

Behaviour of *Heterodera glycines* and *Meloidogyne incognita* infective juveniles exposed to nematode FMRFamide-like peptides *in vitro*

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Received: 29 September 2011; revised: 5 December 2011

Accepted for publication: 6 December 2011; available online: 23 February 2012

Summary – The effects of 1 mM solutions of FMRFamide-like peptides (FLPs) on the behaviours of *Heterodera glycines* and *Meloidogyne incognita* infective juveniles (J2) were examined *in vitro*. Seven FLPs, representing products of five *flp* genes and comprising a variety of amino acid sequences, were tested for their effects on J2 head movement frequency. Distinct differences in species responses were observed. KHEYLRFa and KSAYMRFa caused increased head movement frequencies in each species. In *H. glycines* KHEYLRFa was 2.9-fold more potent than KSAYMRFa. In *M. incognita* the potencies were equal. KHEYLRFa and KSAYMRFa each stimulated *H. glycines* J2 more effectively than *M. incognita* J2. However, two additional FLPs, AQTFVRFa and SAPYDPNFLRFa, were stimulatory in *M. incognita* but not in *H. glycines*. KPNFIRFa, KPNFLRFa and RNSSPLGTMRFa had no effect on either species. Substitution of D-amino acids at any position in KHEYLRFa resulted in decreased stimulation of head movement relative to the native peptide in each species, but all of the D-amino acid KHEYLRFa analogues were stimulatory relative to untreated controls. D-amino acid substitutions in KSAYMRFa eliminated stimulatory activity in *M. incognita* by all analogues except dKSAYMRFa. In *H. glycines*, only KSdAYMRFa and KSAYMdRFa were not stimulatory, and KSAYdMRFa stimulated equal to the native peptide. The remaining four analogues each stimulated relative to controls but below the native peptide level. Analysis of the head movement behaviour of large numbers of J2 of each species demonstrated that behaviours are quite stable and responses to FLP treatment are highly predictable.

Keywords – FLPs, movement, neuromuscular, parasite, pepper, plant-parasitic nematode, soybean.

Plant-parasitic nematodes represent an enormous economic burden on agriculture, with estimates of annual losses persistently in the hundreds of billions of dollars worldwide (Sasser & Freckman, 1987; Chitwood, 2003; Holden-Dye & Walker, 2011). Among the most damaging crop pathogens are the soybean cyst nematode, *Heterodera glycines*, and the southern root-knot nematode, *Meloidogyne incognita* (Holden-Dye & Walker, 2011). Improved efforts to control these pathogens, especially in the context of constraints on the use of chemical nematicides (Chitwood, 2003; Johnston *et al.*, 2010), are dependent upon the development of new control strategies founded upon a basic understanding of plant-parasitic nematode biology. Among the most fundamental aspects of animal biology is coordinated behaviour, and in ne-

matodes such behaviour is essential for the great majority of life processes (Perry & Maule, 2004). Nematode behaviour is highly dependent upon the FMRFamide-like peptides (FLPs), comprising a family of diverse sequences involved with a variety of neuromuscular activities (Kimber & Fleming, 2005; Li, 2005; McVeigh *et al.*, 2005, 2006). Consequently, FLPs are the focus of molecular research to discover alternative controls for nematodes of both animal and plant agricultural importance (McVeigh *et al.*, 2006; Kimber *et al.*, 2007). We present here the first analysis of the effects of direct application of selected FLPs and FLP analogues on the behaviour of plant-parasitic nematodes *in vitro*, and demonstrate species differences in response to these peptides.

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Materials and methods

NEMATODES

Heterodera glycines (NL1-RHp) was raised on soybean (*Glycine max*, cv. Kent) and *M. incognita* (Salisbury Race-1) was raised on pepper (*Capsicum annuum*, cv. PA-136), each grown in sand-filled beakers using a constant moisture system (Sardanelli & Kenworthy, 1997) fed by tap water. Rearing conditions were maintained at 27°C with a 16 h L : 8 h D photoperiod.

Each soybean plant was inoculated with *ca* 1500-2000 *H. glycines* eggs and harvested after 5 weeks. Females and eggs were collected from exposed roots and infective second-stage juveniles (J2) were hatched from eggs on modified Baermann funnels and collected daily. Pepper plants were each inoculated with *ca* 3000 *M. incognita* eggs and harvested after 2 months. Egg masses were picked from roots and J2 were hatched from egg masses and collected as described.

