Peptidomics of the Agriculturally Damaging Larval Stage of the Cabbage Root Fly Delia radicum (Diptera: Anthomyiidae)

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Abstract

The larvae of the cabbage root fly induce serious damage to cultivated crops of the family Brassicaceae. We here report the biochemical characterisation of neuropeptides from the central nervous system and neurohemal organs, as well as regulatory peptides from enteroendocrine midgut cells of the cabbage maggot. By LC-MALDI-TOF/TOF and chemical labelling with 4-sulfophenyl isothiocyanate, 38 peptides could be identified, representing major insect peptide families: allatostatin A, allatostatin C, FMRFamide-like peptides, kinin, CAPA peptides, pyrokinins, sNPF, myosuppressin, corazonin, SIFamide, sulfakinins, tachykinins, NPLP1-peptides, adipokinetic hormone and CCHamide 1. We also report a new peptide (Yamide) which appears to be homolog to an amidated eclosion hormone-associated peptide in several Drosophila species. Immunocytochemical characterisation of the distribution of several classes of peptide-immunoreactive neurons and enteroendocrine cells shows a very similar but not identical peptide distribution to Drosophila. Since peptides regulate many vital physiological and behavioural processes such as moulting or feeding, our data may initiate the pharmacological testing and development of new specific peptide-based protection methods against the cabbage root fly and its larva.

Introduction

The cabbage root fly Delia radicum is a serious pest species on cultivated Brassicaceae (e.g. cabbage, turnip, swede) in the temperate holarctic region. Up to 60–90% of untreated brassica crops can be regionally damaged by a cabbage root fly infestation, while average losses of untreated crop may be somewhat above 20% (see [1], [2] for review). The damaging life stage of D. radicum is the larva also known as cabbage maggot. After hatching from eggs deposited at the root base close to the ground, larvae first feed on smaller rootlets. Later on, with growing size, they also attack its host range and is now attacking rapeseed (Brassica napus L.) in several countries, in Germany and Czech Republic since the mid-1990ies [3,4]. Since rapeseed monocultures have increased considerably due to biofuel and oil production, the now available peptide data may initiate the development of new specific peptide-based protection methods against the cabbage root fly and its larva.
based protection methods against the difficult-to-control cabbage root fly.

**Materials and Methods**

**Insects**  
Adult *D. radicum* were reared at 20°C and an L:D cycle of 16:8 in a small flight cage [10]. Both dry and wet food was provided. The dry food consisted of dextrose, skim milk powder, soy flour and brewer's yeast in a 10:10:1:1 ratio. The wet food consisted of honey, soy flower and brewer's yeast in a 5:5:1 ratio, if necessary diluted with water. For egg deposition, small pieces of swedes were placed into the fly cage. Swede pieces with deposited eggs were then transferred to breeding boxes (Phytoa, Basel, Switzerland) filled with autoclaved bird sand to prevent mould. After approximately three weeks the first pupae appeared on the sand's surface and were transferred to the fly cage again where adult flies eclosed after about one week.  

*Drosophila virilis* were raised on standard *Drosophila* medium at 18°C or 25°C at L:D 12:12.

**Peptide Extraction**  
Larval ring glands (RGs), central nervous systems (CNS) and midgut tissue were dissected on ice in HL3 saline (80 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 20 mM MgCl₂, 10 mM NaHCO₃, 5 mM trehalose, 115 mM sucrose, 5 mM HEPES, adjusted to pH 7.2 with HCl; [11]) using fine forceps and scissors. The tissues were immediately transferred into 40–60°C extraction solution (90% methanol, 9% gradient grade water, 1% trifluoroacetic acid (TFA) (v/v/v)) in an Eppendorf low bind tube and incubated for 30 min at room temperature and sonicated for 10 min in an ultrasonic water bath. After that, the samples were centrifuged at 18,000 g for 15 min and the supernatant (peptides dissolved in extraction solution) was transferred to a fresh Eppendorf low bind tube. 10 µl HPLC grade water was added to the extract and methanol was removed by concentrating the sample to 10 µl in a vacuum centrifuge. The concentrated sample was stored at −20°C until further use.

**Peptide Coupling with 4-sulfophenyl Isothiocyanate (SPITC) for LC/MS**  
Based on the method described by Wang et al. [12], the concentrated samples were dissolved in 8 µl solvent (50% acetonitrile, 0.01% TFA, 49.99% HPLC grade water (v/v/v)) and sonicated for 20 min in an ultrasonic water bath. After that, the samples were centrifuged for 15 min at 18,000 g and the supernatant (peptides dissolved in extraction solution) was transferred to a fresh Eppendorf low bind tube. 30 µg/µl SPITC (4-Isothio-cyanatobenzenesulfonic acid, Sigma-Aldrich) was added to yield a 92 mM SPITC solution. Then, 3 µl buffer (136 mM (NH₄)₂CO₃) was added and after incubating for 30 min at 35°C, the sample was concentrated to a volume of 10 µl by vacuum centrifugation. Then, 20 µl of 0.5% acetic acid were added and the sample was subjected to HPLC.

**Capillary RP-HPLC**  
The concentrated unlabelled samples were dissolved in 40–60 µl eluent A (98% HPLC grade water, 2% acetonitrile, 0.05% TFA (v/v/v)) for 30 min at room temperature and sonicated for 20 min in a water bath. After centrifugation for 15 min at 18,000 g, the supernatant was transferred to a fresh low bind Eppendorf tube and injected into an UltiMate 3000 capillary HPLC system ( Dionex, Idstein, Germany) connected to a Proteiner Fraction Collector (Bruker Daltonik GmbH, Bremen, Germany). SPITC-labelled samples were injected in 0.5% acetic acid. The samples were loaded onto a RP C18 trap column (Acclaim PepMap100 C18, 5 µm, 100 Å) with eluent A at a flow rate of 20 µl/min. Then the flow was switched through the trap column and the analytical RP column (Acclaim PepMap100 C18, 5 µm, 100 Å) with a rate of 2 µl/min. Peptides were eluted with a linear gradient from 4%–60% eluent B (80% acetonitrile, 20% HPLC grade water, 0.04% TFA (v/v/v)) in 30 min. 1 µl sample fraction mixed with 1 µl of matrix solution (half-saturated recrystallised 2-cyano-4-hydroxyxymalic acid in 60% acetonitrile, 40% HPLC grade water, 0.1% TFA (v/v/v)) was spotted every 30 s onto a stainless steel MALDI target plate (Applied Biosystems/MDS SCIEX). The sample was concentrated to a volume of 10 µl in a vacuum centrifuge. HPLC grade water was added to the extract and methanol was removed by concentrating the sample to 10 µl in a vacuum centrifuge. The concentrated sample was stored at −20°C until further use.

