



Ontogenetic shift in the trophic role of the invasive killer shrimp *Dikerogammarus villosus*: a stable isotope study

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Abstract The introduction of the amphipod *Dikerogammarus villosus* in European fresh waters is to date recognized as a threat to the integrity of invaded communities. Predation by *D. villosus* on native benthic invertebrates is assumed as the key determinant of its ecological impact, yet available information describe the species as a primary consumer as well as a carnivore depending on local conditions. Here, we assessed the trophic position (TP) of *D. villosus* in Lake Trasimeno, a recently invaded lentic system in central Italy, using the CN isotopic signatures of individuals captured in winter spanning two orders of magnitude in body size. TP estimations were compared with those characterizing the native amphipod *Echinogammarus veneris* and other

representative invertebrate predators. On average, *D. villosus* showed a trophic position higher than *E. veneris*, and comparable with that of odonate nymphs. An in-depth analysis revealed that large-sized individuals had a trophic position of 3.07, higher than odonates and close to that of the hirudinean predator *Erpobdella octoculata*, while small-sized specimens had a trophic position of 2.57, similar to that of *E. veneris* (2.41). These findings indicate that size-related ontogenetic shifts in dietary habits may per se vary the nature of the interaction between *Dikerogammarus villosus* and native invertebrates from competition to predation. Information collated from published isotopic studies corroborated the generality of our results. We conclude that intra-specific trophic flexibility may potentially amplify and make more multifaceted the impact of the species on other invertebrate species in invaded food webs.

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Introduction

The introduction of aquatic non-indigenous species has long been recognized to have important, disruptive consequences for the structure and function of freshwater ecosystems (Elton 1958; Dudgeon et al. 2006; Gherardi 2007; Strayer 2010; Gallardo et al. 2016; Emery-Butcher et al. 2020). Among other invertebrates introduced in European waters, the killer shrimp *Dikerogammarus villosus* (Sowinsky 1894), a gammarid amphipod of Ponto-Caspian origin, after the opening of the Rhine-Main-Danube Canal has established in German rivers including the Danube, Moselle, and Rhine (Bij de Vaate et al. 2002). Subsequently, the species spread in lentic and lotic environments in France, the Netherlands, Great Britain, and Switzerland (Devin et al. 2001; van der Velde et al. 2002; Koester and Gergs 2014; MacNeil et al. 2010; Rewicz et al. 2017), as well as towards the Baltic region (Bacela et al. 2008; Gusev et al. 2017 and literature cited). In Italy, the killer shrimp appeared in Lake Garda in 2003 (Casellato et al. 2006) and subsequently invaded the northern and central regions of the peninsula (Tricarico et al. 2010).

The predatory behavior of the killer shrimp is generally recognized as a crucial determinant of its ecological impact on other benthic invertebrates, including native amphipods. A number of laboratory trials on the feeding preferences of the gammarid supports this assumption (Krisp and Maier 2005; Kinzler et al. 2008; Pellan et al. 2016; Taylor and Dunn 2017), together with indirect evidences from field investigations highlighting significant declines in native macroinvertebrate assemblages after *D. villosus* invasion (Muskó 1989; Bollache et al. 2004; Noordhuis et al. 2009).

Noticeably, information on the actual impact of the gammarid on benthic communities are less univocal (Hellmann et al. 2017; Koester et al. 2018). In addition, field studies using carbon and nitrogen stable isotopes analysis (SIA hereafter), have emphasized a high variability in dietary habits, with trophic position estimations varying by more than one trophic level from a maximum of 3.7 in the Vistula basin

(Poland) to a minimum of 2.1 in the River Arno (Italy) (Bacela-Spychalska and Van Der Velde 2013; Haubrock et al. 2019).

Indeed, *D. villosus* shows no morphological specialization of the mouthparts for a carnivorous diet (Mayer et al. 2008), and it has been repeatedly indicated to be an omnivore in invaded food webs feeding opportunistically also on vegetal resources (Maazouzi et al. 2007; Truhlar et al. 2014; Boeker and Geist 2015; Jourdan et al. 2016). Accordingly, the species is generally posited to adapt its trophic habits to local environmental conditions, switching between different functional feeding groups depending on season, habitat, and resource availability (Maazouzi et al. 2009; Pellan et al. 2016; Hellmann et al. 2017). Noticeably, Hellmann and colleagues (2015) indicated that the trophic position of *D. villosus* can vary not only among different locations, but also among different ontogenetic stages within the same population, in turn suggesting that the species may play simultaneously multiple functional roles within an invaded food web. So far this aspect of the killer shrimp trophic ecology has generally received scant attention in field studies, even though ontogenetic diet shifts seem to represent a common trophic trait in invasive aquatic species, from crustaceans (Limén et al. 2005; Berezina 2007; Mancinelli et al. 2016; 2017b) to fish (Števo and Kováč 2016; Lee et al. 2018). Accordingly, here we verified whether *Dikerogammarus villosus* is characterized by a high feeding plasticity at the intra-population level related with a size/ontogenetic shift in trophic habits. The alternative hypothesis was that the species maintains constant trophic habits independently from size and ontogenetic stage (as observed for e.g., *Gammarus pulex*: Dick et al. 2013). The trophic position of the killer shrimp was assessed in Lake Trasimeno, a recently invaded lentic system in central Italy (Catasti et al. 2017) using stable carbon and nitrogen isotope analysis. To test the hypothesis, we determined the CN isotopic values in *D. villosus* specimens spanning two orders of magnitude in individual size and assessed the trophic position of different ontogenetic stages of the species using a Bayesian procedure. Estimations were eventually compared with the trophic position characterizing the native amphipod *Echinogammarus veneris* and other representative invertebrate predators.

