Body distribution of toxic peptides in larvae of a pergid and an argid sawfly species

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Abstract

Larvae of most Pergidae and Argidae (Symphyta: Tenthredinoidea) species contain toxic peptides such as pergidin and lophyrotomin. Here, larval hemolymph and organs of the pergid *Lophyrotoma zonalis* and the argid *Arge pagana* were analysed by liquid chromatography-tandem mass spectrometry. The major identified peptides were pergidin and 4-valinepergidin in *L. zonalis*, whereas pergidin and lophyrotomin in *A. pagana*. The storage period prior to chemical analysis was longer for the samples of the pergid than the argid species, which influenced peptide concentrations. In both species, however, the peptides occurred in decreasing order of concentration, first in the hemolymph, then in the integument, while minor amounts of the peptides were detected in other organs such as gut and fat body. By separating the cuticle of the pergid from the remaining integument, the peptides were found in equivalent amounts in each of these two body structures. The results suggest that the peptides play an important role in the defence of these sawfly larvae against predators.

Keywords *Lophyrotoma zonalis*; *Arge pagana*; hemolymph; integument; LC-MS/MS; pergidin; lophyrotomin

Introduction

Insects that use chemical defence against predators store the harmful compounds in specific areas of the body (Dettner 2015). Specialized epidermal exocrine glands are often the site both of production and storage of chemicals produced de novo (Noirot and Quennedey 1974). If an insect sequesters chemicals from its food, the chemicals are in the gut before passing through the gut epithelium, to be stored in the hemolymph or other compartments (Opitz and Müller 2009). Endosymbionts can also produce compounds used by their host in anti-predator defence. The evidence of such systems remains restricted to the genus *Paederus* Fabricius, 1775 (Coleoptera: Staphylinidae) harbouring *Pseudomonas* Migula, 1894 bacterial symbionts that produce the toxin pederin and that are acquired by cannibalism (Kellner 2003; Maleki-Ravasan et al. 2019).

Argidae and Pergidae (Symphyta: Tenthredinoidea) sawfly larvae have been studied by analytical chemistry since the 1970s (Leonard 1972; Oelrichs et al. 1977). Most argid and pergid species are known to contain some or all of the peptides: pergidin (Perg, molecular weight (MW, in g/mol) = 864), 4-valinepergidin (VPerg, MW = 850), dephosphorylated pergidin (dpPerg, MW = 784), and two closely related peptides both called lophyrotomin (LGln, MW = 1039, and LGlu, MW = 1040) (Boevé et al. 2014). The biosynthetic origin of the sawfly peptides remains unknown, but they are most probably produced by endosymbionts (Oelrichs et al. 1999). In the pergid *Lophyrotoma interrupta* (Klug, 1814), the oral discharge of an oily fluid contains about ten times the concentration of lophyrotomin compared to whole larvae (Oelrichs et al. 1992). The hemolymph, integument and gut were shown to be a paralytic feeding deterrent by testing their extracts from the argids *Arge pagana* (Panzer, 1797) and *Arge pullata* (Zaddach, 1859) against ant workers of *Myrmica rubra* (Linnaeus, 1758), which indirectly indicates the presence of peptides (Petre et al. 2007). Nearly no peptides are detected in the excrements of *Arge berberidis* Schrank, 1802 (Boevé and Rozenberg 2019). Aside from these fragmentary data, to date no analysis has been done to determine the precise location of peptides within the body.

Here, we localized the peptides within the larval body of *L. zonalis* (Rohwer, 1910) and *A. pagana* by collecting hemolymph and dissecting organs. The samples were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The results are discussed in an ecological context. Some practical questions about storing samples over a long period are also discussed.

Materials and Methods

Sawfly larvae from two populations were collected in the field: one of *L. zonalis* (30 March 2008; Brisbane, Australia; by JLB), the other of *A. pagana* (16 September 2016; Aalter, Belgium; by Filip De Block). Voucher specimens are kept at the Royal Belgian Institute of Natural Sciences (collection reference codes: P2865 and P4207, respectively). The larvae were weighted on an analytical balance at 0.1-mg precision (Kern ABS 120–4, Kern & Sohn GmbH, Balingen, Germany). Hemolymph samples were collected from live larvae and stored in 100% ethanol. The hemolymph of *L. zonalis* was sticky and collected with bits of filter

