

Trophic preference and preliminary indication of phylloplane fungal influence on the diet of the non-native *Gammarus roeselii* Gervais 1835 (*Amphipoda*, *Gammaridae*) in the sub-lacustrine Ticino river basin (Lombardy, Northern Italy)

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Abstract

Gammarids are one of the most successful invaders in freshwater ecosystems due to both their diet plasticity and high reproductive capacity. One such amphipod, *Gammarus roeselii*, has recently colonised the southern part of the sub-lacustrine Ticino River basin (Northern Italy), where its ecological niche overlaps with the native species *Echinogammarus stammeri*.

In the present paper the food preferences of *G. roeselii* have been investigated, testing the palatability of different food sources: three macrophytes and three different leaf debris. Moreover, an explorative mycological analysis on the three different leaf debris used in the experiment has been performed. Results of the short-term experiment suggest that aquatic plants are less palatable than allochthonous detritus, probably because they can contain secondary metabolites (i.e. tannins) and they have lower nutrient tissues with very high water content. On the contrary, *G. roeselii* showed a clear preference for the oak leaves, resulted colonized by a more abundant fungal biomass and, therefore, more palatable too.

Keywords: *Gammarus roeselii*; trophic behaviour; microfungi; food source

Riassunto

Gli anfipodi gammaridi, grazie alla loro plasticità in termini di dieta e al loro elevato tasso riproduttivo, possono facilmente colonizzare gli ecosistemi di acqua dolce. Uno di essi, *Gammarus roeselii*, ha recentemente

colonizzato la parte meridionale del bacino sub lacuale del fiume Ticino (Nord Italia), andando ad occupare una nicchia ecologica parzialmente sovrapponibile con quella della specie nativa *Echinogammarus stammeri*.

In questo lavoro sono state analizzate le preferenze trofiche della specie *G. roeselii*, tramite un esperimento di laboratorio in cui è stata testata l'appetibilità di differenti risorse: tre idrofite e tre tipologie diverse di detrito fogliare. Il detrito fogliare utilizzato durante l'esperimento, inoltre, è stato analizzato in termini di biomassa fungina. I risultati ottenuti hanno evidenziato come le idrofite siano meno appetibili rispetto al detrito fogliare, probabilmente perché ricche di metaboliti secondari (tannini) con minor valore nutritivo e contenenti molta acqua. È invece emerso come *G. roeselii* manifesti una netta preferenza per le foglie di quercia, risultate colonizzate da una più consistente biomassa fungina e, probabilmente, maggiormente appetibili.

Parole chiave: *Gammarus roeselii*; comportamento trofico; microfunghi; nutrimento

Introduction

Gammarids are one of the most successful invaders in freshwater ecosystems, mainly due to their diet plasticity: they are shredders of allochthonous detritus (Cummins and Klug, 1979), herbivores that use freshwater macrophytes as a food source (Thiébaud et al., 2008), and predators of other macroinvertebrates (Kelly et al., 2002).

Feeding habits are related to food source availability and quality, and to intra- and interspecific competition (Kelly et al., 2002; van Riel et al., 2007). Omnivory is widespread in freshwater gammarids and differences in preferred food sources seem likely (Maier et al., 2011).

Some species, such as the “killer shrimp” *Dikerogammarus villosus* (Sowinsky 1984), have modified mouthparts that are suitable for predator activity, whereas other species, such as *Gammarus roeselii* Gervais 1835, are well-suited for grinding plant material and well-adapted for scraping off fungi and other adherent biofilm layers from the substratum or decaying plant material (Mayer et al., 2009).

In small streams, leaf decomposition is an important process carried out by invertebrates and microorganisms such as *Hyphomycetes* (Hieber and Gessner, 2002; Pascoal and Cássio, 2004). As Suberkropp (1992) stated, freshwater macroinvertebrates have a preference for foliar tissue associated to a rich mycobiota: for example, *Gammarus pseudolimnaeus* grows better on leaf litter colonised by fungi than on uncolonised leaves and it shows a well-defined preference for several fungal assemblages. In particular, the presence of fungi on the surface or inner part of leaves can increase their palatability for shredding invertebrates (Graça, 2001).

Recently, one such successful gammarid invader, *Gammarus roeselii* (Fig. 1), has colonised the southern part of the sub-lacustrine Ticino River basin, from Vigevano (45°20'29.0"N 8°53'03.6"E) to the mouth of the Ticino River (45°20'29.0"N 8°53'03.6"E) (Po river floodplain, Northern Italy), where its ecological niche overlaps with the native species *Echinogammarus stammeri* (Karaman 1931). In this area of 150 km², these two species survive in sympatry with dense populations, and their possible competitive interactions (e.g. food competition) are still unknown (Paganelli et al., 2015).

