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Bladder grasshoppers (Caelifera: Pneumoridae) contain three adipokinetic peptides

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Abstract. The corpora cardiaca (CC) of the pneumorid grasshopper species *Bullacris discolor* contain at least one substance that causes hyperlipaemia in the migratory locust. Isolation of neuropeptides belonging to the adipokinetic hormone (AKH) family was achieved by single-step reversed-phase high performance liquid chromatography (RP-HPLC) of CC extracts and monitoring tryptophan fluorescence. The material of the bladder grasshopper showed three distinct fluorescence peaks with adipokinetic activity in the migratory locust. The peptide sequences were identified by Edman degradation after the N-terminal pyroglutamate residue had been cleaved off enzymatically, and the exact peptide masses were determined by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. Moreover, the assigned peptides were synthesised and natural and synthetic peptides were compared in their behaviour in RP-HPLC. *B. discolor* stores three AKH peptides in its CC: two of those are octapeptides, Schgr-AKH-II (pELNFSTGWamide) and Peram-CAH-II (pELTFTPNWamide), whereas the third peptide is a decapeptide, Phyle-CC (pELTFTPNWGSamide. The concentration of carbohydrates in the haemolymph of *B. discolor* is about 3 times higher than the lipid concentration. Upon injection with synthetic Schgr-AKH-II no adipokinetic or hypertrehalosaemic effect was measurable. A literature survey appears to indicate that an active role of these AKH peptides in substrate mobilisation is only overtly displayed in those caeliferan species that undertake well-defined flight periods.

INTRODUCTION

Endocrine control in insects is mainly achieved by the action of neuropeptides which are synthesised in neurosecretory cells and released from neurohaemal organs, the most prominent of which is the retrocerebral complex, including the corpora cardiaca (CC) (Gäde et al., 1997). The main peptides synthesised by the intrinsic neurosecretory cells of the CC are the adipokinetic hormones (AKH; Gäde, 1996, 2004). These peptides belong to a large peptide family which includes also the red pigment-concentrating hormone (RPCH) of the insects' sister group, the crustaceans, and are called members of the AKH/RPCH peptide family (Gäde, 2004). Peptides of this family are structurally characterised by the following criteria: the chain length is 8 to 10 amino acids; aromatic amino acid residues occur at position 4 (mostly Phe, sometimes Tyr) and 8 (always Trp); at position 9 is always a Gly residue; the termini are blocked by a pGlu residue (N-terminus) and by a carboxyamide (C-terminus). As with many other neuropeptides of invertebrates as well as vertebrates, the peptides of the AKH/RPCH family are pleiotropic in function and they occur in a wide range of insect orders. The main and probably "classical" function in insects is to regulate the level of circulatory metabolites (lipids, carbohydrates, proline) by activating phosphorylases or lipases in the main target cells of the fat body, whereas in crustaceans they cause the aggregation of pigments in epidermal cells resulting in "blanching" of the organism (Gäde et al., 1997; Gäde, 2004; Gäde & Marco, 2006). Other known actions of these pleiotropic peptides are the stimulation of muscle contraction and oxidation of substrates in the flight muscles, inhibition of the synthesis of RNA, fatty acids and protein in the fat body, and even a supportive role in the immune response is discussed (see Gäde, 2004). Many isoforms of AKH peptides have been identified by structural studies. Whereas only one form, code-named Panbo-RPCH, has been sequenced in various decapod crustaceans (Gäde & Marco, 2006), more than 40 members are known from insects (Gäde, 2004; Gäde & Marco, 2006; Gäde et al., 2007a,b), including Panbo-RPCH which is also synthesised in stink bugs (Gäde et al., 2003a; Gäde G., Marco H., Šimek P. & Kodrík D., unpubl. results). AKH/RPCH peptides have been sequenced from all major insect orders. Although the structure of the first insect member of the family, the decapeptide Locmi-AKH-I, was elucidated from migratory and desert locusts over thirty years ago (Stone et al., 1976), the number of caeliferan species investigated is small. Interestingly, two species belonging to the families Pamphagidae and Pyrgomorphidae were found to contain three octapeptide members, whereas in other pyrgomorphid or acridid species one or two octapeptides are associated with one decapeptide (Gäde et al., 1988, 1996; Ziegler et al., 1988; Oudejans et al., 1991; Gäde & Kellner, 1995; Siegert et al., 2000; Taub-Montemayor et al., 2002; Gäde, 2006).

