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Digestive enzyme activity of two stonefly species (Insecta, Plecoptera) and their feeding habits

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ABSTRACT

The digestive enzymes of two stoneflies species, *Hemimelaena flaviventris* and *Isoperla morenica*, were studied for the first time. These species are temporary water inhabitants and exhibit great feeding plasticity. Although they are traditionally referred to as predators, a previous study revealed that *H. flaviventris* incorporates some diatoms into its diet in addition to feeding usually on several prey, and *I. morenica* (in that study under the name of *I. curtata*) only feeds on animals occasionally. The enzymatic activities of digestive amylase, lipase, protease, trypsin and chymotrypsin were determined for each species at the same developmental stage. The results show that *H. flaviventris* has a greater digestive enzymatic pool and higher relative and absolute protease, lipase and trypsin activities than *I. morenica*. The latter has a relative higher amylase activity. As higher amylase activity is typical of phytophagous species and higher protease activity typical of carnivorous species; these results reveal that *H. flaviventris* is a more efficient zoophagous species than *I. morenica*. The ecological implications of these findings, including the higher secondary production of *H. flaviventris* in its habitat, are discussed.

1. Introduction

The digestive tract of insects can be divided in the fore-, mid- and hindgut (Snodgrass, 1935). Most digestion occurs in the midgut, where a variety of enzymes is available in abundance (Engelmann, 1969; Persaud and Davey, 1971; Hori et al., 1981). In certain insects, digestion commences in the foregut by virtue of salivary gland secretions or enzyme regurgitation from the midgut. Rare instances of extra-intestinal digestion have also been reported in some insects (Chapman, 1972). Insect digestive enzymes are all hydrolases, showing general similarities to mammalian enzymes and being classified using standard nomenclature based on the reactions they catalyse. A wide range of digestive enzymes has been recorded in the alimentary canal of insects (House, 1965; Applebaum, 1985; Chapman, 1985a,b; Terra and Ferreira, 1994).

The nature of the enzymes secreted is related to the nature of the meal that an insect can assimilate (Hubert et al., 1999; Agusti and Cohen, 2000; Zeng and Cohen, 2000a, 2000b; Torres and Boyd, 2009). Whereas herbivorous insects secrete more carbohydrases (Day and Powning, 1949; Hori, 1973; Agrawal and Bahadur, 1978), carnivorous insects secrete mainly proteases (Gooding and Rolseth, 1976). Digestive enzymes specific for zoophagous animals include proteases (e.g., trypsin, chymotrypsin, cathepsin), hyaluronidases and phospholipases (Cohen, 1998b, 2000). Digestive enzymes specific for phytophagous animals include amylases and pectinases (Cohen, 1996). For instance, the strictly phytophagous mirid *Poecilocapsus lineatus* (Fabricius, 1798) (Heteroptera) lacks detectable digestive proteases in its salivary gland complex (Cohen and Wheeler, 1998), indicating the inability to use animal protein. On the other hand, the phyto-zoophagous mirids *Lygus lineolaris* (Palisot de Beauvois, 1818) and *Lygus hesperus* Knight, 1917 have trypsin-like and elastase-like enzymes but not chymotrypsin-like enzymes in their salivary gland complexes (Agusti and Cohen, 2000), demonstrating their ability to use animal protein.

Regarding stoneflies, a study on the digestive enzymes of detritus-feeding nymphs of two species of *Pteronarcys* (family Pteronarcyidae) showed a very high proteolytic activity, as found in other aquatic detritivorous insects (Martin et al., 1981). In another study, it was noted that *Pteronarcys* accomplishes cellulose hydrolysis by means of acquired microbial enzymes obtained through the ingestion of microbially conditioned detritus (Sinsabaugh et al., 1985). Few data are available on the digestive enzymes of the order Plecoptera, an amphibiotic insect group whose nymphs inhabit mainly streams and rivers. In these freshwater ecosystems, and particularly in low-order streams, stoneflies (Plecoptera) are one of the most important primary and secondary consumers (Fochetti and Tierno de Figueroa, 2008), as shown by many studies on nymphal feeding describing the gut contents. Two suborders of this group are distinguished: Antarctoperlaria, only present in the Southern Hemisphere, and Arctoperlaria, mainly distributed in the Northern Hemisphere and including the major proportion of the described species (Zwick, 2000; Fochetti and Tierno de Figueroa,

