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ORIGINAL ARTICLE

# Two novel tyrosine-containing peptides ($Tyr^4$) of the adipokinetic hormone family in beetles of the families Coccinellidae and Silphidae

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**Abstract** Novel members of the adipokinetic hormone family of peptides have been identified from the corpora cardiaca (CC) of two species of beetles representing two families, the Silphidae and the Coccinellidae. A crude CC extract (0.3 gland equivalents) of the burying beetle, *Nicrophorus vespilloides*, was active in mobilizing trehalose in a heterologous assay using the cockroach *Periplaneta americana*, whereas the CC extract (0.5 gland equivalents) of the ladybird beetle, *Harmonia axyridis*, exhibited no hypertrehalosemic activity. Primary sequences of one adipokinetic hormone from each species were elucidated by liquid chromatography coupled to electrospray mass spectrometry (LC–MS). The multiple $MS^N$ electrospray mass data revealed an octapeptide with an unusual tyrosine residue at position 4 for each species: pGlu-Leu-Thr-Tyr-Ser-Thr-Gly-Trp amide for *N. vespilloides* (code-named Nicve-AKH) and pGlu-Ile-Asn-Tyr-Ser-Thr-Gly-Trp amide for *H. axyridis* (code-named Harax-AKH). Assignment of the correct sequences was confirmed by synthesis of the peptides and co-elution in reversed-phase high-performance liquid chromatography with fluorescence detection or by LC–MS. Moreover, synthetic peptides were shown to be active in the heterologous cockroach assay system, but Harax-AKH only at a dose of 30 pmol, which explains the negative result with the crude CC extract. It appears that the tyrosine residue at position 4 can be used as a diagnostic feature for certain beetle adipokinetic peptides, because this feature has not been found in another order other than Coleoptera.

**Keywords** Insects · Beetles · Coccinellidae · Silphidae · Adipokinetic hormone family · Mass spectrometry

## Introduction

Neuropeptides fulfil various regulatory functions not only in vertebrates but also in invertebrates. One of the best-researched neuroendocrine systems is the vertebrate hypothalamus-pituitary axis with its key regulatory hormone, the gonadotropin-releasing hormone (GnRH), which is released from the hypothalamus and stimulates the synthesis and release of luteinizing hormone and follicle-stimulating hormone from the pituitary; these hormones, in turn, are finally responsible for gametogenesis and steroidogenesis (see review by Roch et al. 2011). In the invertebrate arthropods an analogous neuroendocrine system exists: the X-organ–sinus gland complex in the eyestalks of decapod crustaceans, and the brain-corpora cardiaca-corpora allata complex of insects (reviewed by Gäde 1997a; Gäde and Marco 2006). The neuroendocrine organs in these groups synthesize peptides of the so-called adipokinetic hormone (AKH)/red pigment-concentrating hormone (RPCH) family, generically referred to as the AKH family. This peptide family is named after the first fully characterized members and their most prominent functions (see reviews by Gäde 1997a; Gäde and Marco 2006), viz. blanching in decapod crustaceans as a result of RPCH that causes pigment aggregation in the epidermal chromatophores, and the release of diacylglycerides or trehalose into the hemolymph as a result of AKH that activates a lipase or a glycogen phosphorylase to break down the stored triacylglycerides or

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glycogen in the fat body cells (see Gäde and Auerswald 2003). Whereas only two octapeptide members of the family have been structurally identified in the Crustacea (code-named Panbo-RPCH and Dappu-RPCH), more than 60 analogues of the family, including Panbo-RPCH, have been structurally characterized from insects of different orders (reviewed by Gäde 2009; and newer structures in Gäde et al. 2009, 2011, 2013; Gäde and Šimek 2010; Marco et al. 2011, 2013; Weaver et al. 2012). The structural signature features of this peptide family are a chain length of eight to ten amino acid residues, aromatic amino acid residues at positions 4 (in the majority of cases, phenylalanine) and 8 (always tryptophan), a glycine is always at position nine (if this is present), and the termini are always post-translationally modified: a glutamine residue at the N-terminus is genetically encoded but modified to pyroglutamic acid, while at the C-terminus of the precursor molecule a glycine residue is encoded followed by a dibasic cleavage site; by the enzymatic action of peptidyl glycine-α-amidating mono-oxygenase this glycine contributes the amide group to form a carboxyamide at the C-terminus of the mature AKH (O'Shea and Rayne 1992). Recently, claims were made for a superfamily which includes GnRH and AKH based on some features of the primary structure but also on the general organization of the preprohormone and a close relationship of the respective receptors (Staubli et al. 2002; Lindemans et al. 2009; Roch et al. 2011; Gäde et al. 2011).

Coleoptera comprise the species-richest order of the insects with about 350,000 described species, which is almost 40 % of all characterized insect species (Gullan and Cranston 2010). They belong to the Endopterygota or Holometabola, thus the larval stage is very different from the adult imaginal stage and between these two stages there is an inactive, non-feeding pupal stage. With respect to fully characterized AKHs the Colorado potato beetle, *Leptinotarsa decemlineata*, was the first species with identified AKHs (Gäde and Kellner 1989): interestingly, the two peptides, Peram-CAH-I and -II (see structures in Table 1), had been identified 5 years earlier as hypertrehalosemic and cardioacceleratory peptides in the American cockroach, *Periplaneta americana* (Scarborough et al. 1984; Witten et al. 1984). In the potato beetle, Peram-CAH-I and -II were shown to be involved in proline metabolism (Gäde 1999). In 1990, the first beetle-specific AKH was isolated and fully sequenced from the corpora cardiaca of the tenebrionid beetles *Tenebrio molitor* and *Zophobas rugipes* and shown to have a hypertrehalosemic effect in larvae and adults, consequently, it was code-named Tenmo-HrTH (Gäde and Rosinski 1990) (see Table 1). Subsequent to this, a number of unique, beetle-specific AKHs were detected in members of the large Scarabaeoidea superfamily, viz. Melme-CC in