In the present study I investigate grasshoppers of the caeliferan family Pneumoridae with the following objectives:

(1) What is the complement of AKH peptides in this family? According to previous work on orthopteroid phy-

logeny the position of the superfamily Pneumoroidea is not undisputed; it has been placed between Pyrgomorphoidea (containing the Pyrgomorphidae which have either two octapeptides and one decapeptide or three octapeptides as AKHs) and Acridoidea (containing the Acrididae, which have either one octa- and one decapeptide or two octa- and one decapeptide, and the Pamphagidae with three octapeptides) (Rowell & Flook 1998; Table 2). In another publication by these authors (Flook & Rowell, 1998: Fig. 6) the Pneumoroidea are placed higher than the Pamphagoidea (containg the families Pamphagidae and Pyrgomorphidae) and closest to the Acridoidea. Thus, without any bias I expected to find in Pneumoroidea either three octapeptides (as in certain Pamphagidae and Pyrgomorphidae) or a decapeptide and either one or two octapeptides as in Acridoidea.

(2) It can be hypothesized that grasshopper species that are either wingless, unable to fly or capable of only very short flights, do not show an overtly hyperlipaemic or hypertrehalosaemic response upon injection with own CC extracts. This hypothesis is based on earlier findings that such grasshopper species did not all display adipokinetic or hypertrehalosaemic effects or only to a marginal extent, although their CC extracts were able to regulate lipid and carbohydrate metabolism in appropriate assay insects, such as locusts and cockroaches (Spring & Gäde, 1987; Ziegler et al., 1988; Gäde & Kellner, 1995; Gäde et al., 1996; Gäde, 2006). Do bladder grasshopper follow this hypothesis?

MATERIAL AND METHODS

Insects

Larvae (5th instar) and adult specimens of both sexes of the pneumorid grasshoppers *Bullacris discolor* (Thunberg, 1810) and a few larvae of *B. unicolor* (Linnaeus, 1758) were collected in the austral spring and summers of 1995, 1998 and 2006 in the Cape Province of South Africa either close to the University of Cape Town in Rondebosch, in the vicinity of Rooiels or close to Clanwilliam (all Western Cape Province of South Africa). Corpora cardiaca were immediately dissected on the day of collection and were kept in 80% methanol at – 20°C until extraction. Some grasshoppers were kept in our insectary in Cape Town for a few days in order to perform biological assays (see below). For heterologous bioassays adult male migratory locusts, *Locusta migratoria*, were used; their rearing is outlined elsewhere (Gäde, 1991).

Isolation of neuropeptides and structural analyses

Methanolic extracts of CC were prepared as described previously (Gäde et al., 1984). The dried material was either taken up in water for bioassaying (see below) or it was dissolved in 15% acetonitrile containing 0.1% trifluoroacetic acid (TFA) for reversed-phase high performance liquid chromatography (RP-HPLC) using columns and equipment as described previously (Gäde, 1985; see also legend to Fig. 1).

Biological activity of HPLC-derived fractions was determined in a heterologous bioassay (in the migratory locust). Biologically active material from such HPLC separations was then digested with pyroglutamate aminopeptidase to remove the N-terminal pyroglutamate residue to make the remaining peptide accessible to Edman degradation sequencing (Gäde et al., 1988), and the resulting mixture of digested and undigested peptides was subsequently separated by RP-HPLC (as above, but

