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The effects of different prey regimes on the proteolytic digestion of nymphs of the spined soldier bug, *Podisus maculiventris* (Hemiptera: Pentatomidae)

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Abstract

The effects of different prey regimes on the performance and digestive physiology of the spined soldier bug, *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae), were assessed. Specifically, *P. maculiventris* nymphs were fed on Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), larvae; Egyptian cotton leafworm (ECW); *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae); larvae; *Calliphora spp*. (CAL) (Diptera: Calliphoridae) pupae or a mixture of the three prey. No differences in development and weight gain were observed when *P. maculiventris* nymphs were fed different prey species (CPB, ECW or CAL). However, an increase in weight gain and a reduction in the duration of the stadia were observed for nymphs fed with a mixture of the three prey. To investigate the physiological background, biochemical analysis were carried out on insects dissected at the end of the feeding assay. We have found that the proteolytic activity in the salivary glands of *P. maculiventris* nymphs was not affected by prey species, whereas the relative activity of these proteases in the midgut depends on the prey. Moreover, gel assays proved that the proteolytic profiles of midguts from *P. maculiventris* nymphs feeding on CPB, ECW and CPB closely resembled those of their prey. All together, these results suggest that *P. maculiventris* may utilize enzymes from the prey they consume that may facilitate the process of digestion.

Keywords: digestion, food quality, predator, prey, *Leptinotarsa decemlineata*, *Spodoptera littoralis*, *Calliphora spp*.

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Introduction

The spined soldier bug (SSB), *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae), is a generalist predator used as a biological control agent in North America and Europe primarily for the control of lepidopteran and coleopteran pests (De Clercq, 2000). As with most beneficial insects, current methods for mass rearing of *P. maculiventris* involve rearing on live insect prey. Hence, the nutritional value or food quality of prey is critical for the fitness of this predator

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(De Clercq *et al*., 1998; Strohmeyer *et al*., 1998; Mahdian *et al*., 2006). Development of immatures, consumption capacity or production of eggs, in response to feeding, has been used to define the food quality of prey (Legaspi & Legaspi, 2004). In addition, specific biochemical parameters in *P. maculiventris* nymphs and adults have been considered to analytically determine food quality, such as lipid and protein profiles (Legaspi *et al*., 2004; Shapiro & Legaspi, 2006) and vitellogenin content in females (Shapiro *et al*., 2000). However, the effect of prey food quality on the digestive capacity of the predator is largely unknown.

Nymphs and adults of *P. maculiventris* mechanically and enzymatically process their prey extra-orally, by inserting the stylet and injecting digestive enzymes through the salivary canal into the prey, then suck the partially digested material into their own gut where digestion is completed (Cohen, 1990, 1995). Protease activity in *P. maculiventris* salivary secretions is mainly based on serine proteases, whereas cysteine proteases and exopeptidases are predominant in the gut (Stamopoulos *et al*., 1993; Bell *et al*., 2005; Álvarez-Alfageme *et al*., 2007). A number of studies have revealed that the quality and quantity of digestive enzymes in phytophagous insects can be altered in response to changes in dietary protein (Broadway & Duffey, 1988; Felton, 1996). However, Habibi *et al*. (2001) showed that, in contrast to phytophagous hemipterans, *P. maculiventris* manifested minimal differences in their salivary protein profile after feeding on different prey. Thus, the capacity to distinguish and respond to dietary stimuli appears to differ between phytophagous and entomophagous hemipterans.

The aim of this study was to examine the effects of different prey regimes on the performance and digestive physiology of *P. maculiventris* nymphs. Specifically, *P. maculiventris* nymphs were fed on Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), larvae; Egyptian cotton leafworm (ECW), *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), larvae; *Calliphora spp*. (CAL) (Diptera: Calliphoridae) pupae or a mixture of the three prey.

Materials and methods

Insects

A laboratory colony of CPB from Ávila (Spain) was reared on potato plants, *Solanum tuberosum* cv. Kennebec. A laboratory colony of ECW was reared on a semi-artificial diet, modified from Poitout & Bues (1970) by the addition of 0.63% (w/w) Wesson's salt mixture. Larvae and pupae of CAL were obtained from a local fishing tackle retailer. Nymphs of *P. maculiventris* were purchased from Koppert Sistemas Biológicos (Almería, Spain) and reared for one generation on larvae of CPB or ECW or pupae of CAL before use in trials. All laboratory colonies were reared in environmental chambers at 25 ± 2°C, 70 ± 10% RH and a 16:8 h (L:D) photoperiod.