**MALDI TOF MS/MS**  
Masses were analysed with a 4800 Plus MALDI TOF/TOF Analyser (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) at a laser wavelength of 355 nm. Settings like laser intensity and the number of sub-spectra per plate spot varied among the samples and were adjusted individually. The device was calibrated with a peptide calibration standard (Applied Biosystems Calibration Mixture 2). Peptides from the LC/MS samples were fragmented by post-source decay (PSD). For direct tissue profiling, both PSD and collision-induced dissociation (CID) were applied depending on sample condition. MS/MS spectra were interpreted using Data Explorer 4.10 software (Applied Biosystems/MDS SCIEX, Foster City, CA, USA).

**Data Base Entry**  
The peptide sequences have been submitted to the Uniprot database (http://www.uniprot.org/); accession numbers are listed in Table 1.

**Immunostainings**  
CNS with and without RG attached were dissected on ice in HL3 saline and immediately fixed in 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS), pH 7.1 for 3.5 h at 4°C. Afterwards tissues were washed 5 times for 10 min in PBT (0.1 M PBS with 0.3% TritonX) on a shaker at room temperature (RT). Precipitation with 10% normal goat serum (Dianova, Hamburg, Germany) in PBT for 4 h at RT on a shaker was followed by the incubation with primary antiserum diluted in PBT and 10% normal goat serum for 2 days at RT on a shaker. The following polyclonal rabbit primary antibodies were used: anti-Dip-AST-A (kind gift of Hans Agricola, Jena, Germany [13]) diluted 1:5000, anti-RFamide (kind gift of Eve Marder, Brandeis, USA [16]) diluted 1:4000, anti-SIFa (kind gift of Peter Verleyen and Liliane Schoofs, Leuven, Belgium [17]) diluted 1:300, anti-DH31 (kind gift of Jan Veenstra, Bordeaux, France [18]), anti-Lem-Tachykinin-related peptide (kind gift of Dick Nessel, Stockholm, Sweden [19]), anti-MIP and anti-PRXa (kind gift of Manfred Eckert, Jena, Germany).
Table 1. Sequences, accession numbers and tissue distribution of the peptides characterised in *D. radicum* larvae.

| Peptide | Sequence* | Mass [M+H]+ | UniProt Accession | CNS | tsPOSb | aPSOsb | SPITC-labeledc | detected in adults [9] |
|---------|-----------|-------------|-------------------|-----|--------|--------|---------------|------------------------|
| **A-type allatostatins** | | | | | | | | |
| AST-A909 | ARPYSFGLa | 909.50 | B3EWJ2 | Y | Y | Y | Y |
| AST-A917 | LPVYNFGLa | 921.43 | B3EWJ8 | Y | Y | Y | Y |
| AST-A922 | NRPYSFGLa | 952.49 | B3EWJ3 | Y | Y | Y | Y |
| AST-A953 | VERYAFGLa | 953.53 | B3EWJ4 | Y | Y | Y | Y |
| **C-type allatostatins** | | | | | | | | |
| AST-C | pQVRYRQcYFNPySdcF | 1904.90 | B3EWJ5 | Y | Y | | Y |
| AST-C | QVRYRQcYFNPySdcF | 1921.87 | B3EWJ6 | Y | Y | | Y |
| **FMRFamide-like peptides** | | | | | | | | |
| FMRFa805 | GDNFMRFa | 885.42 | B3EWJ7 | Y | Y | | Y |
| FMRFa809 | GQDFMRFa | 899.42 | B3EWJ8 | Y | Y | | Y |
| FMRFa825 | PDNMRFa | 925.44 | B3EWJ9 | Y | Y | | Y |
| FMRFa832 | GGNDMRFa | 942.44 | B3EWK0 | Y | Y | | Y |
| FMRFa837 | EQDFMRFa | 971.50 | - | - | Y | | Y |
| FMRFa896 | PGQDFMRFa | 996.48 | B3EWK1 | Y | Y | | Y |
| FMRFa1067 | APQGDFMRFa | 1067.51 | B3EWK2 | Y | Y | | Y |
| FMRFa1077 | TPGQDFMRFa | 1097.60 | B3EWK3 | Y | Y | | Y |
| FMRFa1154 | SAGQDFMRFa | 1154.54 | B3EWK4 | Y | Y | | Y |
| FMRFa1181 | LPEQDFMRFa | 1181.60 | B3EWK5 | Y | Y | | Y |
| FMRFa1185 | SAQQDFMRFa | 1185.53 | B3EWK8 | Y | Y | | Y |
| **Yamides** | | | | | | | | |
| Ya | LPSIGHY | 948.50 | B3EWK9 | Y | Y | | Y |
| **Kinins** | | | | | | | | |
| Kinin | N$VVLGK$KQQRFHSWG | 1741.40 | B3EWL0 | Y | Y | | Y |
| **putative CAPA-peptides** | | | | | | | | |
| CAPA-pyrokinin | AGPSATGWWGFRLa | 1515.81 | B3EWL1 | Y | Y | Y | Y |
| CAPA-pyrokinin2$^{2-10}$ | GPSATGWWGFRPLa | 1444.78 | B3EWL2 | Y | Y | Y | Y |
| CAPA-periviscerokinin-1 | GGGGTSGLFAFPRVa | 1321.72 | B3EWL3 | Y | Y | | Y |
| CAPA-periviscerokinin-2 | AGFLAQQPRLa | 971.59 | B3EWL4 | Y | Y | | Y |
| **putative HUGIN-peptides** | | | | | | | | |
| HUG-pyrokinin | SVOQKPRLa | 973.59 | B3EWL5 | Y | Y | | Y |
| **short neuropeptide Fs** | | | | | | | | |
| sNPF-1$^{6-11}$ | SPSLRLRFa | 974.61 | B3EWL6 | Y | Y | | Y |
| sNPF-1 | AQRSPSRLRFa | 1329.80 | B3EWL7 | Y | Y | | Y |
| **Myosuppressin** | | | | | | | | |
| Myosuppressin | T$D$VHQ$V$FLRFa | 1247.70 | B3EWL9 | Y | Y | | Y |
| Myosuppressin$^{7-10}$ | D$V$DHQ$V$FLRFa | 1146.59 | B3EWM0 | Y | Y | | Y |
| **Corazonin** | | | | | | | | |
| Corazonin | p$Q$TFQYSRGWTNa | 1369.69 | B3EWM1 | Y | Y | | Y |
| Corazonin$^{11-11}$ | FOQ$S$RGWTNa | 1157.56 | B3EWM2 | Y | Y | | Y |
| **SiFamides** | | | | | | | | |
| SiFa | AYRPKFPGNSiFa | 1395.74 | B3EWH1 | Y | Y | | Y |
| **Sulfakinins** | | | | | | | | |
| Sulfakinin | GE$E$QF$D$GY$G$HRFa | 1686.68 | B3EWM3 | Y | Y | | Y |
| Sulfakinin$^{12-14}$ | F$D$GY$G$HRFa | 1186.52 | B3EWM4 | Y | Y | | Y |
| **Tachykinin-related peptides** | | | | | | | | |
| TK$^{12-12}$ | TPTAFYGVRa | 1010.55 | B3EWM5 | Y | Y | | Y |
Scanning Electron Microscopy

