Occurrence and effect of trematode metacercariae in two endangered killifishes from Greece

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1   **Occurrence and effect of trematode metacercariae in two endangered killifishes from Greece**

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21   **Running title:**

22   **Digenean infection of endangered Greek killifishes**

24   **Abstract**

25   We report digeneans (Diplostomidae, Crassiphialinae) in the endangered freshwater fishes *Valencia*

26   *letourneuxi* and *Valencia robertae*, endemics of Western Greece. Digenean metacercariae occurred

in two forms in the abdominal cavity, excysted and encysted, the latter attached to gonads, liver and alimentary tract. Parasites were, using morphological and molecular techniques, identified as two representatives of Crassiphialinae, specifically part of the *Posthodiplostomum-Ornithodiplostomum* clade. The spatial, seasonal and age class variation in parasite prevalence was examined. Autumn parasite prevalence varied between the six populations sampled (18.2% to 100%). Seasonal prevalence at the two sites sampled quadannually peaked in autumn and reached its lowest value in spring; prevalence increased with size to 100% in young adult fish. We did not find a correlation between prevalence and host sex. Overall parasites' weight averaged 0.64% of the host's, while parasite weight increased with host weight. A comparison of relative condition, and hepatosomatic and gonadosomatic indices of infected and metacercariae-free specimens showed that infection did not have a significant effect on host body condition and reproduction. Regarding the parasite's life cycle, planorbid gastropods are proposed as potential first intermediate hosts in view of the host's diet and occurrence data of molluscs in the ecosystem. This is the first record of a diplostomid digenean in valenciid fishes and of representatives of the *Posthodiplostomum-Ornithodiplostomum* clade in a native Greek freshwater fish. Our findings are discussed in conjunction to fish conservation interventions, since parasites may contribute to the decline of endangered species.

Keywords

Digenea; parasite prevalence; host body condition; Planorbidae; Platyhelminthes; Valenciidae

Introduction

The critically endangered freshwater fish *Valencia letourneuxi* (Sauvage, 1880) (Valenciidae) is an endemic of Western Greece and southern Albania (Crivelli 2006). Its sister species, the recently described *Valencia robertae* Freyhof, Kärst and Geiger, 2014, endemic of Central Greece, encompasses the most southern populations previously included in *V. letourneuxi*. Both species are

53 characterized by a fragmented geographical distribution, narrow ecological requirements and low

54 population densities (Barbieri et al. 2000; Kalogianni et al. 2010a). In the last 30 years, both

55 species' geographical range has been reduced, with some populations now being extinct and others

56 in a precarious state (2005 survey data, Kalogianni et al. 2010b; see also Economidis 1991; Bianco

57 et al. 1996; Barbieri et al. 2002a, b, 2015). Their decline has been attributed to the degradation of

58 their habitats due to anthropogenic activities, as well as to predation and competition with the

59 introduced Eastern mosquitofish *Gambusia holbrooki* Girard, 1859 (Bianco and Miller 1989;

60 Barbieri et al. 2000, 2002b; Kottelat and Freyhof 2007). The parasite fauna of these Greek

61 killifishes has never been studied. Parasitization, however, of individuals of a native fish species,

62 either by non-native parasites transmitted from introduced fish (Prenter et al. 2004) or by native

63 parasites, can also potentially contribute to its decline, by influencing host behaviour, survival,

64 growth and fecundity, as well as host population dynamics (Marcogliese 2004). Parasitic organisms

65 are often neglected in the management and conservation of biological resources and ecosystems

66 (Marcogliese 2004). Research on the parasites of endangered species can, however, provide

67 information about their host organisms and the ecological interactions between these organisms

68 (e.g. Whiteman et al. 2007). Parasite community composition can provide valuable information for

69 the management and conservation of aquatic species and habitats, e.g. by contributing to

70 understanding introduction routes (e.g. Huyse et al. 2015). Furthermore, the assemblage of parasites

71 within a host organism potentially reflects that host's trophic position in the food web, as well as

72 the presence in the ecosystem of various other organisms that participate in the life cycles of these

73 parasites. Parasite populations and communities could also be useful indicators of environmental

74 stress, such as eutrophication or acidification, as well as of food web structure and biodiversity

75 (Marcogliese 2005; Vidal-Martínez et al. 2010).

76   A preliminary examination of the abdominal cavity of *V. letourneuxi* and *V. robertae* revealed

77 the presence of digenean trematode metacercariae, possibly belonging to Diplostomidae Poirier,

78 1886. Among parasites, trematodes are the dominant group that causes retarded growth, morbidity

and mortality, especially in juvenile fishes (Shareef and Abidi 2015). Digenean trematodes are widespread around the globe and are characterized by a complex life cycle, often involving a mollusc as first intermediate host, a fish, an amphibian or occasionally a mammal as second intermediate host and piscivorous birds or mammals as definitive hosts (Niewiadomska 2002; Cribb et al. 2003). Digeneans have been shown to induce behavioural changes in their fish secondary host, such as decreasing swimming performance (Coleman 1993) or decreasing predator avoidance (Poulin 1993) resulting in increased predation of the host (Ondráčková et al. 2006). They have also been shown to cause damage to fish host tissues resulting in blindness, inflammatory reactions and perforations in some cases (Sharriff et al. 1980; Niewiadomska 2002; Vianna et al. 2005), though there are also studies reporting no effect of digenean parasitism on fish host condition (e.g. Silva-Souza and Ludwig 2005).

In this study, we further examined the abovementioned metacercariae retrieved from *V. letourneuxi* and *V. robertae* in order to identify these parasites using morphological and molecular techniques, to examine the variation in prevalence between seasons, locations and host sexes and size-classes, and to assess the effect of the metacercariae on host condition and reproduction.

Materials and methods

Sampling methodology and phenotypic characterisation of hosts and parasites

Samples were collected from six sites in Western Greece, in the autumn of 2005, 2006 and 2009; one site hosts *V. robertae* and the other five sites *V. letourneuxi* (Fig. 1 and Table 1). To explore seasonal variation in parasitization, seasonal samplings (July, September, January, May) were conducted at two sites, Mornos and Acheron (sites 4 and 5, hosting *V. robertae* and *V. letourneuxi* respectively, Table 1). All sampling sites were located at lowland semi-lotic streams or canals (elevation range 0-6 m), associated with springs, while distance from sea ranged from 0.4 to 13.5

105 km. Site depth ranged between 0.9 and 1.7 m and site width between 4 and 13 m. All sites were

106 thickly vegetated (surface aquatic vegetation ranged between 40 and 95%). Salinity varied between

107 0.1 and 6.5 ppt and temperature between 14.5 and 25 °C.

