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Bell, P, Down, RE, Matthews, HJ et al. (2 more authors) (2019) Peptidergic control of the crop of the cabbage root fly, Delia radicum (L.) Diptera: Anthomyiidae): a role for myosuppressin. General and Comparative Endocrinology, 278. pp. 50-57. ISSN 0016-6480

https://doi.org/10.1016/j.ygcen.2018.08.001

Peptidergic control of the crop of the cabbage root fly, Delia radicum (L.) Diptera: Anthomyiidae): a role for myosuppressin.

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Abstract

There is much interest in targeting neuropeptide signaling for the development of new and environmentally friendly insect control chemicals. In this study we have focused attention on the peptidergic control of the adult crop of *Delia radicum* (cabbage root fly), an important pest of brassicas in European agriculture. The dipteran crop is a muscular organ formed from the foregut of the digestive tract and plays a vital role in the processing of food in adult flies. We have shown using direct tissue profiling by MALDI-TOF mass spectrometry that the decapeptide myosuppressin (TDVDHVFLRFamide) is present in the crop nerve bundle and that application of this peptide to the crop potently inhibits the spontaneous contractions of the muscular lobes with an IC_{50} of 4.4 x 10^{-8} M. The delivery of myosuppressin either by oral administration or by injection had no significant detrimental effect on the adult fly. This failure to elicit a response is possibly due to the susceptibility of the peptide to degradative peptidases that cleave the parent peptide to inactive fragments. Indeed, we show that the crop of D. radicum is a source of neuropeptide-degrading endo- and amino-peptidases. In contrast, feeding benzethonium chloride, a non-peptide agonist of myosuppressin, reduced feeding rate and increased the rate of mortality of adult D. radicum. Current results are indicative of a key role for myosuppressin in the regulation of crop physiology and the results achieved during this project provide the

basis for subsequent studies aimed at developing insecticidal molecules targeting the peptidergic control of feeding and food digestion in this pest species.

Keywords: Delia radicum, myosuppressin, neuropeptide, crop, mass spectrometry, myoinhibition.

Abbreviations:
FMRFamide-related peptides, FLPs; crop nerve bundle, CNB; matrix assisted laser desorption ionization time of flight mass spectrometry, MALDI-TOF MS; benzethonium chloride, Bztc; 7-amino-4-methylcoumarin , AMC; phosphate buffered saline, PBS; Triton X-100 in phosphate buffer saline, PBST; α-cyano-4-hydroxycinnammic acid, HCCA

1. Introduction

The cabbage root fly, Delia radicum (L.) (Diptera: Anthomyiidae), is a pest of brassicas in Europe and North America and poses a major and chronic threat to the commercial production of brassica crops (Blackshaw et al., 2012). The flies overwinter as pupae and in the spring the emerging females lay their eggs on the soil close to the base of cruciferous plants. Following egg hatch, the larvae feed on the host plant's root system and it is this life stage that is the most damaging (Biron et al., 1998). Insecticide options for controlling D. radicum in brassica crops are now limited, and there is a need to develop alternatives that may replace or at least extend the useful life of conventional insecticides (Myrand et al., 2015). Insect neuropeptides and their receptors are considered important targets for the development of novel pesticides because of their role in the regulation of diverse physiological and behavioural processes (Audsley and Down, 2015; Gäde and Goldsworthy, 2003; Scherkenbeck and Zdobinsky, 2009). One group of peptides that potentially could be utilized is the FMRFamide-like peptides (FLPs), which all share the common RFamide (Arg-Phe-NH_2) C-terminal moiety. In particular, myosuppressins are FLPs with important roles in visceral muscle motility (e.g. heart, gut) in a wide range of insects. The first myosuppressin identified was leucomyosuppressin, isolated from the cockroach Leucophaea maderae showing a digestive enzyme regulatory role in gut of several insects (Holman et al., 1991;

Nachman et al., 1997). This peptide inhibits contractions of the foregut and hindgut in several cockroach species (Periplaneta americana, Leucopheae maderaea and Blatella germanica) and inhibits midgut contractions in Diploptera punctata (reviewed by Orchard et al. 2001). Leucomyosuppressin has also been shown to inhibit foregut peristalsis in vitro in Lepidoptera larvae. When injected into 5th instar Lacanobia oleracea and Spodoptera littoralis it suppresses feeding and reduces survival, which was most likely due to the inhibitory actions on the gut (Matthews et al., 2009).