Since the present investigation focused only on a single population of the killer shrimp sampled in a single season, to verify the generality of our results we reviewed the isotopic literature focusing on the trophic position of *D. villosus*, making an effort to collate information from studies explicitly addressing ontogenetic shifts. In the last decades, SIA has gained popularity as a powerful complement to the study of food webs and of the factors affecting their structure and dynamics (including biological invasions: see Mancinelli and Vizzini 2015; McCue et al. 2020 for recent reviews), allowing robust, large-scale comparisons of species' dietary habits in space and time (e.g., Mancinelli 2012a; Pethybridge et al. 2018; Evangelista et al. 2019; Lang et al. 2020; Liénart et al. in press).

Methods

Study site and samples collection

The study was carried out in Lake Trasimeno (Central Italy), the largest lake in the Italian Peninsula, with a surface area of 128 km² and an average depth of 4.7 m. Further details on lake features can be found in Ludovisi and Gaino (2010) and in Mancinelli et al. (2018). The basin is included in a Regional Natural Park comprised in the Natura 2000 European network as a Site of Community Interest (SCI) and a Special Protection Area (SPA). The macroinvertebrate community of the lake littorals is characterized by a diverse assemblage of epibenthic taxa, including herbivorous/detritivorous crustaceans (e.g., the amphipod *Echinogammarus veneris*, the isopod *Asellus coxalis*, and the decapod *Palaemonetes antennarius*) and gastropods (e.g., *Valvata piscinalis*, *Bithynia tentaculata*, and *B. leachii*), together with predaceous insects (e.g., the coleopterans *Gyrinus caspius*, *G. substriatus*, and odonate nymphs of the genera *Ashna*, *Coenagrion*, *Erythromma*, *Ischnura*, and *Lestes*) and hirudineans (e.g., *Erpobdella octoculata*, *Helobdella stagnalis*, and *Hirudo medicinalis*) (Minelli 1979; VV.AA. 2015; Mancinelli et al. 2020).

Noticeably, during the last century the native lacustrine community has been drastically altered by the introduction of a number of non-indigenous species of different origin, including invertebrates (e.g., *Procambarus clarkii*, *Dreissena polymorpha*,

and *Sinanodonta woodiana*) and fish (e.g., *Lepomis gibbosus*, *Ictalurus melas*, *Carassius auratus*, and *Pseudorasbora parva*) (Dörr et al. 2020; Goretti et al. 2020; Lorenzoni et al. 2020). The killer shrimp appeared in the lake in 2017 (Catasti et al. 2017), and breeding populations are to date established in several areas of the basin (VV.AA. 2020).

In February 2018, invertebrates were collected using a pond net (mesh size = 1 mm) from rocks and floating leaf detritus accumulations in a shallow embayment (approximate depth = 1 m) in the locality of Sant'Arcangelo in the southern sector of the lake (43.089788°N, 12.156246°E). After collection, samples were transferred to the laboratory in lake water using refrigerated containers (4 °C).

Laboratory procedures

In the laboratory, collected specimens were individually identified under a light microscope to the lowest taxonomic level. Immediate identification after collection, when the natural coloration of amphipods was still maintained, allowed a relatively easy differentiation of *Dikerogammarus villosus* specimens, characterized by a striped morph in Lake Trasimeno (VV.AA. 2020), from the native *Echinogammarus veneris*, showing a uniform coloration. Given the high color polymorphism of the killer shrimp (see e.g., Devin et al. 2004a for illustrative examples), an additional trait used for the identification of *D. villosus* was the occurrence of dorsal projections on the 1° and 2° urosome segments, absent in *E. veneris* (Stock 1968; Eggers and Martens 2001; Konopacka 2004). Individuals of the six identified species (see Results) were kept in distilled water for 12 h to clear gut contents and euthanized by thermal shock (−80 °C for 10 min).

Stable isotope analysis

After being euthanized, specimens were oven-dried (60 °C for at least 1 week), and individually weighed to the nearest µg using a micro-analytical balance. Consequently, specimens were individually powdered with a mortar and pestle. Depending on the taxon, subsamples of different sizes were taken (amphipods: 0.73 ± 0.19 mg; isopods: 0.18 ± 0.02 mg; odonate nymphs: 0.61 ± 0.03 mg; hirudineans: 0.79 ± 0.01, means ± 1SD) and pressed into Ultra-Pure tin

capsules (Costech Analytical Technologies). For amphipods, multiple specimens with dry weight identical to the first decimal place were pooled when single individuals did not provide a sufficient mass. Carbon and nitrogen stable isotope values were determined using an Elemental Analyser (Thermo Scientific Flash EA 1112) connected with an Isotope Ratio Mass Spectrometer (Thermo Scientific Delta Plus XP). Isotopic values were expressed in conventional δ notation (as ‰) in relation to international standards (Pee Dee Belemnite for carbon and atmospheric N_2 for nitrogen). Analytical precision based on the standard deviation of replicates of standards (International Atomic Energy Agency IAEA-NO-3 for $\delta^{15}N$ and IAEA-CH-6 for $\delta^{13}C$) was 0.1‰ for both $\delta^{13}C$ and $\delta^{15}N$. Invertebrate taxa showed variable C:N ratios, in some cases > 3.5 (see Tab. 1 in results) thus indicating a non-negligible contribution of lipids to tissue carbon pool (Post et al. 2007). Lipids are depleted in ^{13}C compared to proteins and carbohydrates and can significantly bias $\delta^{13}C$ estimations (Logan et al. 2008). Accordingly, for samples with a C:N ratio > 3.5 , $\delta^{13}C$ values were corrected for lipid content using tissue C:N ratios following the algorithm proposed by Post et al. (2007). Lipid-corrected $\delta^{13}C$ values were used in further analyses.