108 This study was conducted within the frame of a wide scope research programme during 2005-2009,

109 targeting *V. letourneuxi (V. robertae* had not yet been described as a separate species at the time).

110 This research included dietary studies, studies on the effect of *G. holbrooki* Girard, 1859 on *V.*

111 *letourneuxi* and genetic studies. Fish were collected with a D-shaped net (2 mm mesh) from the

112 stream banks at the six sites described above (Fig.1 and Table 1). In five of the six sites sampled

113 (sites 1, 2, 3, 5, 6, Table 1), representatives of *Valencia* are found in association with the introduced

114 *G. holbrooki* (Kalogianni et al., 2010a). After identification, a total of 296 specimens of *Valencia*

115 were anesthetized with quinaldine and preserved in 4% formaldehyde for further laboratory

116 analyses. In the laboratory, for each fish total and standard length (TL and SL, nearest 0.1 mm)

117 were measured, total and net host weight, before and after evisceration respectively (HW and NW,

118 nearest 0.01 mg) as well as liver and gonad weight (LW and GW, nearest 0.01 mg after blotting

119 dry) were also recorded. External surfaces, viscera and musculature of the fish were examined

120 under a Olympus SZX7 stereo microscope for parasitic infection. Parasites were removed, blotted

121 dry and their weight was recorded (PW, nearest 0.01 mg, weighed for all metacercariae of one host

122 specimen together).

123 Parasite prevalence (number of infected fish per total fish examined) was calculated for all

124 metacercariae together (i.e. no species-level morphological identification of the digeneans could be

125 made and these prevalence values are hence not parasite species-specific). Differences in parasite

126 prevalence between sexes and 2 mm size classes (larval size range < 13 mm, juvenile range $\geq$ 13

127 and $\leq$ 17 mm and adults > 17 mm, see Barbieri et al. 2000 and Kalogianni et al. 2010b) were

128 examined pooling data from all the autumn samples. The effect of host sex on the prevalence of

129 parasites was tested by applying chi-square test. Differences in mean standard length between

infected and metacercariae-free specimens were examined using a t-test. The relationship between total PW and HW was examined with Pearson's correlation.

Finally, ANCOVA was used to investigate potential effects of parasitation on the condition and reproduction of the female and male fish hosts, after calculation of the relative condition (NW/SL), the hepatosomatic (LW/NW) and the gonadosomatic (GW/NW) indices of infected and metacercariae-free specimens. Fish standard length was used as a covariate to account for possible size effects. Prior to statistical analysis, values of the above indices were log10 transformed. ANCOVA was conducted with PASW 17 software.

Parasite identification

Parasite identification was done using combined morphological and molecular methods. The digenean metacercariae, excysted when necessary, were stained using paracarmine, dehydrated through a series of alcohols and cleared in beechwood creosote. They were mounted in Canada balsam on glass microscope slides and examined under a Olympus BH2 high-power microscope with interference phase.

Additional samples, used for the genetic characterization of the parasites, were collected from the Acheron site (site 5) in June 2008. We extracted DNA from individual (artificially or naturally) excysted metacercariae found in the abdominal cavity with the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Polymerase Chain Reaction was performed using a GeneAmp PCR system 9700 thermocycler (Applied Biosystems) and Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare), adding 1 µL of each primer (20 µM) (Sigma Aldrich), 2 µL of template DNA and 21 µL of double distilled, autoclaved and filter sterilized water, for a total reaction volume of 25 µL. We amplified fragments of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, and of the nuclear rDNA region (ITS-1, 5.8, ITS-2). Primer combinations were MplatCOX1dF (5'-TGTAAAACGACGGCCAGTTTWCITTRGATCATAAG-3') and MplatCOX1dR (5'-CAGGAAACAGCTATGACTGAAAYAAYAIIGGATCICCACC-3')

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(Moszczynska et al. 2009) for COI and the combinations of D1 (5'-AGGAATTCCTGGTAAGTGCAAG-3') with D2 (5'-CGTTACTGAGGGAATCCTGG-3') (Hillis and Dixon 1991) and 81_f (5'- GTAACAAGGTTTCCGTAGGTGAA-3') (Gustinelli et al. 2010) with ITS2.S_r (5'- CCTGGTTAGTTTCTTTTCCTCCGC-3') (Cribb et al. 1998) for rDNA. These regions (ITS-1, 5.8 and ITS-2) are commonly used for species identification in flatworms (Vanhove et al. 2013; Stoyanov et al. 2017). After an initial denaturation of 2 min at 94 °C, samples were subjected to 35 cycles (40 for 81f – ITS2sr) of 30 s at 94 °C, 30 s (40 s for 81f – ITS2sr) at 50 °C and 60 s (90s for 81f – ITS2sr) at 72 °C. After a final elongation of 10 min (5 min for 81f – ITS2sr) at 72 °C, samples were cooled to 4 °C. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's protocol. Sequencing of both strands was carried out using the same primers as above with an Applied Biosystems 3730 DNA analyser and BigDye version 1.1. Sequences were deposited in NCBI GenBank under accession numbers KY320571-3. Voucher specimens for the genetically characterized parasite population were deposited in the Natural History Museum (London, United Kingdom) (NHMUK 2015.12.2.1) (parasite) and the Natural History Museum Rijeka (Croatia) (PMR VP 3140-2) (host). Sequences were visually corrected and aligned in MEGA v6 (Tamura et al. 2013) with the MUSCLE algorithm and UPGMB clustering method (Edgar 2004) under default conditions. The best fitting substitution model describing molecular evolution of the sequences was selected by TOPALi v2.5 (Milne et al. 2009) based on the Bayesian information criterion. The GTR model (Rodriguez et al. 1990) was used for the rDNA region. Pairwise deletion was used to construct a distance matrix. The phylogenetic position of the collected parasite haplotypes within Diplostomidae (GenBank accession numbers in Table 2, representatives of available diplostomid genera were selected) was inferred based on the rDNA combining the results of a maximum likelihood tree search performed in RAxML 8.7.4. (Stamatakis 2014) with bootstrap values calculated using 1,000 replicates and Bayesian interference performed in MrBayes 3.2 (Ronquist et al. 2011). Posterior probabilities were approximated for 10,000,000 generations, sampled at each 1,000th generation and with a burn-in of

182 10% in two separate runs. Chain stationarity and parameter convergence were checked in Tracer 1.6

183 (Rambaut et al. 2014). Because of its position in a different but related digenean family

184 (Clinostomidae), *Clinostomum complanatum* (Rudoplhi, 1814) collected from *Barbus barbus* (L.)

185 was used as an outgroup. File conversion was carried out using ALTER (Glez-Peña et al. 2010).

