The insecticidal activity of recombinant nemertide toxin α-1 from *Lineus longissimus* towards pests and beneficial species.

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Abstract

The nemertide toxins from the phylum Nemertea are a little researched family of neurotoxins with potential for development as biopesticides. Here we report the recombinant production of nemertide α-1 (α-1), a 65-residue inhibitor cystine knot (ICK) peptide from *Lineus longissimus*, known to target insect voltage-gated sodium channels. The insecticidal activity of α-1 was assessed and compared with the well characterised ICK venom peptide, ω-atracotoxin/hexatoxin-Hv1a (Hv1a). α-1 elicited potent spastic paralysis when injected into cabbage moth (*Mamestra brassicae*) larvae; conferring an ED_{50} 3.90 µg / larva (10.30 nmol / g larva), followed by mortality (60 % within 48 hours after 10 µg injection). By comparison, injection of *M. brassicae* larvae with recombinant Hv1a produced short-lived flaccid paralysis with an ED_{50} over 6 times greater than that of α-1 at 26.20 µg / larva (64.70 nmol / g larva). Oral toxicity of α-1 was demonstrated against two aphid species (*Myzus persicae* and *Acyrthosiphon pisum*), with respective LC_{50} values of 0.35 and 0.14 mg / mL, some 6-fold lower than those derived for recombinant Hv1a. When delivered orally to *M. brassicae* larvae, α-1 caused both paralysis (ED_{50} 11.93 µg / larva, 31.5 nmol / g larva) and mortality. This contrasts with the lack of oral activity of Hv1a, which when fed to *M. brassicae* larvae had no effect on feeding or survival. Hv1a has previously been shown to be non-toxic by injection to the beneficial honeybee (*Apis mellifera*). By contrast, rapid paralysis and 100 % mortality was observed following injection of α-1 (31.6 nmol / g insect). These results demonstrate the great potential of naturally occurring non-venomous peptides, such as α-1, for development as novel effective biopesticides, but equally highlights the importance of understanding the phyletic specificity of a given toxin at an early stage in the quest to discover and develop safe and sustainable pesticides.

Keywords: Nemertide; *Lineus longissimus*; α-1; Hv1a; insecticidal toxin; inhibitor cystine knot.

1. Introduction

Animal toxins are a valuable source of biologically active compounds. Toxins present in venoms or poisons are used by many species to rapidly subdue or kill potential predators and/or prey and comprise a complex cocktail of small molecules, peptides, and proteins, some of which are beginning to find use in therapeutic and agricultural applications (Chen *et al*., 2018; King, 2019). Incorporating natural toxins into the design of novel biopesticides could produce environmentally and toxicologically safer pest control measures, as well as pesticides with molecular targets not yet exploited by currently marketed chemical pesticides. Peptide toxins have been isolated from an impressive number of biological classes, from molluscs, reptiles, and sea anemone to cone snails, sponges, and fungi. Some orders, such as spiders, consist of 10s of thousands of species, each with their own unique blend of toxic compounds (secreted in venom to subdue or kill prey). This wealth of natural compounds permits the selection of toxins with taxonomic specificity, capable of targeting pest species while having no effect on off-target organisms such as natural pollinators, which have taken a beating from the broad action of most widely used chemical pesticides.

The phylum Nemertea (ribbon worms) is comprised of nearly 1300 species, of which many produce a potently toxic mucus which envelops their epidermis. The focus of this work is nemertide α-1 (hereafter α-1), a toxin of the nemertide family of disulfide-rich peptide toxins. α-1, first isolated from the mucus of *Lineus longissimus* by Jacobsson *et al*., (2018), was reportedly highly toxic when injected into *Carcinus maenas* (green crabs). Subsequent synthesis and structural solution by 2D-NMR revealed that α-1 forms an inhibitor cystine knot (ICK) motif by the formation of three interlocking disulfide bridges in its core, in the O1 superfamily cystine framework of I-IV, II-V, III-VI. Injection of chemically synthesised α-1 into cockroaches (*Blaptica dubia*) produced similar results to those seen in green crabs, with responses observed at doses of 1 µg / kg (300 fmol / g) and death or permanent paralysis observed for all animals at doses higher than 7.1 µg / kg. Many ICK peptides have garnered interest for pesticidal development, in part due to the exceptional thermal and proteolytic stability granted to them by their tightly reticulated structure (Herzig & King, 2015). Activity at fmol concentrations makes α-1 an exciting candidate for further research into its potential as a novel biopesticide. Jacobsson *et al*., (2018) also assessed the activity of synthetic α-1 at voltage-gated sodium (Na_v) channels where it was shown to inhibit inactivation, producing sustained non-inactivating currents in cockroach (*Blattella germanica*), fruit fly (*Drosophila melanogaster*) and the parasitic mite *Varroa destructor* Na_vs, indiscriminately of the channels initial state. α-1 also delayed human $Na_v1.1$, $Na_v1.4$, $Na_v1.5$ and $Na_v1.6$ inactivation, but was shown to be 100-fold less selective to tested mammalian channels than to insect channels. The concluding remarks on the data presented by Jacobsson *et al*., (2018), were of its encouraging promise for exploration of α-1 as a bioinsecticide. Being a mucosal toxin, α-1 Is a poisonous component of the mucosal secretion of *L. longisimus,* rather than a venom component, having evolved to be orally toxic towards organisms which ingests it. This may produce interesting differences in the biological activity of α-1 when compared to the hypodermic venom toxins currently consuming most of the research in this area of peptide toxin biopesticide development.

