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Structure elucidation of Mas-AKH as the major adipokinetic hormone in the butterfly *Vanessa cardui* (Lepidoptera: Nymphalidae)*

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Abstract. The presence of adipokinetic activity in crude extracts of corpora cardiaca (CC) from the butterfly (*Vanessa cardui* L., Nymphalidae) was demonstrated by bioassay and Mas-AKH was revealed as the major adipokinetic hormone (AKH) by use of two different technologies of sequence elucidation: HPLC separation of the peptide followed by Edman degradation and Q-TOF mass spectrometry. In contrast to the time- and material-consuming conventional methods of peptide purification and sequencing, substantial structural data of the peptide were confirmed – post factum – from one pCC (pair of CC) by Q-TOF mass spectrometry. Only males of our laboratory colony showed a significant lipid increase in the haemolymph after injection of either crude CC extract (1 pCC equivalent) or 10 pmol of synthetic peptide.

INTRODUCTION

Certain members of the Caelifera and Lepidoptera are known to undertake long-distance migratory flights. From an energetic point of view, such species utilize lipids to provide energy for the contracting flight muscles during long flights (Ziegler & Schulz, 1986; Goldsworthy, 1983; Wheeler, 1989). Lipids, stored in the fat body, are mobilized during flight by the action of small (eight to ten amino acid residues) neuropeptides of the AKH/RPCH (adipokinetic hormone/red pigment concentrating hormone) family. The CC are the source of these peptides, and, compared with most other insect neuropeptides, relatively high quantities are stored in these glands. Members of the AKH/RPCH family have been isolated and structurally studied in several insect orders, including the Lepidoptera (Gäde, 1996; Gäde et al., 1997).

Within lepidopterans, an AKH was first sequenced from the tobacco hornworm moth, *Manduca sexta* (Ziegler et al., 1985) and this nonapeptide was code-named Mas-AKH (pQLTFTSSWGamide). Mas-AKH was also found in the silkmoth, *Bombyx mori* (Ishibashi et al., 1992), whereas the noctuid moth, *Heliothis zea,* contains Mas-AKH and a decapeptide called Hez-HrTH (Jaffe et al., 1986, 1988). Other reports demonstrate that Mas-AKH is very likely present in the true armyworm, *Pseudaletia unipuncta* (Orchard et al., 1991), and the saturniid and the sphingid moths, *Imbrasia cytherea* and *Hippoteon eson*, respectively (Liebrich & Gäde, 1995). *H. eson* contains a second active peak which may be identical to Hez-HrTH.

In contrast to moths, the situation concerning the chemical identity of AKH peptides in butterflies is totally unclear. From two migratory butterfly species, the monarch, *Danaus plexippus*, and the painted lady, *V. cardui*, it is known that CC or head extracts have a lipid-mobilizing effect (Dallmann & Herman, 1978; Herman & Dallmann, 1981). Moreover, CC extracts of the small tortoiseshell butterfly, *Aglais urticae*, had a weak adipokinetic effect when injected into locusts (Gäde, 1990).

In the present paper we started to re-investigate the inventory of AKH peptides in the painted lady butterfly, *V. cardui.* Although we anticipated to find one of the known lepidopteran AKHs as a lipid-mobilizing peptide in the CC of *V. cardui* due to the well-known migratory behaviour of this species (Ebert & Rennwald, 1993) and its taxonomic position in the Lepidoptera, the hypothesis still had to be proven. Previous data have shown that several AKH peptides are not restricted to certain insect families or orders; for example, Scg-AKH can be found in a great number of orthopteroids, as well as in the Hymenoptera (Gäde & Auerswald, 1998). Mas-AKH too, is present in at least one species of the Hymenoptera (Lorenz et al., 1999). The current study shows that Mas-AKH is present as the major adipokinetic hormone in the CC of *V. cardui,* as corroborated by Edman degradation sequencing and its lipid-mobilizing effect in the haemolymph of adult male butterflies. Furthermore, a recently developed method that

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involves time-of-flight mass spectrometry (Verhaert et al., 1997) was employed to detect Mas-AKH from one pair of CC (pCC).