**Table 1** Known peptides of the adipokinetic hormone family sequenced from coleopteran species

| Name of peptide | Primary structure | $MH^+$ | Families and species name |
|---|---|---|---|
| Peram-CAH-I | pEVNFSPNWamide | 973.4526 | Chrysomelidae: *Leptinotarsa decemlineata*, *Chrysolina kuesteri*, *Chrysolina fastuosa*<br>Cerambycidae: *Phryneta spinator*, *Ceroplesis thunbergi*, *Ceroplesis capensis*[a], *Promeces longipes*[a], *Phoracantha recurva*[a], *Morimus funereus*[a], *Leptura maculata*[a] |
| Tenmo-AKH | pELNFSPNWamide | 987.4683 | Tenebrionidae: *Tenebrio molitor*, *Zophobas rugipes*, *Onymacris plana*, *Onimacris rugatipennis*, *Physadesmia globosa*, *Tribolium brevicornis*<br>Meloidae: *Decapotoma lunata*, *Mylabris occulata* |
| Declu-AKH | pELNFSPNWGNamide | 1158.5327 | Meloidae: *Decapotoma lunata*, *Mylabris occulata* |
| Emppe-AKH | pEVNFTPNWamide | 987.4683 | Chrysomelidae: *Chrysolina kuesteri*[a], *Chrysolina fastuosa*[a] |
| Pyrap-AKH | pELNFTPNWamide | 1001.4839 | Tenebrionidae: *Tribolium castaneum*<br>Coccinellidae: *Coccinella septempunctata*[b], *Cheilomenes lunata*[b] |
| Peram-CAH-II | pELTFTPNWamide | 988.4887 | Chrysomelidae: *Leptinotarsa decemlineata* |
| Trica-AKH | pELNFSTDWamide | 992.4472 | Tenebrionidae: *Tribolium castaneum* |
| Melme-CC | pELNYSPDWamide | 1004.4472 | Scarabaeidae: *Melolontha melolontha*, *Geotrupes stercorosus*, *Pachnoda sinuata*, *Pachnoda marginata*, *Dischista cincta*, *Dischista rufa*, *Camenta innocua*, *Trichostetha fascicularis* |
| Scade-CC-I | pEFNYSPDWamide | 1038.4316 | Scarabaeidae: *Scarabaeus deludens*, *Gareta nitens*, *Onitis aygulus*, *Onitis pecuarius* |
| Scade-CC-II | pEFNYSPVWamide | 1022.4730 | Scarabaeidae: *Scarabaeus deludens*, *Gareta nitens* |
| **Nicve-AKH** | pELTYSTGWamide | 937.4414 | Silphidae: *Nicrophorus vespilloides* |
| **Harax-AKH** | pEINYSTGWamide | 950.4367 | Coccinellidae: *Harmonia axyridis* |
| Oniay-CC | pEYNFSTGWamide | 984.4210 | Scarabaeidae: *Onitis aygulus*, *Onitis pecuarius* |
| Trifa-CC | pEINMTTpGWamide | 1012.3958 | Scarabaeidae: *Trichostetha fascicularis* |

Sequence information taken from Gäde (2009) or from [a] Gäde and Marco (2011), [b] Neupert (2007)

Peptide names in bold are peptides characterized in the present study

the cockchafer *Melolontha melolontha* and in the dor beetle *Geotrupes stercorosus* (Gäde 1991a), Scade-CC-I and -II in the dung beetles *Scarabaeus deludens* and *Gareta nitens* (Gäde 1997b), Oniay-CC (together with Scade-CC-I) in the onitine dung beetles *Onitis aygulus* and *O. pecuarius* (Gäde 1997c), a peculiar phosphorylated AKH in the flower scarab *Trichostetha fascicularis* (Gäde et al. 2006) and Trica-AKH in the tenebrionid beetle *Tribolium castaneum* (Gäde et al. 2008) (see Table 1). AKHs that had previously been found in other insect orders were confirmed present (by mass spectrometric methods) in other beetle families: Pyrap-AKH, first identified in the hemipteran bug *Pyrrhocoris apterus* (Kodrik et al. 2000), is present in *T. castaneum* (Gäde et al. 2008) and in the two ladybird beetle species *Cheilomenes lunata* and *Coccinella septempunctata* (Neupert 2007) and Emppe-AKH, first sequenced from the mantids *Empusa pennata* and *Sphodromantis* sp. (Gäde 1991b), is found (together with Peram-CAH-I) in the leaf beetles *Chrysolina kuesteri* and *C. fastuosa* (Gäde and Marco 2011) (see Table 1).

In the current study, AKHs from two species representing two beetle families were investigated. There were a number of compelling reasons to study these two species.

1. No member of the family Silphidae has ever been studied with respect to its neuropeptide complement. The burying beetle *N. vespilloides* is one of the most prominent representatives of this family. It is a model species to study biparental brood care behaviour and recognition of breeding partners (see, for example, Steiger et al. 2007; Haberer et al. 2014). By burying the carcass of a small vertebrate and using this for reproduction, beetles are especially prone to the risk of infection and have developed an innate immune system that changes during development from larval, via pupal to adult stage (Urbanski et al. 2014). AKH is known to interact with the innate immune system in the migratory locust *Locusta migratoria* (Goldsworthy et al. 2002).
2. The harlequin ladybird beetle *H. axyridis* is a native of Asia that was brought into many countries, including the United States, as biological control agent against several aphid species but turned villain because it is so invasive and destroys local diversity of Coccinellidae; it is also a pest insect for certain fruit productions and a nuisance in households, especially during winter aggregations in human homes (Koch and Galvan 2008). *Harmonia axyridis* is found in South Africa, as well, and is spreading rapidly in the country (Stals and Prinsloo 2007). This beetle species is also known to have a large number of genes encoding antimicrobial peptides and proteins which may be in part responsible for the invasive success of the species (Vilcinskas et al. 2012). Moreover, it appears that the presence of a microsporidian parasite which does not harm *H. axyridis*, the host, is infective to other, native ladybird species (Vilcinskas et al. 2013).