paper. That of *A. pagana* was collected with glass capillaries and weighed. Body part samples were obtained from larvae that were frozen, thawed, and dissected in distilled water to isolate the following organs: the digestive tract, or gut, the integument (plus the attached longitudinal muscles), and additional organs such as salivary glands and fat body. Peptide quantities and concentrations per *L. zonalis* larva were unknown in 2008, because procedures of single-larva extraction and LC-MS/MS analysis of the peptides had not yet been developed. Thus, we opted on the side of caution for this species and collected hemolymph and organs from three larvae per sample. In contrast, each sample of *A. pagana* consisted of a single larva. The gut of *L. zonalis* larvae was large enough to be separated in its three parts: foregut, midgut, and hindgut. The cuticle of this species could generally be detached from the rest of the integument (indicating that the larvae were in a pre-moulting phase), to which longitudinal muscles were associated (Table 1). All samples from *L. zonalis* were stored at -30 °C, and those from *A. pagana* at -80 °C, until their extraction.

The extraction procedure was developed by Boevé et al. (2014). In the present study, two extractions were made from each sample of *A. pagana*, and three from each one of *L. zonalis*, independent of the larval fresh weight (FW). The gathered pooled extracts, each making up 3 ml and 4 ml, respectively, were stored at -80 °C. Those from *A. pagana* were diluted 20 times, those from *L. zonalis* 30 times, and these aliquots were then again stored at -80 °C until chemical analysis.

To summarize, there are three events preceding the LC-MS/MS analyses: 1) collection of hemolymph plus dissection of organs, 2) successive extractions of the samples resulting in a pooled extract, and 3) triplicated dilution leading to aliquots. The time elapse between points 1 and 2 was especially long, over nine years for the samples of *L. zonalis* (Table 1).

Equipment and methodology corresponded exactly to those described by Boevé et al. (2018). In short, three aliquots of each pooled extract were analysed by high-performance LC-MS/MS. The LC system (Thermo Fisher Scientific, San Jose, California, USA) was equipped with an Accela 1250 pump and an Accela autosampler. Separation of peptides was performed on a C18-HL Alltima column (150 x 2.0 mm i.d., 3 µm; Grace, Deerfield, Illinois, USA) using a linear gradient from 90% H_2O (with 1% CH_3CN and 0.1% HCOOH)/10% CH_3CN to 10% H_2O in 31 min; the flow rate was 0.2 ml min^{-1}.

Standards of the peptides Perg, VPerg, dpPerg and LGln were purchased from Biosyntan GmbH (Berlin, Germany) at > 95% purity. They were analysed by full MS/MS on the $[M+H]^+$

ions by a Q-Exactive mass spectrometer (Thermo Fisher Scientific). Calibration curves were constructed over four concentrations in the range of 1–1000 ng/ml, and proved to be linear, with r^2 values > 0.9990. Peptide concentrations in the samples were determined by comparing the ratio of a peptide peak area in the sample solution with the ratios of its peak areas in the standard solutions. These concentrations (in ng/ml) were converted to micrograms per sample and averaged over the three replicates, the final concentration was expressed in % of larval FW. Thus, this concentration was calculated by using for *A. pagana* the FW of the larva, and for *L. zonalis* the summed FWs of the three larvae from which the hemolymph and organs were pooled to make a sample.

The single sample of integument including cuticle from *L. zonalis* (Table 1) was discarded when peptide concentrations were summed over organs. Wilcoxon signed-rank tests were performed using VassarStats (Lowry 2019).

Results

The average concentration of the four peptides (Perg, VPerg, dpPerg, and LGln) together was 0.333% FW in *L. zonalis* and 0.116% FW in *A. pagana*. The major compounds were 0.204% FW VPerg and 0.117% FW Perg in the former, and 0.080% FW LGln and 0.033% FW Perg in the latter species.

For *L. zonalis*, the concentration of the four peptides was 0.238% FW in the hemolymph, 0.078% FW in the integument by considering the samples of integument without cuticle plus the samples of cuticle, and 0.016% FW in the gut (Fig. 1). For *A. pagana*, it was 0.095% FW in the hemolymph, 0.018% FW in the integument (i.e. including cuticle), and 0.002% FW in the gut. For this species, the concentration of each LGln and Perg was higher in the hemolymph (0.066% FW LGln and 0.027% FW Perg) than in the integument (0.013% FW LGln and 0.005% FW Perg), and higher in the integument than in the gut (0.001% FW LGln and 0.001% FW Perg) (W = 21, P = 0.05 four times, Wilcoxon signed-rank tests, two-tailed; n = 6 larvae). For both species, the ratio between Perg and LGln concentrations was similar across hemolymph and organs (Fig. 1).