The dipteran myosuppressin peptides identified to date are structurally very similar to leucomyosuppressin (pEDVDHVFLRFamide) except for one amino acid substitution (T or S) at the N- terminus (S/TDVDHVFLRFamide). An antibody specific for the N-terminal region of myosuppressin has been used to identify and localize the peptide to neuronal cell bodies and processes in adult Drosophila melanogaster , while myosuppressin-like material was reported in the house fly, Musca domestica, blow flies Phormia regina and Protophormia terraenovae, and the horse fly Tabanus nigrovittatus, (Angioy et al., 2007; Haselton et al., 2008, 2004; McCormick and Nichols, 1993). In addition, myosuppressin has been identified by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) in the brain and retrocerebral complex, comprising the endocrine glands corpus allatum and corpus cardiacum, from a range of dipteran species (Caers et al., 2015; Hauser et al., 2010; Predel et al., 2010; Rahman et al., 2013; Wegener et al., 2006), including both larval and adult stages of Delia radicum (Audsley et al., 2011; Zoephel et al., 2012). Mass spectrometry has also identified myosuppressin in the crop nerve bundle (CNB) of adult D. radicum and Drosophila suzukii (Audsley et al., 2015).

The crop is present in almost all adult dipterans and has a critical role in the transfer of food to the midgut. Dysfunctionality of the crop can result in profound reductions in survival of the adult fly (Peller et al., 2009; Ren et al., 2014) and therefore targeting the neuronal control of the crop is an attractive strategy in the search for new insect control chemicals.

The crop is an anterior section of the alimentary canal formed by an impermeable cuticle that is shaped into the form of expandable bi-lobed sac connected to the foregut by the crop duct (Imms, 1957). Muscles in the wall of the crop allow it to expand and collapse, and peristaltic waves of contractions of the crop and crop duct allows movement of material from the crop to the midgut through various pumps and

sphincters (Stoffolano et al., 2013; Thomson, 1975) . The crop nerve bundle extends from the retrocerebral complex and branches out over the muscle of the crop lobes. Myosuppressin immunoreactivity has been localised to the adult crop nerve and processes that cover the external surface of the crop of M. domestica, P. regina, D. melanogaster and D. suzukii and the peptide has been shown to reduce the spontaneous contractions of the crop in these insects (Duttlinger et al., 2002; Gough et al., 2017; Haselton et al., 2004; Richer et al., 2000). Interfering with myosuppressin signaling that regulates crop contractions is expected to disrupt the movement and digestion of food, which could potentially lead to the development of more targeted and environmentally safe control measures.

In this study we have investigated the role of FLPs in regulating crop motility of D. radicum. We confirm that myosuppressin is the dominant peptide in the crop nerve bundle and that this FLP is a potent inhibitor of crop muscle contractions. We have also undertaken experiments to assess the potential of myosuppressin and the myosuppresin receptor agonist benzethonium chloride (Bztc) to disrupt gut function in this important pest of brassica crops in our efforts to identify targets for the development of new insect control chemicals.

2. Materials and methods

2.1. Insect maintenance

Delia radicum were reared at 20 °C, a photoperiod of 16L: 8D and 65 % R.H and adults were maintained on a diet consisting of dry yeast powder, sugar, dried skimmed milk powder as previously described (Finch and Coaker, 1969).

2.2. Peptides and chemicals

Myosuppressin (TDVDHVFLRFamide) and truncated short neuropeptide F (sNPF$^{4-11}$, SPSLRLRFamide) were custom synthesized by Biomatik, Cambridge, Ontario, Canada). 7-Amino-4-methylcoumarin (AMC), Dulbecco's Phosphate Buffered Saline (PBS) and Benzethonium chloride (Bztc) were all purchased from Sigma-Aldrich Company Ltd., Gillingham, U.K. AMC-RPPGFSAFK(DNP) and L-Threonine 7-amido-4-methylcoumarin (Thr-AMC) were purchased from Enzo Life Sciences (UK) Ltd, Exeter, U.K. and Insight Biotechnology Ltd., Wembley, U.K., respectively.

2.3. Indirect Immunohistochemistry

Adult flies were anaesthetized with CO_2 and chilled on ice before being dissected in Phormia fly saline (Chen and Friedman, 1975). Samples were fixed in 4% (wt/v) paraformaldehyde at 4°C overnight. Tissues were then permeabilized in 0.3% (v/v) Triton X-100 in phosphate buffer saline (PBST), and blocked by 10% (v/v) goat serum in PBST for 1h at room temperature to reduce non-specific binding. Samples were then incubated with a primary rabbit cross-reactive anti-FMRFamide antibody (1:1000, Peninsula, California) made up in 5% PBST for 48 hours at 4°C. Following incubation, tissues were rinsed five times in PBST, and further incubated in secondary antibody solution containing goat anti-rabbit IgGAlexa Fluor 594 (1:500, Invitrogen) in corresponding blocking buffer overnight at 4 °C. Excess reagent was washed away with PBST and samples were mounted in Dapi-Fluorount-G mounting media (2BScientific, UK) on microscope slides and sealed with nail varnish. Slides were stored in the dark at 4 °C. Immunolabeled samples were analyzed with Zen 2011 viewing software (Zeiss) and pictures taken by Zeiss confocal laser inverted microscope LSM700 (Carl Zeiss, Germany). One set of control samples omitted primary antibody whereas secondary controls were incubated with blocking peptide consisting of primary antibody pre-absorbed with myosuppressin peptide (100 µg/ml).