In the present paper, we investigate the food preferences of *G. roeselii* testing the palatability of different food sources and considering the possible role of phylloplane fungi, the mycota growing inside and on the surfaces of leaves (Langvad, 1980), as a food source or as a modifier of plant resources.



Fig. 1. Specimen of *G. roeselii* used for the laboratory experiments.

Fig. 1. Un esemplare di *G. roeselii* utilizzato per gli esperimenti di laboratorio.

Materials and methods

Laboratory experimental design

In October 2014, specimens of *Gammarus roeselii* were collected in the Gaviola Stream, one of the small right-bank tributaries of the Ticino River (Fig. 2), where we found the highest density of *G. roeselii* compared to other small streams in the sub-lacustrine Ticino River basin (unpublished data).



Fig. 2. Sample site of *G. roeselii* specimens. The Gaviola Stream (grey rectangle; 1:75000), one of the right-bank tributaries of the Ticino River (45°11'27.4"N 09°04'03.7"E, WGS84).

Fig. 2. Sito di campionamento degli esemplari di *G. roeselii*. La roggia Gaviola (rettangolo grigio; 1:75000), uno degli immissari di sponda destra del Fiume Ticino (45°11'27.4"N 09°04'03.7"E, WGS84).

Moreover, based on their availability and abundance in the stream during the survey, we collected six different food sources: three macrophytes [*Myriophyllum spicatum* L., *Elodea nuttallii* (Planch.) H. St. John,

Veronica anagallis-aquatica L.] and well-preserved tree leaf debris from three different species (oak - *Quercus robur* L., maple - *Acer pseudoplatanus* L., black locust - *Robinia pseudoacacia* L.).

Once in the laboratory, the gammarids were acclimatised in dechlorinated tap water and were not fed for 48 hours, while leaf debris and macrophytes were gently cleaned in order to remove the biofilm layer that usually covers aquatic plants and increases their palatability.

This gentle cleaning of the food sources removed and de-activated both superficial inorganic contaminants and fungal propagules that may stick to the leaves. However, it does not influence the fungal communities associated to the leaf debris as they live inside the foliar tissue. Hyphae, mycelia and spores that are systemic in tissues maintain their morphological characteristics (e.g. texture, pigmentation, nutritional value) and their palatability.

For the experiment, we set up a controlled environmental setting with a constant temperature of 18°C and a photoperiod of 10 hours of light and 14 hours of dark per day for a seven-day period.

Each experimental unit (glass Petri dishes: 12 cm diameter, 2 cm high) was filled with 125 ml of dechlorinated tap water, as suggested by Maier et al. (2011). According to Boiché et al. (2010), the use of dechlorinated tap water, instead of water collected in-situ, can prevent possible alterations of the water due to bacteria and nitrogen compounds.

The experimental design included a control with no available food sources and six treatments with *M. spicatum*, *E. nuttallii*, *V. anagallis-aquatica*, *Q. robur*, *A. pseudoplatanus* and *R. pseudoacacia* leaf debris (three replicates for each treatment, overall 21 experimental units). A total of 126 specimens, divided into groups of six adult gammarids of either sex (body length: 10-12mm), were placed in each experimental unit and were fed *ad libitum* with only one food source.

During some preliminary tests, we tried to weigh the food sources at the beginning and at the end of the experiment, but it did not give reliable results because the photosynthesis activity of the hydrophytes produced a biomass increase interfering with the biomass consumed by grazing; for gammarids, the necessity to have the initial weight of the living specimens prevented us from using the dry weight as an accurate parameter to measure small weight increases, therefore in accordance with Pöckl et al. (2003), we used the gammarids' wet weight as an indication of the feeding rate. To avoid significant errors when measuring single individuals, and considering the similar size of the specimens, we fresh-weighted the whole group of six gammarids at the beginning of the experiment and the whole group of gammarids that had survived at the end for each replicate, using a laboratory precision balance (KERN ALJ 220_4; 0,000 g), in order to calculate the mean individual percentage of growth (G): the mean individual initial weight of the six gammarids was subtracted from the mean individual final weight of the surviving gammarids after the 7 day-experiment and then this result was divided by the mean individual initial weight of the six gammarids.

Throughout the experiment, units were checked and dead specimens were removed twice daily, in order to reduce the effect of potential necrophagy, observed occasionally during our preliminary tests.