After three washing steps with PBT, the samples were incubated with affinity-purified goat-anti rabbit or goat-anti mouse Cy3 or Cy5 IgG (Jackson Immunoresearch, Pa., USA) diluted 1:100 in PBT and 10% normal goat serum for 2 days in constant darkness.

Autofluorescence was reduced by three 10 min changes in 100% acetone, and samples were briefly dipped into chloroform and fixed further in 5% glutaraldehyde as above overnight. After washing, the samples were postfixed for 2 h in osmiumtetroxide (1% in 0.1 M Sörensen buffer, pH 7.2). Fixed samples were washed in Sörensen buffer, dehydrated in ethylene-glycol monoethylether over night followed by three 10 min changes in 100% acetone, and critical-point-dried using a Poloran E3000 (Balzer Union). Afterwards, samples were sputtered with gold particles with a sputter coater (Balzer Union), and then examined on a Hitachi S-550 scanning electron microscope.

Results

LC-MS/MS of Ring Gland Extracts

To characterise the sequence of \emph{D. radicum} neuropeptides, we started with an LC-MS/MS analysis of extracts from 10–40 pooled ring glands (2 runs without, 4 runs with SPITC labelling). Automatic PSD peptide fragmentation was based first on a mass list containing the masses obtained by direct profiling (see below) and masses of biochemically identified \emph{Drosophila} peptides, and subsequently on signal intensity. Some of the extracted peptide samples were coupled with 4-sulphophenyl isothiocyanate (SPITC) to direct fragmentation towards y-fragments [12,22]. The selective enhancement of y-fragments after SPITC labelling strongly decreases the complexity of PSD fragmentation patterns. This facilitates the interpretation of fragment spectra in general [23], and also improved de novo sequencing of \emph{D. radicum} peptides considerably.

The LC/MS-analysis revealed the presence of HUG-PK, sNPF-1, sNPF-1\textsuperscript{4–11}, AKH, AKHGK (a processing intermediate of AKH), myosuppressin and corazonin in the ring gland. All peptide sequences were validated by fragmentation (see Table 1). Interestingly, we found and fragmented the [M+H]\textsuperscript{+} adduct of AKH (975.5 Da, Fig. S1), which in \emph{Drosophila} and other insects is only found as a sodium or potassium adduct (e.g. [24,25,26]). SPITC labelling of an unknown peptide ion with the mass of 948.5 Da yielded a full y-fragment spectrum (Fig. 1). Since leucine and isoleucine are mass-identical and cannot be distinguished based on y-fragments, this fragment spectrum indicates the amino acid sequence (L/I)PS(L/I)GHYYamide. The C-terminal amidation is a unique modification of bioactive neuropeptides [27], hence the sequence and occurrence in the ring gland suggest that this peptide -designated here as Yamide- may be stored and released as a bioactive peptide hormone. Yamide shows no sequence-similarity with any hitherto sequenced insect peptide, suggesting it constitutes a new insect peptide family.

Neuropeptides from the Central Nervous System

For identification and sequence analysis of peptides from the larval CNS, we performed LC-MS/MS of a SPITC-labelled and an unlabeled extract of 40 CNS with attached ring glands. Peptides were then identified by aligning the measured fragment-
Figure 1. MS/MS spectrum of Yamide. A+B) SPITC-labelled; C) unlabeled. A+B) The fragment spectrum was divided, therefore the relative intensities vary. y-fragments are labelled with blue lines, b-fragments with green lines. Internal and a-fragments are shown as well.