MATERIAL AND METHODS

Insects and tissue dissection

Vanessa cardui (Lepidoptera: Nymphalidae) larvae were reared on a semi-artificial diet (slightly modified after Nijhout, 1980, by adding leaf-powder of *Cirsium arvense* instead of *Plantago* sp.) at 25°C under long-day conditions (16L : 8D). Imagines were allowed to feed from a 10% sucrose solution. For this study, only adult specimens were used.

Corpora cardiaca/corpora allata (CC/CA) complexes were dissected from either freshly decapitated or previously frozen females and males. The organ complexes were immediately transferred into ice-cold extraction medium (methanol/water/acetic acid, 100/10/1, v/v/v), and stored at –20°C.

Bioassay for adipokinetic activity

One- to two-week old butterflies were used for the bioassay. Animals were separated from the stock culture the night before use and kept isolated in glassine envelopes. Haemolymph samples (1 µl) were taken from the dorsal aorta to measure basal lipid and carbohydrate concentrations. This was repeated 90 min after injection of either synthetic peptide or crude CC extract and HPLC fractions, respectively, dissolved in 6 µl of Ringer (see below). The total sugar concentration of the haemolymph was measured by the anthrone method (Mokrasch, 1954); the total concentration of lipids in the haemolymph was measured by the phosphovanillin method (Zöllner & Kirsch, 1962).

Flight

For experiments investigating metabolite changes during lift-generating tethered flight, males or females were attached to an insect pin using bee wax. The insect was considered to produce lift when the top of the pin went up. For more details see Auerswald et al. (1998). Experiments were performed in a constant room temperature at 25°C. After the butterfly had been attached to the pin, a 1 µl haemolymph sample was taken for determination of control metabolite concentrations. A second 1 µl haemolymph sample was taken immediately after the 5 min flight and a third sample was taken after a 20 min period of rest following the 5 min of flight.

Peptide isolation and sequence determination

Batches of 20–50 CC/CA complexes in 100 µl extraction medium were sonicated and centrifuged. Pellets were resuspended twice in fresh extraction medium and the combined supernatants were dried under a stream of nitrogen and stored dry at –20°C until use. The crude extracts were either resuspended in Ringer (86 mM NaCl, 5.4 mM KCl, 3 mM $CaCl_2$) for the bioassay or dissolved in 20% (v/v) acetonitrile (MeCN) in water containing 0.1% trifluoroacetic acid (TFA) for reversed-phase HPLC as previously described (Lorenz et al., 1999). Peaks were detected with a photodiode array detector (Jasco MD 910) at 214 and 280 nm. An initial HPLC run was carried out with 50 CC/CA, and the peaks were manually collected to identify biologically active fractions. In the subsequent HPLC runs, only bioactive fractions were retained and combined material from a total of 200 CC/CA was used for deblocking of the active peptide. Deblocking of the purified peak material was accomplished by incubating the putative AKH with 1-pyroglutamate aminopeptidase (Boehringer Mannheim, Germany) for 1.5 h at 50°C according to the manufacturer's instructions. After deblocking, the digest was rechromatographed on HPLC to separate the deblocked and the remaining undigested peptide (Lorenz et al., 1999). The sequence of the deblocked peptide was determined by automated Edman degradation. The mass of both native and deblocked peptide was determined using MALDI-TOF mass-spectrometry as previously described (Lorenz at al., 1995). The synthetic Mas-AKH was purchased from Paesel and Lorei (Hanau, Germany).

ESI-TOF mass spectrometry

For nanoflow electrospray ionization (ESI) orthogonal accelaration (OA) time of flight (TOF) on a Q-Tof hybrid ESI-TOF system (Micromass, UK), the CC of one butterfly (pCC) were briefly immersed in 10 µl of methanol/water/formic acid (50 : 48 : 2, v/v/v). After 1 min, a 1 µl sample was removed from the extraction tube and loaded in a gold coated capillary (Micromass type A nanoflow needle). The sample was infused at 50 nl/min (Verhaert et al., 1997). Such a 1 µl sample provided ample analysis time for the acquisition of an extensive MS spectrum as well as for several MS/MS runs.