We envisage that once the primary structure of AKHs of these two beetle species under investigation here is known, this knowledge could be used to start research into structure–activity studies and subsequent peptide mimetics in order to interfere with important aspects of either the metabolic and/or the immune system.

## Materials and methods

### Insects

Adult specimens of both sexes and unspecified age of the burying beetle *N. vespilloides* were provided by Dr Sandra Steiger (Institute of Experimental Ecology, University of Ulm, Germany) and those of the harlequin ladybird beetle *H. axyridis* were collected at the Vredenburg Research Center Campus of the Agricultural Research Council in Stellenbosch, South Africa by Mr Mike Allsopp or were a gift of Ms Ingrid A. Minnaar (Center of Excellence for Invasion Biology, University of Stellenbosch, S. Africa). The corpora cardiaca (CC) of the insects were dissected immediately after receipt of the animals (see below).

Adult male American cockroaches, *P. americana*, of unspecified age were commercially obtained from a supplier in Pretoria, S. Africa and kept in the university insectarium in Cape Town at 25 ± 2 °C, RH at 60 %, 12 h light:12 h dark cycle, and were fed with commercial rat food, Pronutro® and water ad libitum.

### Tissue preparation and peptide isolation

The head capsule of the beetles was opened with a scalpel, the exposed CC removed by microdissection with the aid of a stereomicroscope, placed into 80 % methanol, and extracted as previously outlined (Gäde et al. 1984). Aliquots of such dried methanolic extracts were used for mass spectrometry (see below), or for reversed-phase high-performance liquid chromatography (RP-HPLC) as described previously (Gäde 1985), or were used to perform a heterologous bioassay (see below).

### Biological assay

The CC extracts of *N. vespilloides* and *H. axyridis* and the novel synthetic peptides were tested for hypertrehalosemic potency in *P. americana* according to previously published methods (Gäde 1980).

**Table 2** Biological activity of a crude methanolic extract of corpora cardiaca from *N. vespilloides* and *H. axyridis* in heterologous assays with *P. americana*, and their respective synthetic AKH-family peptides

| Treatment (3 µl) | Hemolymph carbohydrates (mg ml$^{-1}$) | | | | |
|---|---|---|---|---|---|
| | *n* | 0 min | 90 min | Difference | *P** |
| Distilled water | 5 | 12.4 ± 1.6 | 16.8 ± 9.3 | 4.4 ± 4.8 | NS |
| *N. vespilloides* CC (0.3 gland pair equivalent) | 10 | 13.0 ± 1.2 | 19.0 ± 5.0 | 6.0 ± 5.1 | 0.005 |
| *H. axyridis* CC (0.5 gland pair equivalent) | 8 | 16.0 ± 2.4 | 15.8 ± 2.3 | −0.2 ± 3.5 | NS |
| *P. americana* CC (0.1 gland pair equivalent) | 5 | 14.9 ± 1.8 | 38.5 ± 3.3 | 23.6 ± 3.9 | 0.0002 |
| Distilled water | 9 | 14.5 ± 4.2 | 13.6 ± 5.2 | −0.9 ± 2.3 | NS |
| Harax-AKH (10 pmol) | 9 | 12.6 ± 4.2 | 13.2 ± 5.0 | 0.6 ± 3.5 | NS |
| Harax-AKH (30 pmol) | 8 | 13.4 ± 2.4 | 16.9 ± 4.5 | 3.5 ± 3.2 | 0.02 |
| Nicve-AKH (10 pmol) | 7 | 13.8 ± 2.5 | 21.7 ± 5.2 | 7.9 ± 3.5 | 0.0005 |
| *P. americana* CC (0.1 gland pair equivalent) | 8 | 14.9 ± 2.2 | 33.5 ± 5.1 | 18.6 ± 6.1 | 0.00006 |
| Distilled water | 10 | 12.8 ± 3.9 | 13.6 ± 5.4 | 0.8 ± 2.5 | NS |
| Leu$^2$-Harax-AKH (10 pmol) | 12 | 13.6 ± 5.0 | 14.2 ± 6.1 | 0.6 ± 2.3 | NS |
| Leu$^2$-Harax-AKH (30 pmol) | 12 | 13.8 ± 4.3 | 15.8 ± 3.1 | 2.0 ± 4.1 | NS |
| *P. americana* CC (0.1 gland pair equivalent) | 6 | 12.3 ± 4.2 | 26.1 ± 12.1 | 13.8 ± 9.5 | 0.02 |

Data are presented as Mean ± SD

*NS* not significant

* Paired *t* test was used to calculate the significance between pre-and post-injection values

### Liquid chromatography coupled to mass spectrometry

For liquid chromatography (LC)-positive electrospray ionization (+ESI) ion trap mass spectrometry (MS) the dried methanolic CC extract was reconstituted in 50 µl of aqueous 0.1 % formic acid. An aliquot, representing about 1 gland equivalent, was then fractionated and analysed using a Jupiter RP Proteo column (150 mm × 1 mm i.d.; Phenomenex Inc., Torrance, USA) coupled directly to a linear quadrupole ion trap mass spectrometer (LTQ XL; Thermo Fisher, San Jose, USA) equipped with an electrospray ionization source operated at 4 kV as outlined in detail previously (Kodrik et al. 2010).