Investigation of phylloplane fungi

In order to investigate the potential role of fungi in the gammarids' diet, an explorative mycological analysis was performed on the three different types of leaf debris collected in the Gaviola Stream: oak, maple and black locust. Five leaves from each species were gently washed with tap water and then immersed in sterile water (Dickinson, 1971). Subsequently, they were separately (i.e. one leaf in one dish) placed onto 90 mm Petri dishes containing Tap-Water Agar (TWA) to provide the necessary humidity and favour the growth of microfungi living in association with foliar tissue (Waller, 2002). Samples were maintained at room temperature (19±2°C) in natural day/night conditions for 30 days and constantly observed at low magnification (stereomicroscope 10-50x). All resulting fungal colonies were identified on the basis of their

micro-morphology by means of specific taxonomical keys (Domsch et al., 1980; Ellis and Ellis, 1985; Leslie and Summerell, 2006), and the fungal biomass that had developed was expressed in terms of abundance of mycelia with or without fructifications.

Although the aim of this analysis was to investigate fungal colonisation, observations of *Actinomycetes* and any other unidentified bacteria were also recorded.

Statistical analyses

All the statistical analyses were performed using MINITAB 16 software package. Food preferences were investigated through a General Linear Model, an ANOVA procedure in which the calculations are performed using a least squares regression approach to describe the statistical relationship between one or more predictors and a continuous response variable.

Anderson-Darling and Bartlett tests were used prior to GLM to check the normality of data and the homogeneity of variance. Finally, pairwise Tukey post hoc tests were performed in order to determine which set of data mainly contribute to the differences found by GLM.

Results

Food preferences

During the 7-day period, a total of 25% of the specimens died (Tab. 1); however, the results of the experiment indicated that a weight increase was obtained when gammarids were fed with *Q. robur* (40.38%), *A. pseudoplatanus* (15.44%), *V. anagallis-aquatica* (11.69%) and *E. nuttallii* (2.05%), but not when they were fed with *R. pseudoacacia* (-5.85%) or *M. spicatum* (-6.34%) (Tab. 1 and Fig. 3).

A GLM analysis indicated a statistical increase in *G. roeselii*'s weight when they were fed with leaf debris (DF = 3; F = 5.15; p: 0.028) compared to aquatic plants (DF = 3; F = 0.93; p: 0.468). Tukey tests highlighted that only specimens that had been fed with *Q. robur* had a significant weight (Tab. 3).

Tab. 1. Mean initial, final weight and weight percentage growth (\pm standard deviation) recorded at the beginning and at the end of the seven-day experiment. Ni: initial number of specimens; Nf: final number of specimens; CTRL: control groups; E: *E. nuttallii*; M: *M. spicatum*; V: *V. Anagallis-aquatica*; Q: *Q. robur*; A: *A. pseudoplatanus*; R: *R. pseudoacacia*.

Tab. 1. Peso medio iniziale, finale e percentuale di crescita (\pm deviazione standard) registrati all'inizio e alla fine dell'esperimento durato 7 giorni. Ni: numero iniziale di individui; Nf: numero finale di individui; CTRL: gruppo di controllo; E: *E. nuttallii*; M: *M. spicatum*; V: *V. Anagallis-aquatica*; Q: *Q. robur*; A: *A. pseudoplatanus*; R: *R. pseudoacacia*.

	Ni	Total Mean initial weight \pm sd (mg)	Mean individual initial weight (mg)	Nf	Total Mean final weight (mg)	Mean individual final weight (mg)	Mean individual weight growth \pm sd (%)
CTRL	18	200.13 \pm 27.22	11.12	10	103.10 \pm 42.07	10.31	-6.30 \pm 11.58
E	18	205.67 \pm 63.19	11.43	14	157.93 \pm 43.93	11.28	2.05 \pm 21.44
M	18	196.43 \pm 44.42	10.91	14	142.80 \pm 48.45	10.20	-6.34 \pm 15.97
V	18	201.27 \pm 75.97	11.18	15	183.47 \pm 56.17	12.23	11.69 \pm 9.77
Q	18	207.53 \pm 53.19	11.53	12	190.13 \pm 60.79	15.84	40.38 \pm 27.67
A	18	181.60 \pm 37.47	10.09	14	157.37 \pm 38.39	11.24	15.44 \pm 0.65
R	18	270.07 \pm 56.37	15.00	16	223.47 \pm 51.02	13.97	-5.85 \pm 15.43