The MS/MS or tandem mass spectrometry, involves the analysis of fragment ions generated from a selected precursor ion, obtained after collision induced dissociation (CID). In Q-TOF, selection of the precursor ion is achieved with high resolution by setting the first quadrupole analyzer in so-called resolving or narrow bandpass mode only to transmit the ions with the required mass-charge ratio. The quadrupole thus acts as an ion filter; the filtered ion is subsequently fragmented in a collision cell in which (inert) argon gas molecules are made to collide with it. Since not all peptide ions fragment with the same efficiency, the collision energy with which the ions are drawn into the CID cell is typically varied between 20 and 30 V, so that multiple MS/MS spectra are acquired for each precursor ion.

RESULTS

Crude methanolic extracts of *V. cardui* CC/CA complexes were assayed for lipid- and carbohydrate-mobilizing activity in the homologous bioassay. Both females and males were used since Herman & Dallmann (1981) reported that there was no sex-specific difference in the response of *V. cardui* to crude CC extracts. Whereas the males of our colony showed a highly significant increase in total haemolymph lipids after injection of either 1 pCC equivalent of a crude extract (160% compared to the control) or 10 pmol synthetic Mas-AKH (181%), females did not exhibit any significant rise in

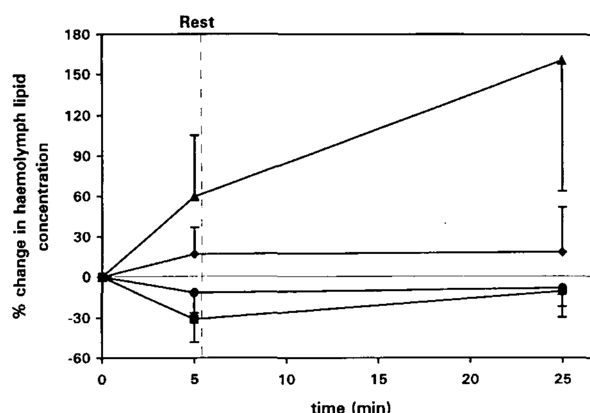

Fig. 1. Changes in female and male total haemolymph lipid and carbohydrate concentrations after 5 min of lift-generating flight and 20 min of rest after 5 min of flight. ▲ – male lipids; ◆ – female lipids; ■ – male carbohydrates; ● – female carbohydrates. Mean values of 4–6 determinates ± SD.

TABLE 1. Adipokinetic and hypertrehalosaemic activity of crude methanolic CC extract of *V. cardui*, or synthetic Mas-AKH.

Treatment	Haemolymph lipids [mg/ml]			
Males	before	after	difference	% change
Control	14.6 ± 4.6	17.1 ± 7.0	2.5 ± 2.9	14
CC extract	23.5 ± 7.2	57.6 ± 11.3	34.1 ± 8.9***	160***
Mas-AKH	14.0 ± 4.9	36.4 ± 13.0	22.6 ± 10.0***	181***
Females				
Control	15.5 ± 5.1	13.1 ± 4.5	–2.4 ± 2.4	14
CC extract	21.5 ± 11.4	25.4 ± 10.3	3.9 ± 10.3	35
Mas-AKH	25.1 ± 10.2	31.8 ± 13.5	6.7 ± 6.6	28
Treatment	**Haemolymph carbohydrates [mg/ml]**			
Males	before	after	difference	% change
Control	18.1 ± 4.0	20.9 ± 2.2	2.8 ± 3.8	20
CC extract	26.9 ± 7.5	28.5 ± 4.4	1.6 ± 6.1	12
Mas-AKH	24.1 ± 5.6	24.4 ± 5.6	0.3 ± 2.1	1
Females				
Control	15.8 ± 4.6	17.1 ± 5.2	1.3 ± 4.8	10
CC extract	16.8 ± 3.8	18.4 ± 5.2	1.5 ± 4.9	11
Mas-AKH	19.1 ± 6.6	18.3 ± 1.7	–0.8 ± 5.1	3

Crude CC extract (1pCC/animal), synthetic Mas-AKH (10 pmol/animal) or Ringer solution for the control groups were injected in *V. cardui* males and females (n = 10). Total haemolymph lipid and carbohydrate concentrations are expressed in mg/ml haemolymph. Results are means ± S.D., with the difference between values before and 90 min after injection, as well as the percent of change. Significance of the difference was calculated (***$p < 0.001$) using paired t-tests.