### Synthetic peptides

The novel adipokinetic peptides elucidated in this study, Nicve-AKH from the CC of the burying beetle and Harax-AKH from the CC of the harlequin ladybird beetle, were custom-synthesized by Pepmic Co., Ltd. (Suzhou, China) and GenScript Corporation (Piscataway, USA), respectively.

## Results

### Presence of hypertrehalosemic activity in the CC extract from the burying beetle and the harlequin ladybird beetle

Injection of 0.3 gland equivalents of a methanolic CC extract from the burying beetle into American cockroaches caused a significant increase in the concentration of total carbohydrates in the hemolymph (Table 2); this increase is about 25 % of what was maximally achieved by injection of 0.1 gland equivalent of a homologous cockroach extract (see Table 2). Surprisingly, 0.5 gland equivalents of the CC from the harlequin ladybird beetle did not increase the total hemolymph carbohydrate concentration in the cockroach significantly (Table 2). It was assumed that the small CC of this beetle contain an AKH in low concentration. To establish this, it was decided to go ahead with structure elucidation of this beetle's putative AKH, as well.

### Mass spectral analyses of CC extracts from *N. vespilloides* and *H. axyridis*

The total ion chromatogram of a CC extract from the burying beetle shows a distinct ion peak at 6.40 min (Fig. 1a) which gave rise to an $[M+H]^+$ ion at *m/z* 937.4 (Fig. 1b). Characteristic $[M+NH_4]^+$ and $[M+Na]^+$ ions were also detected (Fig. 1b). The primary structure was deduced from the tandem $MS^2$ spectrum obtained by collisionally induced dissociation (CID; Fig. 2). From the characteristic b-type and y-type product ions, in conjunction with other diagnostic ions (b–$H_2O$, y–$NH_3$, y–$NH_3$–$H_2O$), and the ions $b_8$–CO (892.3), $b_8$–2 $H_2O$ (884.4), $b_8$–$H_2O$–CO (874.4), $b_8$–3 $H_2O$ (866.4), $b_8$–$H_2O$–CO–2$NH_3$ (840.4), $MH^+$–Trp (751.3), $b_7$–2$H_2O$ (698.3), $b_6$–2$H_2O$ (641.3), $b_5$–2$H_2O$ (540.1) and $y_4$–$NH_3$–2$H_2O$ (396.2), a member of the AKH family could easily be deduced (see the inset in Fig. 2). The only ambiguity of whether an isobaric Leu or Ile residue is at position 2 was resolved (see below) to the presence of Leu. The

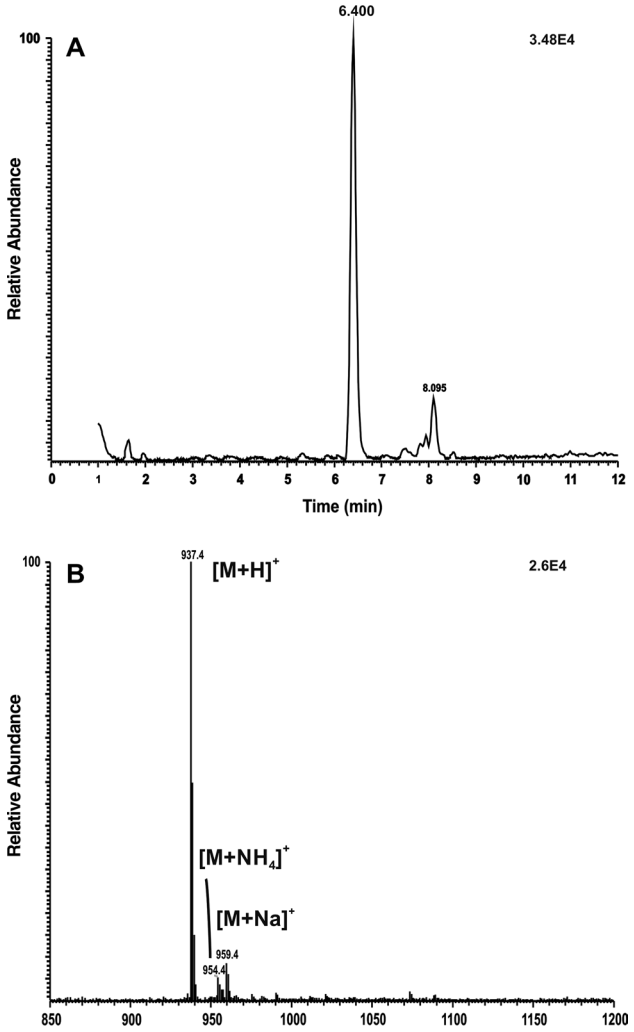


**Fig. 1** Liquid chromatographic (LC)-positive electrospray ionization (+ESI) mass spectrometric (MS) analysis of an extract from corpus cardiacum material of the burying beetle, *N. vespilloides*. **a** Total ion chromatogram (TIC) obtained by LC/MS analysis showing detection of one AKH peptide at 6.40 min. **b** A full scan +ESI mass spectrum recorded from the peak in (**a**), showing $[M+H]^+$ at *m/z* 937.4, $[M+Na]^+$ at *m/z* 959.4, and $[M+NH_4]^+$ at *m/z* 954.4

assigned peptide has not been found before in any insect and therefore, this octapeptide with the structure pGlu-Leu-Thr-Tyr-Ser-Thr-Gly-Trp amide is called Nicve-AKH.