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ation patterns with known peptides from *Drosophila* [25,28] and adult *D. radicum* [9] as well as through manual *de novo* fragment annotation. The data revealed the presence of three A-type allatostatins, one C-type allatostatin with and without N-terminal pyroGlu, 10 FMRFa-like peptides, Yamide, CAPA-PK, CAPA-PK \(^{2,15}\), HUG-PK, CAPA-PVK-1 and -2, sNPF-1 and sNPF-1\(^{1-11}\), myosuppressin, myosuppressin\(^{8-10}\), SIFamide, sulfakinin, sulfakinin\(^{6-14}\), corazonin and corazonin\(^{3-11}\), two tachykinin-related peptides and a peptide very similar to *Drosophila* APK [28]. The sequences of these peptides are given in Table 1, the fragmentation spectra are shown in Fig. S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14, S15, S16, and S17. Since sequences of *D. radicum* prepropeptide genes or ESTs are not available, it is difficult to rationally assign numbers for the different paracopies of the multicopy peptide families AST-A, tachykinin-related peptides and FMRFamides. As a neutral system, we therefore refer to the peptides with their mass as index (e.g. AST-A\(^{909}\) instead of AST-A-1). Instead of the typical C-terminal sequence PRVa, CAPA-PVK-2 from *D. radicum* ends on PRLamide, which has hitherto only been observed in the closely related flesh fly *Neobellieria bullata* as well as locusts [29]. Additionally, we also yielded the sequence of a kinin from direct peptide profiling and fragmentation of ventral ganglion fragments (Fig. 2). This kinin is sequence-identical to the kinin of *Drosophila* species [30].

**Peptides from the Midgut**

Two LC-MS/MS runs of peptide extracts from 20 and 25 *D. radicum* larval midguts respectively led to the identification of four A-type allatostatins, one C-type allatostatin occurring with and without N-terminal pyroGlu, two tachykinin-related peptides and CCHamide\(^{1}\) (Table 1). AST-A\(^{909}\) and AST-A\(^{953}\), the AST-C and the two tachykinins had also been detected in the CNS by LC-MS/MS. AST-A\(^{921}\) has been found in the CNS by Audsley and colleagues [9]. All but one midgut peptide can thus be classified as brain-gut peptides. CCHamide\(^{1}\) was exclusively detectable in the midgut, but represents a brain-gut peptide in *Drosophila melanogaster* [31] and may have escaped detection in the *D. radicum* CNS.

**Direct Peptide Profiling and Fragmentation of Peptide Hormones from Neurohemal Organs**

To identify potential neuropeptide hormones among the characterised peptides, we performed direct peptide profiling of isolated neurohemal tissues from individual larvae. The neurohemal organs associated with the CNS are the major source of neuropeptide hormones in insects. They consist of the corpora cardiaca (CC, containing terminals of secretory neurons with somata in the pars lateralis and pars intercerebralis), and the thoracic and abdominal perisympathetic organs (PSOs, containing terminals of secretory neurons with somata in the thoracic and abdominal neuromeres respectively). The CC also comprise an endocrine compartment containing the adipokinetic hormone (AKH)-producing cells. Scanning electron microscopy shows that the morphology of these organs in *D. radicum* larvae is typical for a cyclorrhaphan (Fig. 3): the CC are fused with the corpora allata and prothoracic gland and form a ring gland (Fig. 3A). Each thoracic neuromere shows a blind-ending thoracic PSO at its dorsal surface as also shown for *Drosophila* and *Calliphora* [32,33]. Unlike *Drosophila*, however, *D. radicum* appears to have four instead of three abdominal PSOs, visible as swellings of the median/transverse nerves (Fig. 3B).

Earlier studies in *Drosophila* and other flies showed that direct mass spectrometric profiling of neurohemal organs leads to specific extraction and detection of peptides, while non-peptidergic signals are largely absent (e.g. [24,25,30,34,35]). A typical direct profile of a larval ring gland is shown in Figure 4A. The masses of 948.5 Da, 974.5 Da, 997.4/1013.4 Da, 1247.6 Da, 1329.8 Da and 1369.6 Da correspond to Yamide, sNPF-14–11, AKH (Na\(^+\) and K\(^+\) adduct), myosuppressin (MS), sNPF-1 and corazonin from the ring gland of various *Drosophila* species [25,30]. *D. radicum* CAPA-PK\(^{2-15}\) (1444.7 Da) and, with much less intensity, CAPA-PK (1515.2

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**Figure 2.** MS/MS PSD spectrum of kinin, obtained by direct profiling of a piece of the ventral ganglion.
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Da) were consistently abundant. Also the AKH processing intermediates AKHGK (1161.6 Da) and AKHGKR (1317.6 Da) could consistently be detected, as well as *D. radicum* HUG-PK (973.6 Da) previously identified by Audsley and colleagues [9] in the adult CC. Subsequent direct PSD/CID fragmentation confirmed the identity of these peptides and the sequence data obtained by LC-MS/MS of CNS extracts (see Table 1). Further consistently detected masses were 939.4 Da, 955.4 Da, 1121.6 Da, 1125.5 Da, 1141.5 Da, 1143.6 Da and 1259.6 Da. None of these masses could be sequenced by direct fragmentation. The monoisotopic peak distribution however suggests that these masses represent peptides which thus remain to be characterised.

The PSOs are very small structures that are very difficult to separate from the larval CNS. Their homolog in adult cyclorhaphan flies is the dorsal sheath of the adult thoracico-abdominal ganglion (TAG) [32,33,36,37,38,39] which is much easier to dissect. A typical profile of this adult dorsal sheath is shown in Figure 4B (anterior “thoracic” region) and Figure 4C (posterior “abdominal” region).