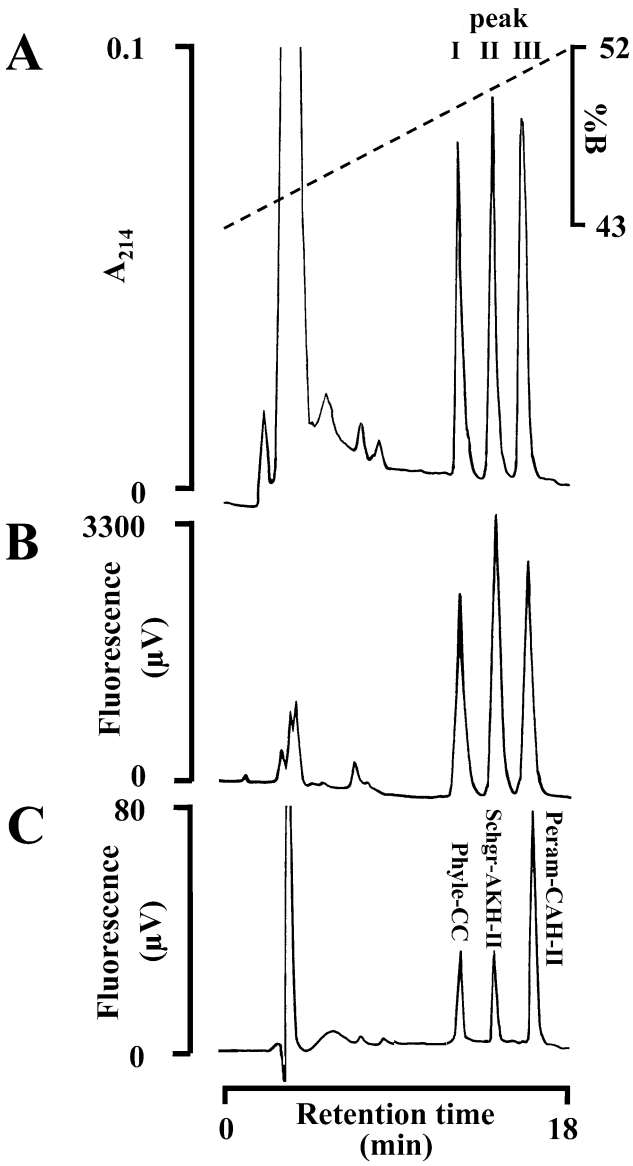

Fig. 1 A. Ultraviolet (214 nm) and B. fluorescence (excitation at 276 nm and emission at 350 nm) profiles of a methanolic extract from four pair equivalents of corpora cardiaca from *Bullacris discolor* applied to a Nucleosil 100 C-18 column. The corresponding fluorescent profile of synthetic peptides run on the same day is shown in C. A linear gradient (solvent A: 0.11% trifluoroacetic acid (TFA); solvent B: 0.1% TFA in 60% acetonitrile) was employed running from 43% to 53% B within 20 min and then from 53% to 70% within 17 min at a flow rate of 1 ml/min. Peak fractions numbered I to III in A were collected and used for further studies.

using a gradient from 33 to 53% B within 40 min). The deblocked peptide was subjected to automated Edman degradation using a model 477A sequencer connected to an on-line model 120 phenylhydantoin amino acid analyser (Applied Biosystems, Foster City, CA, USA).

Biologically active fractions were also analysed by mass spectrometry using a matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) instrument (Voyager-DE^{TM} PRO Biometry Workstation from Applied Biosystems, Inc., Framingham, MA, USA). Samples were prepared in alpha-cyano-4-

TABLE 1. Effect of a crude methanolic extract of corpora cardiaca from the bladder grasshopper *Bullacris discolor* in the migratory locust *Locusta migratoria* and in *B. discolor*.

Metabolite		Haemolymph lipids (mg ml^{-1})				Haemolymph carbohydrates (mg ml^{-1})			
Treatment	n	0 min	90 min	Difference	Statistics*	n	0 min	90 min	Difference
Acceptor: *Locusta migratoria*									
Control (10 µl distilled water)	6	8.7 ± 2.3	9.3 ± 2.0	0.6 ± 0.9	a				
B. discolor extract (0.1 gland pair equivalent)	6	9.8 ± 3.2	41.5 ± 6.8	31.7 ± 5.5	#, b				
Locmi-AKH-I (10 pmol)	6	10.2 ± 1.7	50.3 ± 8.2	40.1 ± 6.5	#, b				
Acceptor: *Bullacris discolor*									
Control (10 µl distilled water)	9	4.9 ± 2.9	6.2 ± 3.5	1.3 ± 3.9		9	17.5 ± 7.1	17.3 ± 6.2	–0.2 ± 3.9
B. discolor extract (0.1 gland pair equivalent)	5	5.0 ± 2.0	6.1 ± 1.7	1.1 ± 0.8		5	14.4 ± 3.7	15.1 ± 5.2	0.7 ± 4.2
Schgr-AKH-II (10 pmol)	11	3.5 ± 0.8	3.5 ± 1.8	0.0 ± 1.6		11	22.8 ± 5.2	23.4 ± 3.0	0.6 ± 6.5

* # Difference between pre- and post-injection significant in two-tailed paired t-test ($p < 0.001$). Different lower case letters indicate significant difference between groups (Kruskal-Wallis test, $p < 0.05$).

hydroxycinnamic acid, and spectra were acquired in positive reflectron mode.

Bioassays

The bioassays in locusts were performed as described previously (Gäde, 1980). Bioassays with bladder grasshoppers were performed as with the locust. In preliminary experiments 5th instar larvae, adult females and males were used and no significant differences in their haemolymph metabolites found; therefore, later a mixed age and sex group was used. Lipids and carbohydrates in the haemolymph were quantified as vanillin-positive (Zöllner & Kirsch, 1962) and anthrone-positive (Spik & Montreuil, 1964) material, respectively .

Synthetic peptides

Synthetic peptides Locmi-AKH-I, Schgr-AKH-II, Peram-CAH-II and Phyle-CC were either purchased from Peninsula Laboratories (Belmont, CA, USA) or were custom-synthesised by solid-phase chemistry by Dr R. Kellner (Merck KGaA, Darmstadt).