The total ion chromatogram of a CC extract from the harlequin ladybird beetle shows a large early-eluting ion peak at 2.72 min (Fig. 3a), which was identified to harbour an AKH peptide. The ion at retention time of 2.72 min has an $[M+H]^+$ ion at *m/z* 950.5 (Fig. 3c). The CID tandem $MS^2$ spectrum of this ion (Fig. 4) gave clearly interpretable b-type and y-type product ions, including $b_8$–CO (905.4), $b_8$–$2H_2O$ (897.5), $b_8$–$H_2O$–CO (887.4), $b_8$–$2H_2O$–CO (869.5), $MH^+$–Trp (764.4), $b_4$–CO (474.3) and $y_4$–$NH_3$–$H_2O$ (414.2). With the ambiguity at position 2, Leu or Ile (resolved below), the assignment of a novel member of the AKH family was otherwise clear (see inset in Fig. 4): the octapeptide pGlu-Ile-Asn-Tyr-Ser-Thr-Gly-Trp amide has never been elucidated from any other animal and is code-named Harax-AKH.

## Confirmation of the assigned peptide sequences

To confirm the accuracy of our assignment of the peptide sequences, two independent sets of experiments were performed which also allowed us to judge unambiguously whether position 2 was occupied by a Leu or Ile residue. Both methods needed the synthetic peptides, which were synthesized and then applied to LC–MS and also to RP-HPLC with fluorescence monitoring (to target the Trp residue of AKHs in position 8). The fluorescent trace of synthetic Nicve-AKH (about 70 pmol) showed clearly one single distinct peak at a retention time of 6.4 min (Fig. 5c). Separation of a methanolic extract of 3.3 CC gland equivalents from the burying beetle *N. vespilloides* resulted in a number of fluorescent peaks; the largest of them was at 6.4 min (Peak 1 in Fig. 5b) and, thus, matched the retention time of synthetic Nicve-AKH. Co-injection of 70 pmol synthetic Nicve-AKH and 3.3 gland equivalents of *N. vespilloides* CC caused the peak at 6.4 min to increase indicating that synthetic and natural peptide co-eluted (Fig. 5a). Similarly, synthetic and natural compound had exactly the same retention time when separated in the LC–MS system and the CID tandem MS spectra of the synthetic peptide showed exactly the same product ions as found earlier (data not shown). A peptide with an Ile instead of the Leu tested here would elute earlier in both RP-HPLC and LC–MS systems (Gäde et al. 2003) and, thus, the $Leu^2$ residue in Nicve-AKH is confirmed.

In the case of the harlequin ladybird beetle co-injection of the crude methanolic extract together with the $Leu^2$ form of Harax-AKH resulted in a clear additional peak at a retention time of 3.10 min in the total ion chromatogram (Fig. 3b) confirming that the natural extract has the $Ile^2$ analogue. Again, this proves that we have correctly assigned the sequence for this peptide. The CID spectra for the synthetic $Ile^2$ peptide also matched the data found for the natural compound (data not shown).

## Functional assays

Homologous bioassays were not performed. In *H. axyridis* it was impossible to work with the hemolymph because it clotted immediately when withdrawn into the glass capillary. From *N. vespilloides* we had, unfortunately, only a few specimens available, and that material was just sufficient for the structural studies.

However, we tested the novel synthetic peptides in a well-known assay system and checked for hypertrehalosemic activity in the cockroach *P. americana*. Compared

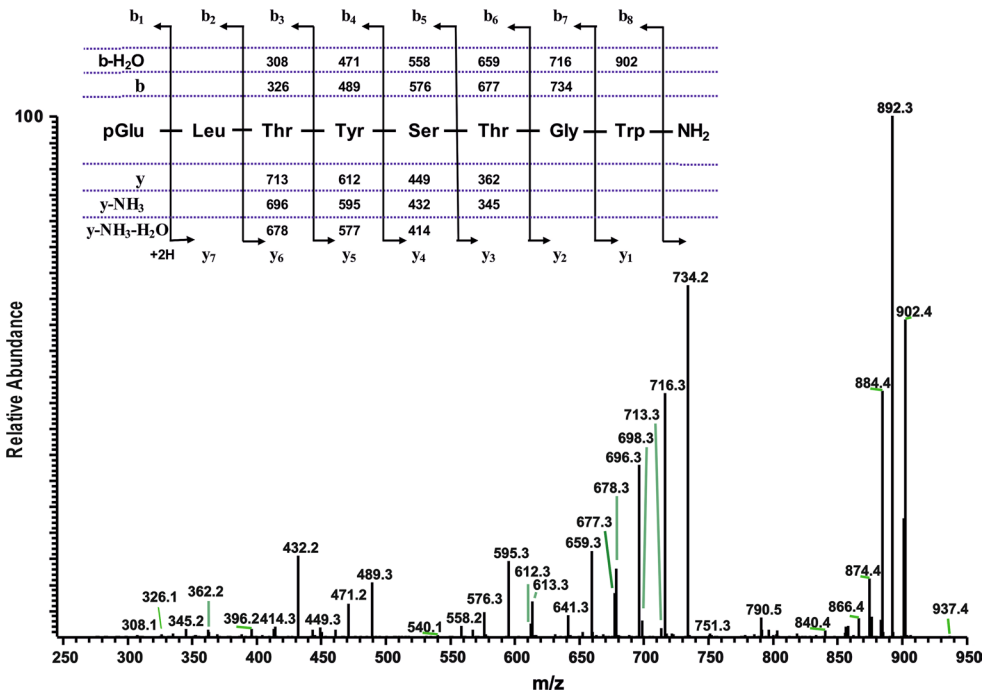


**Fig. 2** A collision-induced dissociation (CID) tandem MS +ESI spectrum of the ion $[M+H]^+ = 937.4$ in Fig. 1b The *inset* shows the proposed peptide sequence, together with the b-type and y-type diagnostic fragment ions observed in the $MS^2$ spectrum