2.4. Mass analysis of crop nerve bundle (CNB)

The CNB from D. radicum were directly transferred onto MALDI-TOF MS plate into 1 µl of HPLC-grade water. Blotting with filter paper removed excess water and 0.5 µl matrix solution (α-Cyano-4-hydroxycinnammic acid (HCCA), Sigma-Aldrich; 10mg/ml in 70% acetonitrile 0.1% trifluoroacetic acid (TFA)) was added and allowed to dry at room temperature. Samples were analyzed using a Voyager DE STR MALDI TOF MS (Applied Biosystems, Warrington, UK). Settings for laser intensity and the number of sub-spectra were adjusted to individual sample. The measured monoisotopic masses ($[M+H]^+$) were compared to the monoisotopic masses of reference peptides calculated using the Applied Biosystems Data Explorer software. A calibration of the Voyager was performed with an external calibration mixture containing des-Arg-bradykinin, angiotensin 1, Glu-fibrinopeptide B and neurotensin (Applied Biosystems) or angiotensin I, angiotensin II, substance P, bombesin, ACTH clip 1-17, ACTH clip 18-39, and somatostatin 28 (Bruker Daltronic) (Audsley et al 2015).

2.5. Metabolism of myosuppressin by crop enzymes

Twenty crops were dissected under Phormia saline and disrupted using a glass homogenizer (0.1 ml glass Wheaton Micro Tissue Grinder, Fisher Scientific) in 100 µl of 0.1 M HEPES buffer (pH 7.5, 10 µM Zn) with 20 upward down strokes. The homogenate was stored in aliquots at 4 °C until required. To study the degradation of myosuppressin, 10 µl of the crop homogenate diluted 5-fold was added to 200 µl of 100 µM myosuppressin in 0.1 M HEPES buffer, pH 7.5 and incubated at 24 ^{O}C. At different time points 20 µl aliquots were removed and added to 5 µl 8% (v/v) TFA to terminate enzyme activity. Each reaction was performed in quadruplicate. Acidified samples were centrifuged (4°C, 12,000 x g for 20 min) and the supernatant was diluted 10-fold with 0.1% TFA prior to reversed-phase high-performance liquid chromatography (Beckman gold chromatography system, Beckman Coulter, U.K. Ltd) using a 150 x 4.6 mm Kinetex reverse phase column (Phenomenex, Macclesfield, U.K.), eluted with a 10-60% acetonitrile 0.1% TFA gradient at a flow rate at 1mL/min over 25 min. HPLC fractions (1 ml) of were collected and concentrated using a Savant Speed Vac concentrator (Thermo Electron, U.K.) to less than 10 µl. The mass of HPLC-purified metabolic breakdown products was determined by MALDI-TOF mass spectrometry using a Voyager DE STR MALDI TOF mass spectrometer. A single 0.5 µl droplet of sample from HPLC fractions was mixed with 0.5 µl of matrix (HCCA) and spotted onto a MALDI-TOF plate. The collected mass spectra fragmentation patterns were compared with those generated by Protein Prospector software (University of California, U.S.A.). The UV (214 nm) peak area (uVmin) of myosuppressin in samples were measured and the reduction in peak area after incubation with crop homogenate at different time periods was used to determine the half-life ($t_{1/2}$) of myosuppressin. Under the separation conditions described above, myosuppressin eluted at 9.6 min.

2.6. Detection of membrane and soluble crop peptidases

To prepare a high-speed membrane and supernatant preparation homogenizing, 15 crops were homogenised in 0.5 ml of PBS,) using a glass homogeniser (Jencons, East Grinstead, U.K.) and 20 up and down strokes of the pestle. The resulting homogenate was centrifuged at 55,000 g for 1 h at 4^{o}C using a Beckman Optima™ MAX bench-top ultracentrifuge and TLA110 rotor (Beckman Instruments Inc, Palo Alto, Ca, U.S.A.). The pellet was re-suspended in 0.5 ml of PBS and both the pellet and supernatant were stored frozen until required. Endopeptidase assays were conducted by measuring the

initial rate of increase in fluorescence from cleavage of the quenched substrate 1.6 µM AMC-RPPGFSAFK(DNP) by 10µl of enzyme in 100µl of MES buffer, pH 6.5 in a 96-well black plastic plate (Corning Life Sciences, High Wycombe, U.K.) using a FLUOstar Omega (BMG LABTECH GmbH, Offenburg, Germany) with the excitation λ set at 330nm and emission λ set at 410nm. The same assay conditions were used for detecting aminopeptidase activity using 1.6 µM Thr-AMC, except that the initial rate of increase in fluorescence was determined with the excitation λ set at 355nm and emission λ set at 460nm. The amount released was calculated from a standard curve of AMC. Activities for both enzymes are expressed as pmol of substrate cleaved/h/crop equivalent.