In this study, a comparison of the insecticidal activity of nemertide α-1 is made to ω-atracotoxin/hexatoxin-Hv1a (hereafter Hv1a), a 37-residue peptide of the ω-HXTX-1 family of ICK toxins, first isolated from the venom of the Australian funnel-web spider *Hadronyche versuta* (Tedford *et al*., 2004). Hv1a is a high-affinity antagonist of insect voltage-gated calcium (Ca_v) channels where it inhibits Ca_v currents (Fletcher *et al*., 1997, Wang *et al*., 1999, Chong *et al*., 2007; Herzig and King, 2015). More recent studies have also suggested Hv1a binds to nicotinic acetylcholine receptors (nAChRs), resulting in positive allosteric modulation (Chambers *et al*., 2019). Like α-1, Hv1a conforms to a disulfide bonding pattern of Cys I-IV, II-V, III-VI (Fletcher *et al.,* 1997). The insecticidal effects of this peptide are limited to invertebrate channels, with no effect seen in vertebrate nerve-muscle preparations or juvenile mice (Fletcher *et al.,* 1997, Tedford *et al.,* 2004). The observed toxicity of Hv1a by injection has been variable, particularly between insect species, with LD_{50} values spanning from 0.77 nmol / g in adult housefly *Musca domestica* (Mukherjee *et al*., 2006) to 18.1 nmol / g in cabbage looper, *Mamestra brassicae*, larvae (Powell *et al*., 2019), demonstrating variation in the toxins ability to bind to and or disrupt Ca_v channels or nAChRs. Hv1a, like most venom peptide toxins, has limited oral activity, having evolved to be hypodermically active. Improvement of the toxins oral activity has been achieved via recombinant fusion to "carrier" proteins, such as plant lectins (Fitches *et al*., 2012; Powell *et al*., 2019) and viral coat proteins (Bonning *et al*., 2013). Critically, neither the toxin nor the recombinant fusion protein is toxic to the European honeybee (*Apis mellifera*) (Nakasu *et al.,* 2014, Powell *et al.,* 2019). As one of the best-studied families of ICK toxins, various ω-HXTX- paralogues have seen use in biopesticide development for over two decades, being transgenically expressed in tobacco plants as early as 1996 (Jiang *et al.,* 1996, Hernandez-Campuzano *et al.,* 2009). GS-omega/kappa-Hxtx-Hv1a, a further member of the ω-HXTX-1 family of ICK toxins, recently saw US EPA approval and commercialisation, demonstrating the potential of peptide toxins and ICKs, leading to a new addition to the insecticide resistance action committee's mode of action classification list, designated for peptide-based biopesticides (Sparks *et al.,* 2020).

The development of crop protection methods utilising natural toxins, be it via conventional spray application, transgenic expression *in planta*, or incorporated into baculovirus systems, could allow safer, more target-specific and sustainable pest control measures to be realised. However, the discovery and selection of appropriate toxin candidates is pivotal to success. Based on the data currently gathered, α-1 has shown considerable promise as a candidate for development as a biopesticide. To further assess this toxins suitability, the present paper reports on the recombinant production of α-1, along with Hv1a, using *Pichia pastoris* as an expression host, followed by characterisation of biological activity in Hemiptera (two aphid species), and Lepidoptera (*Mamestra brassicae* larvae). We show that α-1 is highly potent to insects when delivered either orally or by injection. However, α-1 was also found to be highly toxic when injected into adult honeybees. This is a notable property of any pesticide and should inform

98 further development of α-1 as a novel biopesticide. The reasons behind this broad action and the lessons this can
99 provide for future toxin selection and design are discussed herein.
100

2. Materials and methods

2.1 *Recombinant protein production*

Synthetic constructs encoding for nemertide α-1 and Hv1a, depicted in **Fig 1a**, were generated based on amino acid sequences available on UniProtKB (P0DM24 and P56207, respectively). Gene sequences were codon optimised for expression in *P. pastoris* and synthesised by Integrated DNA Technologies (IDT), with each incorporating their respective predicted 17 residue pro regions and restriction sites for cloning into the yeast expression vector pGAPZαB (Invitrogen). After PCR amplification, gene products were digested with Pst1 and Sal1 and ligated into similarly digested expression vector pGAPZαB, in frame with the N-terminal alpha factor secretion sequence and the C-terminal polyhistidine tag. Plasmid pGAPZαB-proα-1 and pGAPZαB-proHv1a DNAs were transformed into heat shock competent DH5α *E. coli* cells. Sequence-verified clones in the vector pGAPZαB were purified and linearised (*AvrII*) for transformation into *P. pastoris* parental strain SMD 1168H which was carried out using a Pichia EasyComp™ Transformation kit (Invitrogen). Transformants were selected on YPG (1 % yeast extract [w/v], 2 % peptone [w/v], 4 % glycerol ([v/v], 1.5 % agar [w/v]) agar plates containing 100 µg / mL zeocin. Selected clones were screened for protein expression by western blotting culture supernatants derived from 10 mL YPG-zeocin culture grown for 3 days at 30 ˚C, using anti-His antibodies as previously described (Fitches *et al*., 2012).

P. pastoris cells expressing recombinant proteins were used to inoculate 2.5 L sterile basal salt medium. The culture was grown in a 7.5 L Applikon – ez-Control laboratory fermenter BioFlo as previously described (Fitches *et al*., 2012). Secreted proteins were separated from cells by centrifugation (30 min at 7500 *g*, 4 ˚C) and clarified by vacuum filtration through 2.7 and 0.7 µM glass fibre filters (Whatman). Culture supernatant was adjusted to 20 mM sodium phosphate and 0.4 M sodium chloride using a 4X buffer (pH 7.4). Recombinant α-1 and Hv1a were both purified by nickel affinity chromatography on 5 mL HisTrap crude nickel columns with a flow rate of 3-4 mL / min. Bound protein was eluted with 0.2 M imidazole 20 mM sodium phosphate, 0.4 M sodium chloride pH 7.4. Dialysis was carried using 3.5 kDa molecular weight cut off tubing against deionised water using multiple changes to remove all small molecules, and lyophilised. Protein purity was assessed by SDS-PAGE, where α-1 was also quantified by densitometric analysis of protein bands stained for total protein with Coomassie blue. Quantification used known concentrations of GNA as a protein standard with visualisation and analysis carried out using iBright™ software (Thermo Fisher). As Hv1a does not resolve well on polyacrylamide gel, quantification relied on BCA assay, using BSA as a standard protein.