PEPTIDES

FLPs were selected to encompass a variety of sequences and represent a number of *flp* gene products (Li, 2005; McVeigh *et al.*, 2005; *flp-1*: KPNFIRFa, KP NFLRFa, SAPYDPNFLRFa; *flp-3*: RNSSPLGTMRFa; *flp-6*: KSAYMRFa; *flp-14*: KHEYLRFa; *flp-16*: AQTFVRFa). All non-labelled FLPs were custom synthesised by Biomatik (Wilmington, DE, USA). Each peptide was dissolved in sterilised HPLC-grade deionised-distilled water, and aliquots were dried with vacuum centrifugation and stored at -20°C. For the behavioural assays, peptide aliquots were dissolved in tap water to prepare a peptide stock solution and used either directly in the assay or diluted as appropriate and refrigerated for use within 2-3 h. Unused peptide stock was stored at -20°C and could be used after a single thaw with no effect on activity in the bioassay.

INFECTIVE JUVENILE BEHAVIOUR

Aliquots of freshly collected J2 were examined with a dissecting microscope to estimate J2 density (J2 μl^{-1}) in each collection. Density was then adjusted to between 3 and 7 J2 μl^{-1} , a range found to provide optimal numbers of J2 for observation. An aliquot (5 μl) of the adjusted J2 collection was transferred to a well of an Ibidi μ -Slide (Ibidi μ -Slide Angiogenesis, uncoated chamber for cell microscopy, www.ibidi.com) and immediately examined

at 40 \times with an inverted microscope (Nikon Eclipse 2000-S, Nikon Instruments, Melville, NY, USA) to verify worm numbers and condition. Then 45 μl of treatment (peptide solution, tap water control) was quickly added to the well and the μ -Slide cover attached to prevent evaporation. Worms in the covered μ -Slide were incubated at room temperature for 15 min prior to assay.

Treatment effects were quantified by observing anterior movement of individual worms using the inverted microscope at 40-100 \times . An individual movement is defined as any change in direction from a previous position, or any continuation in the same direction interrupted by a momentary pause (Masler, 2007), and movements in any plane were recorded. All movements of an individual worm were counted for 1 min per trial, and three movement trials were performed for each worm. The average of the three trials was used as a single observation (individual worm score replicate). A minimum of five replicates was used for each treatment for each experiment, and experiments were repeated 2-5 times. Results are expressed as head movement frequencies (movements min^{-1}) and are summarised across experiments.

PEPTIDE DIGESTION

J2 collected as above were used as a source of extracts for proteolytic digest assays. Worms were mechanically disrupted with 0.5 mm zirconia-silica beads in sterile deionised-distilled water using a Bead Beater high-speed shaker (BioSpec Products, Bartlesville, OK, USA) and the aqueous extracts were processed as previously described (Masler, 2010). Preparations were examined at 40 \times and 100 \times to assure that all worms were disrupted, and were centrifuged at 40 000 *g*, 20 min, 5°C. Total supernatant protein was determined (microBCA, Pierce, Rockford, IL, USA), and aliquots dried by vacuum centrifugation were stored at -20°C.

KHEYLRFa and KSAYMRFa substrates were synthesised (AnaSpec, Fremont, CA, USA) as the FRET-modified sequences QXL520-KHEYLRf-K(5-FAM)a and QXL520-KSAYMRF-K(5-FAM)a, which emit fluorescent signals upon peptide cleavage. Substrates were dissolved in 50% aqueous acetonitrile and dried as aliquots as above.

Digest reactions were carried out at 27°C in 384-well assay plates (flat bottom, non-treated; Corning, Corning, NY, USA) in 100 mM Tris, pH 7.8 assay buffer, 25 μl reaction volume. Nematode extract aliquots were dissolved directly into assay buffer at 0.08-0.1 μg extract protein (μl reaction mixture) $^{-1}$. FRET-peptide substrate

aliquots were dissolved in DMSO prior to addition to the reaction mixture (4 pmol final concentration of peptide substrate; 2% DMSO in all reactions). Reactions were initiated by the addition of substrate and monitored for 60 min for increasing fluorescence (Ex 490 nm, Em 520 nm; SpectraMAX EM fluorescent plate reader; Molecular Devices, Sunnyvale, CA, USA). Activity is expressed as $V_{\max} \text{ min}^{-1} \mu\text{g}^{-1}$.