2.7. Crop Bioassay

Three-day old adult females were deprived of food and water for a 24 h period prior to use to ensure that their crop was devoid of food. Flies were then fed with a 4 ul droplet of blue-colored 1M sucrose mixture (Natural Blue Food Colouring, Ocado). As soon as each fly has stopped feeding, it was anesthetized with CO_2 and the crop was exposed under a drop of Phormia physiological saline. The crop duct was cut from the proventriculus (cardia) and transferred immediately into 40 µl of saline in a cavity slide for viewing using a stereo dissecting microscope (GXM-XTL, GT Vision Ltd, Stansfield, Suffolk, U.K.). For routine assays, contractions were counted by direct observation using the following protocol. After allowing 1 min for acclimatization, to determine the basal rate, the saline was replaced by test solutions using the 'two-pipette transfer system' which limits disturbance to the crop tissue (Stoffolano et al., 2013). After a 1 min adjustment period, contractions were counted for the following minute and compared to the basal contraction rate for each tissue. Test solutions were washed out with physiological saline to observe the recovery of muscle activity. A further procedural control was performed where saline was substituted with carrier saline only. Each crop was used only once. Dose-response plots were generated and analyzed using Prism version 7 (Prism Software Corporation, U.S.A.). The graphic presented in Fig. 4b was generated by video recording the experiment using a GXCAM camera attached to the microscope (see supplementary Fig. 3 and videos in the supplementary section). Crop movement in the video was determined by adding a vector that crossed the crop

and using AviLine software (http://biolpc22.york.ac.uk/avianal/avi_line/) to record changes in pixel brightness in successive frames as described by Norville et al., (2010).

2.8. Survival and food intake assays

Adult females (3-5 days post eclosion) were transferred to 25 x 95 mm vials containing either 6 ml of 5% sucrose (wt /vol) and 2% agar (wt/vol) (control diet) or the same sucrose/agar mixture containing 5 mM Bztc (treatment diet). For both control and treatment groups, the diet was replenished weekly until all flies were dead. For measuring food intake, 0.5% bromophenol blue (wt/vol, Sigma-Aldrich) was included in both the control and the treatment (5 mM Bztc) sucrose/agar diet. Flies were allowed to feed for 24 h before pairs of females were transferred to 6 mm diameter glass tubes and fed from a drop (10 µl) of 5% (wt/vol) sucrose solution without added dye. After 24h, the flies were removed and the empty tubes were washed with 300 µl of distilled water. Pooled washes from three tubes, containing excreta from six flies, were measured at the absorbance of 595 nm wavelength using a SpectraMax 340PC Microplate Reader Spectrophotometer (n=6)

All flies were 3-5 days post-eclosion and were maintained at 26°C, 12:12 light regime and 65% relative humidity.

2.9 Injection of adult D. radicum with myosuppressin

Females (2 days post-eclosion) were anaesthetized under CO_2 and injected with either 1 µl of PBS (controls) or 1ul PBS containing 6.4 µg of myosuppressin. Flies were monitored twice daily until all flies had died.

2.10. Statistical analysis

All graphs and statistical analyses were performed using GraphPad Prism 7 for Windows. Survival curves were compared by the Kaplan-Meier log-rank survival analysis for each treatment group.

3. Results

3.1. Peptidergic innervation of the D. radicum crop

Immuno-staining of a whole mount preparation of the crop of adult D. radicum using antibodies recognizing the RFamide epitope of myosuppressin revealed prominent

innervation by a network of immunoreactive fibres extending over the central region of the crop sac with individual projections reaching towards the lobes (Fig. 1). These immuno-reactive fibres originate from two axons emanating from the retrocerebral complex (Fig. 2A) that travel along the lateral sides of the crop duct (Fig. 2B) towards the crop sac. Reaching the base of the crop, they undergo prominent division (Fig. 1A). Figure 2C shows stained cells of the retrocerebral complex as well as processes that project over the proventriculus (Fig. 2D) and terminate on the surface of the anterior midgut. No differences were noted between male and female crop preparations. The antibody specificity was confirmed when tissues were incubated either with secondary antibody alone or with antibody pre-absorbed with peptide, which abolished the immuno-reactivity (supplementary Figure 1). The staining in the midgut is the result of cross-reactivity to FLPs present in the enteroendocrine cells.

3.2. Mass analysis of crop nerve bundle (CNB) peptides

In the mass range of 500-2500 Da two prominent monoisotopic mass ion peaks m/z, 974.7 and 1247.8 were present in the mass spectra obtained from single tissue extracts of the CNB (Fig. 3). These signals correspond to the monoisotopic masses of myosuppressin (TDVDHVFLRFamide) and $sNPF^{4-11}$ (SPSLRFamide), respectively. The sodium adduct of the myosuppressin ion (m/z, 1269.8) was also present (Fig. 3).