2.2 *Protein characterisation*

Proteins were analysed by SDS-PAGE on 17.5 % acrylamide gels, prepared with 5X loading buffer (312.5 mM Tris–HCl pH 6.8, 10 % SDS [w/v], 25 % β-mercaptoethanol [v/v], 50 % glycerol [v/v], 0.01 % bromophenol blue [w/v]) and denatured at 100 ˚C for 5 min before loading. Analysis by western blotting was carried out as described previously

(Fitches *et al*., 2012) with anti-His antibodies. LC-MS analysis of digested α-1 was carried at the Proteomics Analysis Facility within Durham University, using protein in excised gel slices digested with trypsin, as previously described by Powell *et al*., (2019).

2.3 ***Insect cultures***

Mamestra brassicae (cabbage moth), were maintained at the Biosciences Dept., Durham University, UK. Insects were reared on standard lepidopteran diet, as described by Bown *et al.*, (1997), and maintained at 25 ˚C, 65 % relative humidity, in a 16:8 h light:dark regime. *Myzus persicae* (peach potato aphid) were reared on oilseed rape (*Brassica napus* L. cv Apex), at 22 ˚C, 50 % RH, under a 16:8 h light:dark regime. *Acyrthosiphon pisum* (pea aphid) were cultured on plants of *Vicia faba* (broad bean cv. Sutton Dwarf) under similar conditions to *M. persicae*. *Apis mellifera* workers were supplied by Fera Science Ltd. Home Apiary.

2.4 ***Injection bioassays***

Injection bioassays were carried out using newly eclosed fifth instar larvae of *M. brassicae* (approx. 50-70 mg). Larvae were briefly anesthetised with CO_2 prior to injection of 5 µl of protein solution (sodium phosphate buffer: 20 mM sodium phosphate pH 7.4), delivering 1.25 – 50 µg of α-1 or 10 – 40 µg Hv1a toxin per larva (n=15 per dose) at the anterior dorsal region, directly behind the head capsule. Controls were injected with 5 µl sodium phosphate buffer. The larvae were monitored post injection to assess both paralysis and mortality. Expulsion of the toxin following injection of higher doses of α-1 prevented derivation of reliable LD_{50} data. As such, the level of paralysis 20 minutes or 1-hour post injection was used to determine a median effective dose (ED_{50}) for Hv1a and α-1, respectively. The difference in period of assessment is due to the highly different phenotypic response and duration of reaction to the two toxins. Mortality was also recorded daily for 2 days. Newly emerged adult *A. mellifera* (approx. 100 mg) were anesthetised by cooling on ice before injection under the 5^{th} abdominal segment with 2 µL SPB solutions containing 20 µg or 40 µg of ProNα-1 (3 replicates of 10 bees per dose). Controls were injected with 2 µL SPB. Injections were conducted using a Hamilton syringe with a 33-gauge custom fine needle.

2.5 ***Feeding bioassays***

Adult apterous (non-winged) pea and peach potato aphids were fed on artificial liquid diet (AD) (Prosser & Douglas, 1992) for 24 and 48 h, respectively to produce nymphs for feeding assays. Stock solutions of α-1 and pro-Hv1a were prepared by resuspending proteins in SPB before being added to sterile AD at a ratio of 1:4. Day 1 nymphs were fed on AD containing recombinant α-1 at 0.1 to 0.8 mg / mL or Hv1a at 0.2 to 2 mg / mL. As control treatment, aphids

were either fed AD with an equivalent volume of SPB to the protein treatments or no diet at all. Survival of nymphs was assessed daily for 3-4 days. Artificial diets were replaced every 2 days.

Newly eclosed fifth instar *M. brassicae* larvae (60-100 mg), having been starved overnight, were droplet fed 5 µl of aqueous solution containing 5 – 30 µg α-1 toxin in 10 mM sodium phosphate buffer pH 7.4 and 10 % sucrose [w/v]. Control groups were fed 5 µL 10 % sucrose solution (2.5 µl SPB, 2.5 µl 20 % sucrose aqueous solution). Larvae were droplet fed and monitored for phenotypic response / mortality 1 hour and 24 hours post feeding. ED_{50} was assessed by the number of larvae paralysed 1-hour post feeding. Data were analysed and median dose values calculated using Graphpad Prism (Graphpad USA) Kaplan-Meier survival analysis.