Insect bioassays

Feeding assays were performed with third instar *P. maculiventris* nymphs placed singly in ventilated plastic dishes (30 mm height × 90 mm Ø) that contained filter paper and cotton soaked with water and were maintained in an environmental chamber as described above. Two to three larvae of CPB, ECW, pupae of CAL or a mixture of the three prey were provided daily until *P. maculiventris* nymphs reached the fifth instar. Twenty replicas were made for each prey regime, and the weight of each *P. maculiventris* nymph were recorded before release (newly molted, less than 12 hours, third instar nymphs) and at the end of the bioassay (fifth instar nymphs allowed to feed for 24 hours). The weight gain of *P. maculiventris* nymphs was calculated as the difference between the initial and the final weight.

At the end of the assay, *P. maculiventris* nymphs were placed at −20°C for about 5 min and then dissected in ice-cold dH_2O and salivary glands and midguts extracted for enzymatic determinations. Midguts were homogenised in 500 µl of 0.15 M NaCl, centrifuged at 10,000 g for 5 min and the supernatants individually frozen to provide 18–20 samples of each feeding regime. Salivary glands were pooled in groups of two to provide 9–10 samples, homogenised in 150 µl of 0.15 M NaCl, centrifuged, and the supernatants stored frozen until needed.

Digestive protease assays

Unless otherwise stated, insect protease activities were determined at 30°C, at their optimum pH of activity in 1 ml of reaction mixture. Total protein in midgut extracts was determined according to the method of Bradford (1976) with BSA as the standard. All substrates were purchased from Sigma Chemical Co. (St Louis, Missouri, USA).

The protease activities present in the salivary glands and midgut of *P. maculiventris* nymphs were determined following the conditions described by Álvarez-Alfageme *et al*. (2007). All assays were performed using 30 µl of salivary gland extract or 20 µl of midgut extract. Trypsin-like activity was assayed using 1 mM BApNa (Nα–benzoyl-DL-arginine-p-nitroanilide) as substrate and incubating for 24 h at pH 9.0 (salivary glands) or pH 10.0 (midgut), chymotrypsin-like activity using 0.25 mM SA_2PPpNa (N-succinyl-(alanine)$_2$-proline-phenylalanin-p-nitroanilide) as substrate and incubating for 24 h at pH 9.5 (salivary glands) or pH 10.0 (midgut), cathepsin B-like activity using 50 µM ZAA_2MNA (N-carbobenzoxy-alanine-arginine-arginine 4-methoxy-β-naphthyl amide) as substrate and incubating for 24 h (salivary glands) or 4 h (midgut) at pH 7.0 with buffer that contain 1 mM L-cysteine, leucine aminopeptidase-like activity using 1 mM LpNa (L-leucine p-nitroanilide) as substrate and incubating for 24 h at pH 7.0 (salivary glands) or 2 h at pH 7.5 (midgut), and carboxypeptidase B-like activity using 1 mM HA (hippuryl-L-arginine) as substrate and incubating for 24 h at pH 7.0 (salivary glands) or 7.5 (midgut). Spectrophotometric measurements were made using a Hitachi U-2000 spectrophotometer (Tokyo, Japan), and readings taken at 410 nm for pNa substrates, 520 nm for ZAA_2MNA, and 570 nm for HPA and HA by the ninhydrin procedure.

Zymograms

Electrophoretic detection of proteolytic forms was performed by 0.1% (w/v) gelatine-containing, 0.1% (w/v) SDS, 12% (w/v) polyacrylamide gel electrophoresis under non-denaturing conditions (Lantz & Ciborowski, 1994) using a Bio-Rad Mini-Protean II Electrophoresis Cell system. The ratio of acrylamide to bis-acrylamide was 37.5 : 1. Samples of salivary glands and midguts of *P. maculiventris* nymphs contained 3 µg of total protein, whereas CPB and ECW

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Table 1. Performance and digestive protease activity of *P. maculiventris* nymphs feeding on *L. decemlineata* (CPB) larvae, *S. littoralis* (ECW) larvae, *Calliphora spp.* (CAL) pupae or a mixture (MIX) of the three prey.