3.3. Inhibition of crop muscle contractions by myosuppressin and Bztc

Myosuppressin inhibited spontaneous contractions of semi-isolated preparations of adult D. radicum crop in a dose-dependent manner (Fig. 4) with an apparent EC_{50} of 4.4×10^{-8} M. Spontaneous contractions were recovered when the peptide solution was removed and washed from crop preparations with physiological saline (Fig. 4B). The application of the non-peptide agonist Bztc to the isolated crop tissue also reduced the frequency of spontaneous contractions, but was less potent (EC_{50} 7.2×10^{-6} M) than myosuppressin (Fig. 4A). Importantly, the crop tissues recovered from inhibitory effect of 1 and 10 µM Bztc, but not 100 µM Bztc, when washed with fresh saline. $sNPF^{4-11}$ had no significant effect on spontaneous contractions of the crop even at high concentrations (10^{-4} M) (Fig. 4A).

3.4. In vivo effect of peptides and Bztc

Injection of myosuppressin into adult female D. radicum had no effect on survival compared to control (saline injected) flies (Kaplan-Meier log-rank survival analysis,

P=0.667, 0.416) (supplementary Fig. 2). In contrast, there was a significant difference in the survivorship between the controls and flies maintained on 5 mM Bztc/agar diet (Log-rank test, P<0.0001) (Fig. 5A). The survival median was 5 days for Bztc-fed flies, whereas control flies lived for up to 12 days. All the flies fed with Bztc died by day 10, whereas it took 27 days for all the control flies to die. When Bztc was included in the diet containing a food dye, the amount of colored food passing into the faeces was much less than that occurring in the absence of the agonist, suggesting reduced consumption of food (Fig. 5B).

3.5. Degradation of myosuppressin by crop peptidases

HPLC with uv detection was used to monitor the reduction in 2 nmoles myosuppressin when incubated with crop homogenate. Myosuppressin degradation by the crop peptidases was rapid with an estimated half-life of c. 2 min (R^2= 0.9398). MALDI-TOF mass analysis of HPLC fractions identified a number of degradation products (Table 1, Fig. 6), five of which retained the amino terminus (N-terminus) and six had Phe-amide at the carboxy terminus (C-terminus). One peptide fragment (DVDHVFLR) was truncated at both termini. The mass spectrometric data indicated the involvement of crop aminopeptidases as well as endopeptidase activity capable of cleaving the Arg-Phe peptide bond. In support of this hypothesis, we used fluorogenic aminopeptidase and endopeptidase substrates to show that the crop possessed both peptidase activities (Fig. 6). These enzyme activities were measured in both a soluble and a membrane fraction separated from each other by high-speed centrifugation. Around 85% of the endopeptidase (85 ± 2 pmoles/h) and 68% of the aminopeptidase activity (3.11 ± 0.08 pmole/h) were located in the soluble fraction.

4. Discussion

The release of regulatory peptides in response to external and internal cues is well known to have direct impact on feeding activity from the control of levels of digestive enzymes in response to food stimuli to effectively maneuvering a food bolus through the gut via coordinated muscle contractions (reviewed by Audsley and Weaver, 2009, Spit et al., 2012). Previous studies have demonstrated that myosuppressins have a role in the regulation of feeding in several insect species. Leucomyosuppressin increased

food intake in the cockroach Blattella germanica (Aguilar et al., 2004), while injection of myosuppressin into Spodoptera littoralis resulted in anti-feeding behavior (Vilaplana et al., 2008). In the pea aphid, Acyrthosiphon pisum, myosuppressin suppressed feeding resulting in mortality, most likely due to the inhibition of gut motility preventing the movement of food (Down et al., 2011). Feeding the non-peptide myosuppressin agonist Bztc to adult M. domestica and D. suzukii resulted in early mortality suggesting that myosuppressin signaling has potential as an insecticide target (Gough et al., 2017; Haselton et al., 2004).

In the present study we established the presence of myosuppressin and sNPF$^{4-11}$ within the CNB of D. radicum and provide several pieces of evidence in support of myosuppressin, but not sNPF$^{4-11}$, as an important regulator of crop function in this pest species. Myosuppressin was demonstrated in the CNB of D. radicum by direct peptide profiling using the same approach used by Audsley and colleagues (2015) to show the presence of myosuppressin in the CNB of D. suzukii. The current findings however differ from the previous study by the co-occurrence of myosuppressin and sNPF$^{4-11}$. Our identification of these two peptides were based on monoisotopic peaks $(M+H]^{+}$) that are in accordance with the peptide sequences and masses reported by Zoephel et al. (2012) and Audsley et al. (2011) in their peptidomics studies of the larval and adult central nervous system of D. radicum, respectively. Commercially available antiserum recognizing the C-terminus of FMRFamide was used to support the claim that myosuppressin/ sNPF$^{4-11}$ neurons extend to the crop muscle of flies (Gough et al. 2017). Myosuppressin and sNPF$^{4-11}$, as well as other insect FLPs, share the Arg-Phe-amide sequence with FMRFamide and are expected to cross-react with FMRFamide antibodies. Consistent with this expectation, pre-incubation of the antiserum with synthetic myosuppressin blocked the staining of the D. radicum nervous system. The immuno-staining of the axons in the crop nerve that project from the retrocerebral complex to the crop muscle and spread over the surface of the crop is consistent with previous reports on the spatial distribution of FLPs in other dipteran species including the housefly M. domestica (Haselton et al., 2004), the fruitfly D. melanogaster (Dickerson et al., 2012), blowfly P. regina (Richer et al., 2000), horn fly H. irritans and stable fly Stomoxys calcitrans (Meola et al., 1996). The widespread occurrence of FMRFamide-like immunoreactive material in the central and stomatogastric nervous system and enteroendocrine cells of dipterans suggests a