186 Phylogenetic trees were rendered by FigTree 1.4.2 (Rambaut and Drummond 2009) and edited in

187 Adobe Photoshop CS6.

188

Results

Parasite identification

191 Digenean metacercariae were found in the abdominal cavity of *V. letourneuxi* and *V. robertae,*

192 while isolated cysts were also found in some fishes beneath the lens of the eye. Parasites were

193 identified as members of Diplostomidae, subfamily Crassiphialinae. This was based on the

194 morphological characters of the 'neascus' (a characteristic type of diplostomid metacercariae, see

195 Niewiadomska 2002), such as the presence of a bipartite body, with a reserve bladder consisting of

196 a ramified median and two lateral canals forming a net-like structure in the forebody, and a

197 developed hindbody with unconnected excretory canals. Since only immature gonads were present,

198 further identification to genus or species level was not possible, because the classification is based

199 on the size of testes and the absence/presence of an ejaculatory pouch (Niewiadomska 2002).

200 For the genetic identification of the parasites, sequences from nine metacercariae (only specimens

201 from the abdominal cavity were available for molecular work) were obtained and two different

202 rDNA haplotypes were recorded, with a length of 1159 base pairs and a pairwise difference of

203 4.8%. These haplotypes did not correspond with the distinction between encysted and excysted

204 metacercariae. Only one corresponding COI haplotype was recorded with a length of 531 base

205 pairs, due to low amplification success. The pairwise distances in the entire dataset ranged from

206 0.5–37.8 % in the rDNA regions and from 0.5–33.2 % in the COI region (pairwise deletion).

207 Phylogenetic analyses of rDNA did not cluster the two haplotypes of metacercariae infecting Greek

killifishes together. Genetic distances between both haplotypes surpassed those between other sequences considered to belong to different species. This indicates the presence of two parasite species. They clustered with representatives of *Posthodiplostomum* Dubois, 1936 and *Ornithodiplostomum* Dubois, 1936 placed among other basal lineages of this clade. Both methods produced the same tree topology for rDNA (Fig. 2). The analyses confirmed the previously observed polyphyly of *Posthodiplostomum*.

Host-parasite ecology

Metacercariae occurred in two forms (encysted and excysted, Fig. 3), the encysted form usually in groups attached to the anterior end of the gonad, adjacent to the mesentery and at the area between the liver and the digestive tract, with parasites varying in size. Of a total of 296 fishes, 219 were found to be parasitized, corresponding to a high overall metacercaria prevalence of 73.99% (not separated between parasite forms).

Parasite prevalence, in the six *Valencia* populations studied, varied in autumn from 18.18% to 100% (Table 1; maximum prevalence was 100% for both *V. robertae* and *V. letourneuxi*). Seasonal prevalence remained high throughout the year at the two sites sampled quadannualy (Table 1) ranging between 74.3% and 100% (Fig. 4). In these two sites, prevalence peaked in autumn with all fish being parasitized (100%) and then decreased in the winter to 82.3%, due to the appearance of a group of metacercariae-free fish, ranging in size from 13 to 36 mm SL (juveniles and adults, see Kalogianni et al. 2010b). In the spring, prevalence further decreased to 74.3%, reaching its lowest value. Parasite prevalence in function of the host's sex (ratio 5:4 in favor of females), was 52.88% and 63.15% in host females (n=52) and males (n=42) respectively, but this difference was not statistically significant ($\chi^2 = 0.792$; $df = 1$; $P = 0.374$).

Parasite prevalence increased gradually with host size (SL, Fig. 5), from a prevalence of 0% for the larval length classes 6-8 and 8-10 mm to a maximum prevalence value of 100% in the 22-24 mm adult length class (larval size range < 13 mm, juvenile range ≥ 13 and ≤ 17 mm and adults > 17

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mm, see Barbieri et al. 2000 and Kalogianni et al. 2010b). For fish lengths > 24 mm these prevalence values remained stable, with one exception for the adult length class 24-26 mm (prevalence 75%, Fig. 5). Mean SL values for infected and metacercariae-free fish differed, i.e. mean SL of infected fish was 19.70 ± 0.92 S.E. (n = 58); mean SL of metacercariae-free fish was 13.64 ± 0.55 S.E. (n = 36); this difference was statistically significant (t = -4.846, $P < 0.0001$).

Mean parasite:host weight ratio was 0.0064 ± 0.0010 S.E., with parasite weight averaging 0.64% of the host weight. Parasite weight and host weight were positively correlated (n = 9, rho correlation = 0.882, $P = 0.002$, see Fig. 6) for fish < 600 mg, with a mean parasite:host weight ratio of 0.0076 ± 0.0009 S.E. and percentage mean 0.76%; however, there was no correlation for the larger specimens (mean ± S.E. parasite:host weight ratio of 0.0009 ± 0.0007 and percentage mean 0.09%, n = 2).

The study of the condition, gonadosomatic and hepatosomatic indices showed no statistically significant differences between infected and metacercariae-free specimens, in either males or females ($p > 0.05$).

Discussion

Parasite identification

This study is the first record of valenciids as second intermediate hosts for diplostomid parasites, being heavily infected by their metacercariae. No other endoparasites were found in this study. (Dactylogyridean monogenean gill parasites were also found – at very low prevalence and infection intensity – but fall outside of the scope of this study.) Dominance of (immature) endoparasites in general, and of trematode metacercariae in particular, in killifishes has been previously observed. It was suggested to demonstrate the importance of these fishes as intermediate or paratenic hosts (Nezhybová et al. 2017). Pairwise uncorrected genetic distances between the retrieved rDNA haplotypes (4.8%) suggest the existence of two different diplostomid species (Georgieva et al.