	P. maculiventris nymphs feeding on			
	CPB	ECW	CAL	MIX
Performance[1]				
Weight gain (mg fw)	37.0 ± 3.3^{a}	41.3 ± 2.3^{ab}	41.3 ± 2.8^{ab}	49.7 ± 2.5^{b}
Duration N3–N5 (days)	7.5 ± 0.2^{a}	7.6 ± 0.1^{a}	7.3 ± 0.1^{ab}	6.9 ± 0.1^{b}
Protease activity in salivary glands[2]				
Trypsin (BApNa)	2.5 ± 0.5^{a}	4.9 ± 0.9^{a}	2.2 ± 0.6^{a}	4.7 ± 1.5^{a}
Chymotrypsin (SA_2PppNa)	2.0 ± 0.4^{a}	3.9 ± 0.9^{a}	2.4 ± 0.6^{a}	2.9 ± 0.6^{a}
Cathepsin B (ZAA_2MNA)	0.7 ± 0.1^{a}	0.6 ± 0.1^{a}	0.7 ± 0.1^{a}	0.6 ± 0.1^{a}
Leucine aminopeptidase (LpNa)	1.7 ± 0.6^{a}	1.1 ± 0.2^{a}	2.0 ± 0.5^{a}	1.1 ± 0.2^{a}
Carboxypeptidase B (HA)	37.5 ± 7.5^{a}	20.0 ± 3.4^{a}	45.0 ± 10.0^{a}	35.5 ± 8.9^{a}
Protease activity in midgut[b]				
Trypsin (BApNa)	0.6 ± 0.1^{a}	17.7 ± 2.0^{c}	0.9 ± 0.1^{a}	4.5 ± 1.1^{b}
Chymotrypsin (SA_2PppNa)	0.3 ± 0.1^{a}	18.4 ± 2.1^{c}	0.2 ± 0.1^{a}	4.8 ± 1.4^{b}
Cathepsin B (ZAA_2MNA)	16.7 ± 2.0^{a}	18.5 ± 2.3^{a}	3.3 ± 0.8^{b}	14.5 ± 2.5^{a}
Leucine aminopeptidase (LpNa)	68.0 ± 8.5^{a}	86.6 ± 6.5^{a}	15.0 ± 1.9^{b}	67.9 ± 12.7^{a}
Carboxypeptidase B (HA)	12.7 ± 1.2^{a}	12.6 ± 1.3^{a}	6.5 ± 0.8^{b}	10.8 ± 1.9^{a}

[a] Assays were performed with third instar nymphs of *P. maculiventris* feeding on different prey until they reached the fifth instar. Data are the mean ± SE ($n = 18$ for CPB, 19 for BAW, 20 for CAL and 18 for MIX).
[b] Protease activity is expressed as nmoles of substrate hydrolyzed min^{-1} mg^{-1} protein. Data are the mean ± SE ($n = 9$–10 for salivary glands and 18–20 for midguts).
Row means followed by the same letter are not significantly different from each other (Student-Newman-Keuls test, $P \leqslant 0.05$).

midgut extracts and CAL pupae full body extracts contained 2, 0.5 and 3 µg of total protein, respectively. After migration at 4°C, gels were transferred to a 2.5% (v/v) aqueous solution of Triton X-100 for 30 min at room temperature, to allow renaturation of the proteases. Gels were then placed in the following activation buffers for 24 h at 30°C: 0.1M Tris-HCl and 10 mM L-cysteine, pH 7.0; or 0.1 M glycine-NaOH, pH 9.0. Proteolysis was stopped by transferring the gels into a staining solution [0.3% (w/v) Coomassie Blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid]. The gels were destained in 25% (v/v) methanol and 10% (v/v) acetic acid. Bands of proteolytic activity were visualised against the blue background of the gel.

Statistical analysis

Weight gain, duration of stadia and protease activities of *P. maculiventris* nymphs fed with different prey species were analyzed by means of the Student-Newman-Keuls-test. Differences between treatments were considered significant at the $P \leqslant 0.05$ level.