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2008). Arctoperlaria is subdivided in two groups: Euholognatha, with mainly phytophagous and detritivorous nymphs, and Systellognatha, whose nymphs have been traditionally considered predators (Hynes, 1976; Merrit and Cummins, 1984). Analyses of gut contents have confirmed the phytophagous/detritivorous behaviour of Euholognatha, but Systellognatha may exhibit a greater variability. Ontogenetic shifts on the ingested food have been repeatedly noted in this group of insects, with nymphs being more detritivorous when smaller and mainly carnivorous when larger (Berthélemy and Lahoud, 1981; Lucy et al., 1990; Céréghino, 2006; Bo et al., 2008). Moreover, in a recent study conducted in a temporary stream in the southern Iberian Peninsula, the analyses of the gut contents of three species of Systellognatha over their entire life cycle showed a carnivorous gradient (López-Rodríguez et al., 2009). These species were *Hemimelaena flaviventris* (Pictet, 1842), *Guadalgenus franzi* (Aubert, 1963) and *Isoperla morenica* Tierno de Figueroa & Luzón-Ortega, in press [that in this study appeared under the name of *I. curtata* Navás, 1924 before the study of Tierno de Figueroa et al. (in press)], the first being the most carnivorous, feeding mainly on Chironomidae (Diptera), whilst the nymphs of *I. morenica* ingested mainly diatoms.

In the present paper, the results of a study on the digestive enzymes of *H. flaviventris* and *I. morenica* are presented and related to data on the nymphal feeding and secondary production of these species obtained in a previous study (López-Rodríguez et al., 2009). Along with supporting information on digestive enzymes in Plecoptera, these complementary studies shed light on the potential assimilation capacity of trophic resources in this insect group. In addition, the fact that both species coexist in space and time under the same environmental conditions and are closely related from a phylogenetic point of view makes this comparative study particularly interesting.

2. Material and methods

2.1. Experimental animals

Nymphs of both species, *H. flaviventris* and *I. morenica* (which reach approximately the same body length, the former being slightly larger) were collected in Río Despeñaperros (Sierra Morena, Jaén, Spain; 38°22′22.98″N, 3°30′26.25″O, 560 m.a.s.l.), a Mediterranean-type seasonal stream, on May 17th, 2010. On the sampling date, the abiotic characteristics of the study site were as follows: O_2 saturation = 61.4%; O_2 concentration = 5.72 mg/L; pH = 7.85; conductivity = 219.8 μS/cm; discharge = 0.234 m^3/s; and temperature = 16.10 °C. The duration of the nymphal development of *H. flaviventris* is approximately 5 months, whilst that of *I. morenica* is 6 months. As these species have a relatively similar univoltine life cycle (López-Rodríguez et al., 2009), they were collected at approximately the same developmental stage. Samples were immediately placed in liquid nitrogen and stored at −80 °C until analysis.

2.2. Treatment of the samples

Due to the small size of the animals and their digestive tracts, enzymatic determinations were performed using the whole individuals. Animal samples (approximately 8–10 individuals per sample, five replicates per species) were homogenised on ice in ice-cold distilled water (1/4, w/v) with an electric homogeniser (Heidolph Instruments). The homogenates were centrifuged at 30,000 *g* for 30 min at 4 °C in a model 3 K30 Sigma centrifuge. After centrifugation, the supernatant was collected and frozen at −80 °C until analysis.

2.3. Enzymatic determinations

The proteolytic enzymatic activity determination was performed using several pH values over the physiological range of the digestive system (pH 2, 3, 4, 6, 7, 8, 9, 10 and 12). The assay was a modification of the methods described by Anson (1938) and Walter (1984) for acid and basic proteases, respectively. Stauffer universal buffer (Stauffer, 1989) adjusted to different pHs was employed. As in previous assays, the enzymatic reaction mixture consisted of 30 μL of extract and 600 μL of haemoglobin (1%) or casein (1%) in Stauffer buffer with the appropriate pH for acid or basic proteases, respectively. Samples (in triplicate) were incubated for 30 min at 37 °C. A control for each sample was performed by adding the extract after the incubation time. The reaction was stopped by the addition of 270 μL of 20% trichloroacetic acid (TCA) (w/v) to the control and experimental samples. After being kept for 20 min in an ice bath, the samples were centrifuged at 5000 *g* for 10 min, and the absorbance of the supernatant was measured at 280 nm. L-tyrosine was used as a standard. The total proteolytic activity was estimated from the sum of the different activities at different pH values.

Trypsin (E.C. 3.4.21.4) activity was assayed following the method of Faulk et al. (2007). As in previous assays, the enzymatic reaction mixture consisted of extract, N-benzoyl-DL-arginine-4-nitroanilide (BAPNA) 1 mM in Tris–HCl (50 mM) and $CaCl_2$ (20 mM, pH 8.2) buffer as substrate. The production of 4-nitroaniline (ε 8800 M^{-1} cm^{-1}) was monitored at 410 nm at 30 °C.