to the response elicited after injection of own CC material, 10 pmol of Nicve-AKH was about 42 % active (Table 2). In contrast, 10 pmol of the synthetic Harax-AKH elicited no increase in carbohydrates in the hemolymph of cockroaches (Table 2). It required 30 pmol of Harax-AKH to achieve a small but significant hypertrehalosemic effect that was about 19 % of the response to own cockroach extract (Table 2). When an analogue of Harax-AKH was tested that had a Leu residue at position 2 instead of the Ile, no significant hypertrehalosemia was recorded, irrespective of the dose of 10 and 30 pmol injected (Table 2).

## Discussion

The major aim of this investigation was the structural characterization of putative AKHs in two different families of beetles, one of which (Silphidae) had not been previously studied, and to compare it with the AKHs from other beetle families. In order to achieve this, we first had to ascertain whether the dissected neurohemal tissue, the CC, exhibited any activity associated with AKHs. We utilized a well-established heterologous assay in cockroaches and measured the released amount of carbohydrates, thus a hypertrehalosemic response, because *P. americana* mobilizes trehalose as main blood sugar. Measurable and significant hypertrehalosemia was caused by injection of 0.3 gland equivalents of the burying beetle but even 0.5 gland equivalents of the ladybird beetle were not active. It was assumed that the CC of the ladybird beetle, which are very small, produced very few molecules of an AKH and, moreover, this AKH may have a primary structure that was quite different from that of the cockroach AKHs, hence, making it very unlikely for the ladybird beetle AKH to interact sufficiently with the cockroach AKH receptor. These results on the presence/putative absence or low quantity of AKHs in the CC of both species encouraged us to use once again LC–ESI–MS methodology, in conjunction with the comparison of RP-HPLC and MS properties of synthetic and natural endogenous material to unequivocally identify the AKHs in each species. In comparison to a number of Blattodea, Orthoptera, Hemiptera, Lepidoptera (see Gäde 2009) and also some Coleoptera (see Table 1), the two species studied had only one AKH, and in each case it was an octapeptide. At present, only one decapeptide has been elucidated from a beetle species, i.e. the peptide code-named Declu-AKH which is found in beetles of the family Meloidae (blister beetles; Gäde 1995) together with the octapeptide Tenmo-HrTH (see Table 1). The two peptides investigated in this study are novel members of the still-growing AKH family, thus, they have not been found previously in any species. Therefore, they have been named Nicve-AKH and Harax-AKH, respectively, according to the species from which they were isolated. Both peptides are structurally quite similar and do not differ in the 5 amino acids at the C-terminus, effectively differing from each other only in positions 2 and 3 (see Table 1). Both peptides display quite an unusual feature for a member of the AKH family: usually the amino acid at position 4 is a Phe residue; in both peptides investigated presently this residue is a Tyr