Results

No differences in development and weight gain were observed when *P. maculiventris* nymphs were fed different prey species (CPB, ECW or CAL) (table 1). However, an increase in weight gain and a reduction in the duration of the stadia were observed for nymphs fed with a mixture of the three species. To investigate the physiological background, biochemical analysis were carried out on insects dissected at the end of the feeding assay. Biochemical analysis showed that the activities of the proteases (trypsin-, chymotrypsin-, cathepsin B-, leucine aminopeptidase- and carboxypeptidase B-like) present in the salivary glands of *P. maculiventris* nymphs were not affected by the different prey regimes, whereas the activities in the midgut were determined by the prey (table 1). Thus, trypsin- and chymotrypsin-like activities were high in *P. maculiventris* midguts when the nymphs were fed on ECW, low when fed on CPB or CAL and intermediate when fed with a mixture of the three species. Likewise, *P. maculiventris* nymphs fed on CPB, ECW or the MIX diet presented higher midgut cathepsin B-, leucine aminopeptidase- and carboxypeptidase B-like activities than when fed with CAL.

Gel assays were performed to look for differences in protease forms in salivary glands and midgut of *P. maculiventris* nymphs fed with different prey. The proteolytic profile in salivary glands was not affected by the different prey regimes, and the protease forms were different to those present in the prey (fig. 1a). On the contrary, the midguts from *P. maculiventris* nymphs feeding on CPB, ECW, CAL or a mixture of the three prey presented different proteolytic profiles, and the banding patterns closely resembled those of their prey (fig. 1b). Minimal differences in banding patterns were obtained when the zymograms with *P. maculiventris* salivary glands and midguts were performed at two different pHs, 7.0 and 9.0 (data not shown). With respect to the effect of pH in the protease forms of the prey, different banding patterns were obtained for CPB at pH 7.0 and 9.0, whereas similar results were observed with ECW (figs 1a, b). No protease forms were detected with CAL at any of the pH tested (figs 1a, b).

Discussion

The involvement of serine proteases, especially trypsin and chymotrypsin, in extra-oral digestion of heteropteran predators is well documented (Boyd, 2003; Bell *et al*., 2005; Oliveira *et al*., 2006). We have found that, in addition to these two activities, cathepsin B-, leucine aminopeptidase- and carboxypeptidase B-like activities were also detected in the salivary glands of *P. maculiventris* nymphs. Bell *et al*. (2005) also provided evidences for the presence of cysteine

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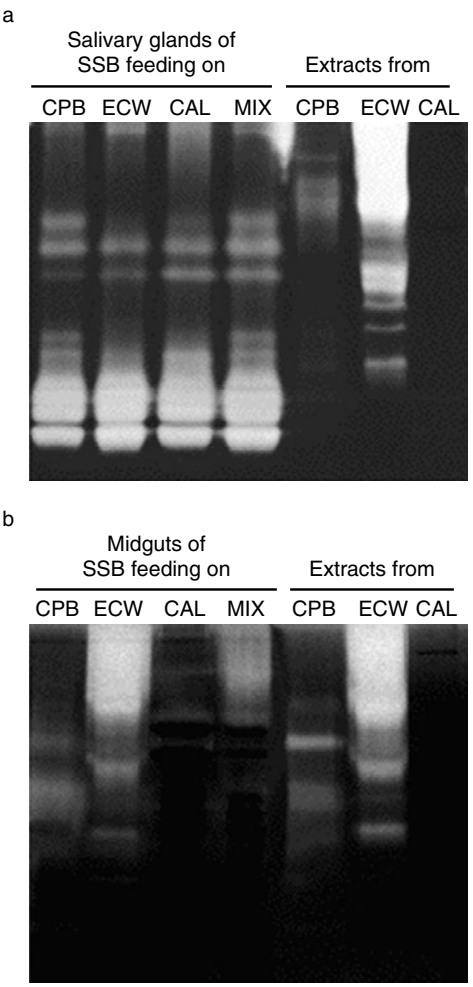

Fig. 1. Gelatin-containing SDS-PAGE gels of salivary glands and midgut of *P. maculiventris* (SSB) nymphs feeding on *L. decemlineata* (CPB) larvae, *S. littoralis* (ECW) larvae, *Calliphora spp*. (CAL) pupae or a mixture (MIX) of the three prey. Extracts from CPB, ECW and CAL larval midgut and CAL full body were also analyzed. Gels were incubated for 24 h at 30°C with (a) 0.1 M glycine-NaOH, pH 9.0 or (b) 0.1M Tris-HCl and 10 mM L-cysteine, pH 7.0.