Lophyrotoma zonalis larvae contained a similar peptide concentration in the cuticle (0.025% FW VPerg and 0.013% FW Perg) compared with the rest of integument plus the

layer of longitudinal muscles (0.024% FW VPerg and 0.013% FW Perg; Fig. 1). For both species, nearly no peptides were detected in the fat body and salivary glands (Fig. 1). The weight was only quantifiable for the hemolymph of *A. pagana*, and it reached a mean ± standard deviation of 14.5 ± 2.8 mg FW, thus representing 21% of the larval FW (Table 1).

Discussion

The chemical profiles of the hemolymph and integument were dominated by VPerg and Perg in *L. zonalis*, and LGln and Perg in *A. pagana*. These profiles are the same as those of whole larvae of the corresponding species (Boevé et al. 2014). Thus, the distribution of the peptides within the body qualitatively reflects that of a whole larva. In theory, considering either all body compartments together or a whole larva should lead to equivalent peptide concentrations. This, however, was not the case, since the concentration of the summed hemolymph and organs for each species *L. zonalis* and *A. pagana* was lower (0.333 and 0.116% FW, respectively) than from a whole larva (0.719 and 0.162% FW, respectively; Boevé et al. 2014). For each of the species, this difference may be due to variation between populations (Boevé et al. 2014, 2018), to a loss of compounds during collection of the hemolymph and dissection of larvae, and/or to a degradation of compounds during the subsequent storage of samples and pooled extracts. The period between gathering the samples and their extraction was much longer for *L. zonalis* (112 months) than *A. pagana* (10 months), and the loss in peptide concentration was also higher in *L. zonalis* than *A. pagana*. Thus, a long storage period of biological samples in ethanol may cause peptide degradation. In natural conditions, however, these compounds are known to be stable to enzymatic degradation (Oelrichs et al. 2001).

For both species, the highest peptide yields were from the hemolymph, followed by the integument. In *A. pagana*, the FW of the hemolymph represented ca. 20% of the larval FW. By measuring the FW of an *A. pagana* larva just after moulting and of its exuvia, it was found that the exuvia represented ca. 2% of the larval FW (J-L Boevé, unpublished result). These results combined with those gathered for *L. zonalis* lead to the conclusion that the cuticle, although of a low FW, contains high amounts of toxic peptides. This is in agreement with defensive function of the peptides (Oelrichs et al. 1999, Petre et al. 2007). Predators,

187 especially small ones, first come into contact with the insect's integument, and if biting, then
188 with its hemolymph. Toxic peptides in *L. zonalis* and *A. pagana* were detected in low
189 quantities in the gut and some other organs, and nearly none were detected in the
190 excrements (Boevé and Rozenberg 2019), which supports the idea that they play a defensive
191 role against attacking predators. Other peptides with an antimicrobial activity can be found
192 on the cuticle of other hymenopterans (Otti et al. 2014). More research about the toxic
193 peptides is needed to understand their function in relation to their body distribution, as well
194 as whether and, if so, how the compounds are transported within the larval body.
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196 ## Compliance with ethical standards