Literature search

Isotopic studies reporting on the killer shrimp were searched using the online databases ISI Web of Science, Scopus, and JSTOR (last access date: November 20th, 2020). We adopted a multiple search criterion, using the terms “*Dikerogammarus villosus*” or “killer shrimp” in combination with “stable isotopes” to search in all available fields. Additional information were found by checking the references of collected papers, and performing general searches in Google Scholar. The papers identified in the search (13 in total) were screened by a three-step approach using a title/abstract/full text procedure (Mancinelli et al. 2017a and literature cited therein). In order to be selected, publications had to (1) be performed in the field; laboratory studies were omitted, although they had their references scrutinized; (2) analyze the stable isotopes at least of nitrogen and (3) include an estimation of the trophic position of *D. villosus* or, alternatively, its nitrogen isotopic signature together with those of taxa located at the first or second trophic level. Data were extracted from tables and figures of 10 studies meeting the criteria; figures were digitized after a five-fold enlargement and converted to numerical format (estimated error: 0.02%) using the

Table 1 List of sampled taxa reported in order of abundance

Species	Order	N	Dry weight	$\delta^{13}C$	$\delta^{15}N$	C:N
<i>Dikerogammarus villosus</i>	Amphipoda	169	9.1 ± 15 (0.4/27.7)	− 16.2 ± 2.3 (− 19.1/− 13.6)	7 ± 2.1 (4.7/9.4)	5 ± 0.8 (4.3/5.9)
<i>Erythromma</i> sp.	Odonata	12	5.6 ± 1.5 (3.4/8)	− 17.6 ± 0.8 (− 18.5/− 15.7)	7.2 ± 8.8 (7/7.4)	3.9 ± 2.3 (3.6/4.2)
<i>Echinogammarus veneris</i>	Amphipoda	6	3.8 ± 2.4 (1.6/7.7)	− 18.5 ± 0.6 (− 19/− 17.5)	5.3 ± 1 (4.2/6.3)	6.4 ± 0.8 (5.7/8)
<i>Asellus aquaticus</i>	Isopoda	5	1.5 ± 0.7 (0.6/2.4)	− 18.5 ± 1.4 (− 19.9/− 16.3)	5.1 ± 0.8 (4.5/6.5)	5.2 ± 0.7 (4.2/5.9)
<i>Ischnura</i> sp.	Odonata	4	3 ± 0.4 (2.7/3.6)	− 17.4 ± 0.2 (− 17.6/− 17.2)	7.4 ± 0.4 (6.8/7.8)	3.8 ± 0.1 (3.6/3.9)
<i>Erpobdella octoculata</i>	Arhynchobdellida	3	7.3 ± 3.5 (3.3/9.8)	− 20.6 ± 0.2 (− 20.8/− 20.5)	11.5 ± 0.8 (10.7/12.2)	4.3 ± 0.2 (4.1/4.5)

Additional information on individual dry weights (in mg), carbon and nitrogen isotopic values (in ‰), and C:N ratios are included as mean ± SD, with min–max values in parentheses. $\delta^{13}C$ values were corrected considering C:N ratios (see text for details). $\delta^{13}C$, $\delta^{15}N$, and C:N values were determined in the totality of sampled individuals for all taxa with the exception of *Dikerogammarus villosus*, for which CN isotopic and elemental concentrations were determined in a subsample of 54 individuals whose individual dry weight ranged between 0.4 and 27.6 mg

freeware WebPlotDigitizer (ver. 4.3; <https://automeris.io/WebPlotDigitizer/>).

The majority of the studies explicitly reporting *D. villosus* trophic position (see Results) used the equation $TP = (\delta^{15}N_{\text{consumer}} - \delta^{15}N_{\text{baseline}}) / \Delta^{15}N + \lambda$ (Vander Zanden and Rasmussen 1999), where $\delta^{15}N_{\text{consumer}}$ is the nitrogen isotopic signature of the killer shrimp, $\delta^{15}N_{\text{baseline}}$ and λ are the nitrogen isotopic signature and the trophic position of a baseline taxon, while $\Delta^{15}N$ is the trophic level fractionation of $\delta^{15}N$ (3.4‰; Post 2002). For the sake of comparison, we adopted the same formula and $\Delta^{15}N$ for those studies where the trophic position of *D. villosus* was not explicitly calculated using the isotopic signatures of the killer shrimp ($\delta^{15}N_{\text{consumer}}$) and those of taxa with $\lambda = 1$ or 2 ($\delta^{15}N_{\text{baseline}}$).