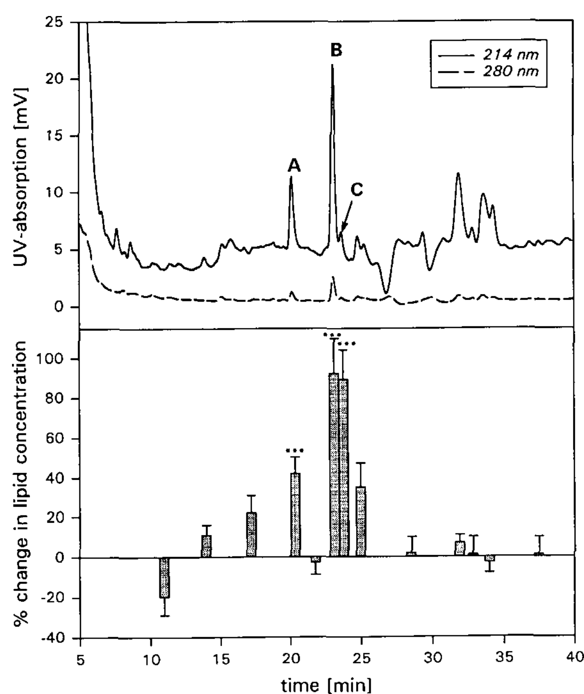

Fig. 2. HPLC run of 50 *V. cardui* CC/CA methanolic crude extract monitored at 214 and 280 nm. The Capcell Pak C18 column was developed with a linear gradient of 0.115% TFA in water (solvent A) and 0.1% TFA in MeCN (solvent B), running from 20% to 32% B within 40 min at a flow rate of 0.2 ml/min. Peak fractions were collected manually and aliquots of 1 CC were tested in the homologous bioassay. The change in total haemolymph lipids is given in percent in the histogram below (n = 10). Significance of lipid changes is marked by ***$p < 0.001$, using a paired t-test.

haemolymph lipids (Table 1). Furthermore, no change in the haemolymph total carbohydrate content of females and males was observed (Table 1). During 5 min of lift-generating flight, the level of lipids in the haemolymph of males increased by about 60% compared to the control (rest), whereas no significant increase was observed in female haemolymph lipids. Levels of haemolymph carbohydrates in females and males were slightly lower than at rest (Fig. 1). After 20 min of rest following a 5 min flight, the lipid concentration in the haemolymph of males further increased (150% compared to the control), but lipid concentration in females and carbohydrate levels in the haemolymph of females and males did not change significantly. Based on these results, we selected only males for use in further bioassays and studied only lipid mobilization.

Crude methanolic extracts of batches of 50 CC/CA were purified on a RP-HPLC system using a gradient of 20 to 32% MeCN. Three peaks, labelled A, B, and C in order of elution time (Fig. 2), showed absorption both at 214 and 280 nm and demonstrated significant bioactivity. In this paper, we focus on the structure elucidation of the bioactive compound that comprises the most abundant peak, B.

Under the HPLC conditions used, peak B eluted at a similar retention time as synthetic Mas-AKH (at about 22 min). As Mas-AKH also showed a strong lipid-increasing effect in the bioassay (Table 1), the co-elution of Mas-AKH and peak B strengthened our assumption that both compounds represent identical peptides.