AMINO ACID SUBSTITUTIONS

A series of D-amino acid substituted analogues were synthesised (Biomatik) for both KHEYLRFa and KSAYMRFa and aliquots prepared as above. Sequences prepared were dKHEYLRFa, KdHEYLRFa, KHdEYLRFa, KHdEYLRFa, KHEYdLRFa, KHEYLdRFa and KHEYLRdFa, and dKSAYMRFa, KdSAYMRFa, KSdAYMRFa, KSAdYMRFa, KSAYdMRFa, KSAYMdrFa and KSAYMRdFa.

NEMATODE AND ASSAY STABILITY

Consistency of nematode behaviour and robustness of the assay were examined by analysing all individual worm movements (individual trials as described above) rather than trial means. For each worm, the standard deviation of the mean of three trials was used as a single metric. Means of the trial standard deviations of all worms within three treatments (water control, 1 mM KHEYLRFa, 1 mM KSAYMRFa) were determined for each species, and compared to assess consistency or changes in levels of variation.

DATA ANALYSIS

Head movement frequency data and FLP digestion data were analysed using JMP Statistical Discovery software (SAS Institute, Cary, NC, USA) and GraphPad Prism (GraphPad Software, LaJolla, CA, USA). Data are expressed as mean \pm SEM, and means are compared using either one-way ANOVA with Tukey's post-test or Student's *t*-test with Mann-Whitney post-test.

Results

FLP STIMULATION OF HEAD MOVEMENT

Mean head movement frequencies were determined for J2 of *H. glycines* and *M. incognita* following 15 min *in vitro* exposure to 1 mM solutions of seven

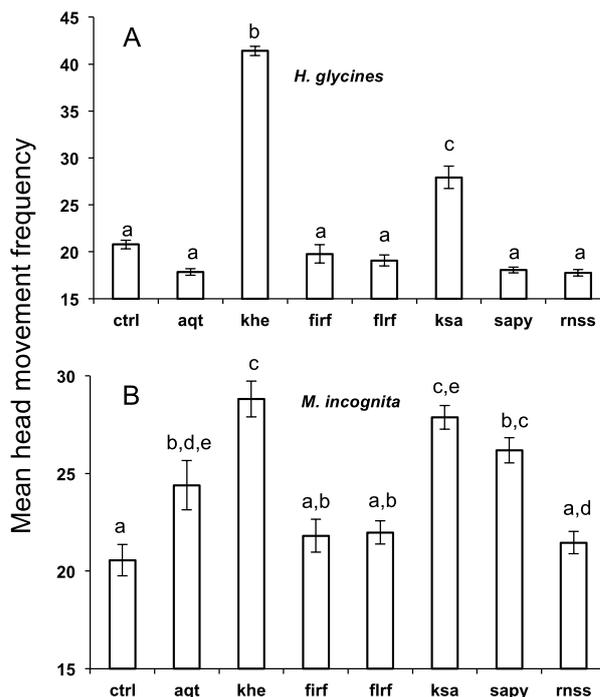


Fig. 1. A: *Heterodera glycines*; B: *Meloidogyne incognita*. Effect of seven different nematode FLPs on the frequency of head movement in infective juveniles (J2) of *H. glycines* and *M. incognita*. J2 were incubated *in vitro* in a 1 mM solution of the indicated FLP and observed using an inverted microscope at 40 \times . For each worm, head movement was monitored (see Materials and methods) at 1-min intervals (trials) and the head movement frequency (movements min^{-1}) recorded. The average frequency of three separate trials for each worm was used as a single replicate and each bar represents the mean head movement frequency \pm SEM of a minimum of 14 separate observations (worms). All means were compared by one-way ANOVA with Tukey's post-test. Means followed by different letters are significantly different ($P < 0.05$). FLP abbreviations: ctrl: control; aqt: AQTfVRFa; khe: KHEYLRFa; furf: KPNFIRFa; flrf: KPNFLRFa; ksa: KSAYMRFa; sapy: SAPYDPNFLRFa; rns: RNSSPLGTMRFa.

different nematode FLPs (Fig. 1). Behavioural responses to FLP treatment in *H. glycines* were more distinct than in *M. incognita*. Frequency was increased significantly ($P < 0.05$) in *H. glycines* only by KHEYLRFa and KSAYMRFa (Fig. 1A). KHEYLRFa stimulated head movement frequency two-fold over controls and, relative to controls, KHEYLRFa was 2.9-fold more stimulatory than KSAYMRFa ($P < 0.05$). None of the other five FLPs had any significant effect on *H. glycines* head movement.