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

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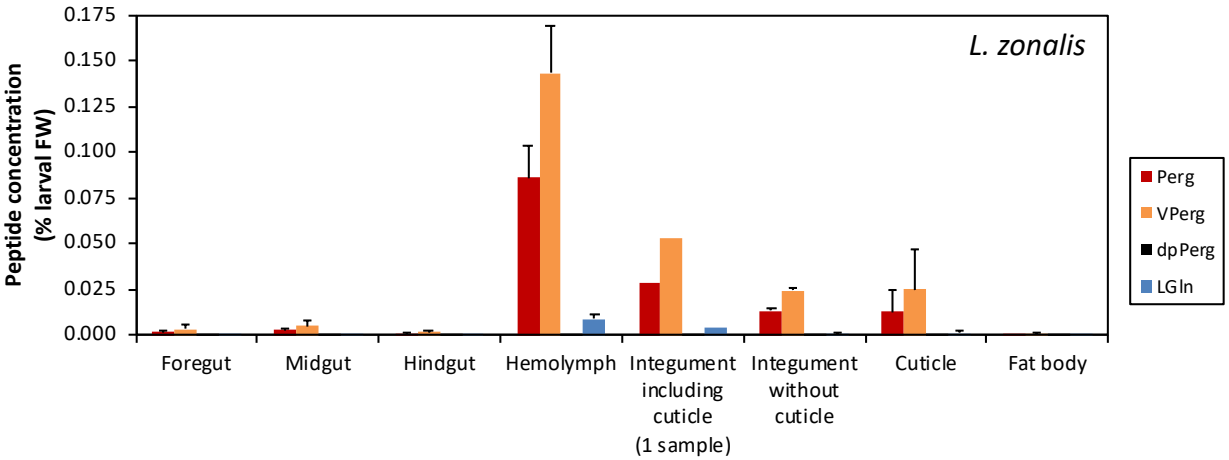
Table 1 Data about the samples of *Lophyrotoma zonalis* and *Arge pagana* sawfly larvae, analysed by LC-MS/MS

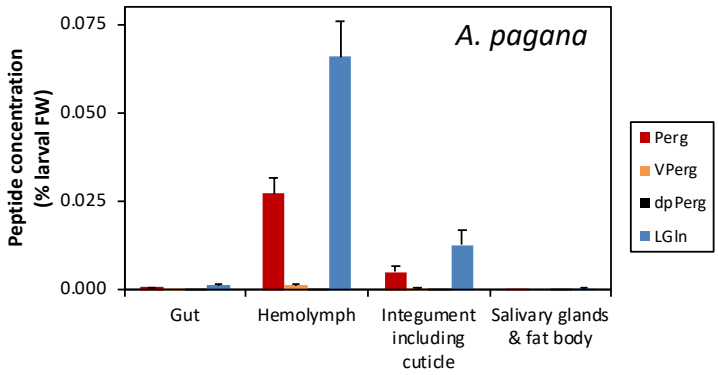
	L. zonalis	***A. pagana***
Larval fresh weight (mg)	152.1 ± 32.8	68.6 ± 14.4
Date of dissection	March–April 2008	September 2016
Gut	–	6 [1]
Foregut	5 [3]	–
Midgut	5 [3]	–
Hindgut	5 [3]	–
Hemolymph	5 [3]	6 [1]
Integument including cuticle	1 [3]	6 [1]
Integument without cuticle	3 [3]	–
Cuticle	3 [3]	–
Salivary glands & fat body	–	6 [1]
Fat body	4 [3]	–
Date of extraction	August 2017	July 2017
Date of dilution	August 2017	July 2017
Date of LC-MS/MS analysis	August 2017	July 2017

The larval fresh weight is given as mean ± standard deviation. The date of dissection corresponds to the period of collecting hemolymph and dissecting organs. For samples of hemolymph, organs or organ parts, the number of samples analysed by LC-MS/MS is given without square brackets, and each sample comprised a number of larvae that is given between square brackets. Note that samples of integument include associated longitudinal muscles. (–) No sample analysed. Dates are given for the extractions leading to pooled extracts, the dilutions leading to aliquots, and the LC-MS/MS analyses

Figure caption

Fig. 1 Peptide concentrations in the larval organs and hemolymph of *Lophyrotoma zonalis* and *Arge pagana*. The peptides are pergidin (Perg), 4-valinepergidin (VPerg), dephosphorylated pergidin (dpPerg), and lophyrotomin (LGln). The data are given as mean and standard deviation of percentage of larval fresh weight (FW). Details about the organs and hemolymph are given in Table 1. Note that in *L. zonalis* a single sample corresponded to the integument including cuticle


L. zonalis
Peptide concentration (% larval FW)
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
Foregut
Midgut
Hindgut
Hemolymph
Integument including cuticle (1 sample)
Integument without cuticle
Cuticle
Fat body
Perg
VPerg
dpPerg
LGIn


A. pagana
Peptide concentration (% larval FW)
0.075
0.050
0.025
0.000
Gut
Hemolymph
Integument including cuticle
Salivary glands & fat body
Perg
VPerg
dpPerg
LGIn