general regulatory role for FLPs in regulating feeding and digestion in this group of insects.

Consistent with an important role for myosuppressin in regulating crop function, the synthetic peptide powerfully inhibited the spontaneous contractions of the D. radicum crop musculature. Myoinhibition was observed with nM doses of peptide (EC_{50}, 44 nM) and the effect was immediate and long lasting, but reversible. Such potency is typical of insect peptide receptors and compares well with the potency (EC_{50}, 40 nM) of myosuppressin at activating two cloned G protein-coupled receptor genes (DmsR-1 and DmsR-2) from D. melanogaster expressed in mammalian cell lines (Egerod et al., 2003a; Johnson et al., 2003). Both DmsR1 and DmsR-2 are expressed in the crop of D. melanogaster, but DmsR-1 appears to be more important for myosuppressin signal transduction in the crop of this fruit fly (P. Bell, unpublished data, Chintapalli et al., 2007). In contrast, $sNPF^{4-11}$ failed to elicit a myoinhibitory response when applied to the crop at concentrations even as high as 0.1 mM leading us to conclude that myosuppressin probably works alone to inhibit D. radicum crop contractions. At present we have no functional information for the CNB $sNPF^{4-11}$.

When fed or injected into adult D. radicum, myosuppressin had no measurable effect on feeding behaviour or mortality. This lack of a response could have resulted from a failure to reach target gut tissues and/or rapid inactivation. Myosuppressin was rapidly degraded ($t_{1/2}$< 2min) when incubated with a homogenate of the D. radicum crop. MALDI-MS revealed a complex mixture of myosuppressin fragments that suggested multiple initial attacks by aminopeptidase and endopeptidase enzymes. Indeed, we confirmed the presence of both aminopeptidase and endopeptidase activities predominantly in a soluble fraction of the crop homogenate. A very similar pattern of rapid degradation was reported for leucomyosuppressin (pEDVDHVFLRF-NH_2) by gut juices of two moths, Lacanobia oleracea and Spodoptera littoralis (Matthew et al., 2009; Down et al., 2011). A structure-activity study of the inhibitory activity of N-terminally truncated myosuppressin peptides on adult D. melanogaster crop contractions showed that removal of the N-terminal tripeptide resulted in loss of activity (Dickerson et al., 2012). When tested the same peptides were tested on larval gut, only the parent 10-mer peptide gave a full inhibitory response. A similar study of leucomyosuppressin inhibition of the cockroach (Leucophaea maderae) hindgut

identified VFLRFamide as the core fragment, although this activity was at least two orders of magnitude below that of the intact peptide (Nachman et al., 1993). In conclusion, myosuppressin is susceptible to rapid breakdown by gut peptidases present in the crop. Many of the fragments generated are expected to have weak or no agonist activity on the D. radicum crop. These studies emphasise the need for myosuppressin analogues that are resistant to degradation by gut peptidases when testing as oral activity.

Benzethonium chloride (Bztc), a quaternary ammonium salt, was the first non-peptide compound to be described as a myosuppressin analogue capable of mimicking the myoinhibitory actions of myosuppressin on heart, visceral and skeletal muscle from different a variety of insect species, including the crop of dipterans such as M. domestica, P. regina, or D. melanogaster and D.suzukii (Duttlinger et al., 2002; Gough et al., 2017; Haselton et al., 2004; Lange et al., 1995; Richer et al., 2000). The evidence for Bztc being a myosuppressin agonist included shared structural features and the competitive displacement of radioactively labelled myosuppressin from both high- and low-affinity myosuppressin receptors in locust oviduct membranes (Lange et al., 1995) . In the present study, the inhibitory effect of Bztc was 100-fold less potent compared to myosuppressin and the recovery of spontaneous contractions after the Bztc was replaced with saline was noticeably slower compared to the peptide. Our results are in accordance with findings reported by Stoffolano et al., (2013) and Lange et al., (1995), where in both instances Bztc reversibly inhibited muscle contractions. Furthermore, Richer (et al., 2000) described Bztc action in mM range to be equivalent to myosuppressin peptide, terminating spontaneous crop contractions in the blowfly P. regina. However, it remains unclear how Bztc mimics the effect of myosuppressin on muscle contractions. Egerod et al., (2003) could not demonstrate that heterologously expressed Dms-R1 and Dms-R2 of D. melanogaster were activated by Bztc in a specific manner and it is possible that some of the physiological effects of this quaternary ammonium salt results from its weak surfactant properties. Using dye-labelled food to follow food ingestion and excretion in adult D. radicum, we showed that Bztc had a significant effect on food intake which probably contributed to the toxicity of the chemical as revealed by a strong reduction in life-span. A fuller understanding of the mechanisms leading to this toxicity is required before we can conclude that myosuppressin signalling is being targeted.