In *M. incognita*, AQTFVRFa, KHEYLRFa, KSAYMRFa and SAPYDPNFLRFa each stimulated head movement frequency ($P < 0.05$; Fig. 1B). By contrast to *H. glycines*, KHEYLRFa and KSAYMRFa were similar in frequency stimulation. Both were statistically the same ($P < 0.05$) as SAPYDPNFLRFa, and KSAYMRFa and SAPYDPLFLRFa were similar in effect to AQTFVRFa ($P < 0.05$). Three FLPs, KPNFIRFa, KPNFLRFa and RNSSPLGTMRFa, each had no significant effect on head movement frequency.

Since KHEYLRFa and KSAYMRFa were the only FLPs that stimulated activity in both *H. glycines* and *M. incognita*, they were examined further. Dose responses revealed that *H. glycines* was more sensitive to stimulation by either FLP than was *M. incognita* (Fig. 2). KHEYLRFa stimulation of *H. glycines* was significantly higher ($P < 0.001$) than in *M. incognita* at both 0.5 mM and 1 mM (Fig. 2A). Although the raw data means at 0.25 mM were apparently different ($P < 0.05$), the control means were also different ($P < 0.05$) in the KHEYLRFa experiments. When data were normalised by subtracting control means from treated means and re-analysed by Student's *t*-test, no differences were observed at 0.25 mM and lower, but species differences were maintained ($P < 0.001$) at both 0.5 mM and 1 mM KHEYLRFa. For KSAYMRFa, means were significantly different ($P < 0.001$) at 1 mM but not at 0.5 mM (Fig. 2B).

BIOCHEMICAL COMPARISONS

Two features that can affect FLP activity, stability of the peptide and amino acid sequence, were examined with KHEYLRFa and KSAYMRFa. Preparations from *H. glycines* and *M. incognita* each digested QXL520-KHEYLRf-K(5-FAM)a at similar rates ($1.58 \pm 0.46 V_{\max} \text{ min}^{-1} \mu\text{g}^{-1}$, *H. glycines*; $1.26 \pm 0.59 V_{\max} \text{ min}^{-1} \mu\text{g}^{-1}$, *M. incognita*), and each species also digested QXL520-KSAYMRf-K(5-FAM)a at similar rates (*H. glycines*, $10.41 \pm 3.05 V_{\max} \text{ min}^{-1} \mu\text{g}^{-1}$; *M. incognita*, $10.44 \pm 4.18 V_{\max} \text{ min}^{-1} \mu\text{g}^{-1}$). In each species, the rate of KSAYMRFa digestion was significantly higher ($P < 0.001$) than that of KHEYLRFa.

Substitution of D-amino acids at each of the seven positions in KHEYLRFa resulted in decreased ($P < 0.05$) activity for each of the seven analogues, relative to the native peptide, in both *H. glycines* and *M. incognita* (Table 1). However, all seven KHEYLRFa analogues stimulated head movement above control levels in each species. In *M. incognita*, no differences in activity were observed among any of the analogues. In *H. glycines*,