3. Results

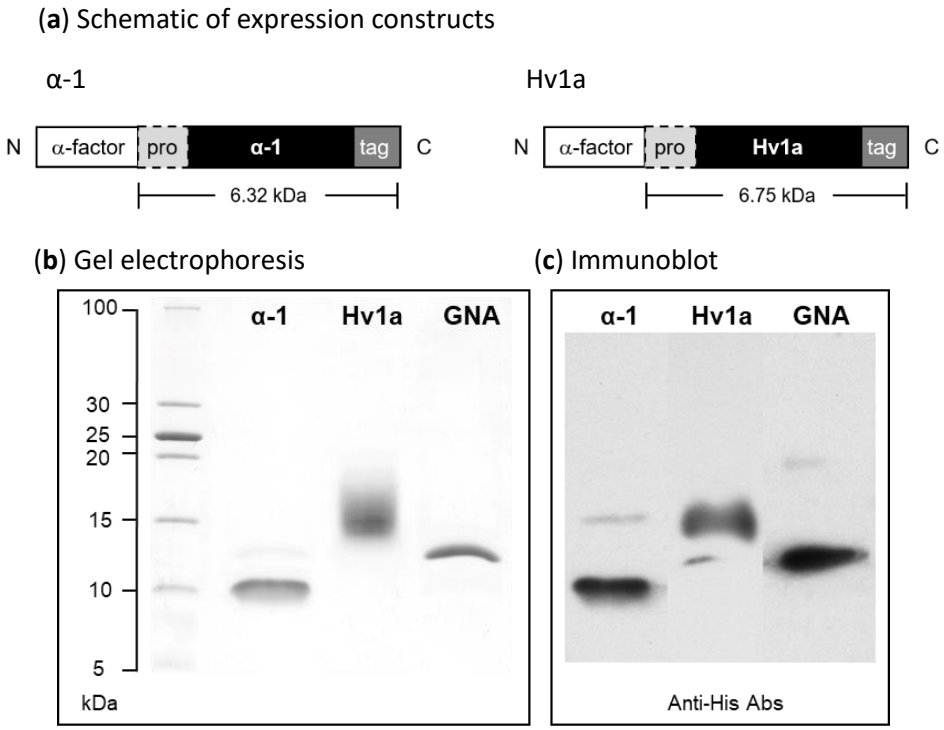

Figure 1. a Schematic of constructs encoding recombinant proteins produced in the yeast *P. pastoris*. The α-factor pre-pro-sequence directs expressed protein to the yeast secretory pathway enabling purification from fermented culture supernatants. Tag denotes the presence of a six-residue histidine sequence that allows recombinant protein detection by western blotting and purification by nickel affinity chromatography. **b** Separation of purified recombinant proteins on SDS-PAGE gels stained for total protein: α-1, Hv1a, GNA standard (6 µg total protein loaded in all wells). **c** Western analysis (composite blots of recombinant proteins using anti-His antibodies) of purified recombinant protein (200 ng loaded in all wells). Location of protein mass markers are as depicted in **b**.

3.1 ***Production of recombinant protein in P. pastoris***

Recombinant α-1 and Hv1a were produced by benchtop fermentation using selected transformed *P. pastoris* clones. A six-residue N-terminal histidine tag incorporated into the expression cassettes allowed single step nickel-affinity purification of the expressed peptides and detection by western analysis. Purified α-1 stains as a single polypeptide of approx. 10 kDa on gels stained for total protein, greater than the predicted molecular mass of α-1 including the pro-region of 6.32 kDa (**Fig 1b**). The α-1 peptide reacted positively with anti-His antibodies (**Fig 1c**) and data from LC-MS analysis confirms the presence of full-length protein and demonstrated that the pro region of the peptide is not cleaved during expression in *P. pastoris* (**Fig 2**). The peptide expressed at approx. 20 mg / L culture supernatant and more than 90 % was recovered by nickel affinity purification, followed by dialysis and lyophilisation. As shown in **Fig 1a**, purified Hv1a does not separate well, running as a smear on polyacrylamide gel, and this is thought to be due to heterogonous glycosylation by *P. pastoris* cells and/or incomplete denaturation by SDS. Whilst both proteins were found to be glycosylated by periodic acid-Schiff stain (data not shown), the single band observed for recombinant α-1 may be indicative of homogeneous glycosylation. The predicted mass of Hv1a after cleavage of the pro-region occurs is 6.75 kDa, whereas the expressed product separates as a peptide of approx. 15 kDa which reacts positively with anti-His antibodies. Previously published N-terminal sequencing of Hv1a has shown that the pro-region is cleaved and recovered as intact mature peptide (Powell *et al.*, 2019).

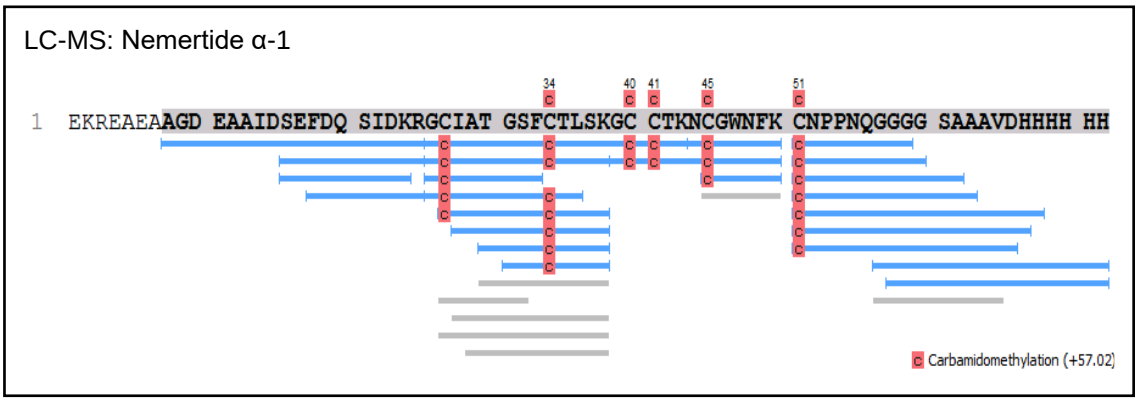

Figure 2. LC-MS data obtained from digests of purified recombinant α-1. Blue bars depict identified peptides and grey bars are sequence tags. The presence of N-terminal residues AGDEAAIDSEFDQSIDKR provide evidence that the pro region of the peptide in not cleaved during expression in *P. pastoris*. Details of peptide fragments are presented in supplementary table 1.