Acknowledgements:

PB, REI and NA received generous support from the Department for Environment, Food and Rural Affairs (Defra), project code PH0453. REI was also supported by the European Union's Horizon 2020 under grant agreement 634361.

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Table 1

Monoisotopic masses ($[M+H]^+$) and sequences of myosuppressin and hydrolysis products identified in HPLC fractions after incubation with peptidases from the crop of adult Delia radicum.

Myosuppressin fragment	Amino acid sequences	$[M+H]^+$
1-10 (intact)	TDVDHVFLRFNH_2	1247.6
1-9	TDVDHVFLR	1101.5
1-8	TDVDHVFL	945.4
1-7	TDVDHVF	832.3
1-6	TDVDHV	685.3
1-5	TDVDH	586.2
2-10	DVDHVFLRFNH_2	1146.6
3-10	VDHVFLRFNH_2	1031.5
4-10	DHVFLRFNH_2	932.5
5-10	HVFLRFNH_2	817.4
6-10	VFLRFNH_2	680.4
7-10	FLRFNH_2	581.3
2-9	DVDHVFLR	1000.5

Figure legends

Fig.1. Immunostaining of the crop of D. radicum using an antibody recognising the RFamide epitope. A) Whole mount showing FLP material in a network of filaments covering the central region. Enteroendocrine cells of the midgut are also visible with this antiserum. B) Higher magnification view of the region highlighted by the square box in (A).

Fig. 2. Immunostaining of neuronal FLP peptides in whole mounts of the foregut, retrocerebral complex and crop duct of adult D. radicum. A) Axons on the surface of the oesophagus enter the retrocerebral complex. Stained axons run across the proventriculus surface where they divide passing over the anterior midgut (A) and along crop duct surface (B) to the crop lobes. C and D) Confocal z-stack images of the retrocerebral complex showing prominently stained. D) Immunostained axons (arrow) originating in the retrocerebral complex cover the proventriculus.

Fig.3. Mass spectrum of direct analysis of a single tissue of the D. radicum crop.

Fig. 4. Inhibition of crop contractions. A) The effect of myosuppressin, $sNPF^{4-11}$ and Bztc on the spontaneous contractions of the crop. Data are expressed as the % inhibition of the contractions counted in a 1 min period after the addition of the agonist as described in the methods section. Values are the mean of 5 determinations using fresh tissues for each determination. Non-linear regression analysis (GraphPad Prism 7.01) was performed to calculate EC_{50} values. B) Graphical representation of the inhibition of crop contractions by 10 µM myosuppressin and recovery after washing with fresh saline. Muscle contractions generated tissue movement that was video recorded (see supplementary Fig.3 and videos) and analysed as described by Norville et al., (2010).

Fig. 5. The effect of feeding Bztc to adult D. radicum. A) 5 mM Bztc in the diet increases mortality rate. B) Bztc reduces ingestion and excretion of sucrose/food dye. The amount of dye in the faeces after 24 h of feeding was determined spectrophotometrically (595 nm) and the results are expressed as the mean ± SEM (n = 6). Differences in the means values are statistically significant (t-test, $P < 0.001$).

Fig.6. Predicted scissile peptide bonds of myosuppressin and the structures of substrates used to measure endopeptidase and aminopeptidase activities of the crop. Arrows indicate aminopeptidase (1) and endopeptidase (2) cleavages.