The profiles of the thoracic region showed many different mass peaks, most of which corresponded to FMRFa-like peptides, while the posterior region is enriched in masses corresponding to CAPA peptides. With the exception of FMRFa1099, all FMRFamide-like peptides identified by LC-MS/MS in whole CNS extracts could also be detected in the thoracic dorsal sheath preparation. This may suggest that FMRFa1099 represents a degradation or processing intermediate of FMRFa1115. A consistent mass peak of 971.5 Da indicates the presence of FMRFa1071 (EQDFMFRA) reported from adult *D. radicum* [9]. This peptide had not been found by LC-MS and could not be fragmented. Also APSQDFMRFa with an oxidised mass of 1113.5 Da characterised by Audsley et al. [9] from adult cabbage root flies was not found by LC-MS/MS of CNS extracts. However, a matching mass peak consistently occurred in direct profiles of the thoracic preparation but could not be fragmented - it may thus equally well represent the oxidised form of the mass-identical TPGQDFMRFa ( = FMRFa1097). The peaks corresponding to FMRFa1097 and FMRFa1154 gave higher signal intensities than other FMRFa-like peptides, suggesting that the peptides are encoded in three and two copies in the *fmrf* prepropeptide gene respectively (e.g. [24,25]). Alternatively, if APSQDFMRFa also occurs in the larva albeit undetected, the peak at 1097.6 Da represents the integrated intensity of both APSQDFMRFa and TPGQDFMRFa.

The profiles of the abdominal dorsal sheath preparation only showed four peaks, corresponding to CAPA-PVK-1 and -2, CAPA-PK and a mass of 2217.2 Da. The same preparation in other fly species show also three CAPA peptides [24,34] plus-at least in *Drosophila* species- a non-amidated cleavage product in the 2200 Da range (CAPA precursor protein B (CPPB)) [25,30]. While a direct fragmentation could not be achieved, it is therefore likely that the peak at 2217.2 Da represents the CPPB of *D. radicum*.

**Distribution Pattern of Peptidergic Cells**

To compare the general cellular architecture of peptidergic systems in *D. radicum* larvae with that of other flies, we performed immunofluorescent stainings with a host of peptide antisera.

**Peptidergic neurons in the CNS and ring gland.** AST-A IR: Clusters of AST-A IR cell bodies and descending neurites are prominent in the brain and ventral ganglion (Fig. 5A-C), and are highly similar in number and morphology to the bilateral pairs of AST-A PMP, LP and LT neurons in the brain, and the DMA, VMA, LA and LAa neurons in the ventral ganglion of larval *Drosophila* [40,41]. *D. radicum* has, however, further pairs of AST-A IR brain neurons, e.g. in the posterior protocerebrum (Fig. 5B). Like in *Drosophila*, the LAa neurons send neurites to the hindgut through segmental nerve 8/9 and the ring gland is devoid of AST-A IR; neurites projecting towards the ring gland could not be detected (Fig. 5A). Also in adult *Calliphora*, posterior AST-A IR LAa-like neurons innervate the hindgut, and the CC are devoid of AST-A IR [42].

**SIFamide IR:** Two pairs of strongly SIFamide-immunoreactive somata are located in the pars intercerebralis (Fig. 5D-E). Their axons project to contralateral parts of the protocerebrum and descend through the entire ventral ganglion. Thus, the pattern is identical to that of the larval SIFamide neurons in *Drosophila*.
Figure 4. Typical spectra from direct peptide profiling. (A) Profile of the larval ring gland, (B) the anterior (thoracic), and (C) posterior (abdominal) portion of the adult dorsal sheath of the TAG. For some peptides, sodium and potassium adducts are visible besides the typical [M+H]+ adducts.

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the ring gland staining represents HUG-PK and CAPA-PK 2–15, pattern was very similar to that described in the stained a complex pattern of somata with broad arborisations in further very weakly stained cells at the midline. D-E) Sfia immunoreactivity. D) Dorsal overview (maximum projection) of the two pairs of Sfia neurons in the protocerebrum, that send contralaterally descending fibres through the ventral ganglion. E) Close-up showing the arborisation pattern of the descending fibres. Somata are marked by arrows. F-G) FMRFa-like IR. F) Dorsal overview (maximum projection) of the whole CNS. Immunoreactive somata are most prominent in the dorsal protocerebrum and the thoracic neuropeutic neurons. G) Detail of the thoracic neuropeutic neurons. The thoracic PSOs are strongly stained in a varicose fashion likely due to stored peptide vesicles (asterisks). A pair of strongly stained TNV neurons is visible in each neuromere (arrows), H-I) PG-1-like IR. H) Dorsal overview (maximum projection) of the whole CNS. Immunoreactive somata are most prominent in the dorsal protocerebrum, suboesophageal ganglion and the anterior abdominal neuromeres. I) Detail showing the ventral ganglion with four pairs of strongly stained median/transverse nerves in the anterior abdominal neuromeres. The swellings along the median and transverse nerves represent the abdominal PSOs (arrows). J) Detail showing the brain, ring gland and suboesophageal ganglion. Somata in the protocerebrum are visible. The strong immunoreactivity in the ring gland is due to innervation by the CC-MS neurons (arrows) in the suboesophageal ganglion. K) Detail showing the ventral ganglion with a pair of strongly stained neurons (arrows) anterior to the Va neurons. This pair seems to be a different cell type than the Va neurons due to differences in shape and the lack of neurohemal projections. L) Detail showing the ventral ganglion with four pairs of strongly stained Va neurons (arrows) in the anterior abdominal neuromeres.

Figure 5. Wholemount immunostainings of the larval CNS. A-C) AST-A immunoreactivity. A) Dorsal overview (maximum projection). Arrows point to prominently stained somata in the ventral ganglion. Exit neurites innervating the hindgut are marked by an asterisk. B) Detail of the dorsal protocerebrum showing a pair of strongly labelled PMP-like neurons (asterisks) and a strongly labelled neuron not described in Drosophila (arrow). C) Detail of the thoracic and first abdominal neuromeres, showing the strongly labelled MA (asterisks) and LA (arrows) neurons, and the used antiserum recognises not only FMRFamide-like peptides, but (less strongly) also other peptides with a C-teratal RFamide (sNPF, sulfakinin, myosuppressin, neuropeptide F). Relatively weak FMRFamide IR is visible in the ring gland and in bilaterally symmetric somata in the pars intercerebralis (Fig. 5F-G). Since sNPF-1 and myosuppressin are the only RFamides found via mass spectrometry in the ring gland, at least part of this IR is likely to be attributable to sNPF-containing secretory or myosuppressin neurons. A pair of strongly stained secretory neurons is visible in each of the three thoracic neuromeres (Fig. 5G). These neurons innervate the thoracic PSOs and appear to be homolog to the FMRFamide-like peptide expressing neurons of Drosophila melanogaster [41,43]. Similar neurons have also been described in larvae of Lucilia cuprina [35], Sarcophaga bullata [39] and Calliphora erythrocephala [33].