3.2 ***Biological Activity of α-1 and Hv1a***

3.2.1 *Injection toxicity of α-1 and Hv1a in* M. brassicae

The biological activity of α-1 was assessed via injection into newly eclosed fifth instar *M. brassicae* larvae. Injection of α-1 with doses ranging from 1.25 – 50 µg / larva resulted in immediate spastic paralysis of the larvae. The period of

spastic paralysis lasted for up to 48 hours post injection. Larvae were able to recover from injections of low doses (5-10 µg / larva), but at a dose of 10 µg, 60 % of larvae died within 48 hr of injection. The level of spastic paralysis of larvae injected with higher doses (15-25 µg / larva) caused for the expulsion of the injected toxin from the point of injection (Fig. 4). As a result, calculating an LD_{50} was not considered appropriate, as it would be unreliable and would not allow for direct comparison with values reported for other peptide toxins. Therefore, the ED_{50} was calculated from the level of phenotypic response (spasm and/or paralysis) observed 1-hour post injection and a median effective dose of 3.90 µg (10.30 nmol / g larva) was derived from dose response assays. Injections of 10 – 40 µg of Hv1a produced a phenotypic response, with flaccid paralysis observed in response to all doses. This response was shorter lived than that of α-1, leading to ED_{50} measurements being taken at 20 minutes post injection. The derived Hv1a injection ED_{50} of 26.22 µg / larva (64.70 nmol / g larva) is some 6.3 times greater than that obtained for α-1.

3.2.2 *Oral toxicity of α-1 and Hv1a to A. pisum and M. persicae*

The oral toxicity of α-1 and Hv1a were assessed by feeding day 1 nymphs of both *A. pisum* and *M. persicae* on diets containing different concentrations of each toxin, ranging from 0.1 – 0.8 mg / mL for α-1 and 0.2 – 2 mg / mL for Hv1a. Control groups were fed either diet with no added protein, or no diet at all. As shown in **Fig 3**, rapid dose dependent mortality was observed in both aphid species in response to orally delivered α-1. A similar spastic paralytic response as observed for injected *M. brassicae* larvae was observed in both aphid species and complete mortality occurred within just 3-4 days of feeding in both species at the lowest dose of 0.1 mg / mL. The highest dose of α-1, 0.8 mg / mL, produced total mortality in *A. pisum* groups within just 24 hours, with an LC_{50} (day 2) of 0.14 mg / mL. In *M. persicae* α-1 produced mortality more slowly, with complete mortality at 0.8 mg / mL occurring at day 3, with an LC_{50} (day 2) of 0.35 mg / mL, over 2 times greater than that seen in *A. pisum*. Dose dependent mortality was also observed in aphids fed on Hv1a solutions, though with a slower response. At a dietary concentration of 2 mg / mL, Hv1a caused total mortality in *A. pisum* by day 3 and *M. persicae* by day 4, with total mortality only occurring at the higher doses (≥ 0.6 mg / mL), with *M. persicae* and *A. pisum* surviving to days 7 and 8 of the assay, respectively (**Fig 3**). Day 2 LC_{50} values for Hv1a were more than 6-fold greater than those for α-1, though a similar discrepancy between the species was seen at 1.01 mg / mL for *A. pisum* and 2.34 mg / mL for *M. persicae*.