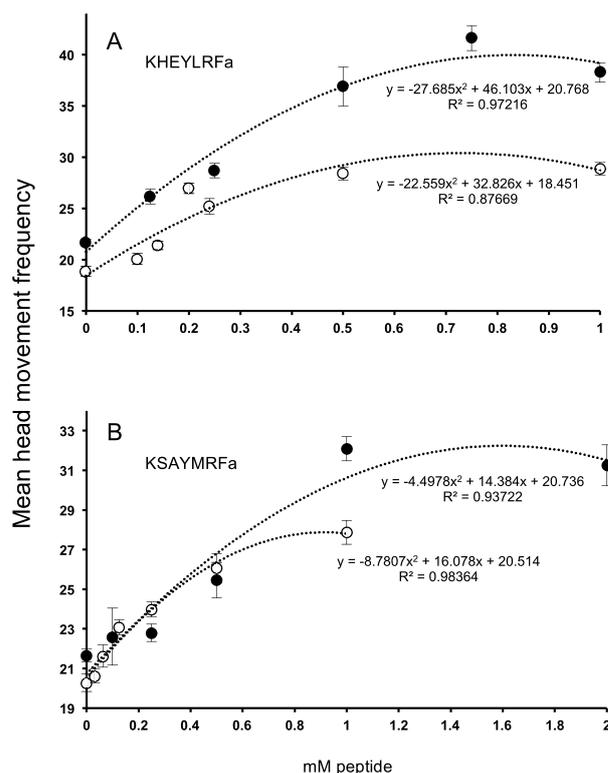


Fig. 2. Effect of varying doses of two different nematode FLPs on the frequency of head movement in infective juveniles (J2) of *Heterodera glycines* and *Meloidogyne incognita*. J2 were incubated in either KHEYLRFa (A) or KSAYMRFa (B) at the indicated doses and observed using an inverted microscope at 40 \times . For each worm, head movement was monitored (see Materials and methods) at 1-min intervals (trials) and the head movement frequency (movements min^{-1}) recorded. The average frequency of three separate trials for each worm was used as a single replicate and each data point represents the mean head movement frequency \pm SEM of 5-7 separate observations (worms). Dose response data are best fit by 2nd order polynomial lines. Selected means (see text) were compared between species by Student's *t*-test.

KHEYLRdFa and dKHEYLRFa were each more active ($P < 0.05$) than KdHEYLRFa, and KHEYLRdFa was also more active than KHEYdLRFa ($P < 0.05$).

With KSAYMRFa, substitution at any position resulted in decreased activity ($P < 0.05$) in each species except for KSAYdMRFa in *H. glycines*, which was not different from the native peptide (Table 2). In *H. glycines*, neither KSdAYMRFa nor KSAYdMRFa stimulated activity above the control. All other analogues were stimulatory, and dKSAYMRFa and KSAYMRdFa were nearly identical. In *M. incognita*, six of seven analogues failed to stim-

Table 1. Effect of KHEYLRFa and D-amino acid analogues on the in vitro head movement frequency of infective juveniles of two plant-parasitic nematodes.

Treatment	<i>Heterodera glycines</i>		<i>Meloidogyne incognita</i>	
	Mean frequency	% of control	Mean frequency	% of control
Control	21.19 ± 0.61 ^a	–	20.45 ± 0.47 ^a	–
KHEYLRFa	40.52 ± 0.65 ^b	183	28.89 ± 0.68 ^b	141
dKHEYLRFa	31.48 ± 1.20 ^{ce}	142	22.17 ± 0.68 ^c	108
KdHEYLRFa	26.12 ± 0.93 ^d	118	22.76 ± 0.82 ^c	116
KHdHEYLRFa	27.83 ± 1.06 ^{cde}	125	23.69 ± 0.63 ^c	116
KHEdYLRFa	29.38 ± 0.81 ^{cde}	132	22.67 ± 0.53 ^c	111
KHEYdLRFa	27.33 ± 0.85 ^{cd}	123	22.09 ± 0.52 ^c	108
KHEYLdRFa	30.81 ± 1.47 ^{ce}	139	22.79 ± 0.42 ^c	111
KHEYLRdFa	32.10 ± 0.89 ^e	145	22.05 ± 0.45 ^c	108

Data are expressed as the mean ± SEM of a minimum of 14 replicates except *M. incognita* KHEYLRFa (n = 12). All means were compared by one-way ANOVA with Tukey's post-test. Within each species, means followed by different letters are significantly different ($P < 0.05$).

Table 2. Effect of KSAYMRFa and D-amino acid analogues on the in vitro head movement frequency of infective juveniles of two plant-parasitic nematodes.

Treatment	<i>Heterodera glycines</i>		<i>Meloidogyne incognita</i>	
	Mean frequency	% of control	Mean frequency	% of control
Control	21.37 ± 0.41 ^a	–	20.77 ± 0.63 ^a	–
KSAYMRFa	32.00 ± 0.66 ^b	133	27.52 ± 0.85 ^b	133
dKSAYMRFa	27.52 ± 0.45 ^{cdf}	122	24.21 ± 0.81 ^c	117
KdSAYMRFa	28.36 ± 0.85 ^{cd}	125	22.76 ± 0.59 ^{ac}	110
KSdAYMRFa	23.03 ± 0.57 ^{ae}	107	23.57 ± 0.63 ^{ac}	113
KSAdYMRFa	25.93 ± 0.95 ^{cef}	118	23.86 ± 0.85 ^{ac}	115
KSAYdMRFa	29.43 ± 0.77 ^{bd}	127	23.38 ± 0.68 ^{ac}	113
KSAYMdRFa	24.88 ± 0.77 ^{af}	114	22.79 ± 0.47 ^{ac}	110
KSAYMRdFa	27.38 ± 0.80 ^{cdf}	122	21.33 ± 0.62 ^{ac}	103

Data are expressed as the mean ± SEM of a minimum of 14 replicates. All means were compared by one-way ANOVA with Tukey's post-test. Within each species, means followed by different letters are significantly different ($P < 0.05$).

ulate head movement. Only dKSAYMRFa was stimulatory ($P < 0.05$). No analogue was inhibitory in either species.