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2013; Stoyanov et al. 2017). Unfortunately, the metacercarial lifestage does not allow morphology-based species-level distinction. Therefore, parasite haplotypes could not be linked with specific phenotypic characters; neither was there a link with being encysted or not. The collected parasite species are considered as representatives of *Posthodiplostomum* or *Ornithodiplostomum* based on the results of phylogenetic analysis (Fig. 2). Interestingly, haplotype 1 takes a basal position within the *Posthodiplostomum-Ornithodiplostomum* clade and most likely represents a hitherto unsequenced phylogenetic lineage. Low maximum likelihood bootstrap values of deeper nodes compared to the Bayesian inference posterior probabilities highlight the unresolved position of several diplostomid genera and an insufficient number of already published sequences. Moreover, the tree also indicates that *Posthodiplostomum* is not monophyletic and in need of revision (Locke et al. 2010b; Athokpam and Tandon 2014; García-Varela et al. 2016). However, according to Stoyanov et al. (2017) the uncertain phylogenetic positions of *Posthodiplostomum* species is often the result of incorrect morphological identification due to low quality of specimens, creating confusion even in available molecular data. Although previous studies based on barcoding approaches reported cryptic diversity in the family (Locke et al. 2010a, b; Georgieva et al. 2013), complicated morphological identification and the lack of information about adult stages makes formal species description challenging as it depends on high quality stained material. Four species of *Posthodiplostomum* (*P. brevicaudatum* (von Nordmann, 1832); *P. centrarchi* Hoffman, 1958; *P. cuticola* (von Nordmann, 1832); *P. minimum* (MacCallum, 1921)) and one of *Ornithodiplostomum* (*O. scardinii* (Schulman in Dubinin, 1952)) respectively, have been reported from more than 70 freshwater fish hosts, mostly cyprinids and cobitids, in Europe (Sonin 1986; Stoyanov *et al*. 2017). To the best of our knowledge this is the first report of a freshwater species native to Greece found infected by either of both abovementioned diplostomid genera (see e.g. Stoyanov et al., 2017). Our results enrich the list of the known digenean fauna in Greece, following the record of *Diplostomum spathaceum* (Rudolphi, 1819) from ten different fish host species in Lakes Volvi and Vistonis (Kalfa-Papaioannou and Sinis 1985).

Host-parasite ecology

Within the framework of this study, differences were found in the prevalence of the reported diplostomid infection of fish between the various *Valencia* populations studied. These cannot probably be attributed to variation in habitat features, such as temperature, salinity or surface vegetation cover, as all the habitats of the target species are spring-fed streams and wetlands that are rather stable both hydrologically and thermally; nor to a variation of fish population densities, evident from data published elsewhere (Kalogianni et al. 2010a). Therefore, we assume that this spatial variation in the parasite prevalence could be related to a spatial variation of the primary host (gastropod) densities, as well as of that of the definitive host. Parasite prevalence also remained high throughout the year, a range similar to that reported elsewhere for *Posthodiplostomum cuticula* (Ondráčková et al. 2004a) or other digeneans (Mbokane et al. 2015; Kondo et al. 2016). Maximum prevalence (100%) was observed in autumn, and then prevalence decreased in winter, due to the presence of both juvenile and adult metacercariae-free fish. This trend, common to both species of *Valencia*, is thought to be attributed to water temperature as an important factor for the emergence of cercariae from the snail, corresponding to the highest propagation of second intermediate hosts (Chubb 1979; Ondráčková et al. 2004b). Alternatively, the metacercariae-free juvenile fish in the winter samples could be the product of late recruitment at the end of autumn (the reproductive period of both target species extends to late October, see Barbieri et al. 2000; Kalogianni et al. 2010a) not yet parasitized due to limited exposure time. The presence of metacercariae-free mature adults (over 30 mm) in the winter samples of both species, on the other hand, could be attributed to parasite mortality induced by an adaptive immune response of the fish host (for a review of immune responses induced in teleost fish by digenean metacercariae see Alvarez-Pellitero 2008). Parasite prevalence may also be related to gastropod availability that is at its lowest in winter and spring, as it has been shown in a seasonal dietary study on *V. letourneuxi* that also included benthic data (Kalogianni et al. 2010b).

The linear correlation between parasite weight and host weight indicates that infection occurs early in the life of the fish and then the parasites grow with the host and/or fish accumulate metacercariae as they grow. The positive relation between the length of the host and the prevalence of the parasite observed in the target species is most likely a result of temporal accumulation of parasitation, as larger fish could be exposed repeatedly to infection for a longer time than younger fish (Saad-Fares and Combes 1992; Paes et al. 2010). This is also supported by the presence of different stages of digenean metacercariae in the target species, as suggested also in various cyprinids or cobitids (Ondráčková et al. 2004a). Finally, there were no significant differences in the susceptibility of infection between males and females, as reported also for other freshwater fish species infected with diplostomid digeneans (Flores and Semenas 2002; Machado et al. 2005). Digenean metacercariae have been also found, in various freshwater species, subcutaneously in the trunk region and head, in fins, gills, the eyes and muscle tissue, as well as in viscera (Sonin 1986; Niewiadomska 2002). In the two host species of this study, metacercariae were found mostly in the visceral cavity, in association with the gonads and the digestive tract and liver, but also beneath the lens of the eye. Hence, it seems a systemic infection. This distribution of the parasite therefore corresponds with previous studies and it is correlated with the high infection level reported (Kvach *et al*. 2017).

Previous studies have shown that the natural definitive hosts of *Posthodiplostomum* and *Ornithodiplostomum* are piscivorous birds, with planorbid or lymnaeid gastropods the most common first intermediate hosts (Miller 1954; Niewiadomska 2002; Faltýnková et al. 2008; Nguyen et al. 2012). Planorbids and lymnaeids were the only gastropods that were found at both Louros, Mornos and Acheron *Valencia* habitats during a benthic faunal study conducted in summer 2009 (unpublished data). However, only planorbid availability reflected the variation of parasite prevalence between these three populations, with the Louros habitat having both lower planorbid availability and lower parasite prevalence. Furthermore, benthic macroinvertebrate data collected seasonally at the Mornos habitat to assess food availability for a dietary study on *V. robertae* (then *V. letourneuxi*) showed that planorbids were the only gastropod taxon available throughout the year

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(Kalogianni et al. 2010b). In addition, that study showed that the target species consumed only three gastropod prey categories (Planorbidae, Valvatidae and Physidae), with Lymnaeidae being absent from its diet and Planorbidae being the most frequent and abundant gastropod in the species diet. Since the planorbids reflect variation in metacercariae prevalence and figure as preferable prey of valenciid fishes, we assume high contact rates. We therefore suggest planorbids as the first intermediate host of the collected digenean species.

Parasite effects on the host species and conservation implications

The results of the current study show that there was no correlation between the diplostomid infection of the target species and the condition and reproduction of the hosts, as reported also elsewhere (Paes et al. 2010; Gholami et al. 2011), though there are studies that have shown a negative correlation between the abundance of *Posthodiplostomum* sp. and the relative condition factor of its hosts (Lucký 1970).

The absence of any detectable differences on fish condition and reproduction between infected and metacercariae-free *Valencia* specimens leads us to tentatively assume that this endoparasite is not pathogenic to its host. Given the short lifespan of the target species (two or three years in the wild, Barbieri et al. 2002b) and the fact that trematode species may live for more than a year in the fish host and even for the whole lifespan of the host (Kalantan et al. 1987; Dias et al. 2006), it appears that this host-parasite relationship bears the characteristics of a strategy in which the parasite does not affect fish survival, fitness and reproduction. However, the observed eye infection by diplostomid metacercariae could affect fish vision and thus increase its predation by birds, as reported for other diplostomids (Seppälä et al. 2005).