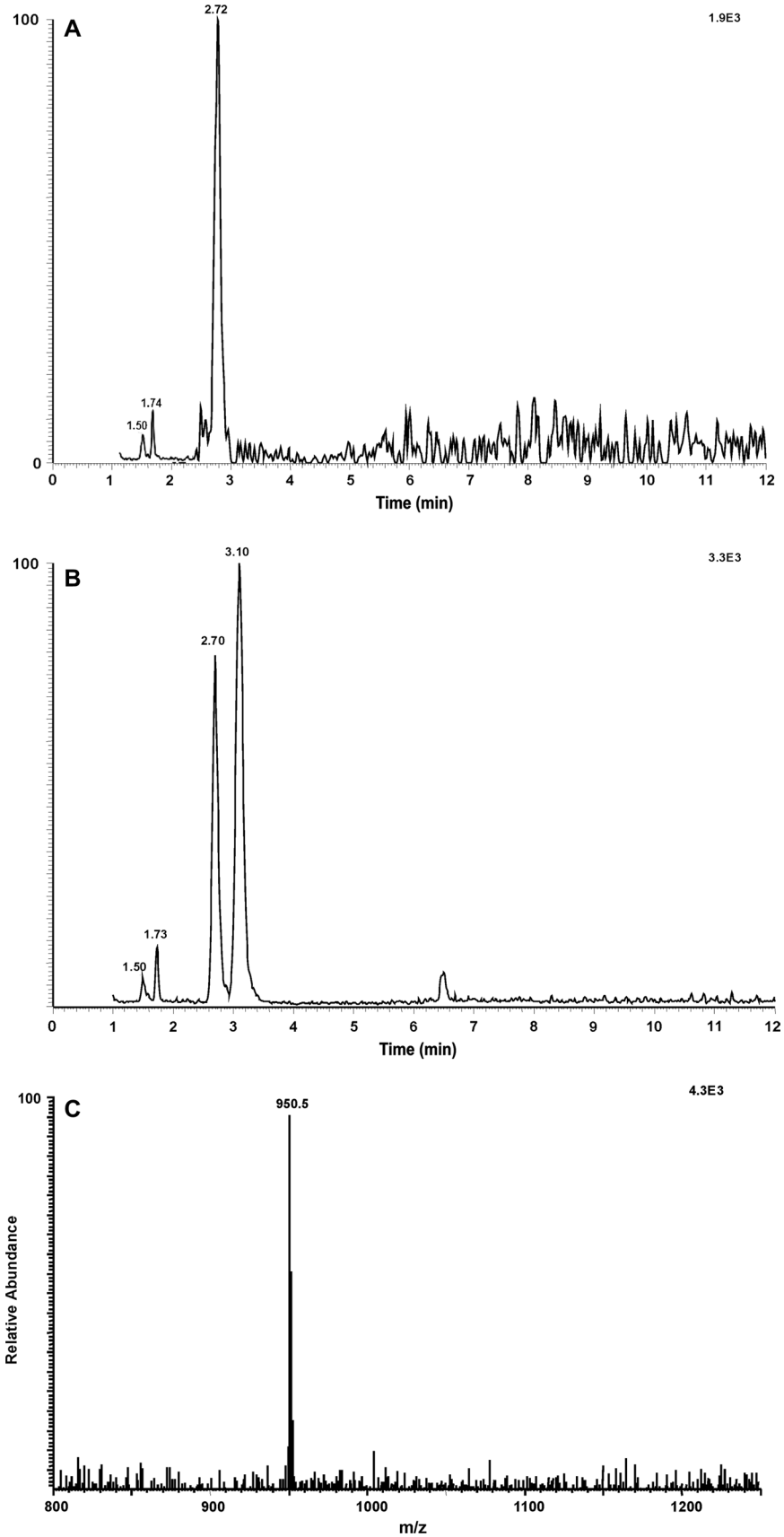


**Fig. 3** Liquid chromatographic (LC)-positive electrospray ionization (+ESI) mass spectrometric (MS) analysis of an extract from corpus cardiacum (CC) material of the ladybird beetle, *H. axyridis*. **a** Total ion chromatogram (TIC) obtained by LC/MS analysis showing detection of an early-eluting AKH peptide at 2.72 min. **b** A TIC of a mixture of crude CC extract and a synthetic peptide analogue of Harax-AKH with Leu in position 2 revealed two peaks: one AKH peptide at 2.70 min (=the native Harax-AKH with $Ile^2$) and one at 3.10 min corresponding to synthetic $Leu^2$-Harax-AKH. **c** A full scan +ESI mass spectrum recorded from the peak in (**a**), showing $[M+H]^+$ at *m/z* 950.5

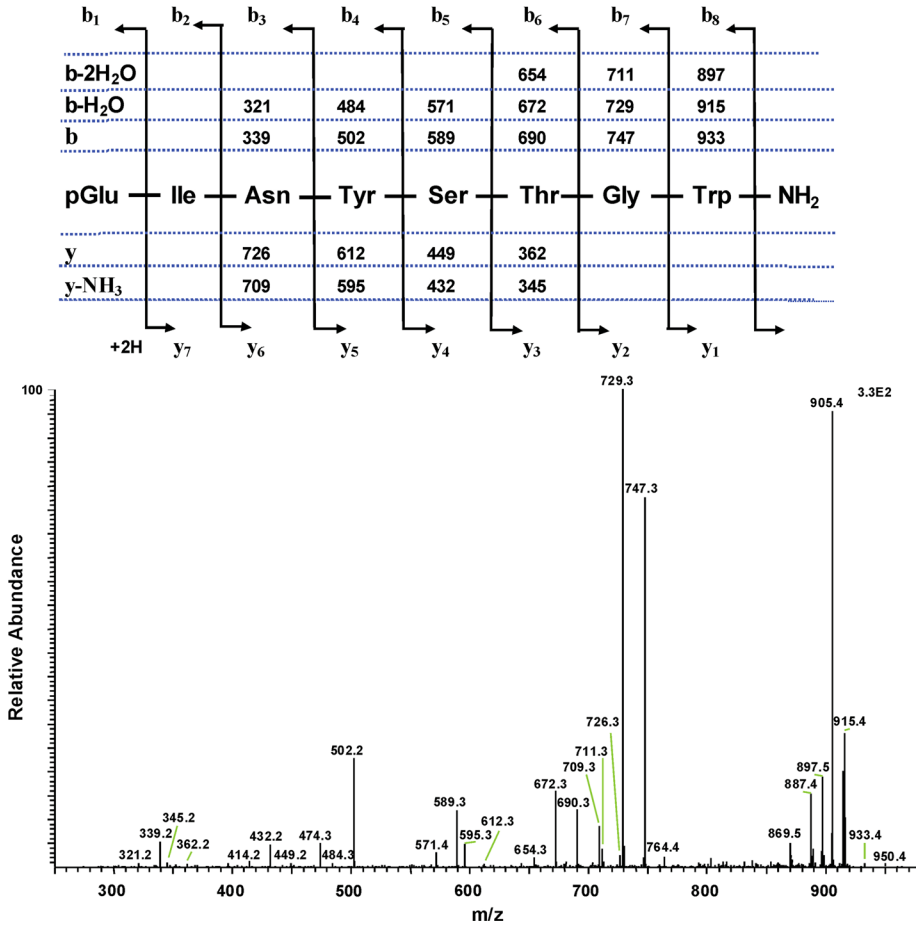


**Fig. 4** A collision-induced dissociation (CID) tandem MS +ESI spectrum of the ion $[M+H]^+ = 950.5$ in Fig. 3c The inset shows the proposed peptide sequence, together with the b-type and y-type diagnostic fragment ions observed in the $MS^2$ spectrum

having an extra phenolic hydroxy group. The change from Phe to Tyr requires only a single base change in the genetic code and can be explained by point mutation. Previously, three other members of the AKH family have been shown to have this feature: the peptides Melme-AKH, Scade-CC-I and Scade-CC-II (see Table 1 for structures). All are octapeptides and all are occurring in the CC of beetles. Whereas up to now this feature was only known for the large superfamily Scarabaeoidea that contains dor beetles, scarabaeid and onitine dung beetles, we have now extended this to two more beetle families, the Silphidae and Coccinellidae. At present it may be safe to state that a Tyr residue at position 4 may be used as a signature feature of beetle AKHs.