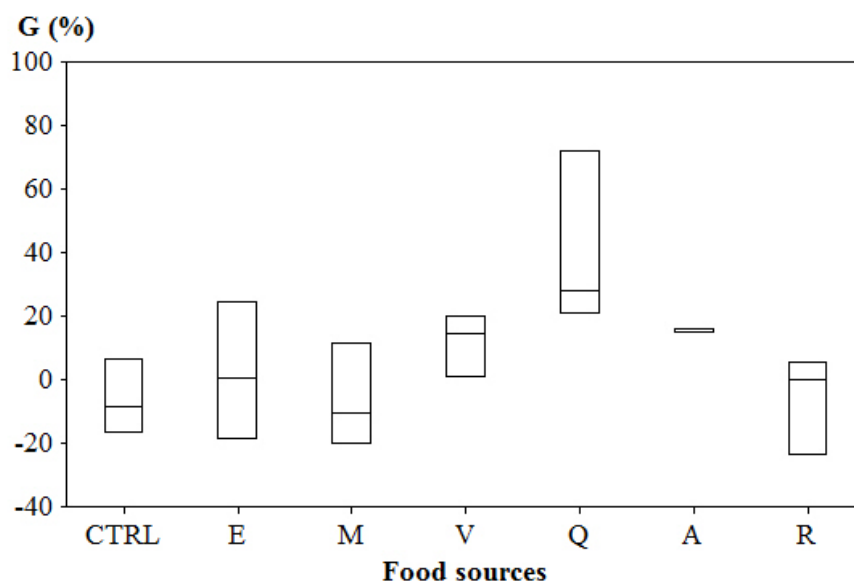


Fig. 3. Weight percentage growth (G) of *Gammarus roeselii* registered at the end of the experiment (7days). Letters indicate type of food: E: *E. nuttallii*; M: *M. spicatum*; V: *V. Anagallis-aquatica*; Q: *Q. robur*; A: *A. pseudoplatanus*; R: *R. pseudoacacia*; CTRL: control groups.

Fig. 3. Percentuale di incremento di peso (G) di *Gammarus roeselii* registrato alla fine dell’esperimento (7giorni). Le lettere indicano la tipologia di cibo: E: *E. nuttallii*; M: *M. spicatum*; V: *V. Anagallis-aquatica*; Q: *Q. robur*; A: *A. pseudoplatanus*; R: *R. pseudoacacia*; CTRL: gruppi di controllo.

Tab. 2. Results of pairwise Tukey post hoc tests (T) and relative p-values (NS: not significant; *: $p < 0.05$) of the gammarids’ weight increase when fed with different food sources compared to the control groups. CTRL: control groups; E: *E. nuttallii*; M: *M. spicatum*; V: *V. Anagallis-aquatica*; Q: *Q. robur*; A: *A. pseudoplatanus*; R: *R. pseudoacacia*.

Tab. 2. Risultati del Tukey post hoc test (T) e relativa significatività statistica (NS: non significativo; *: $p < 0.05$) dell’incremento di peso dei gammaridi alimentati con le diverse fonti di cibo comparati con i gruppi di controllo. CTRL: gruppi di controllo; E: *E. nuttallii*; M: *M. spicatum*; V: *V. Anagallis-aquatica*; Q: *Q. robur*; A: *A. pseudoplatanus*; R: *R. pseudoacacia*.

	T-test	p value	Statistical significance
CTRL vs E	T=0.66	p: 0.90	NS
CTRL vs M	T=-0.003	p: 1.00	NS
CTRL vs V	T=1.43	p: 0.51	NS
CTRL vs Q	T=3.38	p: 0.03	*
CTRL vs A	T=-1.57	p: 0.44	NS
CTRL vs R	T=0.032	p: 1.00	NS

Phylloplane fungi

A total of 9 fungal taxa were isolated from the leaf samples (Tab. 3).

Two species grew on all tree samples: *Alternaria alternata* (Fr.) Keissl (Fig. 4a), a common saprophyte of plant material, and *Cladosporium cladosporioides* (Fresen.) G. A. de Vries, a ubiquitous fungus and secondary invader of damaged plants. The former species had abundant and fructifying mycelium, and the latter had a low presence of single conidiophores.

In addition to these two phylloplane fungal species that grew on all samples, *Q. robur* was also colonised by another four taxa: the highly abundant *Fusarium verticillioides* (Sacc.) Nirenberg, which is able to establish a

Tab. 3. Microfungi and microorganism colonisers of leaves grazed by gammarids. Five leaves from each species were investigated. The quantity of mycelia and/or fructification that had developed were expressed in terms of abundance: +: single or few fungal structures; ++: abundant fungal structures emerging from the leaves; +++: very abundant fungal structures, almost totally covering the surface of leaves.