A similar fraction containing about 400 pmol of peak B material was enzymatically deblocked with pyroglutamate

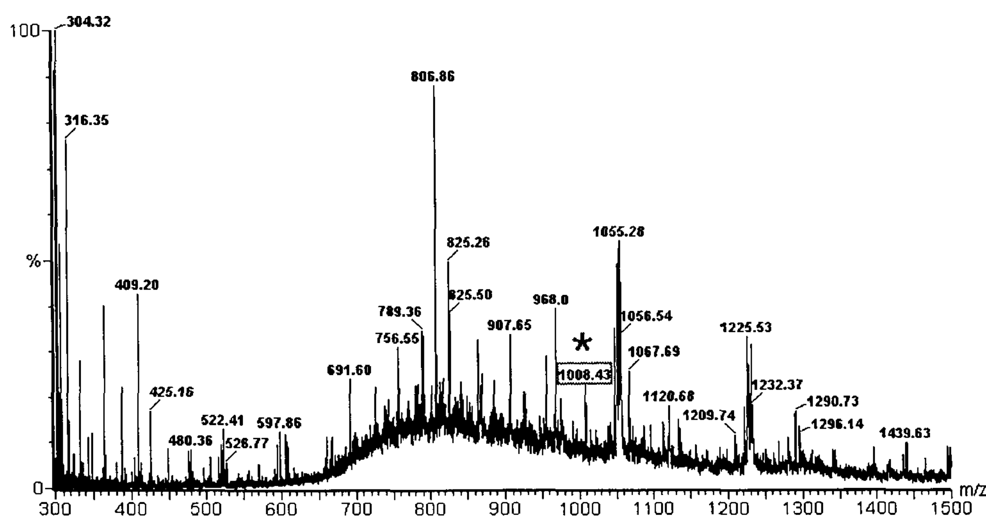

Fig. 3. Q-TOF MS spectrum of 1 µl preparation of one pair of CC (pCC) of *V. cardui*, showing multiple peptide ions. The ion marked with the asterisk was selected for MS/MS analysis (Fig. 4).

aminopeptidase. A yield of about 30% of the deblocked peptide was obtained after HPLC analysis of the digest. This was subsequently sequenced by automated Edman degradation to reveal the sequence (pQ)LTFTSSWG which is identical to Mas-AKH. The amount of peptide present was estimated at ca. 2 pmol/CC.

MALDI mass spectrometry of both undigested and deblocked peptide showed masses of 1008.18 Da and 897.01 Da ($M + H^+$), respectively, which corresponded well with the theoretical values of 1008.11 and 897.91 Da ($M + H^+$) for Mas-AKH and des-pGlu-Mas-AKH.

To further confirm the identity of the hormone, the 1008.43 Da peptide that originated from the peptide mix of one pair of CC (pCC) of *V. cardui* (Fig. 3) was selected in the nanoflow ESI-TOF experiment. Subjection of the peptide to fragmentation at collision energies of 20 to 30 V yielded tandem MS spectra, one of which is shown in Fig. 4 showing clear typical y'' and b-series of ions corresponding to the amino acid sequence pQLTFTSSWGamide which is that of Mas-AKH. A similar spectrum was obtained when the 504 Da peak (from this isotopic distribution, a clearly doubly charged ion $n + 2 H^+$) was singled out for MS/MS (not shown).

DISCUSSION

V. cardui was used in the present AKH study since this butterfly species exhibits large scale migration in spring from Southern Europe or Northern Africa to Middle and Northern Europe to establish one to several new generations there. Overwintering in Germany or other parts of Middle Europe has never been observed and it is assumed that in late summer, butterflies return South for overwintering in a reproductive diapause (Ebert & Rennwald, 1993). Furthermore, painted lady butterflies can be easily bred on an artificial diet in the laboratory and thus experimental animals are available all year round.

Our present data show that *V. cardui* contains the same adipokinetic hormone as found in several moth species, namely Mas-AKH. Although not the only adipokinetic hormone in this species, Mas-AKH is the most abundant and seems to play an important role in haemolymph lipid regulation. Despite both sexes containing about the same amount of Mas-AKH in their CC, as judged from HPLC runs of crude CC extracts (not shown), in our experiments only males showed a significant increase in haemolymph lipids upon injection of either CC extracts or synthetic Mas-AKH. Interestingly, upon lift-generating flight for 5 min an increase in haemolymph lipids was also observed only in males. Twenty minutes after a flight of 5 min we did not find a replenishment of the lipid concentration in the haemolymph, but a further increase in the lipid level. This indicates that during the resting period after flight, when butterflies could not feed in our experimental set-up, even more lipids were mobilized from the fat body. Carbohydrates seem to be important only during beginning of flight (see Fig. 1).