Chymotrypsin (E.C. 3.4.21.1) was assayed following the method of Faulk et al. (2007), and as in previous assays, the enzymatic reaction mixture consisted of extract and N-benzoyl-L-tyrosine ethyl ester (BTEE) 0.566 mM in Tris–HCl 0.1 M, NaCl 25 mM pH 7.8 buffer as substrate. The hydrolysis of BTEE (ε 964 M^{-1} cm^{-1}) was recorded at 256 nm and 37 °C.

Lipase (E.C.3.1.1.3.) activity was measured following the method of Faulk et al. (2007), and as in previous assays, the enzymatic reaction mixture consisted of extract and 4-nitrophenyloctanoate as substrate (0.35 mM) in Tris–HCl pH 7.4 buffer 0.5 mM, sodium taurocholic acid (TA) 6 mM, NaCl 1 M. Nitrophenol generation (ε 16300 M^{-1} cm^{-1}) was measured at 400 nm and 30 °C.

α-Amylase (E.C.3.2.1.1.) activity was assayed with a commercial kit (Chemelex, S.A., 08420, Barcelona, Spain) by measuring the generation of 4-chloro-2-nitrophenol (CNP) from the hydrolysis of 2-chloro-4-nitrophenol-α-D-maltotrioside ($CNPG_3$) at 405 nm and 37 °C.

For all enzymatic determinations, one unit of activity was defined as 1 μmol of product generated per mL per min.

To estimate the enzyme's specific activities, the protein content of the extracts was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

All biochemicals including substrates and coenzymes were obtained from Sigma-Aldrich Chemical Co. (USA). All other chemicals were provided by Merck (Darmstadt, Germany) and were of reagent grade.

2.4. Statistical analysis

The results are expressed as means ± SEM. Multifactorial analysis of variance (ANOVA, SPSS version 13.0 for Windows software package 5) was employed to assess the significance of differences between the species. When F values were significant ($p<0.05$), the differences between species were compared using Duncan's multiple range test (Duncan, 1955).

3. Results

Table 1 shows the main results obtained after the determination of the different enzymatic activities in *I. morenica* and *H. flaviventris*, expressed per ml of extract. In addition, this table shows the soluble protein concentration in the whole body extract. The protein content of the extracts was similar for both species. *H. flaviventris* displayed significantly higher lipase and general proteolytic activities than *I. morenica*. In addition, the trypsin and chymotrypsin activities were

Table 1
Digestive enzymatic activity (per ml extract) and soluble protein in two species of Plecoptera.

Species		*Isoperla morenica*	*Hemimelaena flaviventris*
Amylase	(mU/mL)	485.7 ± 111.0	433.2 ± 67.2
Lipase	(mU/mL)	171.1 ± 13.2*	325.3 ± 18.5
Proteases	(mU/mL)	101.6 ± 13.8*	204.3 ± 29.6
Trypsin	(mU/mL)	92.4 ± 15.9*	236.2 ± 46.7
Chymotrypsin	$(mU/mL).10^{-2}$	51.6 ± 5.3*	78.6 ± 10.6
Soluble protein	(mg/mL)	5.7 ± 0.7	5.3 ± 0.4

Protease activity is the sum of the proteolytic activity at pHs 2, 3, 4, 6, 7, 8, 9, 10 and 12. One unit of activity was defined as 1 μmol of product generated per mL per min. Values are the mean ± SEM (n = 5).
* Significant differences between species for the same digestive enzyme ($p<0.05$).

significantly higher in *H. flaviventris*. The digestive specific activity results (per mg protein) displayed a trend similar to that expressed per ml; *H. flaviventris* had significantly higher protease and lipase specific activities than *I. morenica*. With respect to the amylase activity, the values were greater in *I. morenica* (Fig. 1). The trypsin and chymotrypsin activity results show that *H. flaviventris* had significantly higher values for the trypsin activity. No significant differences were found for the chymotrypsin activity between the two species (Fig. 2).

Fig. 3 presents the protease activities determined at different pHs. At acid pHs, both species showed similar values; these values were lower than those found for the protease activity determined at basic pHs. From pH 7, *H. flaviventris* displayed significantly higher values than *I. morenica*.