proteases and carboxypeptidases in the salivary glands of this species. It has been reported that the salivary composition of phytophagous hemipterans may vary in response to diet composition of the host plant (Miles, 1987; Habibi *et al*., 2001). However, information regarding the capacity of entomophagous hemipterans to respond to dietary stimuli is very scarce. Habibi *et al*. (2001) showed that *P. maculiventris* manifested minimal differences in banding patterns of the saliva proteins after feeding on different prey. Our results go further, indicating that the proteolytic activity in the salivary glands of *P. maculiventris* nymphs was not affected by prey species. It has been suggested that variable salivary composition in phytophagous hemipterans might indicate induction of new proteins in preparation for continued feeding on a new host plant (Habibi *et al*., 2001). However, generalist predators may consume a large variety of prey species in a short period of time, and changes in salivary composition in respond to dietary stimuli of one specific prey may not represent a utility for the consumption of the next prey.

Gut proteolysis in *P. maculiventris* is mainly performed by cysteine proteases, aminopeptidases and carboxypeptidases (Stamopoulos *et al*., 1993; Bell *et al*., 2005; Álvarez-Alfageme *et al*., 2007). Biochemical analysis showed that, in contrast to what happen with salivary glands, the relative activity of these proteases in the midgut depends on the prey. Thus, nymphs fed on CPB, ECW or the MIX diet presented higher midgut cathepsin B-, leucine aminopeptidase- and carboxypeptidase B-like activities than when fed with CAL. Likewise, trypsin- and chymotrypsin-like activities were detected in the *P. maculiventris* midgut when the nymphs were fed on ECW or the MIX diet, but very low levels were found when fed on CPB or CAL. Interestingly, trypsin- and chymotrypsin-like activities are the major digestive proteases in ECW (Lee & Anstee, 1995), whereas CPB mainly rely on cysteine proteases (Thie & Houseman, 1990) and only traces of proteolytic activity were found in CAL pupae (data not shown). Moreover, the zymograms proved that the proteolytic profiles of midguts from *P. maculiventris* nymphs feeding on CPB, ECW and CPB closely resembled those of their prey. All together, these results suggest that *P. maculiventris* may utilize enzymes from the prey they consume. However, none of the main proteases present in the salivary glands, and presumably injected in the prey, were recovered in the gut.

Nymphs of *P. maculiventris* fed with a mixture of the three prey attained higher weights and shorter developmental time than those reared on a single prey. In predation studies where a choice of prey is presented simultaneously, polyphagous predators can improve their fitness or fecundity by feeding on mixed diets. For example, *Podisus nigrispinus* (Dallas) displayed higher egg and nymphal production on a diet of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) and *Musca domestica* L. (Diptera: Muscidae), compared with diets consisting of either species exclusively (Zanuncio *et al*., 2001). Under this scenario, polyphagous predators are expected to select prey that would be of the best nutritional quality (Strohmeyer *et al*., 1998; Legaspi & Legaspi, 2004). However, prey selection may be determined by other factors, such as prey size and mobility (De Clercq *et al*., 2002) and the avoidance of toxic unpalatable prey (Traugott & Stamp, 1996). The results of the present study indicate that, as a consequence of the extra-oral digestion, the digestive proteases of the prey are sucked by *P. maculiventris* nymphs into its own gut. Thus, when consuming a variety of prey, a broader range of proteases will be available that may facilitate the process of digestion. Likewise, the overall performance of *P. maculiventris* reared on insect-free artificial diet is inferior to those reared on live prey (De Clercq *et al*., 1998; Wittmeyer & Coudron, 2001). Differences in nutritional quality, lack of feeding stimulants and diet presentation may explain these results (De Clercq *et al*., 1998). Still, the use by the predator of prey-derived enzymes may also help to explain the higher nutritional value of prey over artificial diets.

In summary, the effects of different prey regimes on the performance and digestive physiology of *P. maculiventris* nymphs have been assessed. It is noteworthy that *P. maculiventris* apparently utilizes enzymes from the prey they consume that may facilitate the process of digestion. Physiological analysis would be particularly informative for

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insight into which combinations of prey enhance the efficiency of digestion and what physiological processes are involved.

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