NEMATODE BEHAVIORAL STABILITY

Worm-to-worm variation was rather stable. The averages of the three-trial standard deviations for *H. glycines* and *M. incognita* in control conditions were the same (Table 3), as were the averages for the 1 mM KSAYMRFa treatments. Averages for the three-trial standard deviations for *H. glycines* and *M. incognita* treated with 1 mM

KHEYLRFa were different, however ($P < 0.05$), with the *H. glycines* average 33% greater than that of *M. incognita*. This was the only significant difference among the six means compared. Analysis of worm responses (worm head movement frequency average vs worm three-trial standard deviation) across all treatments revealed little correlation between the frequency of head movement and the variation among trials. For *H. glycines* (n = 1262 individual worms) $r^2 = 0.092$ for both linear and second-order polynomial models, and for *M. incognita* (n = 845 individual worms), r^2 values were 0.092 and 0.091, respectively.

Table 3. Stability of head movement behaviour of infective juveniles exposed to 1 mM FLPs in vitro.

Treatment	Mean trial variation	
	<i>Heterodera glycines</i>	<i>Meloidogyne incognita</i>
Control	2.04 ± 0.08 ^{1,a} (308)	2.22 ± 0.09 ^{1,a} (135)
KSAYMRFa	2.57 ± 0.27 ^{1,a} (31)	2.55 ± 0.20 ^{1,a} (56)
KHEYLRFa	3.48 ± 0.37 ^{2,b} (42)	2.61 ± 0.22 ^{1,a} (42)

Data are expressed as the mean ± SEM of (n) replicates. All means were compared by Student's *t*-test with Mann-Whitney post-test. Within each species, means followed by different letters are significantly different ($P < 0.05$). Between species, means followed by different numbers are significantly different ($P < 0.05$).

Discussion

Nematode FLPs are particularly important for regulating motor and sensory activities (Marks & Maule, 2010; Holden-Dye & Walker, 2011). Their effects on behaviour, identified through genetic (*e.g.*, silencing) and direct exposure experiments, include regulating pharyngeal (feeding) and body wall (locomotion) musculatures in free-living (Reinitz *et al.*, 2000; Rogers *et al.*, 2001; Li & Kim, 2010), animal-parasitic (Brownlee *et al.*, 1996; Reinitz *et al.*, 2000, 2011) and plant-parasitic (Kimber *et al.*, 2007; Masler, 2007; Dalzell *et al.*, 2010) nematodes. Consequently, FLPs are important targets for novel parasitic nematode control strategies (Kimber & Fleming, 2005; Marks & Maule, 2010; Holden-Dye & Walker, 2011). The present work provides the first extensive analysis of behavioural responses of plant-parasitic nematodes directly exposed to nematode FLPs in an assay system useful for FLP physiological screening.

In vitro behaviours of *H. glycines* and *M. incognita* J2, measured by changes in the frequency of head movement, were significantly affected by FLP exposure, and responses differed among specific FLPs and between species. KHEYLRFa was a more effective stimulant than KSAYMRFa in *H. glycines*, whereas the two FLPs were equally stimulatory in *M. incognita*. While reasons for this are not clear, one possible explanation is suggested by the different digestion rates of KSAYMRFa and KHEYLRFa. Rates of digestion, and ostensibly *in vivo* half-lives, vary widely among FLPs exposed to different nematode extracts (Masler, 2010). If the *in vivo* half-life of KSAYMRFa in *H. glycines* is shorter than that of KHEYLRFa, behavioural effects may be lower. Although the *in vitro* digestion rates of the two FLPs in *M. incognita* were

similarly different, *in vivo* exposure of KHEYLRFa and KSAYMRFa to different tissue environments may require different susceptibilities of the peptides to endogenous protease and protease inhibitor combinations.