Females of our laboratory-reared colony do not fly very well at any time. On the contrary, vitellogenesis and ovarian maturation started immediately after emergence and first eggs were layed already three days after imaginal moult. Egg laying continued for at least two weeks. In reproductively-active females migration behaviour is suppressed (oogenesis-flight-syndrom; Johnson, 1969) and, therefore, animals may be less responsive to lipid-mobilizing stimuli. In migratory insect species, juvenile hormone (JH) is the primary hormone responsible for the regulation of both migration and reproduction (Ramaswamy et al., 1997). Juvenile hormone induces vitellogenin production in the fat body of freshly emerged females of *V. cardui* (Herman & Dallmann, 1981) and Monarch females, *D. plexippus* (Herman, 1975). In the true armyworm, *P. unipuncta*, JH was not only implicated in ovarian development of females (Cusson & McNeil, 1989), but it was shown that the rate of JH biosynthesis was lower in both sexes under conditions that signal the advent of habitat deterioration prior to migration (Cusson et al., 1990). With regard to peptides, adipokinetic hormones may also be involved in the regulation of vitello-

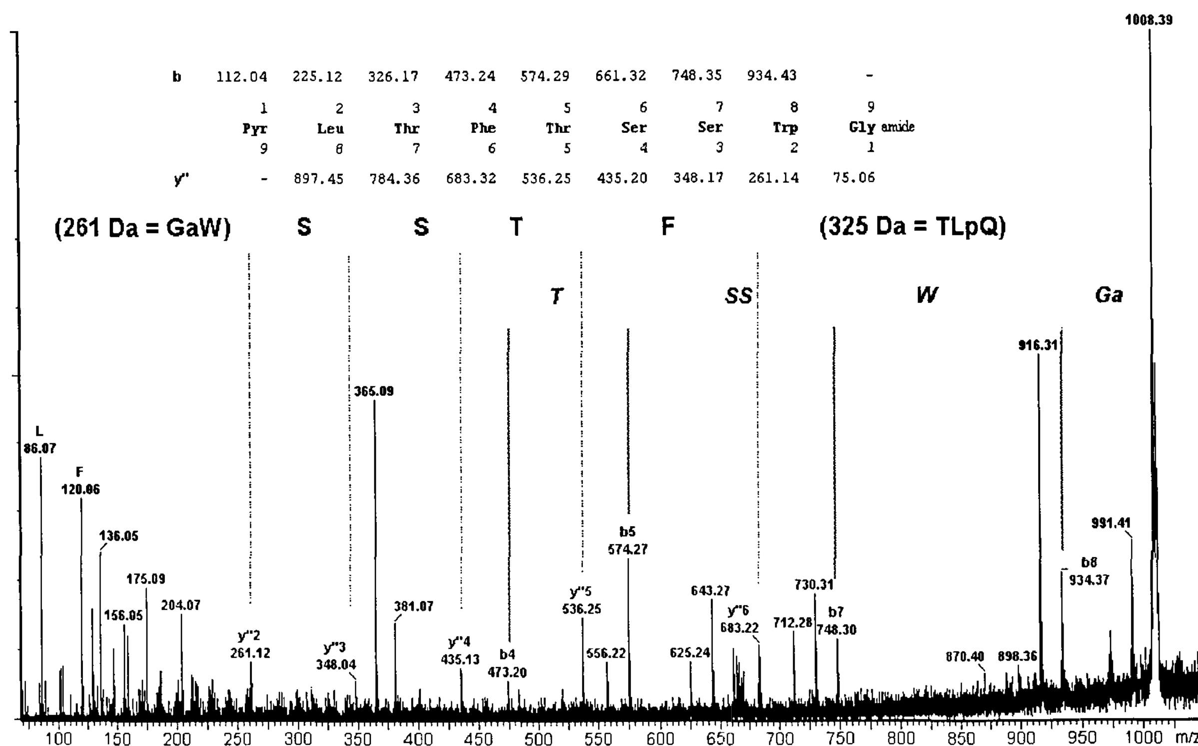

Fig. 4. Q-TOF MS/MS spectrum of the 1008.43 precursor ion. Table insert shows theoretical calculated masses for b and y″-ions of Mas-AKH, which has an identical precursor mass. The presence of complementary y″- and b-ion series in the spectrum confirms this structure. The sequence which can be readily read from the y″-series is typed in normal font (from C- to N-terminus); the sequence directly deducible from the complementary b-series is typed in italic font. The missing part of the sequence could be puzzled together from the ions in the low molecular weight region (more prominent in a spectrum obtained at higher collision energy), showing immonium ions indicative of the presence of I/L, F, pQ, W, T, S (as well as the clear absence of H, Y, P). Note that most b-ions are accompanied by one or more fragment ions characterized by a loss of one to three molecules of H_2O (–18, –36, –54). For the b6-ion, these "neutral loss"-fragments are even more prominent than the ion itself, which is buried in the noise. This is due to the presence of Ser and Thr in the fragment, two residues which tend to readily lose H_2O. The ion at –17 from the precursor is indicative of the C-terminal amide, which is known to exhibit loss of NH_3. Abbreviations: pQ, pyroglutamate; Ga, glycine-amide.

genesis. Lom-AKH I has been shown to inhibit vitellogenin production (and protein synthesis in general) in *Locusta migratoria* (Carlisle & Loughton, 1986; Moshitzky & Applebaum, 1990). In the same species, injection of an adipokinetic factor led to a higher increase in haemolymph lipids in males than in females (Gäde, 1980). From this result, it was suggested that the female-specific vitellogenin may affect the lipoprotein responsible for haemolymph diacylglycerol transport.