**PRXamid IR:** PRXamide IR labels pyrokinins and periviscerokinin ones ending on either PRLa and or PRVamid [21]. Prominent PRXamide IR is visible in the CC part of the ring gland, and the abdominal PSOs/transverse nerves 1–4 (Fig. 5H-L). The MS data and the situation in Drosophila melanogaster [23] indicates that the ring gland staining represents HUG-UK and CAPA-PK7–15, while the asP staining represents both CAPA-PK and CAPA-PVKs. Large neurosecretory cells (Fig. 5J) in the suboesophageal neuromeres -like highly homologous of the huin-expressing CG-MS-1 and capa-expressing CC-MS-2 cells of Drosophila [44,45,46]- provide the immunoreactivity of the ring gland. Each of the four abdominal PSOs (see Fig. 3) is innervated by a pair of neurones homolog to the Va neurons of Drosophila (Fig. 5K-L, [47]). The number of Va neuron pairs thus matches that of abdominal PSOs like in Drosophila melanogaster larvae which, however, only have three PSOs and Va neuron pairs respectively [32].

**Diuretic hormone-31 (DH31) IR:** The antiserum against DH31 stained a complex pattern of somata with broad arborisations in both the brain and ventral ganglion (Fig. 6A-C). Again the overall pattern was very similar to that described in the Drosophila melanogaster maggot [48]. The ring gland is innervated by DH31-positive neurites that most likely originate from somata in the pars intercerebralis. Unlike most peptidergic terminals that end in the CC tract, these DH31-immunoreactive neurites end in a neurite meshwork in the region of the corpora allata. This opens the possibility that DH31 may play a role in the regulation of juvenile hormone synthesis or release.

**Myoinhibiting peptide (MIP) IR:** The pattern of MIP immunoreactivity in the brain and ventral ganglion (Fig. 6D-F) was again strongly reminiscent to the situation in Drosophila melanogaster [49]. Strongly stained neurons and descending neurites are prominent in the brain (Fig. 6D). In the protocerebrum, one pair of neurites projects contralaterally dorsal to the foramen (Fig. 6E). One pair of median cells in the suboesophageal neurones, and a pair of lateral cells in the thoracic and all but the last abdominal neurones are stained (Fig. 6F). In larval Drosophila melanogaster, similar cells express also CCAP [50]. MIP immunoreactivity also occurred in intrinsic endocrine cells of the glandular CC part of the ring gland (Fig. 6E). Since MIP's could not be detected in the ring gland by mass spectrometry in D. radicum and other fly species [9,25,30,51], and since in all insects the intrinsic endocrine cells produce AKH, it seems unlikely that the MIP IR in the ring gland represents the occurrence of MIPS. The antiserum recognises the C-terminus of Pea-MIP (GGWamide), and we thus rather assume a cross-reaction with AKH (ending Wamide) in the ring gland, while the staining in the CNS is more likely to be MiP-specific.

**Tachykinin-related peptide (TK) IR:** Bilaterally symmetric TK-immunoreactive cells are situated in both brain and ventral ganglion (Fig. 6G-H). No TK-IR was observed in neurohemal organs. Again, the number, pattern of somata and projections is very similar to the situation in Drosophila [19,52] and also the blowfly Calliphora vomitoria [53], with prominent descending neurites originating in the brain and running along a lateral tract throughout the ventral ganglion. Unlike in Drosophila and Calliphora, however, TK-immunoreactive somata were not discernible in the suboesophageal ganglion.

**Pigment dispersing factor (PDF) IR:** Two different clusters of PDF immunoreactive neurons could be observed (Fig. 6I-K). One cluster of four cells is located in each half of the brain, sending projections to the dorsal protocerebrum and to the putative larval optic neuropile (Fig. 6J). The number, morphology and PDF IR identity these cells as homologs of the lateral neurons (LNs) of larval Drosophila melanogaster [54]. The second group of PDF-immunoreactive cells is located in abdominal neurones 8 and 9, again identical to the situation in Drosophila melanogaster [41,54]. Like in the fruit fly [54], their axons exit the ventral ganglion through segmental nerve a8 and innervate the hindgut (Fig. 6K, Fig. 7A). Both LNs and the abdominal neurons also occur in the housefly, though additional PDF-immunoreactive neurons neither found in Drosophila melanogaster nor D. radicum have been described for larval Musca domestica [55].

**Peptidergic endocrine cells.** Tachykinin-like-immunoreactive endocrine cells (EECs) occurred in a region possibly presenting the anterior-middle midgut junction, and scattered throughout the posterior midgut (Fig. 7B). In larval
Figure 6. Wholemount immunostainings of the larval CNS. A-C) DH31 immunoreactivity. A) Dorsal overview (maximum projection). Large strongly stained somata in the protocerebrum and further smaller cells are visible, as well as paired lateral neurons in the ventral ganglion. B) Detail of the protocerebrum and ring gland. Several DH31-immunoreactive fibres project over the whole ring gland and branch intensely around the corpora.
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Drosophila melanogaster, tachykinin-like immunoreactive EECs have only been found in the posterior midgut; more anterior parts seem to be devoid of tachykinin-like IR [52,56]. Both in Drosophila and D. radicum larvae, the highest density of Tk-IR cells in the posterior midgut is seen in the short portion closest to the hindgut. 

Like in Drosophila melanogaster [40], AST-A-immunoreactive EECs are located in the posterior midgut, and AST-A-IR neurites from the CNS innervate the hindgut (Fig. 8A). The AST-A-IR EECs are apically elongated and teardrop-shaped, thus reminiscent of the typical structure of open type EECs (Fig. 8A).