Data analysis

In general, values reported in the paper are expressed as means \pm 1SD if not otherwise specified. For parametric statistical analysis, data were tested for conformity to assumptions of variance homogeneity (Cochran's C test) and normality (Shapiro-Wilks test) and transformed when required. A Shapiro-Wilks test was also used to verify the normality of the size frequency distribution of collected *D. villosus* specimens. One-way Permutational Analysis of Variance (PERMANOVA; Anderson 2005) was run on a Euclidean distance matrix calculated on individual CN isotopic signatures with 9999 permutations of residuals within a reduced model to test for dissimilarities in isotopic values among taxa. PERMANOVA was also used to confirm the significance of the differences between size groups after a preliminary cluster analysis with complete linkage performed on the Euclidean distance matrix indicated a discontinuity in the isotopic signatures of the specimens analyzed (see Results).

The trophic positions of gammarids, odonate nymphs and hirudineans were estimated implementing a one-baseline, two-discrimination factor Bayesian model run with 2 parallel chains and 40,000 adaptive iterations, using the isopod *Asellus aquaticus* as isotopic baseline (see also Annabi et al. 2018 for an example). The species, together with the confamilial *Proasellus coxalis*, is a herbivorous/detritivorous consumer widely distributed in Italian lentic and lotic waters and plays a significant role in leaf detritus

processing in lacustrine environments (Marcus et al. 1978; Costantini et al. 2005). Accordingly, a trophic level (λ) = 2 was assigned to *A. aquaticus*, assuming $\lambda = 1$ for basal resources, $\lambda = 2$ for primary consumers, etc., with the trophic enrichment factors (TEF hereafter) $\Delta^{13}C = 0.57 \pm 1.58\text{‰}$ and $\Delta^{15}N = 3.40 \pm 1.04\text{‰}$ (Post 2002). Even though a species-specific TEF measurement is available from the literature (i.e., Hellmann et al. 2015), here we used a general TEF value to make more comparable the trophic positions estimated for the different taxa analyzed in the study, as well as those collated for *D. villosus* from the literature.

All statistical analyses were performed in the R statistical environment (R Development Core Team 2020). Specifically, PERMANOVA was run in the package *vegan* (version 2.5–6; Oksanen et al. 2019), while trophic positions were estimated using the *tRophicPosition* package (version 0.7.7; Quezada-Romegialli et al. 2018; 2019). The function “*compareTwoDistributions*” available in the aforementioned package was used to statistically compare in a Bayesian context TP values estimated for the different taxa and size groups.

Results

Six taxa were captured in total, including the gammarid amphipods *Dikerogammarus villosus* and *Echinogammarus veneris*, the isopod *Asellus aquaticus*, odonate larval stages, and the hirudinean *Erpobdella octoculata* (Table 1). Only six and five adult specimens of *A. aquaticus* and of *E. veneris* were respectively collected, while *D. villosus* dominated in abundance with 169 individuals varying in size by two orders of magnitude (Table 1; see also Fig. S1 in the online material). Among predators, 16 odonate nymphs were captured belonging to the genus *Erythromma* and, to a minor extent, *Ischnura* (four specimens). In addition, three individuals of the hirudinean *Erpobdella octoculata* were collected (Table 1).

On average, *Dikerogammarus* showed $\delta^{15}N$ values lower by 4.5‰ than *E. octoculata*, but close to the overall isotopic values of odonate nymphs (Table 1). *Ischnura* individuals were significantly smaller than *Erythromma* (Table 1; t-test for separate variance estimates: $t = 5.28$, $P = 0.0001$, adjusted