Parasites are a potential risk factor in conservation initiatives targeting native species, such as population enhancement, assisted migration or reintroduction actions. Digenean colonization of non-native areas depends on the strategy of larval stages, highly productive asexual reproduction, host specificity, level of virulence in intermediate hosts and measure of similarity of environmental

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conditions between source or recipient localities (see Bauer 1991; Kennedy 1993). Based on the enemy release hypothesis, introduced endangered host species could profit of parasite loss (Genner et al. 2008) or be affected by spill-back of parasites from alien hosts in an introduction locality (McCallum and Dobson 1995; Daszak et al. 2000; Holt et al. 2003). On the other hand, a scenario suggesting a greater pathogenetic effect of co-introduced parasites on native hosts was also documented (naïve host hypothesis) (Anderson and May 1992; McCallum and Dobson 1995; Hudson et al. 1998). In this respect, translocations of endangered species to reestablish or to help recover populations could introduce parasites, harmless to the reintroduced population, but pathogenic to the already present naïve conspecifics or other sympatric species (see Daszak et al. 2000; Britt et al. 2004). Therefore, even the seemingly harmless diplostomid digeneans reported here should be considered carefully in the context of the conservation-related release or translocation of *Valencia* populations. Finally, no specimens of the introduced mosquitofish *G. holbrooki*, examined in two water systems where the species is sympatric with *Valencia* species (Acheron and Louros, unpublished data), were found infected by the same diplostomid metacercariae. This indicates that the parasites were not introduced locally through the mosquitofish nor that it could pose a threat to the native species, acting as a reservoir for these parasites. A similar absence of metacercariae of digenean parasites has been also reported in the only available study on mosquitofish parasites in Europe, namely in *G. holbrooki* from eight Mediterranean river mouths in Spain and France (Benejam et al. 2009).

Conclusion

This study showed no negative effect of metacercaria infection on *Valencia* species. Furthermore, seasonal differences in digenean prevalence and parasite accumulation over fish age were documented, with no differences in infection parameters between host sexes. It also confirmed the need for a revision of the complicated taxonomy of diplostomids and their unresolved phylogenetic classification. While we suggest planorbids to be potential first intermediate hosts, further

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390 investigations reconstructing the life cycle of the here reported parasites are required, in order to

391 understand the ecological parameters of infection of their secondary host, as well as to identify the

392 other host taxa. Such information is important to the understanding of parasite-host interactions, as

393 well as to the planning or implementation of appropriate conservation measures for the endangered

394 fish species, targeted in this study, as well as other vulnerable fish hosts of these parasites.

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Compliance with ethical standards

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

414 **Ethical approval:** All applicable international, national, and/or institutional guidelines for the care

415 and use of animals were followed.

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Table 1 Features of the six sites sampled in autumn, number of fish examined, Standard length range and parasite prevalence (%) in the total sample, in females and males. The two sites that were also sampled seasonally are marked with an asterisk. Sites 1, 2, 3, 5 and 6 host *V. letourneuxi*; Site 4 hosts *V. robertae.* Veg denotes Vegetation cover.

	Site	Date	Latitude	Longitude	Veg (%)	Temp (°C)	Salinity (ppt)	n fish	SL range (mm)	Parasite prevalence (%)		
										% Total	% ♀	% ♂
1	Vlychos	Oct 2005	38° 54' 54" N	20° 52' 32" E	50	21.4	3.5	20	8.8 - 24.8	20.00	27.27	12.50
2	Louros	Oct 2005	39° 10' 31" N	20° 45' 53" E	95	15.8	0.2	16	9.7 - 24.8	50.00	33.33	71.43
3	Arachthos	Oct 2005	39° 05' 30" N	21° 02' 13" E	70	17.8	0.4	11	10.0 - 24.5	18.18	16.67	20.00
4	Mornos*	Sep 2006	38° 24' 26" N	21° 55' 03" E	80	25.0	0.3	23	16.5 - 56.0	100.00	100.00	100.00
5	Acheron*	Sep 2006	39° 14' 55" N	20° 28' 50" E	40	17.2	3.2	16	12.7 - 20.0	100.00	100.00	100.00
6	Kypseli	Sep 2009	39° 17' 15" N	20° 32' 35" E	60	16.9	0.1	9	7.0 - 29.2	55.56	40.00	75.00

Table 2 List of digenean species (Diplostomatidae) obtained from GenBank with their accession numbers for the ITS1-5.8S-ITS2 rDNA region and for the COI sequences retrieved, their host species and the country where the species were collected.