Strongly stained myoinhibitory peptide (MIP)-IR cells are densely located in a relatively short midgut portion possibly representing the anterior-middle midgut junction (Fig. 8B). Thus, these cells are another common attribute of D. radicum and Drosophila larvae. Smaller MIP-IR EECs occur in the middle and posterior midgut, whereas in Drosophila these portions of the gut show only weak MIP immunoreactivity.

Numerous diuretic hormone 31 (DH31)-IR EECs are located in the posterior portion of the anterior midgut, where they are largest and show open EEC type-like cytoplasmic extensions (Fig. 9). Smaller and more roundish DH31-IR cells are also found in the middle midgut and posterior midgut (Fig. 9).

In general, the pattern of immunoreactivity in the larval midgut is very similar to that described in Drosophila melanogaster for AST-A [40,56], while smaller differences occur for TK-, MIP-and DH31-immunoreactive EECs. Like in the fruit fly larva [56], also the larval D. radicum hindgut is innervated by PDF-immunoreactive neurites which do not reach the midgut (Fig. 7A).

Yamide is also Present in Drosophila Species and Represents an Eclosion Hormone-related Peptide

C-terminal amidation is a unique modification of regulatory peptides, and is generated from a C-terminal glycine residue by a specific set of enzymes occurring in peptidergic cells [27]. Peptides originating from the break-down of proteins therefore do not carry an amidation signal. The presence of a Yamide signal in direct profilings of the neurohemal ring gland suggests that this peptide is released as a neurohormone, while its amidation may suggest bioactivity. Since peptides are evolutionarily strongly conserved, it would be surprising if Yamide, a peptide without published homologs in other insect species, only occurred in D. radicum. An unrestricted blast search based on the sequence LPSIGHYa identified a highly similar sequence only for Drosophila species outside the melanogaster group (Fig. 10A). The identified sequence in the non-melanogaster fruitflies represents a short peptide stretch of the respective eclosion hormone precursor. This stretch is N-terminally joined to the signal peptide, and C-terminally extended by KR, the processing signal for prohormone convertases. It is also present in the EH prepropeptide of melanogaster fruitflies and the relatively closely related mosquitoes and moths, yet with a differing sequence and without an amidation signal (Fig. 10A). This predicts that for the Drosophila species outside the melanogaster group, a Yamide is produced during the normal processing of eclosion hormone. In Drosophila, eclosion hormone is stored and released from the ring gland, which predicts that also Yamide should be stored in this neurohemal organ. To test this, we directly profiled the ring gland of wandering L3 larvae of Drosophila virilis by MALDI-TOF MS. In all preparations (n = 16), a prominent peak of the predicted mass 785.43 Da was detectable (Fig. 10B). Tandem MS of this peak yielded a complete fragmentation spectrum indicating the sequence LPSIGHYa and thus confirming the presence of Yamide in Drosophila virilis (Fig. 10C).

Discussion

We have chemically characterised 38 peptides (including variants of different size and N-terminal pyroglutamination) from the nervous system, neurohemal organs and midgut of larval D. radicum. Of these peptides, sNPF [57], HUG-pyrokinin [58], kinins [59,60,61,62], CAPA-PVKs [44,63], AST-A [64] and AKH [65,66,67] have important effects on feeding and diuresis in Drosophila and other Dipterans. Since it is likely that the function of these peptides is conserved within the Diptera, their signalling pathways are potential targets for a chemical control of D. radicum larvae. The available peptide sequence data for D. radicum maggots and adults now allow physiological and pharmacological studies with native peptides in this species, and may possibly provide a platform for the future development of peptide-based protectants against cabbage maggot infestation.

The SPITC Labelling Approach Strongly Improved MALDI-TOF/TOF De Novo Sequencing

Peptide fragmentation by mass spectrometry has largely substituted traditional peptide sequencing methods since it in principle allows de novo sequencing of peptides from very little material. A caveat for MALDI-based mass spectrometric peptide fragmentation is that the obtained fragmentation patterns can be very complex due to the many different types of fragments that are generated. These include N- and C-terminal fragments, immuno- nium ions and internal fragments, sometimes accompanied by satellite peaks caused by the loss of water or ammonia. In species with sequenced genome this is rarely a problem, since the whole fragment spectrum can be predicted from the respective candidate gene. Not surprisingly, most insect species with characterised
Figure 7. PDF and tachykinin-like immunoreactivity in the larval gut. A) PDF-immunoreactive neurites innervate the hindgut, but do not reach the midgut or Malpighian tubules. B) Tachykinin-like-immunoreactive EECs are visible in a region possibly representing the anterior-middle midgut junction (asterisk). Further immunoreactive cells are scattered throughout the posterior midgut. The most posterior midgut portion is shown enlarged in the inset. PV = proventriculus, aMG = anterior midgut, pMG = posterior midgut. Scale bars = 150 μm.

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Figure 8. AST-A and MIP (AST-B) immunoreactivity in the larval gut. A) AST-A immunoreactive EECs are located in the posterior midgut, their typical tear-drop like shape is visible in the insets. On the hindgut, AST-A immunoreactive neurites are labelled (asterisks). B) MIP-immunoreactive EECs are densely located in the anterior middle midgut (inset), smaller cells are visible throughout the posterior middle and posterior midgut. PV = proventriculus, aMG = anterior midgut, pMG = posterior midgut, HG = hindgut. Scale bars = 150 μm.