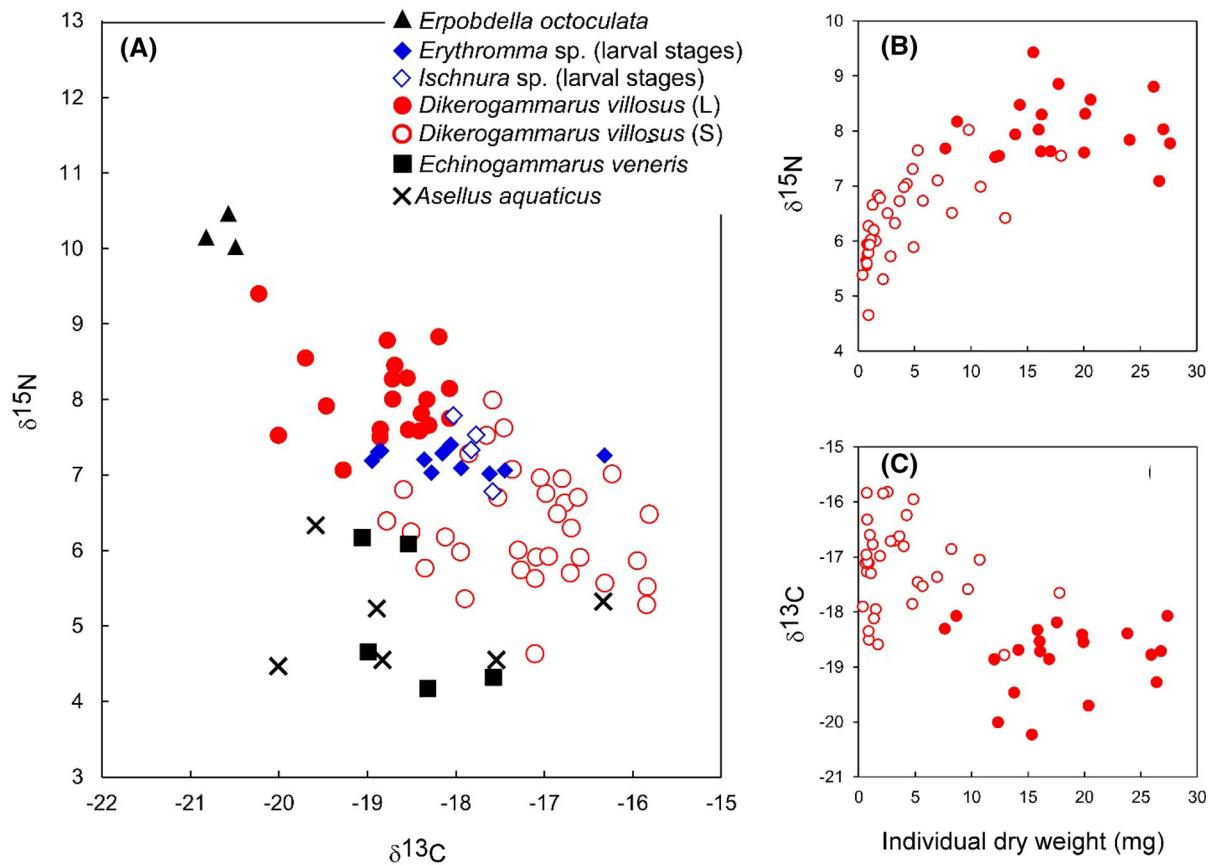


Fig. 1 (A) Bi-plot of individual $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the amphipods *Dikerogammarus villosus* and *Echinogammarus veneris*, Odonata larval stages, and the hirudinean *Erpobdella octoculata*. See Table 1 for summary statistics. For *D. villosus*, full and empty circles identify large-sized (L) and small-sized

(S) individuals included in the two size groups recognized by cluster analysis and PERMANOVA (see Fig. S2 in the online information). The relationships between the individual dry weight of *D. villosus* specimens and the respective $\delta^{15}\text{N}$ (insert B) and $\delta^{13}\text{C}$ values (insert C) are also reported

d.f. = 13.91); nonetheless, negligible differences between the two taxa were verified in terms of isotopic values [1-way PERMANOVA on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, factor “taxon”: Pseudo-F = 0.42, $P(\text{perms}) = 0.42$, d.f. = 1.14]. Accordingly, no distinctions were made between the two taxa in subsequent analyses.

$\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in individual *D. villosus* varied considerably (Table 1), suggesting a significant trophic plasticity. In particular, $\delta^{15}\text{N}$ varied by more than 4.5‰, shifting from values close to *E. octoculata*, to signatures comparable with those of *A. aquaticus* (Fig. 1A). In addition, $\delta^{15}\text{N}$ values showed a non-linear increase with amphipod body size (Fig. 1B), while $\delta^{13}\text{C}$ varied considerably, with small-size gammarids being more ^{13}C -enriched compared with large-size individuals (Fig. 1C).

A cluster analysis followed by a PERMANOVA revealed two distinct groups of *D. villosus* individuals with statistically different isotopic values (see Fig. S2 in supplementary online material). The first group included small-sized specimens with an individual dry weight of 3.78 ± 0.69 mg (range 0.39–17.98 mg); the second comprised large-sized gammarids with an average dry weight of 18.03 ± 1.33 mg (range 7.74–27.65 mg). Accordingly, the two size groups showed significantly different trophic positions ($P = 0.03$; Fig. 2). In particular, small-sized *D. villosus* showed a TP value not statistically different from that estimated for *E. veneris* (2.57 ± 0.14 vs. 2.41 ± 0.19 ; $P = 0.19$; mean \pm 1SD; see also Tab. S1 for additional statistical information on the robustness of the estimations). Conversely, large-sized killer

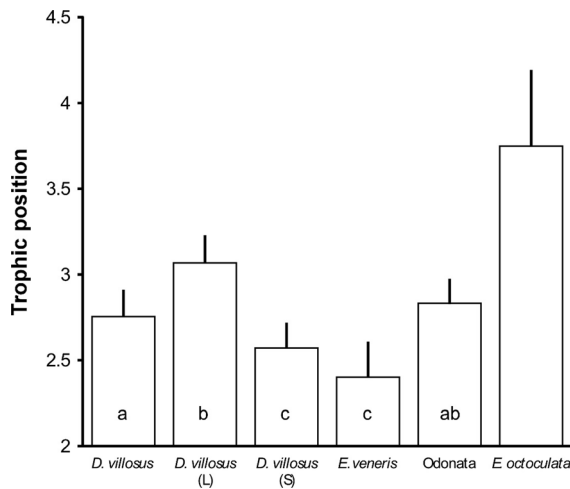


Fig. 2 Trophic positions (TP) of the amphipods *Dikerogammarus villosus* and *Echinogammarus veneris*, Odonata larval stages, and the hirudinean *Erpobdella octoculata*. For *D. villosus*, TP values for large-sized (L) and small-sized (S) individuals included in the two groups differing significantly in their isotopic values after cluster analysis and PERMANOVA (see Fig. S2 in the online information) are also reported. Bars showing identical letters were not statistically different (α level = 0.05)

shrimps showed a TP of 3.07 ± 0.15 , similar to the trophic position of odonate nymphs (2.83 ± 0.14 ; $P = 0.12$), but significantly lower than that of *E. octoculata* (3.75 ± 0.44 ; $P = 0.03$).