Parasite species	Host species	Country	ITS1-5.8S-ITS2 rDNA	COI mtDNA
Alaria mustelae Bosma, 1931	*Mustela frenata* Lichtenstein, 1831	USA	JF820609.1	KT254032.1
Austrodiplostomum ostrowskiae Dronen, 2009	*Dorosoma cepedianum* (Lesueur, 1818)	USA	KT728782.1	KR271028.1
Bolbophorus confusus (Krause, 1914)	*Pelecanus onocrotalus* Linné, 1758	Israel	AY242851.1	-
Clinostomum complanatum Rudolphi, 1814	*Triturus carnifex* (Laurenti,1768)	Italy	KM518257.1	JF718595.1
Diplostomum mergi Dubois, 1932	*Radix auricularia* Linnaeus, 1758	Czech Republic	KR149499.1	KR149528.1
Diplostomum paracaudum (Iles, 1959)	*Gadus morhua* Linnaeus, 1758	Denmark/Germany	KJ889013.1	JQ639176.1
Diplostomum spathaceum Rudolphi, 1819	*Larus ridibundus* Linné, 1766	Czech Republic	KR269765.1	JX986895.1
Ornithodiplostomum sp. 1 Dubois, 1936	*Percina caprodes* (*Rafinesque, 1818*)	Canada	HM064937.1	HM064748.1
Ornithodiplostomum sp. 2 Dubois, 1936	*Notemigonus crysoleucas* (Mitchill, 1814)	Canada	HM064939.1	HM064764.1
Ornithodiplostomum sp. 3 Dubois, 1936	*Pimephales promelas* (Rafinesque, 1820)	Canada	HM064942.1	HM064784.1
Ornithodiplostomum sp. 4 Dubois, 1936	*Pimephales promelas*	Canada	HM064945.1	HM064785.1
Ornithodiplostomum sp. 5 Dubois, 1936	*Notemigonus crysoleucas* (Mitchill, 1814)	Canada	FJ469595.1	KT831368.1
Ornithodiplostomum sp. 6 Dubois, 1936	*Pimephales promelas* (Rafinesque, 1820)	Canada	HM064946.1	HM064790.1
Ornithodiplostomum scardinii (Schulman in Dubinin, 1952)	*Scardinius erythrophthalmus* (Linnaeus, 1758)	Czech Republic	KX931443.1	KX931425.1
Posthodiplostomum brevicaudatum 1(von Nordmann, 1832)	*Perca fluviatilis* (Linnaeus, 1758)	Czech Republic	KX931428.1	KX931418.1
Posthodiplostomum brevicaudatum 2	*Gasterosteus aculeatus* (Linnaeus, 1758)	Bulgaria	KX931429.1	KX931419.1
Posthodiplostomum centrarchi 1 (Hoffman, 1958)	*Lepomis gibbosus* (Linnaeus, 1758)	Bulgaria	KX931441.1	KX931421.1
Posthodiplostomum centrarchi 2	*Lepomis gibbosus*	Slovakia	KX931442.1	KX931423.1
Posthodiplostomum sp. 1 Dubois, 1936	*Channa punctata* (Bloch, 1793)	India/Canada	KF738447.1	HM064795.1
Posthodiplostomum sp. 2 Dubois, 1936	*Channa argus* (Cantor, 1842)	Japan/Canada	AB693170.1	HM064798.1
Posthodiplostomum sp. 3 Dubois, 1936	*Lepomis gibbosus*	Canada	HM064957.1	HM064821.1
Posthodiplostomum sp. 4 Dubois, 1936	*Morone americana* (Gmelin, 1789)	Canada	HM064960.1	HM064844.1
Posthodiplostomum sp. 5 Dubois, 1936	*Lepomis gibbosus*	Canada	HM064958.1	HM064857.1
Posthodiplostomum sp. 6 Dubois, 1936	*Micropterus salmoides* Lacepède, 1802	Canada	HM064962.1	HM064864.1
Posthodiplostomum sp. 7 Dubois, 1936	*Perca flavescens* Mitchill, 1814	Canada	HM064961.1	HM064865.1
Tetracotyle xenentodoni Chakrabarti, 1970	*Tetracotyle xenentodoni* (Hamilton, 1822)	India/Canada	KU316948.1	HM064876.1
Tylodelphys aztecae García-Varela, Sereno-Uribe, Pinacho-Pinacho, Hernández-Cruz, Pérez-Ponce de León, 2015	*Podilymbus podiceps* (Linnaeus, 1758)	Mexico	KT175388.1	KT175369.1

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Tylodelphys mashonensis (Beverley-Burton, 1963)

Clarias gariepinus Burchell, 1822   Tanzania   KC685363.1   KR863382.1

Figure Captions

Figure 1 Location of sampling sites in Western Greece. Mornos hosts *Valencia robertae*, while Vlychos, Arachthos, Louros, Kypseli and Acheron host *V. letourneuxi*.

Figure 2 Phylogenetic tree based on rDNA fragments from 29 haplotypes of Diplostomatidae. Posterior probabilities for Bayesian inference, (before slash) and bootstrap percentages for maximum likelihood (behind slash) are shown. Clades that neither yield a support value higher than 80 nor of 50 under BI or ML, respectively, are collapsed. The haplotypes obtained in this study are called Haplotype 1 and 2.

Figure 3 Encysted and excysted metacercariae of diplostomatid digenean from the abdominal cavity of *V. letourneuxi* and *V. robertae* (Scale bar 250 μm), OS – oral sucker; PS – pseudosuckers; GP – genital pore; VS – ventral sucker.