Figure 1

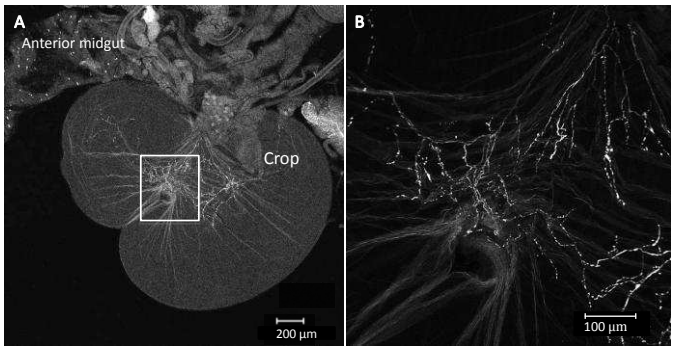


Figure 2

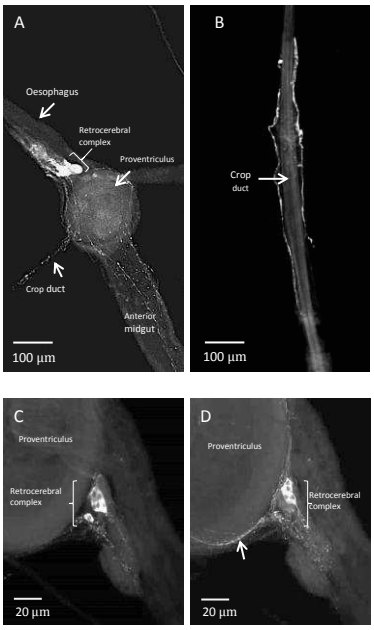

A
Oesophagus
Retrocerebral complex
Proventriculus
Crop duct
Anterior midgut
100 µm
B
Crop duct
100 µm
C
Proventriculus
Retrocerebral complex
20 µm
D
Proventriculus
Retrocerebral Complex
20 µm

Figure 3

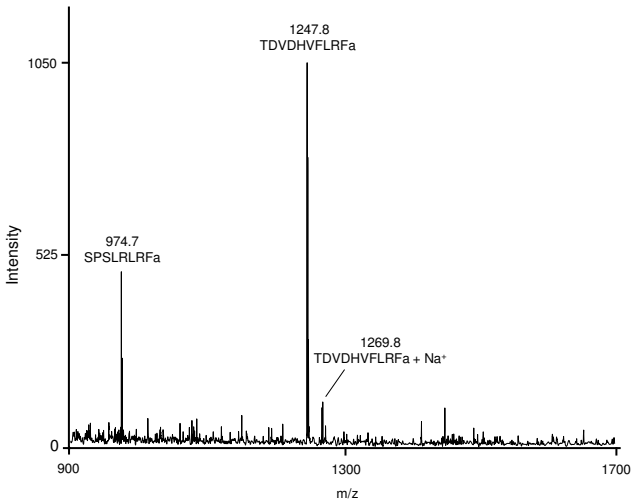

1247.8
TDVDHVFLRFa
1050
974.7
SPSLRLRFa
525
Intensity
1269.8
TDVDHVFLRFa + Na+
0
900
1300
1700
m/z

Figure 4

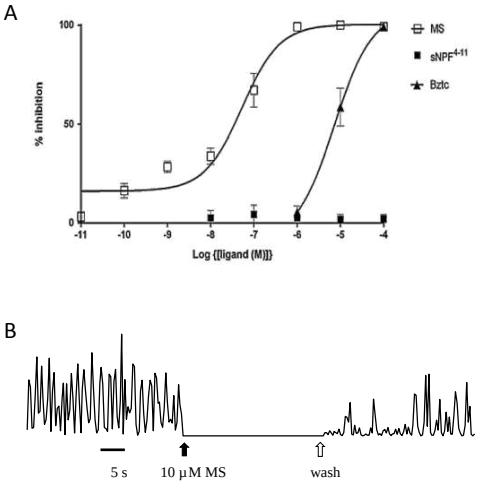

A
100
50
0
% inhibition
-11
-10
-9
-8
-7
-6
-5
-4
Log {[ligand (M)]}
MS
sNPF4-11
Bztc
B
5 s
10 µM MS
wash

Figure 5

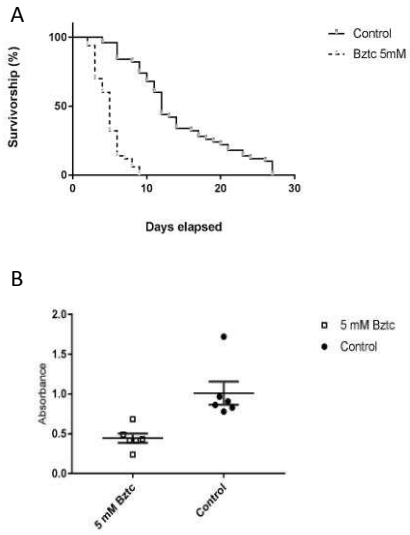
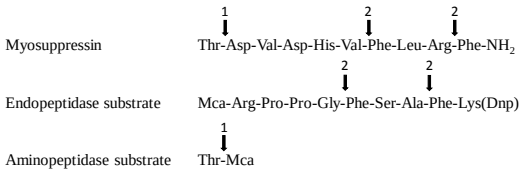

A
Survivorship (%)
100
50
0
0
10
20
30
Days elapsed
Control
Bztc 5mM
B
Absorbance
2.0
1.5
1.0
0.5
0.0
5 mM Bztc
Control
5 mM Bztc
Control

Figure 6


1
2
2
Myosuppressin
Thr-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH2
2
2
Endopeptidase substrate
Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)
1
Aminopeptidase substrate
Thr-Mca