The literature search and the subsequent selection procedure identified ten isotopic studies on *D. villosus* published between 2006 and 2019 (Tab. 2), including information on its trophic position either directly or providing $\delta^{15}\text{N}$ values for the killer shrimp and baseline taxa (5 studies per category). All the publications providing trophic position estimations used the equation by Vander Zanden and Rasmussen (1999) with the exception of Haubrock et al. (2019), where a Bayesian approach identical to the one adopted in the present study was used. The study by Haubrock and colleagues was also the only carried out in southern Europe (i.e., in the River Arno, Central Italy) while the remaining were all located in lakes and rivers of central European countries (Germany, the Netherlands, Poland, Switzerland, and Austria). 9 literature sources provided a total of 25 trophic position values estimated on bulk samples (Table 2A), corresponding to a mean TP of $2.38 (\pm 0.71 \text{ SD})$, but varying three-fold between 1.7 and 4.9 independently from the system (i.e., river or lake: t-test for separate variance

estimates: $t = -0.52$, $P = 0.61$, adjusted d.f. = 13.45) or the season [1-way ANOVA, factor “season” with three levels (“spring”; “summer”, and “autumn”): $F_{2,21} = 0.05$, $P = 0.94$ after excluding the TP value from Bacela-Spychalska and van der Velde (2013), providing no indication of the sampling season].

Only three studies performed in Germany and the Netherlands estimated the trophic position of *D. villosus* considering the ontogeny of analyzed individuals (i.e., adult vs. juvenile; Table 2B). Over the 13 investigated locations the two developmental stages of the killer shrimp showed a two-fold variation in trophic position (Table 2B; adults min-max range: 1.9–3.5; juveniles: 1.5–3.3), yet adult *D. villosus* were generally characterized by TP values significantly higher than juveniles (2.36 ± 0.45 vs. 2.14 ± 0.49 , mean \pm SD; t-test for paired samples: $t = 5.65$, $P = 0.0001$, 12 d.f.).

Discussion

The results of the present study suggest that the individuals of a single population of *Dikerogammarus villosus* simultaneously play, depending on body size, two distinct functional roles within an invaded food web, shifting from omnivorous feeding habits that are predominantly plant-based and similar to the native amphipod *Echinogammarus veneris*, to a markedly predaceous strategy comparable with those characterizing odonate nymphs.

Dikerogammarus villosus showed a bipartite pattern in the isotopic signatures of its specimens (Fig. 1, and Fig. S1), and, in turn, a dual trophic position (Fig. 2). These findings corroborate and extend the results of other studies where the effect of size on the isotopic values of *D. villosus* has been addressed explicitly (see references in Tab. 2B). In particular, Koester and colleagues (2016) found in the majority of ten populations of *D. villosus* analyzed in the River Rhine a size-related positive shift in $\delta^{15}\text{N}$ with increasing body size, even though no attempts were made to consider the trophic position of the species, either estimated on bulk samples or, most importantly, on different ontogenetic stages of the species. Here, the first group included small-sized *D. villosus* specimens, the majority less than 10 mg in weight. Transformed using the allometric equation proposed in Dobrzycka-Krahel et al. (2016), i.e., dry

Table 2 Summary of published isotopic investigations on *Dikerogammarus villosus* in invaded systems

(A) <i>D. villosus</i> bulk samples										
Reference		System	Location	Notes	Season	$\delta^{15}\text{N}$	N	$\delta^{15}\text{N}_{\text{baseline}}$	TP	
van Riel et al. (2006)	The Netherlands	River	Waal	1996–98	Summer	14.7	10		2.6	
			Waal	2001–03	Summer	10.6	33		2.7	
Brauns et al. (2011)	Germany	Lake	Langer See	Shore	Autumn	9.2	NA		2.3	
			Langer See	Retaining wall	Autumn	9.1	NA		2.5	
Gergs et al. (2011)	Germany	Lake	Constance	2005	Autumn	15.1	NA	5.2 (2)*	4.9	
			Constance	2006	Autumn	6.2	NA	6.1 (2) ^o	2	
Bacela-Spychalska and Van Der Velde (2013)	Poland	Lake	Zegrzynski		NA		1		3.7	
Brandner et al. (2013)	Germany	River	Danube		Summer	11.6	6	2.8 (2)*	2.8	
Koester and Gergs (2014)	Switzerland	River	Untere Lorze	Hagendorn	Spring	6.1	16	1.9 (2)**	1.9	
			Untere Lorze	Maschwanden	Spring	6.6	17	1.8 (2)**	1.9	
Rothhaupt et al. (2014)	Germany	Lake	Constance	Site1	Summer	8.5	NA	1.8 (2)#	1.8	
			Constance	Site3	Spring	8.2	NA	1.7 (2)#	1.8	
			Constance	Site4	Summer	8.2	NA	2.3 (2)#	2.3	
			Constance	Site5	Summer	8.3	NA	1.7 (2)#	1.7	
Koester et al. (2016)	Austria	River	Rhine	Site1	Autumn	9.7	20	2.3 (1)§	2.3	
			Rhine	Site2	Autumn	10.6	20	1.9 (1)§	1.9	
	Germany	River	Rhine	Site3	Autumn	8.7	20	2.1 (1)§	2.1	
			Rhine	Site4	Autumn	9.8	20	2.1 (1)§	2.1	
			Rhine	Site5	Autumn	9.1	20	2.3 (1)§	2.3	
			Rhine	Site6	Spring	12.5	20	3.5 (1)§	3.5	
			Rhine	Site7	Autumn	9.0	20	2.2 (1)§	2.2	
			Rhine	Site8	Autumn	7.3	20	2.1 (1)§	2.1	
	Germany	Lake	Constance	Site9	Autumn	6.9	20	2.1 (1)§	2.1	
			Constance	Site10	Autumn	12.0	20	1.9 (1)§	1.9	
Haubrock et al. (2019)	Italy	River	Rhine tributary		Spring	9.3	11		2.1	
			Arno		Spring	9.3	11		2.1	
(B) <i>D. villosus</i> ontogenetic stages										
Reference	Country	System	Location	Notes	Season	$\delta^{15}\text{N}$	N	Size	$\delta^{15}\text{N}_{\text{baseline}}$	TP
van Riel et al. (2006)	The Netherlands	River	Waal	2001–03	Summer	11.3	5	adults > 12 mm		2.9
			Waal		Summer	10.5	22	juveniles		2.7
Hellmann et al. (2015) +	Germany	River	Elbe		Autumn	11.0	17	adults > 9 mm		1.9
			Elbe		Autumn	8.2	NA	juveniles		1.5
		River	Rhine		Autumn	12.7	18	adults > 9 mm		2.6
			Rhine		Autumn	11.7	NA	juveniles		2.4
Koester et al. (2016)	Germany	River	Rhine	Site1	Autumn	9.8	12	adults > 9 mm	2.3 (1)§	2.3
			Rhine		Autumn	9.6	8	juveniles		2.2
		River	Rhine	Site2	Autumn	10.7	17	adults > 9 mm	1.9 (1)§	2.0
			Rhine		Autumn	10.3	3	juveniles		1.8
		River	Rhine	Site3	Autumn	8.7	19	adults > 9 mm	2.1 (1)§	2.1