Figure 4 Seasonal variation of the size frequency distribution of infected and metacercariae-free specimens in Mornos and Acheron habitats that host *V. robertae* and *V. letourneuxi* respectively. Number of specimens and parasite prevalence values (%) are also shown. The two groups of metacercariae-free fish of the winter sample are marked with asterisks.
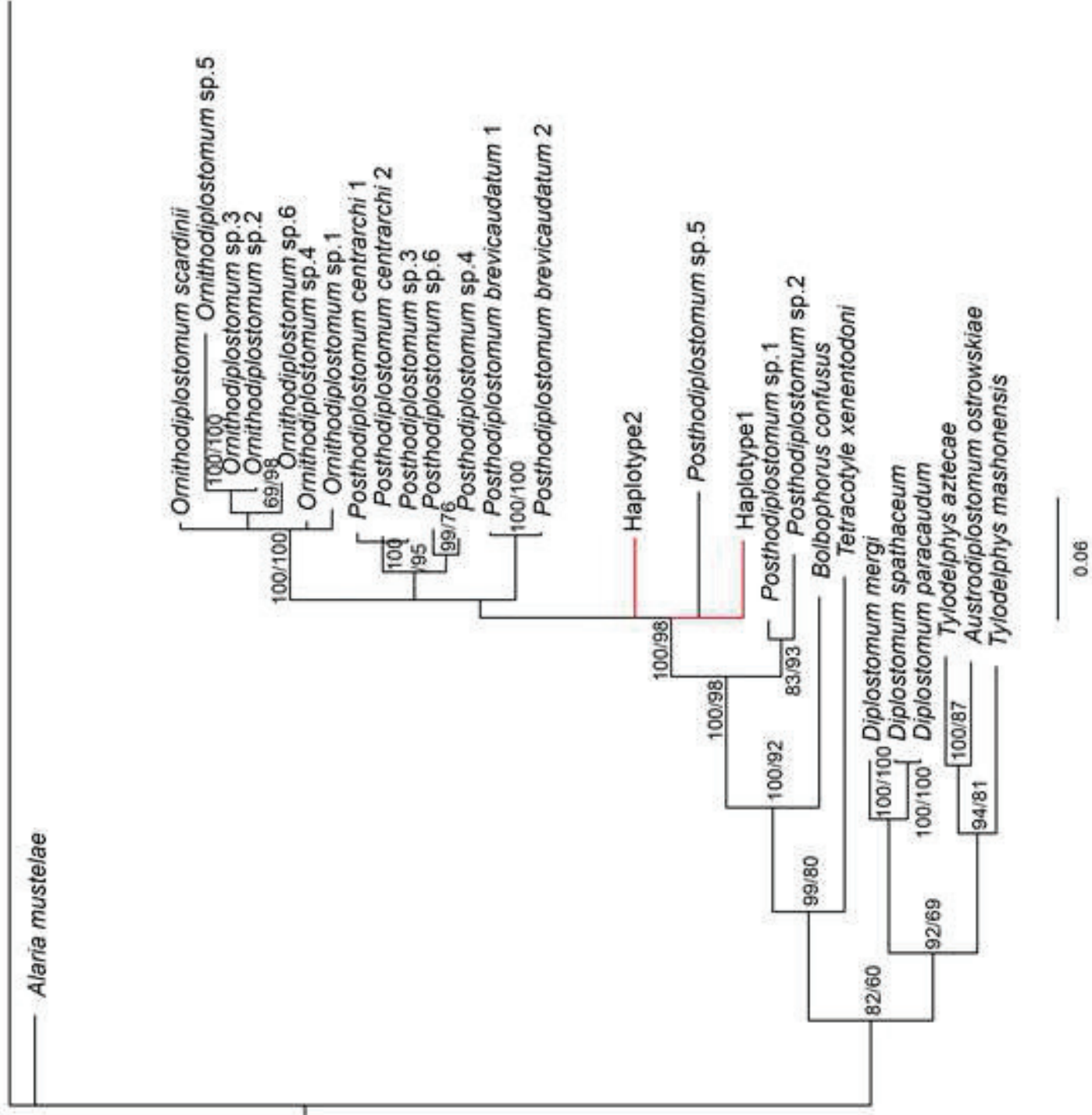
Figure 5 Size frequency distribution of metacercariae-free and infected *V. robertae* and *V. letourneuxi* specimens of the autumn samplings (n = 94). Maximum parasite prevalence (100%) was first observed at the 22-24 mm length class (marked with asterisk).
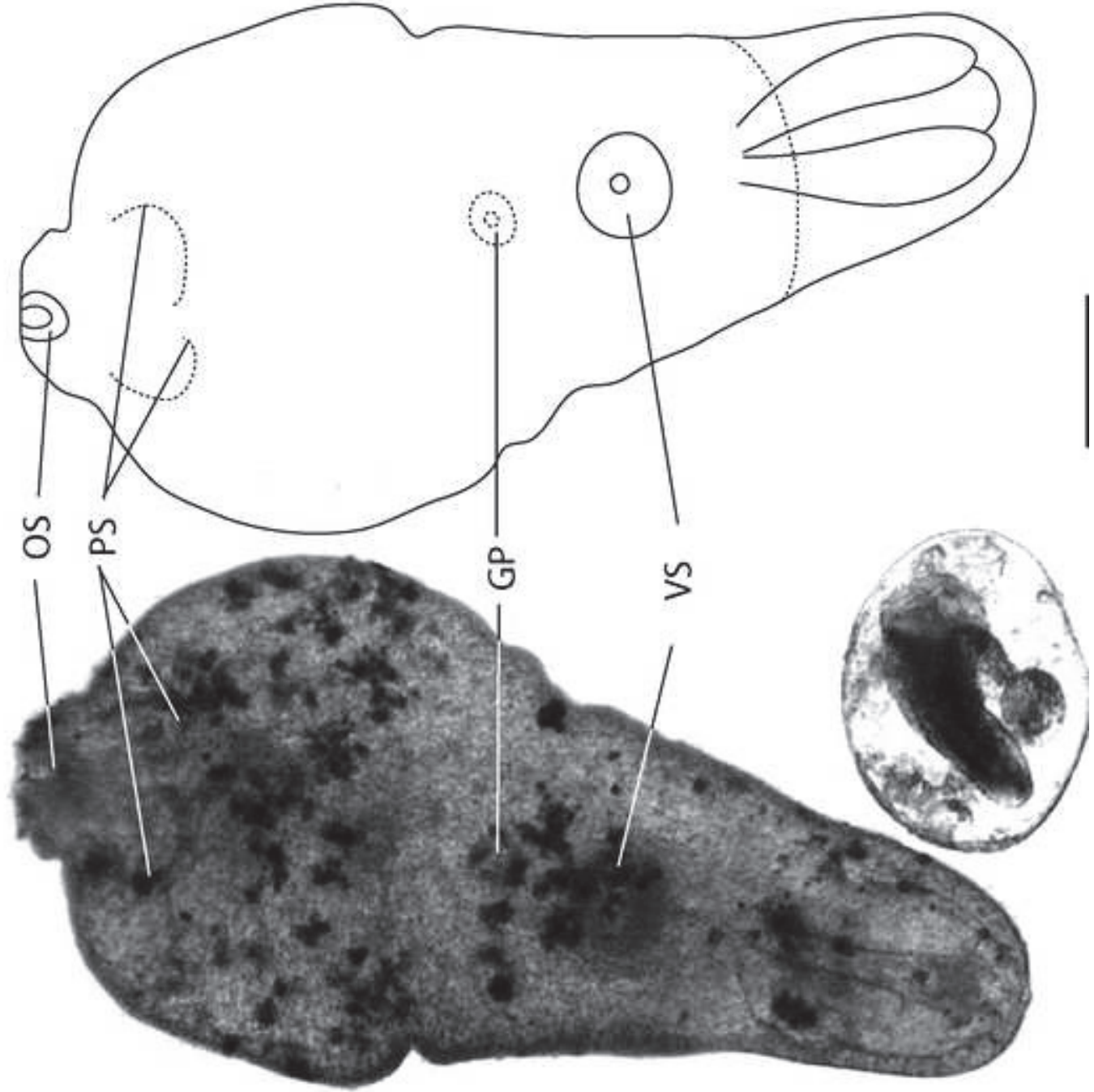
Figure 6 Parasite weight (PW) and host weight (HW, i.e. total fish host weight) positive relationship for fish < 600 mg.
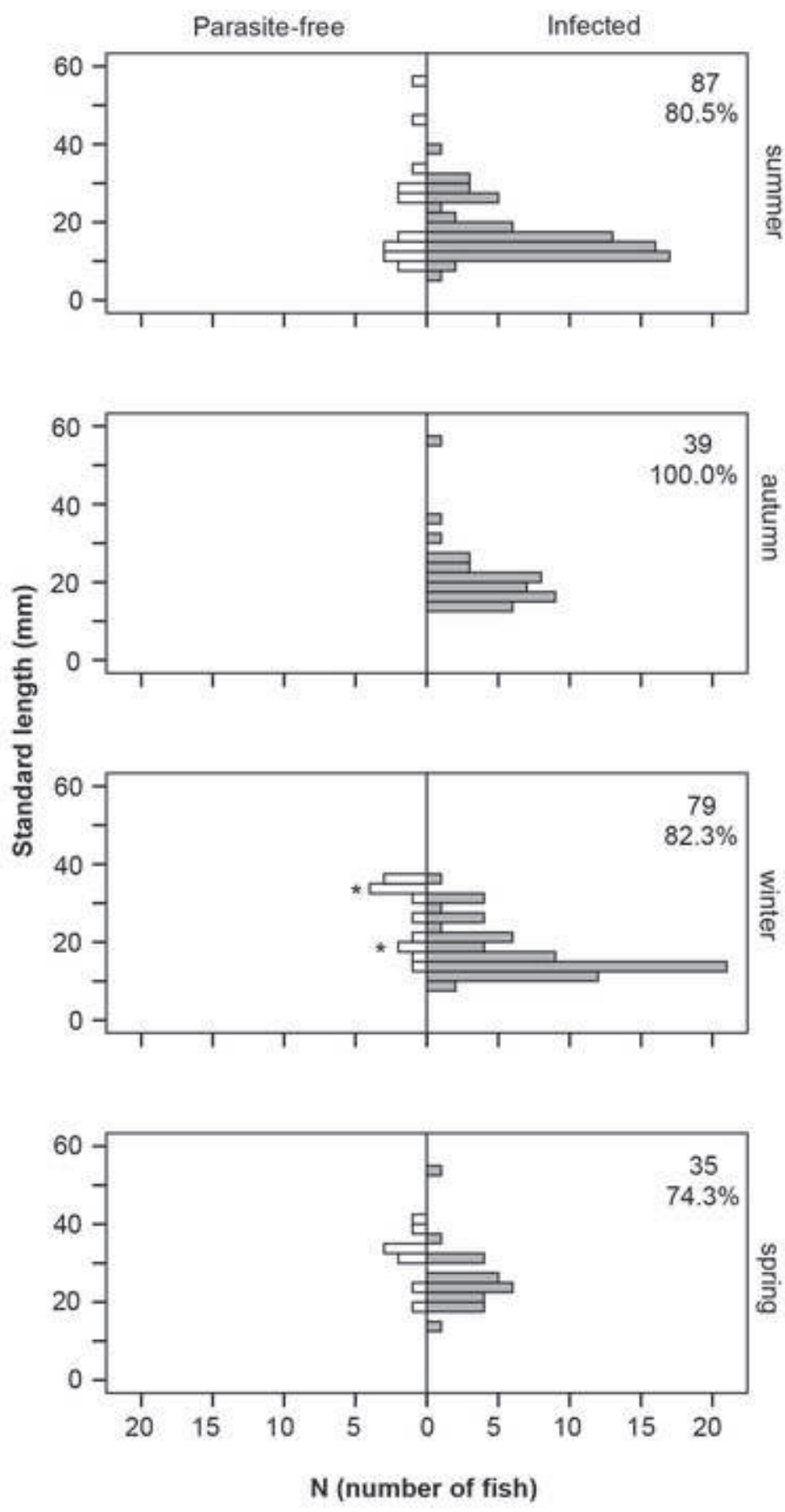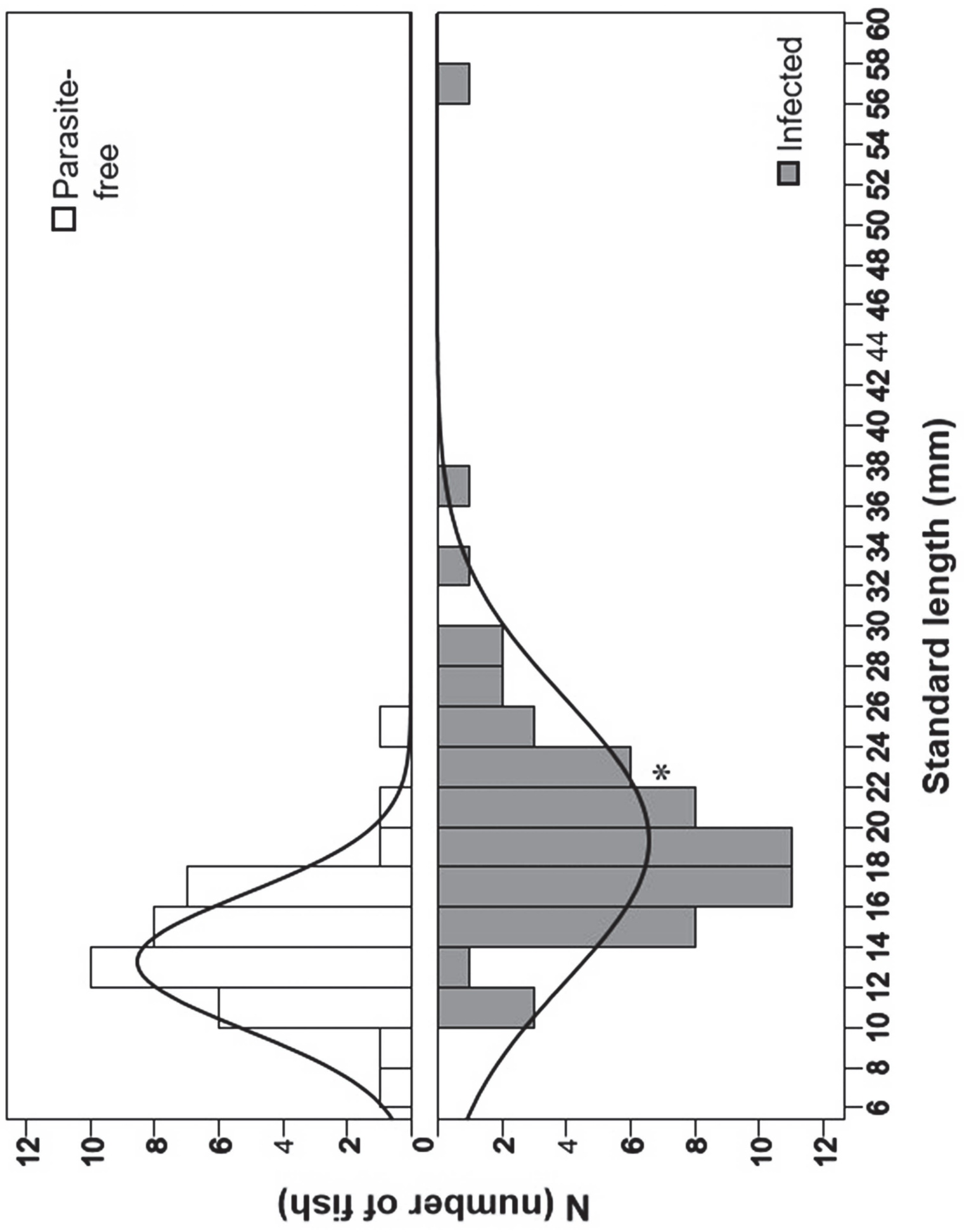
Figure1

Figure2

Figure 2

Figure3

OS

PS

GP

VS

Figure4

Figure5

Figure 5

Figure6