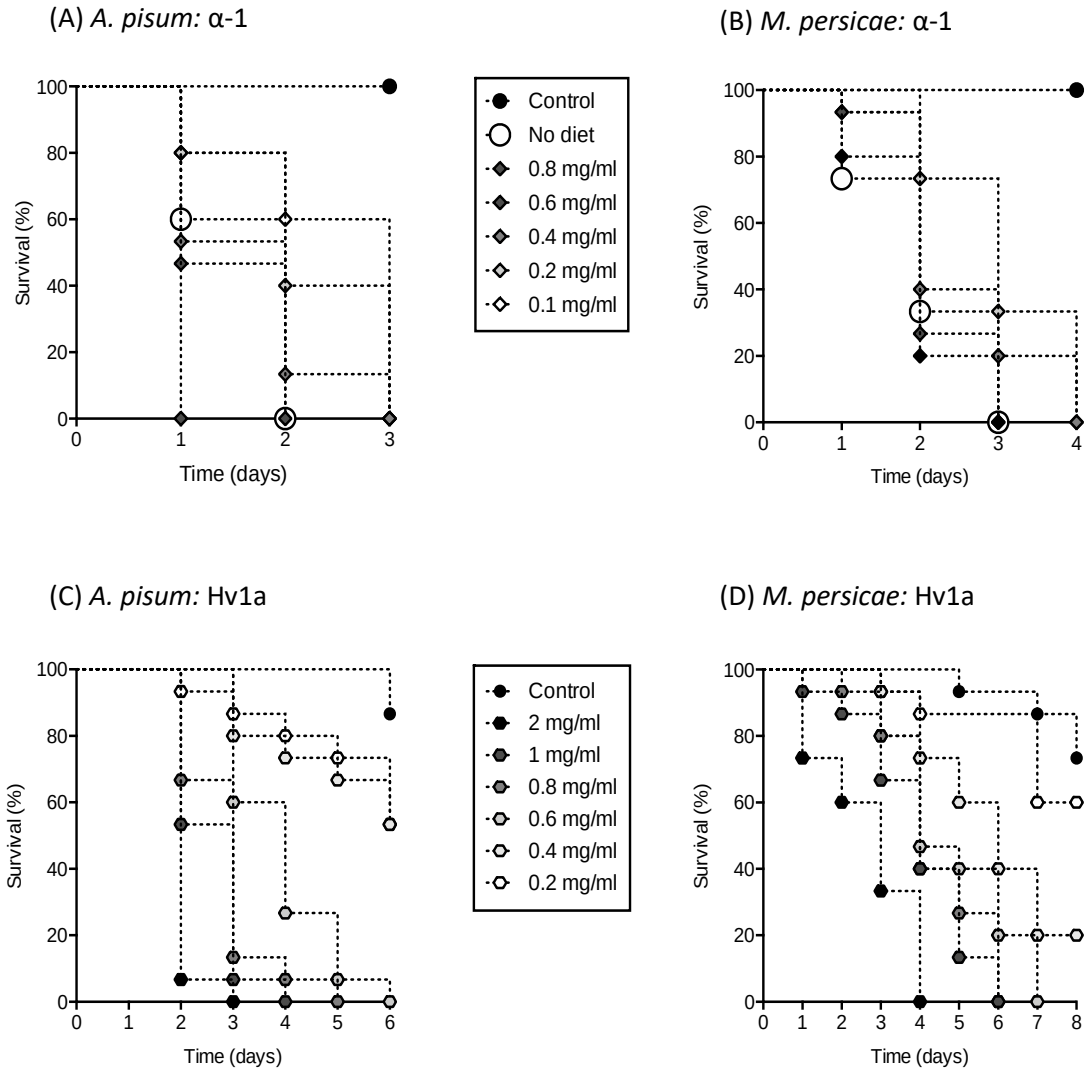

Figure 3. Aphid feeding assay. Kaplan-Meier survival plots of *A. pisum* (A & C) and *M. persicae* (B & D) (*n* = 15 aphids per replicate) fed on diets containing either α-1 (A & B) or Hv1a (C & D), control (no added protein) or no diet at all.

3.2.3 *Oral toxicity of α-1 to M. brassicae*

Feeding *M. brassicae* larvae on droplets containing 5 – 30 µg of α-1 resulted in a rapid response (10 – 20 minutes) and provided direct evidence of oral activity towards lepidopteran larvae. After ingestion, writhing of the insect occurred, followed within 1 hour by paralysis. Although at higher doses the toxin was expelled by vomiting shortly after ingestion, paralysis still ensued, and at ≥ 20 µg (52.8 nmol / g larvae), complete larval mortality occurred within 24 hr. The median effective dose was calculated from the number of larvae showing a paralytic response after 1 hour (**Table 1**); ED_{50} = 11.93 µg / larva (31.5 nmol / g larva).

3.2.4 *Injection results of α-1 in A. mellifera*

To investigate the effects of α-1 on non-target pollinator species, injections were carried out with newly emerged honeybee (*A. mellifera*) workers. Bees (approx. 100 mg) injected with α-1 experienced spastic paralysis within 30 minutes of injection, similar to that observed for injected *M. brassicae* larva. All α-1 treated bees (lowest dose 20 µg (31.6 nmol / g insect) subsequently died within 24 hours post-injection as compared to 97 % survival of the control group.

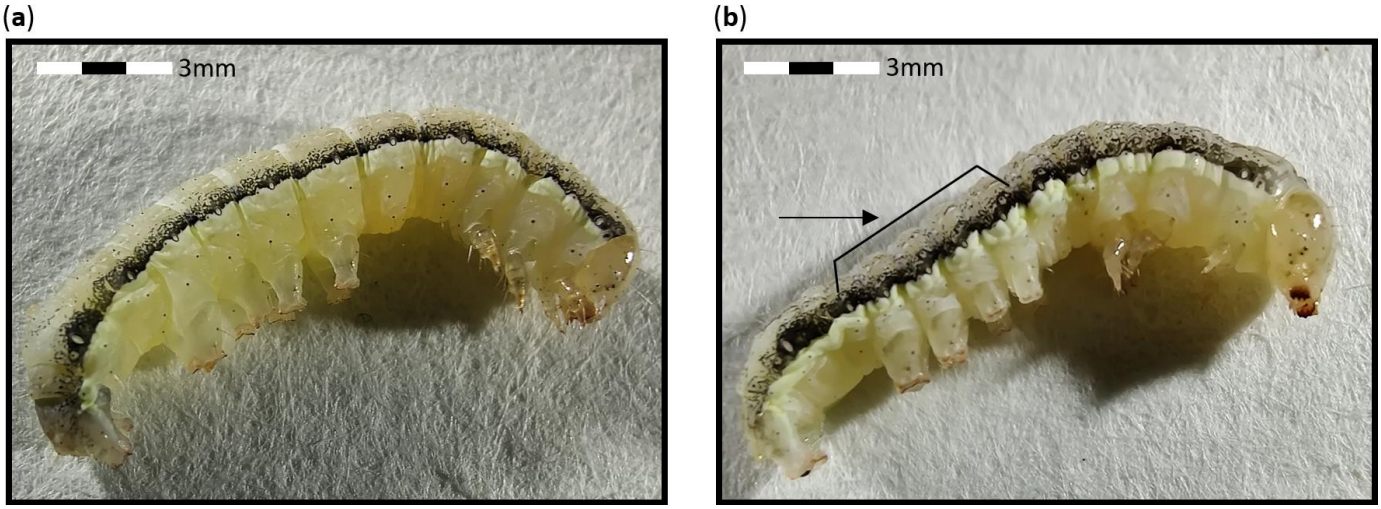

Figure 4. a *Mamestra brassicae* 5th instar larva, anaesthetised with CO_2 gas. **b** *Mamestra brassicae* 5th instar larva, 1 hour post injection with 25 µg of α-1. Larva is unable to right itself. All limbs are in spasm, and the mandibles are extremely active. The skin is contorted all along the lateral side (arrow).