Table 2 continued(B) *D. villosus* ontogenetic stages

Reference	Country	System	Location	Notes	Season	$\delta^{15}\text{N}$	N	Size	$\delta^{15}\text{N}_{\text{baseline}}$	TP
			Rhine		Autumn	7.5	1	juveniles		1.6
		River	Rhine	Site4	Autumn	9.9	3	adults > 9 mm	2.1 (1)§	2.2
			Rhine		Autumn	9.7	17	juveniles		2.1
		River	Rhine	Site5	Autumn	9.2	15	adults > 9 mm	2.3 (1)§	2.4
			Rhine		Autumn	8.7	5	juveniles		2.1
		River	Rhine	Site6	Spring	12.6	20	adults > 9 mm	3.5 (1)§	3.5
			Rhine		Spring	12.0	1	juveniles		3.3
		River	Rhine	Site7	Autumn	9.8	2	adults > 9 mm	2.2 (1)§	2.6
			Rhine		Autumn	8.9	21	juveniles		2.2
		Lake	Constance	site8	Autumn	7.3	9	adults > 9 mm	2.1 (1)§	2.2
			Constance		Autumn	7.2	12	juveniles		2.1
		Lake	Constance	Site9	Autumn	6.9	15	adults > 9 mm	2.1 (1)§	2.1
			Constance		Autumn	6.9	5	juveniles		2.1
		River	Rhine tributary	Site10	Autumn	12.1	16	adults > 9 mm	1.9 (1)§	1.9
			Rhine tributary		Autumn	11.6	4	juveniles		1.7

The first part of the table (A) includes references where *D. villosus* signatures were assessed on bulk samples, while the second (B) includes studies considering the ontogenetic stage of analyzed individuals (i.e., adult vs. juvenile). In part B the original, individually-resolved data by Koester et al. (2016) were grouped as juveniles and adults to compare them with data provided by van Riel et al. (2006) and Hellmann et al. (2015), taking the sizes provided in the latter publication as a reference. Additional information on the country, location, system, season, nitrogen isotopic signatures of *D. villosus* ($\delta^{15}\text{N}$ = mean, N = number of analyzed samples) are reported. The table includes studies where the trophic position of *D. villosus* was not explicitly calculated; for the sake of comparison, a TP value (reported in bold) was estimated using the isotopic signatures of *D. villosus* and of a baseline species (*: *Dreissena polymorpha*; ° = *Centropilum luteolum*; **: *Ancylus fluviatilis*; #: *Limnomysis benedeni*; §: periphyton; trophic level λ provided in round brackets) using the equation provided in Vander Zanden and Rasmussen (1999; see text for further details)

+ : TP estimated using a $\Delta^{15}\text{N} = 2.93$

weight = $0.0674 \times \text{total length}^{2.6917}$ (expressed respectively in mg and mm), this reference individual mass corresponds to a length of 6.4–6.5 mm roughly matching the lower size limit for adults (Devin et al. 2004b). Thus, the observed differences in isotopic signatures may be due to an ontogenetic dietary shift. Growth in gammarid amphipods is essentially a continuous process, yet gonadal maturation generally induces a strong metabolic and physiological trade-off between investments in somatic or reproductive tissues. Accordingly, sexual maturity coincides with abrupt morphological changes and variations in energetic requirements, the latter in turn reflecting in ontogenetic diet shifts (Hartnoll 1982; Summers et al. 1997; Longo and Mancinelli 2014; Shi et al. 2020).

In the present study sexes were not determined; as male *D. villosus* are generally larger than females

(Devin et al. 2004b) it is possible that also sex may have contributed to the observed pattern, even though available information do not support this hypothesis (Maazouzi et al. 2009; Sahm et al. in press).