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Figure 9. DH31 immunoreactivity in the larval gut. Parts of the midgut containing stained EECs are enlarged in the insets. DH31-immunoreactive EECs are located throughout the middle and posterior midgut, and are also densely distributed around the presumptive anterior-middle midgut junction. PV = proventriculus, aMG = anterior midgut, pMG = posterior midgut, HG = hindgut. Scale bars = 150 µm. doi:10.1371/journal.pone.0041543.g009
Figure 10. Yamide in Dipterans and moths. A) Alignment of the eclosion hormone prepropeptides of different fly, mosquito and moth species with D. radicum Yamide (aligned at position 30), generated with Jalview 2 [77]. The predicted signal peptide cleavage site locates at aligned position 29. In all Drosophila species, the Yamide-aligning sequences are followed by a dibasic cleavage site (KR, at aligned position 40), which is absent in mosquitoes and moths. An amidation signal (G) precedes this cleavage sites in fruitflies outside the melanogaster group, while the flies of the melanogaster group possess the sequence TH instead. B) Direct mass spectrometric profile of the ring gland of a wandering third instar larva of Drosophila virilis. Several mass peaks are visible, which above 900 Da represent known neuropeptides. The mass peak at 785.45 corresponding to Drosophila virilis Yamide typically showed a high relative intensity comparable with that of the abundant sNPF-14–11 and AKH peptide hormones. C) Combined post-source and collision-induced decay spectrum of the mass peak at 785.45 Da reveals the identity of Drosophila virilis Yamide.
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peptidomes belong to those (still rather rare) species with a sequenced genome. Considerable peptidomic data for species without sequenced genome or EST data banks exist only for large insects such as cockroaches, locusts and blowflies for which enough peptide could be extracted for traditional Edman sequencing (see [60]) or de novo mass spectrometric sequencing [69,70,71]. Since SPTC labelling directly fragmentates towards y-fragments [12,22], it strongly decreased the complexity of the PSD fragmentation pattern in this study. This allowed us to characterise a substantial (so clearly not the full) complement of peptides present in the comparatively small cabbage root fly larvae for which no genomic or suitable EST sequences are available. Our results confirm and extend previous results from adult cabbage root flies [9], suggesting that the neuropeptide complement does not change quantitatively between the maggot and adult fly. Also in Drosophila, the peptide complement does not change qualitatively during postembryonic development [24,25,28]. Nevertheless, our sequence data differ from those of Audsley and colleagues for Drosophila fmrf

sequence data is in support of the phylogenetically grouping of Anthomyiidae, Sarcophagidae and Calliphoridae within the Calyptratae, a sister group of the Ephydroidea (Drosophila and allies) [75].

In light of the above, the restricted occurrence of the Yamide in Anthomyiidae and Drosophila-species outside the melanogaster group is remarkable. Our mass spectrometric data show that Yamide is stored in higher concentrations in the ring gland, but this might simply be a consequence of its C-terminal glycine (absent in e.g. Drosophila melanogaster) which is amidated due to co-processing and co-packaging with eclosion hormone. We therefore assume that Yamide represents an evolutionary caprice without functional advantage, at least until a receptor for this peptide family has been identified.

Supporting Information

Figure S1 MS/MS spectrum of unlabeled AKH. (TIF)
Figure S2 MS/MS spectrum of AST-A909, SPTC-labelled. (TIF)
Figure S3 MS/MS spectrum of FMRFa885, SPITC-labelled. (TIF)
Figure S4 MS/MS spectrum of FMRFa899, SPITC-labelled. (TIF)
Figure S5 MS/MS spectrum of FMRFa996, unlabelled. (TIF)
Figure S6 MS/MS spectrum of FMRFa1097 with an oxidised methionine (1113.5 Da), SPITC-labelled. (TIF)
Figure S7 MS/MS spectrum of FMRFa1154, unlabelled. (TIF)
Figure S8 MS/MS spectrum of FMRFa1181, SPITC-labelled. (TIF)
Figure S9 MS/MS spectrum of FMRFa1185, SPITC-labelled. (TIF)
Figure S10 MS/MS spectrum of CAPA-PK, SPITC-labelled. (TIF)
Figure S11 MS/MS spectrum of CAPA-PVK-1, SPITC-labelled. (TIF)

D. radicum Shows Fly-typical Presence and Distribution of Peptide Hormones

In general, the peptide families identified in D. radicum are common in cyclorrhaphan fly species [e.g. [24,25,28,30,31,35,51,71]], and the peptide hormone complement in neurohemal organs and enteroendocrine cells is typical for this fly group [24,25,30,56,31,35,34,51]. Moreover, our anatomical results emphasise that the gross distribution and projection patterns of the immunostained peptidergic neurons and enteroneocrinoid cell are not change qualitatively between the maggot and adult fly. Also in Drosophila, the peptide complement does not change qualitatively during postembryonic development [24,25,28]. Nevertheless, our sequence data differ from those of Audsley and colleagues for Drosophila melanogaster [9,28]. Thus, our sequence data is in support of the phylogenetically grouping of Anthomyiidae, Sarcophagidae and Calliphoridae within the Calyptratae, a sister group of the Ephydroidea (Drosophila and allies) [75].

In light of the above, the restricted occurrence of the Yamide in Anthomyiidae and Drosophila-species outside the melanogaster group is remarkable. Our mass spectrometric data show that Yamide is stored in higher concentrations in the ring gland, but this might simply be a consequence of its C-terminal glycine (absent in e.g. Drosophila melanogaster) which is amidated due to co-processing and co-packaging with eclosion hormone. We therefore assume that Yamide represents an evolutionary caprice without functional advantage, at least until a receptor for this peptide family has been identified.
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Author Contributions

Conceived and designed the experiments: JZ WR CW. Performed the experiments: JZ WR KHR JK CW. Analyzed the data: JZ WR CW. Contributed reagents/materials/analysis tools: JZ KHR. Wrote the paper: JZ WR CW.

Figures

Figure S12 MS/MS spectrum of CAPA-PVK-2, SPITC-labelled. (TIF)
Figure S13 MS/MS spectrum of SIFa, SPITC-labelled. (TIF)
Figure S14 MS/MS spectrum of sulfakinina6-14, SPITC-labelled. (TIF)
Figure S15 MS/MS spectrum of TK1116, SPITC-labelled. (TIF)
Figure S16 MS/MS spectrum of TK1010, SPITC-labelled. (TIF)
Figure S17 MS/MS spectrum of APK, SPITC-labelled. (TIF)
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