Treatment	***A. pisum* LC_{50} (Day 2)**	***M. persicae* LC_{50} (Day 2)**	***M. brassicae* injection ED_{50}**	***M. brassicae* droplet ED_{50} (1 hour)**
α-1	0.14 (0.1 - 0.19)	0.35 (0.28 - 0.44)	3.89 (2.17 – 6.97) 1 hour	11.93 (10.73 – 13.25)
Hv1a	1.01 (0.87 - 1.17)	2.34 (2.06 - 2.66)	26.22 (23.83 – 28.84) 20 minutes	-

Table 1: LC_{50} (mg / mL) and ED_{50} (µg / g larva) values calculated from survival of aphids and the paralytic response of cabbage moth larvae after feeding and injection (n = 15 per dietary concentration or droplet dose). Confidence intervals (95 %) are provided in parenthesis.

4. Discussion

The purpose of this study was to assess the pesticidal properties of recombinant nemertide α-1, an ICK peptide toxin first isolated from the mucus of the bootlace worm *L. longissimus* (Jacobsson *et al*., 2018). The well-characterised funnel-web spider venom ICK peptide Hv1a was also produced to enable comparison of injection and oral toxicity to lepidopteran larvae and two species of aphids (*M. persicae* and *A. pisum*). Due to the constrained topological structure of ICK peptides, *P. pastoris* was selected as an appropriate expression host, capable of correctly forming the disulfide bridge cross-links, crucial to ICK peptide functionality. The predicted masses of α-1 with pro-region and Hv1a following removal of the pro-region are 6.32 and 6.75 kDa, respectively. The inclusion of a secretory signal (α-factor) sequence and His_6 tag allowed for straightforward purification from culture supernatants, and two protein bands of approx. 10 and 15 kDa reacted positively with anti-His antibodies, corresponding to α-1 and Hv1a, respectively (**Fig 1**). Previous attempts at expressing Hv1a without the N-terminal pro-region produced non-functional peptide (Powell *et al*., 2019); thus, the respective toxin pro-regions were incorporated into the yeast expression cassettes for both recombinant peptides.

To date, little research to explore the range and potential of nemertean toxins for use in medical or agricultural applications has been conducted. The work carried out by Jacobsson *et al.*, (2018) indicates that nemertide α-1 could be an effective new biopesticide, with exceptionally high toxicity towards invertebrates, measured in the fmol / g range in crabs, and 2 – 4 pmol / g in cockroaches. By comparison, the most potent arachnid venom toxins are active in the 10 – 100 pmol / g range (Wang *et al.,* 1999; Figueiredo *et al.,* 1995). Our results support the findings of Jacobsson *et al.*, (2018) via demonstrating high levels of toxicity and remarkable phenotypes in insects exposed to recombinantly produced α-1. Upon injection of α-1 into *M. brassicae* larvae, spastic paralysis was observed within 20 minutes at doses ranging from 10 – 50 µg / larva. At the higher doses (15 – 25 µg / larva) paralysis persisted for up to 48 hours, and > 50 % of larvae died within 24 hr (60 % mortality at 10 µg, 100 % mortality at ≥ 15 µg). Rapid expulsion of the toxin at doses greater than 15 µg during spasm prevented derivation of a reliable LD_{50} value. However, an ED_{50} of paralysis (1-hour post-injection) of 3.89 µg α-1 / larva (10.3 nmol / g larva) was derived from responses to a range of injection doses. We have previously reported that the injection of Hv1a produced mortality in *M. brassicae* and reported an LD_{50} 7.3 µg / larva (18.1 nmol / g larva) (Powell *et al.,* 2019). When injected, Hv1a produced a flaccid paralytic effect in injected larvae. This paralysis was short-lived, with 66 % of larvae injected with 40 µg appearing to recover within 1 hour, though such high doses did produce mortality after 24 hours (40 µg caused 73 % mortality after 24 hours). Thus, an ED_{50} of paralysis for Hv1a (20 minutes post-injection) was calculated at 26.22 µg / larva, notably, more than six times greater than that of α-1.

The α-1 toxin was also shown to be orally toxic to *M. brassicae* larvae when fed in droplet form and to two species of aphid in artificial diet assays. Acute oral activity of α-1 was demonstrated against the specialist *A. pisum* and

generalist *M. persicae* aphid species, with day 2 LC_{50} values of 0.14 and 0.35 mg / mL respectively, and total mortality at the lowest dietary concentration of 0.1 mg / mL observed at days three and four, respectively. Mortality occurred later than in aphids withheld diet, indicating that mortality was due, at least in part, to toxicity rather than solely anti-feeding effects. By contrast, whilst Hv1a was also found to be orally toxic to both aphid species, slower declines in survival and higher doses were required to induce 100 % mortality, with day 2 LC_{50} values 6-fold higher as compared to α-1 (**Table 1**). Bonning *et al*., (2013) previously reported similar results for Hv1a's oral toxicity to aphids (Bonning *et al.,* 2013), although they used only one concentration of Hv1a (0.2 mg / mL) which had been expressed in *Escherichia coli*. A lower toxicity of both toxins towards *M. persicae* than *A. pisum* is likely the result of increased xenobiotic detoxification mechanisms of this species, resulting from its generalist feeding nature (Ramsey *et al*., 2010). *M. brassicae* larvae fed on single droplets containing 5 – 30 µg of α-1 displayed a phenotypic response, although unlike injection, oral delivery produced a paralytic effect without spasm. An oral ED_{50} of 11.93 µg / larva (31.5 nmol / g larva) was derived, and acute oral toxicity was indicated by a rapid expulsion of droplets containing 10 – 30 µg of α-1 by treated larvae. Despite this, paralysis occurred at all doses above 10 µg / larvae, with 100 % mortality occurring at doses ≥ 20 µg within 24 hours. This oral activity sets α-1 apart from previously studied ICK toxins, as, despite the high stability and injection toxicity of ICK peptides, many are much less potent when orally delivered and this is generally attributed to an inability to traverse the insect gut epithelium. Although this is the case with most venom peptides, many crude venoms do produce surprisingly high oral insecticidal activity (Guo *et al*., 2018). The absence of oral activity of Hv1a against *M. brassicae* larvae has previously been shown (Fitches *et al.*, 2012), with no mortality or reduction of larval growth when fed droplets containing 9.6 µg Hv1a / larva daily for four days. Improvements to the oral activity of Hv1a have been demonstrated, utilising fusion to carriers such as snowdrop lectin (*Galanthus nivalis* agglutinin) (Fitches *et al*., 2012) or viral coat proteins (*Pea enation mosaic virus* coat protein) (Bonning *et al.,* 2013), capable of delivering the toxin across the insect gut epithelium, allowing access to the CNS. The presence of α-1 in the mucosal epidermal layer of *L. longissimus* immediately differentiates the toxin from venom ICK peptides, having evolved to be orally rather than hypodermically active. Clearly, the natural oral toxicity of α-1 may circumvent the need to develop delivery systems for development as a new biopesticide.