A Tyr at position 4 also has consequences for receptor recognition. Previous work clearly shows that in such peptides where the hydrophobic $Phe^4$ residue is replaced by a polar $Tyr^4$, a severe loss in activity results when tested in the migratory locust (Gäde 1997d). Thus, we expect both peptides to exhibit a very high $ED_{50}$ in the hyperlipemic bioassay, but this remains to be seen in future studies. The $Tyr^4$ also does not bring the peptide in the preferred conformation for the cockroach receptor: The Melme-CC $Asn^7$ analogue (pGlu-Leu-Asn-Tyr-Ser-Pro-Asn-Trp amide) has a 50-fold increased $ED_{50}$ compared to the endogenous cockroach peptide (Gäde 1992).

The current data show that both of the novel synthetic peptides are active in the American cockroach but that Nicve-AKH seems to be a better fit to that receptor than Harax-AKH. This may well be related to the fact that the first three N-terminal amino acids (pGlu-Leu-Thr-) in Nicve-AKH match those in the cockroach peptide Peram-CAH-II (pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp amide). It also became clear that the Ile at position 2 in Harax-AKH cannot be replaced by Leu, since such a synthetic AKH analogue was not active at all in the cockroach bioassay system at the doses tested.

In contrast to the receptors in cockroaches and locusts, the receptors in onitine and scarabaeid beetles do recognize their endogenous $Tyr^4$ containing peptides very well and respond with an increase of the amino acid proline—which is used as substrate to power the contraction of the flight muscles during flight activity—after injection of low doses of synthetic peptide (5 pmol) (Gäde 1997b, c). The invasive species *H. axyridis*, as well as the burying beetle *N. vespilloides,* disperse by flight and we predict that such flight bouts are possible by producing energy through the

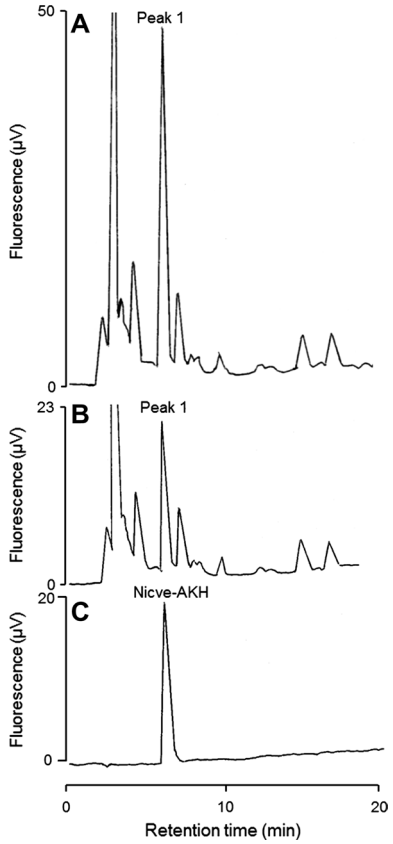


**Fig. 5** Reversed phase-HPLC separation of a crude CC extract of *N. vespilloides* and the comparative elution of the synthetic peptide Nicve-AKH. **a** Methanolic crude extract of 3.3 pairs of *N. vespilloides* corpora cardiaca co-injected with 70 pmol of synthetic Nicve-AKH peptide resulted in a single peak (Peak 1). The mixture was applied to a C-18 column, monitored by fluorescence (excitation at 276 nm and emission at 350 nm). The column was developed with a linear gradient of 43 % to 53 % B in 20 min at a flow rate of 1 ml/min. **b** Methanolic crude extract of 3.3 pairs of *N. vespilloides* corpora cardiaca only, applied to the RP-HPLC system under the same chromatographic conditions as in (**a**). **c** 70 pmol synthetic Nicve-AKH only, applied to the RP-HPLC system under the same conditions as in (**a**)

oxidation of proline as shown for a number of beetles (see review by Gäde and Auerswald 2002). This has to be verified experimentally.

It was surprising to see that the Coccinellidae *H. aryxidis* synthesizes Harax-AKH, because we anticipated finding Pyrap-AKH as in the other two species of Coccinellidae previously analysed, *Cheilomenes lunata* and *Coccinella septempunctata* (see Table 1). Out of the eight amino acids five are different between Harax-AKH and Pyrap-AKH and only position 1 (pyroGlu), 3 (Asn) and 8 (Trp-amide) do not differ. All three genera, *Harmonia*, *Cheilomenes* and *Coccinella,* belong to the same sub-family (Coccinellinae) of the family Coccinellidae. A recent phylogenetic study on ladybirds and the monophyly of sub-families based on five primarily ribosomal loci find all three genera in the grouping of Coccinellinae but the genera were all on other branches, thus no sister-group relationship, for example, between *Cheilomenes* and *Coccinella* was established (Magro et al. 2010), which would concur with the findings in AKH structure. Thus, at present we have no explanation for this diversity other than the fact that in certain cases even species of the same genus express different AKHs as, for example, experienced in *Tribolium castaneum* and *T. brevicornis* (Gäde et al. 2008).

In conclusion, we have characterized two novel octapeptides from the AKH family in the CC of a species each of the coleopteran families Silphidae and Coccinellidae which contain the polar amino acid tyrosine instead of the hydrophobic phenylalanine at position 4 from the N-terminus. Such peptides are notoriously not very active in a locust hyperlipemic or a cockroach hypertrehalosemic assay system because of different receptor requirements. They are, however, active in proline oxidation and, hence, it is suggested that the two investigated beetle species rely on a proline-based metabolism during periods of strenuous activity.

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

**Ethical approval** All applicable international, national and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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