Tab. 3. Microfunghi e microorganismi colonizzatori delle foglie consumate dai gammaridi. Sono state analizzate 5 foglie per ciascuna specie. La quantità di micelio sviluppatasi è stata espressa in termini di abbondanza: +: poco abbondante; ++: abbondante; +++: molto abbondante, ricoprente la quasi intera superficie fogliare.

Fungal taxa and microorganisms	<i>Quercus robur</i>	<i>Acer pseudoplatanus</i>	<i>Robinia pseudoacacia</i>
<i>Alternaria alternata</i> (Fr.) Keissl.	+++	+++	++
<i>Botrytis cinerea</i> Pers.	-	+++	-
<i>Chaetomium globosum</i> Kunze	-	-	+
<i>Cladosporium cladosporioides</i> (Fresen.) G.A. de Vries	+	+	+
<i>Epicoccum nigrum</i> Link	+	-	+
<i>Fusarium verticillioides</i> (Sacc.) Nirenberg	+++	-	-
<i>Periconia byssoides</i> Pers.	-	-	+
<i>Phoma eupyrena</i> Sacc.	++	-	+
<i>Trichoderma atroviride</i> P. Karst.	+	-	+
Actinomycetes	-	-	+++
Bacteria	-	-	+

wide range of interactions with plants, from parasitic to devastating; the moderately abundant *Phoma eupyrena* Sacc., a potentially phytopathogenic fungus; the scarce *Epicoccum nigrum* Link, an early secondary invader of plants; *Trichoderma atroviride* P. Karst, a cosmopolitan fungus primarily living in soil. *Robinia pseudoacacia* shared five of its seven taxa with *Q. robur*, but all fungi showed a low presence except for *A. alternata*. Among these fungi, *Periconia byssoides* Pers., often isolated from blackened areas of leaf and stems, and *Chaetomium globosum* Kunze (Fig. 4b), a cellulolytic fungus of soil and decaying plants, were only observed on *R. pseudoacacia*, which also hosted *Actinomycetes* and Bacteria.

Finally, *Botrytis cinerea* Pers. (Fig. 4c), a cosmopolitan pectinolytic fungus which damages leaves and other parts of various plants, developed with high fertile biomass exclusively on *A. pseudoplatanus*.

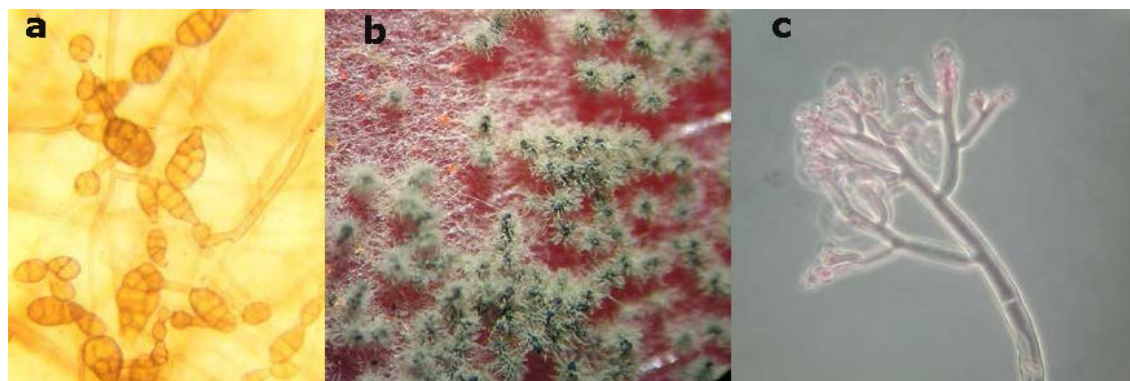


Fig. 4. a. Conidia of *Alternaria alternata* (40x); b. Ascomata of *Chaetomium globosum* (5x); c. Conidiophore of *Botrytis cinerea* (25x).

Fig. 4. a. Conidi di *Alternaria alternata* (40x); b. Ascomi di *Chaetomium globosum* (5x); c. Conidioforo di *Botrytis cinerea* (25x).

Discussion and conclusion

The Gaviola Stream, with its highly abundant aquatic vegetation, provides a suitable habitat for both gammarids that are present, i.e. the native *E. stammeri* and the alien *G. roeselii*, as demonstrated by their dense populations found during our surveys.