A primary consideration in the development of novel bioinsecticides should be of their effects upon non-target organisms. Thus, we tested α-1 for its activity against adults of the European honeybee, *Apis mellifera*. Injection of α-1 (lowest dose: 20 µg) into honeybee workers produced similar spastic paralysis to that observed in cabbage moth larvae. Injection doses resulting in 100 % mortality within 24 hours were a comparable 32 nmol / g honeybee and 53 nmol / g cabbage moth larva, indicating that the activity of α-1 is comparable between the species. Like many commercial pesticides, α-1 targets Na_v channels, a family of channel proteins that have been highly conserved among insects (Dong 2006; King *et al*., 2008; Silva & Scott, 2020). Sequence conservation, particularly within the voltage-sensing and pore regions, often targeted by ICK peptides, may contribute to the indiscriminate action between

species of Na_v targeting compounds. By contrast, Hv1a has a reported LC_{50} of 18 nmol / cabbage moth larva but produces neither a paralytic response nor mortality in honeybees at comparable injection doses (Powell *et al*., 2019). The Ca_v channels targeted by Hv1a, have experienced relatively low levels of conservation through insect evolution and, while the repertoire of calcium channel subunits is often small in insect genomes, alternative splicing is utilised to expand the number and diversity of proteins produced (King *et al*., 2008), producing taxonomic specificity in those compounds which target them. Indeed, this helps in part to explain the varied published toxicities of Hv1a between different species (Mukherjee *et al.*, 2006; Wang *et al.,* 1999; Powell *et al*., 2019). More recent studies into the targets of hexatoxins have shown that they act as positive allosteric modulators of insect nicotinic acetylcholine receptors (nAChR) (Chambers *et al.*, 2019). Like Ca_v channels, there is high diversity in insect nAChR receptor subunits and the presence of multiple nAChR subtypes; notably the honeybee has been shown to possess a larger number of nAChR subunits as compared to the fruit fly *Drosophila melanogaster* and mosquito *Anopheles gambiae* (Jones *et al.*, 2006), and this may help to explain the lack of toxicity of Hv1a towards honeybees (Nakasu *et al.,* 2014).

5. Conclusion

The wealth of natural chemistries found in venomous and poisonous animals provides a rich source of lead compounds for biopesticide discovery and design. Here we have shown that α-1 has comparable injection and ingestion toxicity towards aphids and lepidopteran larvae and is notably more orally toxic as compared to the already well studied ICK venom derived peptide, Hv1a. This is likely the result of α-1's role as a secreted mucosal toxin, having evolved for predation and/or protection (Thiel & Kruse 2001; Göransson *et al*., 2019). Nevertheless, α-1 exemplifies the potential of such mucosal toxins for deployment as novel insecticides in a research space currently dominated by the study of venom toxins that are typically vastly more potent by injection than ingestion. Voltage gated Na channels are critical for electrical signalling in most excitable cells and as such have, and remain to be, a major target for pesticides including DDT, pyrethroids and more recently indoxacarb (Silver *et al*., 2018). However, the indiscriminate action of such pesticides towards Na_v highlights the importance of an early understanding of toxicity of novel peptides, such as α-1, towards non-target species. Furthermore, as highlighted by Kadala *et al.,* (2019) who reported differential sensitivity of honeybee and the bumblebee (*Bombus terrestrius*) towards pyrethroids, the structural homology of toxin binding sites may not be the only factor in determining non-target effects. Whilst we have shown α-1 to be similarly toxic by injection towards honeybees and lepidopteran larvae we have not tested for oral activity towards *A. mellifera*. On the basis of comparable injection and oral toxicity towards cabbage looper larvae we would anticipate that α-1 would also be similarly orally toxic to honeybees. However, it would be interesting to assess the toxins oral activity towards this species, as toxicity does not always translate between the two routes of delivery (Guo *et al*., 2018). The activity of α-1 towards other beneficials would also be an interesting pursuit, as *A. mellifera* is in no way a proxy for all beneficial insects. It may be worth noting that application approaches that avoid negative impacts on non-target species may offer opportunities for practical uses of α-1 as a biopesticide. For example, via transgenic expression of α-1 in fibrous plant material away from pollen and nectar, or application situations where contact with beneficials is negligible, i.e., indoor greenhouse application or domestic use.

We conclude by highlighting the importance of discovering novel naturally derived peptide toxins for development of biopesticides to alleviate challenges associated with ever increasing instances of resistance development in target pest species. However, an early understanding of the potential for negative impacts upon non-target pests is equally imperative and integral to the development of safer, more sustainable pest control strategies.

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Compliance with ethical standards

Conflict of interest: The authors declare they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals.

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