KSAYMRFa and KHEYLRFa are, in fact, expressed differently. In the potato cyst nematode *Globodera pallida* and in *M. incognita*, KHEYLRFa is expressed primarily in anterior neurons associated with head musculature (Kimber *et al.*, 2002; Johnston *et al.*, 2010), whereas KSAYMRFa is expressed in anterior neurons and in posterior lumbar ganglia in *M. incognita* (Kimber *et al.*, 2002). Also, in cyst and root-knot nematodes, *flp-6* genes encode more copies of KSAYMRFa than *flp-14* genes encode KHEYLRFa (McVeigh *et al.*, 2005; Kimber *et al.*, 2007). These quantitative and qualitative expression differences may be accompanied by different degradation kinetics. More comprehensive biochemical analyses of FLPs as substrates, and analyses of nematode protease complements, are required to draw any firm conclusions.

While *M. incognita* was less sensitive than *H. glycines* to KHEYLRFa and KSAYMRFa, it was more broadly responsive to other FLPs. AQTFFVRFa is an intriguing example; it inhibits locomotion in *Ascaris suum* (Reinitz *et al.*, 2011), and pharyngeal pumping in *C. elegans* (Rogers *et al.*, 2001), had no effect on *H. glycines* and stimulated *M. incognita*. Also, it is the only FLP without a tyrosine at position 4 to have an effect on movement in our assays.

The complex natures of FLP physiology and metabolism prevent specific conclusions to be drawn from such observations. The current assay does not provide information on explicit interactions between FLPs and their receptors, especially since nematode FLP receptors are promiscuous (McVeigh *et al.*, 2006; Li & Kim, 2010), nor does it describe *in vivo* metabolic conditions associated with specific anatomical locations. While the possibility of procedural artefacts must be considered, the assay provides a convenient and rapid high level tool for screening FLPs and has the capacity to reveal subtle species differences.

D-amino acid substitution at any KHEYLRFa position decreased stimulatory activity in *M. incognita*, and all substitutions had the same effect. In *H. glycines*, all substitutions also decreased activity relative to the native peptide, but the effects were not as uniform as in *M. incognita*. Substitution at the C-terminus was significantly less effective than at two internal positions, and substitution at the N-terminus was less effective than substitution at His₂. With KSAYMRFa there were again no positional

variations in *M. incognita*, while in *H. glycines* substitution at Met₅ did not decrease activity relative to the native peptide. The absence of inhibition of head movement by any of the substituted analogues may not be surprising since there should be endogenous levels of KHEYLRFa and KSAYMRFa present in the treated worms. In fact, silencing of *Gp flp-6* encoding KSAYMRFa in *G. pallida* profoundly decreased motility, perhaps through dysfunction in body wall muscle coordination (Kimber & Fleming, 2005; Kimber *et al.*, 2007). It would be interesting to treat such animals with a KSAYMRFa soak to see if some locomotory functions are restored.

The reduced ability of modified FLPs to stimulate head movement relative to the native peptide does suggest that binding to receptors might have been compromised. An alternative explanation is that the presence of D-amino acid substitutions decreased peptide stability in the nematode. However, this is unlikely since D-amino acid substitution is used to stabilise peptides *in vivo* (Hong *et al.*, 1999) and has been shown to reduce KSAYMRFa degradation in nematode extracts (Masler, 2010).

The behaviour of plant-parasitic nematodes *in vitro* is remarkably stable. Under control conditions, worm-to-worm variability in head movement frequency is highly predictable and is, in fact, the same for both *H. glycines* and *M. incognita*. This should allow detection of subtle changes in nematode behaviour. For example, beyond measuring changes in mean head movement frequency in response to specific treatments and using these measurements to compare species, changes in response variability can also be analysed. This provides additional metrics with which to compare species.

The importance of FLP regulation of nematode neuromuscular control to the maintenance of normal physiology and survival of parasitic nematodes is evident, and provides targets for novel intervention (Kimber & Fleming, 2007; Holden-Dye & Walker, 2011). However, the sheer complexity of the FLP signalling systems in parasitic nematodes requires an equally complex and comprehensive research approach (McVeigh *et al.*, 2006). Although it would not be responsible to project *in vivo* actions based solely upon *in vitro* observations (Kimber *et al.*, 2007), high-level (whole animal) experimentation can reliably detect qualitative and quantitative changes in behavioural responses, guiding the selection of FLPs for further biochemical analysis. The close coupling of organismal and molecular approaches will continue to provide necessary insight into the complexity of FLP regulatory systems.

Acknowledgement

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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