Although Dehedin et al. (2013) discovered that *G. roeselii* and another two crustacean species (*Asellus aquaticus* and *Gammarus pulex*) prefer the submerged leaves of aquatic plants to other food sources, our results reveal a substantial rejection for *M. spicatum* and *E. nuttallii*. *Myriophyllum spicatum* is known to be a tannin-rich aquatic plant and a low quality food source (Li et al., 2004; Orav-Kotta et al., 2009; Wong et al., 2010). It contains a high level of phenolic, which can reduce the growth rate and feeding rate of herbivores (Choi et al., 2002; Boland et al., 2008; Qiu and Kwong, 2009). Aquatic plants can also contain secondary metabolites (i.e. tannins) and they have lower nutrient tissues with higher water content than other plants. Tannins are a deterrent for macroinvertebrates and they can also interfere with the growth and development of some herbivorous insects (Smolders, 2000; Choi et al., 2002; Elger and Willby, 2003; Parker and Hay, 2005).

Furthermore, other studies have demonstrated that *Elodea nuttallii*, despite its high nitrogen content, can produce chemical defences (i.e. flavonoids) against herbivorous freshwater macroinvertebrates (Erhard et al., 2007) and has an intense allelopathic activity against epiphytes (Erhard and Gross, 2006). All these traits might explain the low palatability of these aquatic plants.

A small weight increase was observed for *V. anagallis-aquatica*, notwithstanding the lack of statistical relevance.

The generalised preference for allogenous detritus vs aquatic plants that emerges from our investigations is mainly supported by the marked palatability shown for oak leaves, because the growth of the gammarids' weight was low and not statistically significant for maple leaves, and negative for locust leaves. This food preference disagrees with the observations by Macneil et al. (1997), who stated that another gammarid species, i.e. *Gammarus pulex*, had a very low shredding capacity for oak leaves because they are hard and rich in tannins and thus not very palatable.

Macroinvertebrates generally consume hard leaves less than soft leaves (Li and Dudgeon, 2008; Ratnarajah and Barmuta, 2009). Moreover, according to Abelho (2008) and Foucreau et al. (2013), soft leaves are readily available to macroinvertebrates, whereas hard leaves may be used as a reserve of organic matter for the rest of the year.

In our study, the oak leaves hosted a very abundant phylloplane fungal biomass; fungi are notorious for being an important component of the food supply to many animals (Shaw, 1992; Graça et al., 1993; Graça, 2001; Sterner and Elser, 2002; Cross et al., 2005), due to their high levels of proteins and vitamins. As reported by Reddy and Das (1983) and Aßmann et al. (2011), fungi improve the nutritional quality of leaf debris for shredders, and different fungi may have different nutritional values, with some species being more beneficial and others detrimental to population growth. Furthermore, the presence of fungi on the surface and inner part of leaf debris makes it more palatable or energy-rich, increasing the level of N and P (Aßmann et al., 2011).

The nutritional value of phylloplane fungi cannot be neglected: they are an important source of carbohydrates, proteins, minerals, nucleoproteins and glycoprotein structural components of the whole fungal body (Bowman and Free, 2006).

Considering the gammarids' clear preference for oak detritus and the high abundance of the phylloplane fungi within these leaves, we hypothesise that fungi play a role in this selective choice.

Another two characteristics that can favour grazing on allogeous leaves are: (i) leaf debris is much more digestible than aquatic plants because bacteria have already started decomposing the organic matter (Abelho, 2001; Abelho, 2008); (ii) long exposure to stream water may reduce the effect of the toughness and tannins of leaf debris (Foucreau et al., 2013; Cornut et al., 2015).

The preference for leaf debris over macrophytes is probably due to the different food sources we chose to use in our experiment and may also be related to local conditions which can make the former food source more palatable than the latter.

In conclusion, this study can be considered as a preliminary assessment of the trophic behaviour of *G. roeselii* to be followed by future experiments that will compare the competition for food between the non-native species *G. roeselii* and the native *E. stammeri*. Furthermore, the mycological investigation we carried out could become the basis for future inoculation tests to delineate the fungal potentiality as a rich food source and/or as a useful modifier of plant resources, in order to provide a better understanding of the largely unknown influence of fungi on animal communities.

Acknowledgements

We express our thanks to Dr. Nicola M.G. Ardenghi for the identification of the freshwater plants and to Charlotte Buckmaster for the linguistic revision of the English text.

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