Considering the total digestive enzymatic activity in percentages (Fig. 4), the amylase activity of *I. morenica* accounted for more than 60% of the total enzymatic activity, whereas in *H. flaviventris*, this percentage was approximately 40%. In contrast, the protease activity in *H. flaviventris* was approximately twice that of *I. morenica*.

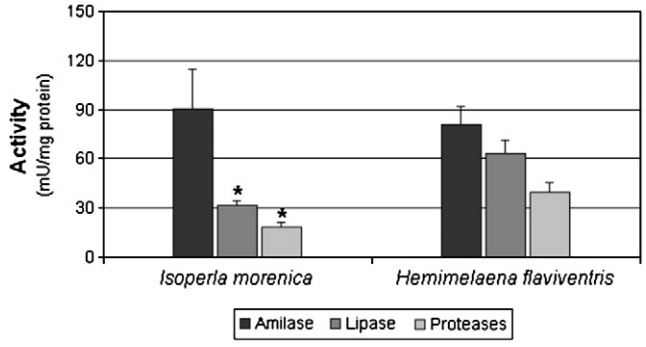

Fig. 1. Digestive enzyme specific activity (per mg protein) in the studied species of Plecoptera. Values are the mean ± SEM (n = 5). *Significant differences between species for the same digestive enzyme ($p<0.05$).

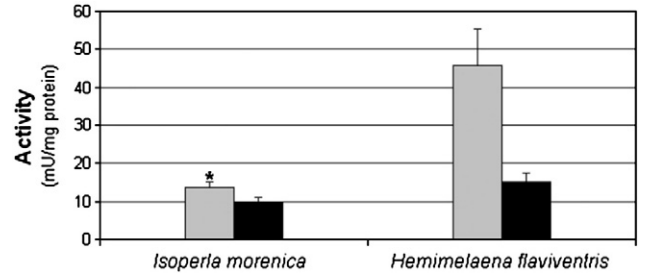

Fig. 2. Trypsin and chymotrypsin specific activities in the studied species of Plecoptera. Values are the mean ± SEM (n = 5). *Significant differences between species ($p<0.05$).

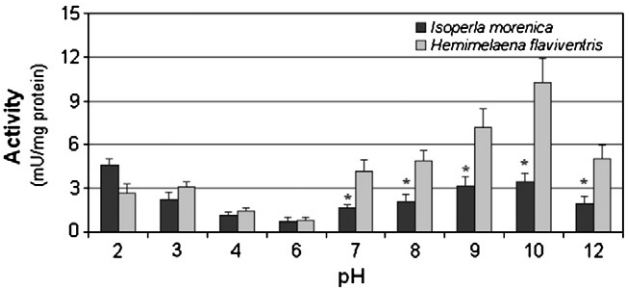

Fig. 3. Protease specific activity profile at different pHs for the studied species of Plecoptera. Values are the mean ± SEM (n = 5). *Significant differences between species for the same pH ($p<0.05$).

Finally, *H. flaviventris* presented a greater proportion of trypsin (approximately 20% more than *I. morenica*, Fig. 5).

4. Discussion

Understanding the functioning of the digestive machinery helps to explain the utilisation of nutrients at the digestive level (Glass et al., 1989; Hidalgo et al., 1999; Kolkovski, 2001). In addition, the pattern of digestive enzymes can reflect the feeding habits of an animal (Hofer and Köck, 1989).

Considering the total digestive enzymatic activities (Table 1, Fig. 1), the values detected for *H. flaviventris* were higher than those found in *I. morenica*. Moreover, our study revealed that the lipase and protease specific activities (both absolute and relative) were greater in *H. flaviventris*, whereas the relative amylase activity was greater in *I. morenica* (Figs. 1, 4). Studies carried out in insects and in other animals have demonstrated that greater amylase activity is typical of

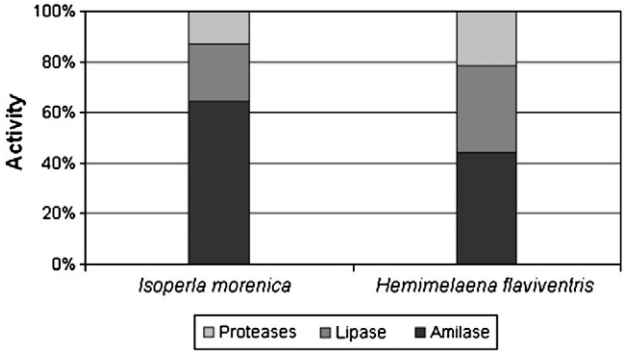

Fig. 4. Relative percentages of digestive activity in the studied species of Plecoptera.