Small-sized killer shrimps showed a trophic position similar to that of *E. veneris* (2.57 vs. 2.41, respectively; Fig. 2). *Dikerogammarus villosus* juveniles are very efficient consumers of plant material before they reach maturity (Rewicz et al. 2017); similarly, *E. veneris* is predominantly a primary consumer; however, as generally observed for other gammarids in freshwater and brackish habitats, it can include animal items in the diet, especially in the winter season (Gophen 1979; see also Mancinelli 2012a, b). The similar trophic position, and the generally comparable carbon isotopic values, suggest that small-sized *D. villosus* may compete with *E.*

veneris for trophic resources, a hypothesis supported also by their similar sizes [*E. veneris* reaches a maximum size of 10–12 mm (Gophen 1979; Herbst and Dimentman 1983)]. The sampling method used in this investigation allowed neither a quantitative analysis of the macrobenthic assemblage characterizing the study site, nor a robust assessment of the relative densities of *D. villosus* and *E. veneris* in Lake Trasimeno; however, the disproportionality in the number of specimens collected for the two species is remarkable (Table 1). *Echinogammarus veneris* is the only native gammarid characterizing the littoral macrobenthic communities of Lake Trasimeno (VV.AA. 2015). The species was found at high abundance in 2015 and 2016 (Mancinelli et al. 2018; 2020); the extremely reduced occurrence recorded in 2018 at the same location (this study) coincides with the recent establishment of *D. villosus* and suggests that the latter may have played a negative impact on *E. veneris* abundance, as hypothesized for other native amphipods in other investigations (Muskó 1989; Bollache et al. 2004; Noordhuis et al. 2009). Besides representing an important food source for macroinvertebrates, fish, birds and amphibians, *E. veneris* also plays a major role in leaf detritus processing in lacustrine environments (Mancinelli et al. 2002, 2007). Significant variations in leaf detritus decomposition rates have been suggested to occur in freshwater systems after the arrival of *D. villosus* (Truhlar et al. 2014; Jourdan et al. 2016); thus, potential post-invasion variations in key functions of the benthic system of Lake Trasimeno deserve to be thoroughly addressed in future investigations.

In contrast with small-sized *D. villosus*, large-sized individuals showed a trophic position analogous to that of damselfly nymphs, and comparable with that of the hirudinean *Erpobdella octoculata* (Fig. 2). For odonates, in particular, the $\delta^{13}\text{C}$ ranges indicate a potentially high overlap in the prey included in the diet and, in turn, competition or even intra-guild predation. Odonata larvae are generalist predators including a wide spectrum of invertebrate and vertebrate secondary consumers in their diet (Fischer 1964; Thompson 1978; Johnson 1991), while *E. octoculata* is a macrophagous opportunistic predator preying on a wide spectrum of benthic invertebrates, including chironomids, amphipods, insects, and conspecifics

(Dall 1983). Our results indicate that the trophic niche of large-sized *D. villosus* may overlap those of damselfly nymphs and, to a minor extent, of hirudineans, competing for food or even preying on both groups of native predators. While laboratory experiments indicate that the killer shrimp prey actively on hirudineans (Krisp and Maier 2005), information on odonate nymphs are less univocal: *D. villosus* is generally recognized to include them in its diet (Rewicz et al. 2014), yet laboratory trials showed low or negligible predation, a discrepancy likely to be related with the experimental design, as influenced by e.g., differences in sizes between prey and *D. villosus* individuals (Krisp and Maier 2005; Lipinskaya and Makarenko 2019 and literature cited).

Future investigations deserve to focus on the double, size-dependent role of *D. villosus* as an intra-guild competitor and predator for native primary consumers as well as for native invertebrate predators. It is apparent that a thorough analysis of the size structure of the invader' populations will be necessary to assess the relative prevalence of one role in respect with the other. To date, available information are relatively scant (but see Devin et al. 2004b), yet they indicate for the species an almost continuous reproduction, with juveniles present throughout the year, and a generally bi- or multi-modal size frequency distribution, as clearly suggested also by the present study (Fig. S1). Accordingly, to test how constant the functional duality highlighted here for the killer shrimp is, future studies need to include multiple seasonal samplings (i.e., temporal data), since the present investigation, as most of the isotopic studies carried out in the past (Table 1), focused only on a single season, thus providing only a snapshot of the trophic habits of the species.

The impact of omnivores on food webs and ecosystems is still under debate, due to the difficulties in predicting their ultimate functional effects on the general structure and dynamics of food webs (Wootton 2017). This difficulty is particularly challenging for invasive omnivores, as their direct and indirect effects on multiple trophic levels through resource consumption limit a robust assessment of their ecological impact on invaded communities (Gallardo et al. 2016; Tumolo and Flinn 2017). Even though limited in space and time, the individual-scale investigation carried out

in the present study, and the results of the literature search, clearly indicated that at least two groups of specimens with distinct functional roles coexist as “ecological species” (sensu Polis 1984) within the same *D. villosus* population, reflecting potential simultaneous interactions with taxa located at different trophic levels. Obviously, the possibility for populations of the killer shrimp to adapt to local conditions, as generally assumed, is not ignored (as testified in Table 1 by the remarkable variability in trophic position estimations). Indeed, the present study adds an intraspecific dimension to the omnivorous nature of *D. villosus*, stressing the necessity to explicitly take into consideration its potentially multiple functions related to body size and, in turn, ontogenetic stage, for an ecologically meaningful assessment of the ecological impact of the gammarid in invaded communities (Médoc et al. 2018).

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