun. 2008	15.0–33.6			2.4–14.5			6.0–20.7			4.2–17.7			0–4.0			1.6–12.9			17.19–36.38			6.0–20.7			0–7.6			0–4.0					
<i>L. gibbosus</i>	2			1			0			1			1			1			4			2			0			0					
MD	11.8			5.9			0.0			5.9			5.9			5.9			23.5			11.8			0.0			0.0					
Jul. 2008	2.0–35.6			0–28.9			0–16.2			0–28.9			0–28.9			0–28.9			9.1–47.8			2.0–35.6			0–16.2			0–16.2					
<i>L. gibbosus</i>	0			4			6			1			1			0			1			1			0			0					
Brateş	0.0			26.7			40.0			6.7			6.7			0.0			6.7			6.7			0.0			0.0					
Jul. 2008	0–18.0			0–31.8			19.8–64.3			0–31.9			0–31.9			0–18.0			0–31.84			0–31.8			0–18.0			0–18.0					
<i>L. gibbosus</i>	6			19			20			8			3			0			12			4			0			2					
Crapina	6.7			21.3			22.5			9.0			3.4			0.0			13.5			4.5			0.0			0.0					
Jul. 2008	2.9–14.2			14.0–31.0			15.0–32.4			4.4–17.0			0.7–9.9			0–3.6			7.73–22.3			1.4–11.4			0–3.6			0.1–8.3					

considering the geographic origin of the species involved. Firstly, we have an Asian parasitic species, one Asian host (*P. parva*) and one North-American host (*L. gibbosus*). Although our data fits the rare model of the sympatric host having developed efficient defense mechanisms against its sympatric parasite (Morand *et al.*, 1996), the data we have are not enough to support this hypothesis. We believe the more likely explanation in this case is the general resistance of *P. parva* to various parasite infections observed by some authors (Hanel *et al.*, 2011). In fact, similar low levels of prevalence and intensity of *L. cyprinacea* infection in *P. parva* to those in our study have been reported in the literature (Margaritov and Kiritsis, 2011). The above-mentioned parasite resistance actually refers to the resistance to the possible damaging effects of the parasite, the topmouth gudgeon being otherwise known as a healthy carrier for different parasite species (Spikmans *et al.*, 2013) or being a host for Unonid glochidia, a relation which some authors define more as commensalism (phoresy) than parasitism (Douda *et al.*, 2012). Secondly, we have a host-parasite combination, *L. gibbosus* and *L. cyprinacea*, which have lived in sympatry in Europe for more than 100 years, and a newly arrived host species population (*P. parva*). Looking at our data this way, it fits the general model of a parasite species being more effective at infecting the sympatric rather than the allopatric host, showing that the parasite has had more time to develop adaptations for infecting the sympatric host species (Lemoine *et al.*, 2012). However, the differences observed in the parasitological parameters between our host species could also be explained by ecological factors (local conditions, proximity of host and parasite in time and space, *etc.*), not only by co-evolutionary driven physiological compatibility, as discussed above (Poulin, 2006). In fact, the results discussed in the next paragraph suggest that the ecological conditions could be an important factor explaining our data.

Besides the difference in prevalence between the two host species, our data showed significant differences in the infection prevalence in *L. gibbosus* within the same aquatic system, between two consecutive surveys (June and July 2008). These results are probably explained by the temporal variability of parasite outbreaks. It is known that infestations with *L. cyprinacea* are more prevalent in the summer months, with water temperatures ranging from 25 to 28 °C, when this parasite finds excellent conditions for reproduction (Raissy *et al.*, 2013). Another possible explanation of these results refers to microhabitat differences between species resulting in different probabilities of the parasite infection.

> HOST SITE ATTACHMENT PREFERENCE

Although *L. cyprinacea* seems to have a random choice for attachment on the body host, we noted that the parasites were fixed especially on the fins and skin. These observations were previously reported in the literature (Barzegar and Jalali, 2009; Jalali *et al.*, 2008; Kim *et al.*, 2002). One of the hypotheses proposed to explain this copepod attachment preference is that fins offer greater protection against currents and tissues at the base of the fins may be more easily penetrated (Gutierrez-Galindo and Lacasa-Millan, 2005; Iqbal *et al.*, 2012).

> HOST SIZE-LEVEL OF INFESTATION CORRELATION

Two of the studied *L. gibbosus* populations exhibited a significant positive correlation between the host size and the intensity of infection, results which are in agreement with the studies conducted by Gutierrez-Galindo and Lacasa-Millan (2005) and Pérez-Bote (2000). On the other hand, one of the *P. parva* populations exhibited a negative correlation coefficient, which may be due to the development of acquired immunity in older fish specimens (Kanwal *et al.*, 2012; Tasawar *et al.*, 2009).

This is one of the few studies providing parasitic parameters of infections of *Lernaea cyprinacea* in *Lepomis gibbosus* and *Pseudorasbora parva* in an environment where both host species are invasive. We found significant differences in prevalence and intensity between

the two host species, but further studies are necessary to determine if the observed differences are generated by host-parasite coevolution in new environments, by one of the species (*P. parva*) exhibiting a general resistance to parasite infections, by extrinsic factors affecting both the host and the parasite populations, or maybe by a combination of any of the above-mentioned factors.

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