Levels of haemolymph lipids in *V. cardui* were high compared to many other insect species (e.g., *Gryllus bimaculatus*, Gäde & Rinehart, 1987; *Decapotoma lunata*, Gäde, 1995; *Melolontha melolontha*, Gäde, 1991). In the Monarch, however, lipid levels are even higher, especially before migration (Dallmann & Herman, 1978), suggesting that high titers of haemolymph lipids are common to migrants. The rather high variability in basic titers of haemolymph lipids of females and males of *V. cardui* (see Table 1) may have resulted from variations in the nutritional status of the experimental animals as well as from their responses to handling. Starvation and handling both provoke an increase in haemolymph lipid concentrations (Arrese et al., 1996). Experiments using animals from the field have to show whether haemolymph lipid concentrations are higher and more consistent in migrating specimens of *V. cardui* than in the laboratory-reared females. It can be expected that in migrating females levels of haemolymph lipids will increase upon injection of AKH, similar to the situation in our laboratory-reared males.

In the present study, we used two different technologies to elucidate the primary sequence of an AKH peptide. Edman degradation demands purity of the peptide to a high degree and further, deblocking of AKHs prior to N-terminal sequencing. Deblocking with pyroglutamate aminopeptidase often involves considerable loss of peptide. In the present case, about 100 pmol of purified, deblocked peptide was needed to get the full sequence by automated Edman degradation; this amount relates to an extract from ca. 200 butterflies. A more elegant method appears to be nanoflow ESI-OA-TOF mass spectrometry. Acquisition of a mass spectrum can be accomplished in less than one hour and a gland from a single butterfly was sufficient for the sequence analysis. It has to be mentioned, however, that our prior knowledge of the primary sequence facilitated the spectra analyses. In the tandem MS spectra, for example, one is not able to discriminate between leucine and isoleucine. Tandem mass spectra at different collision energies gave complementary data of

sequence information. The Q-TOF MS method had previously been performed on locust and cockroach AKH-related peptides (Verhaert et al., 1997; Verhaert & De Loof, 1998), but CC of a locust or a cockroach may contain up to 200 times higher amounts of AKH than the butterfly CC. Also in that case, knowledge of the primary sequences facilitated the spectra analyses. Nevertheless, the present experiments may demonstrate the power of modern mass spectrometry in the analysis of minute amounts of neuropeptides. Current improvement of the various peptide MS analyzing software will greatly facilitate de novo sequence interpretation of peptide MS spectra. We anticipate, that Q-TOF MS will help us to identify the second AKH (peak A) of *V. cardui*.

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