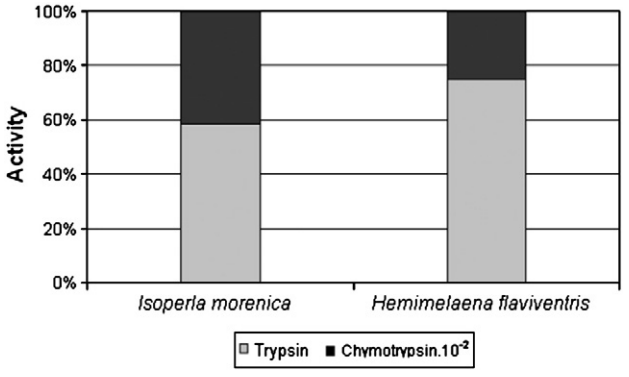

Fig. 5. Relative percentages of trypsin and chymotrypsin activities in the studied species of Plecoptera.

phytophagous animals (Cohen, 1996; Hidalgo et al., 1999; Furné et al., 2005; De Almeida et al., 2006; Ferro Correa et al., 2007), whilst greater protease activity is characteristic of carnivorous animals (Cohen, 1998b, 2000). Thus, from a physiological point of view, *H. flaviventris* can be considered a more efficient carnivore or zoophagan than *I. morenica*. These results support the data obtained in a previous study analysing gut contents (López-Rodríguez et al., 2009).

As previously noted, lipase activity was greater in *H. flaviventris* (Figs. 1, 4). Insect lipases are more difficult to study than proteases and carbohydrases because of their lower activities, and thus, in general, lipid digestion is poorly understood in insects (Arrese et al., 2001; Arrese, 2010). Nevertheless, studies in fishes have shown increased lipase activity in animals with carnivorous habits (Hidalgo et al., 1999; Furné et al., 2005). Consequently, our results seem to demonstrate the efficiency of *H. flaviventris* as a zoophagous species.

The specific enzymatic activities determined at different pHs (Fig. 3) did not indicate the existence of cysteine proteinase activity, which requires an acid pH. Particularly, cathepsin activity has been described in hemipteran insects (Houseman and Downe, 1983). The activities found at basic pH should correspond to trypsin, chymotrypsin, and other proteases (probably elastase and carboxypeptidase), enzymes previously investigated in other insect studies (Boyd, 2003).

In our study, the trypsin and chymotrypsin activities were specifically determined (Fig. 2). The presence of trypsin-like and chymotrypsin-like enzymes is indicative of an insect's ability to access structural or other insoluble proteins (Cohen, 1993, 1998a, 2000). The presence of trypsin-like enzymes and the lack of chymotrypsin-like and elastase-like enzymes in the salivary gland complex are common in heteropteran predators (Cohen, 1993, 1998a, 2000; Agusti and Cohen, 2000). Trypsin-like enzymes are endoproteases that attack proteins at arginine and lysine residues, whereas chymotrypsin-like enzymes, which are also endoproteases, attack proteins at aromatic residues (e.g., tryptophan).

The fact that *H. flaviventris* contained a greater proportion of trypsin (approximately a 20% more than *I. morenica*, Fig. 5) is in agreement with the results found in other studies of other insects and vertebrates (Yetty et al., 2003) and would support a feeding specialisation closer to zoophagy for this species than for *I. morenica*.

Finally, our research highlights that the *H. flaviventris* digestive enzymatic pool is greater than of *I. morenica*. This difference could be the reason why *H. flaviventris* has a higher annual secondary production in this same stream than *I. morenica*, as shown in a previous study by López-Rodríguez et al. (2009). In this study, it was found that the annual secondary production of *H. flaviventris* was 1.91 g DM m^{-2} y^{-1}, whereas that of *I. morenica* was 0.38 g DM m^{-2} y^{-1}. That study had already pointed out that animal matter accounted for an extremely low percentage of production in *I. morenica* but was the main contributor to production in *H. flaviventris*, based on an estimation of the assimilation efficiency of both species. Our current results support this idea from an enzymatic point of view.

In conclusion, the comparative study of the digestive enzymatic activities (proteases, lipases and amylase) in the species *I. morenica* and *H. flaviventris* indicates that the latter has a greater efficiency as a zoophagan than the former. Moreover, this result supports the data obtained in a previous study of these species' gut contents and on the contribution of each trophic resource to each species' secondary production (López-Rodríguez et al., 2009). Thus, this study allows the integration of ecological and physiological information to better understand the trophic behaviour of these two species, which constitute two of the main components of the benthic community of a typical seasonal Mediterranean stream.

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