RESULTS AND DISCUSSION

Presence of adipokinetic neuropeptide material

A crude methanolic extract of 0.1 pair equivalent of a CC extract from *B. discolor* had lipid-mobilising activity in locusts (Table 1). Compared with the maximal possible response for lipid release in locusts, which was achieved by injecting 10 pmol of the endogenous peptide Locmi-AKH-I, the increase after injection of the bladder grasshopper extract amounted to about 80%. The total vanillin-positive material in the haemolymph of the bladder grasshopper was about 50% lower than in the locust and an injection of 0.1 pair equivalent of endogenous material did not result in an increase of circulating lipids (Table 1). The total anthrone-positive material in the haemolymph of the bladder grasshopper was about 3-fold higher than its lipid content; injection of endogenous gland material, however, had no significant effect on the concentration of carbohydrates in the haemolymph of the bladder grasshopper (Table 1).

As a first summary it can be stated that the bladder grasshopper *B. discolor* contains one or more substances in its CC that appear to regulate metabolite levels, a function attributed to members of the AKH family of peptides and, secondly, that the endogenous material in the CC of the bladder grasshopper has no obvious effect on mobilisation of substrates when conspecifically injected. Such results had previously also been achieved with a number of other grasshopper species (for example, Spring & Gäde, 1987; Ziegler et al., 1988; Gäde & Kellner, 1995; Gäde, 2006).

Peptide purification, characterisation and conformation

A typical chromatogram resulting from RP-HPLC fractionation of a methanolic extract from 4 pair equivalents of CC from *B. discolor* is presented in Fig. 1A and B. These traces represent the absorbance at 214 nm (Fig. 1A) and fluorescence (Fig. 1B), which is characteristic of the presence of the amino acid tryptophan (commonly at position 8 in all members of the AKH peptide family). Peaks numbered I to III (Figs 1A and B) were collected and tested for adipokinetic activity in 5 migratory locusts at a concentration of 0.1 gland equivalent.

The material with retention times of 12.4 min (peak I), 14.2 min (peak II) and 16.2 min (peak III) in the *B. discolor* CC extract (Fig. 1A, B) was active in causing hyperlipaemia in locusts (results not shown); such peak material was selected for further analyses to elucidate their primary structure. Material from peak I, II and III was N-terminally deblocked enzymatically by pyroglutamate aminopeptidase (see Fig. 2 as example), and the deblocked peptides were analysed for their primary sequence by performing the Edman degradation reaction. The results for each peptide are given as pmoles (in brackets): Peak I = Leu (26)-Thr (18)-Phe (22)-Thr(15)-Pro (13)-Asn (14)-Trp (4)-Gly (11)-Ser (5); peak II = Leu (133)-Asn (121)-Phe (128)-Ser (91)-Thr (70)-Gly (68)-Trp (2) and peak III = Leu (109)- Thr (71)-Phe (82)- Thr (52)- Pro (44)- Asn (35)-Trp (2).

Material from each peak was also subjected to MALDI-TOF mass spectrometry. Each peptide gave the

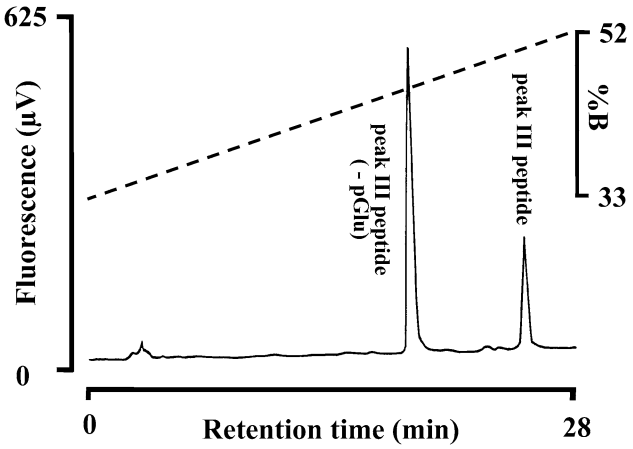

Fig. 2. Fluorescent profile of peak III material which was cleaved by pyroglutamate aminopeptidase. The less hydrophobic material which was the deblocked peptide was used for Edman degradation studies. Note that the intact (undigested) peptide, which is more hydrophobic, elutes later than its digested counterpart. HPLC conditions were as stated in Fig. 1 except that the gradient ran from 33 to 53% B in 30 min.

characteristic two mass peaks with 16 mass units difference (Fig. 3); these mass pairs can easily be interpreted as the mass signature for members of the AKH family, i.e. $[M+Na]^+$ and $[M+K]^+$ ions, while the $[M+H]^+$ ion is absent (König et al., 2005). Taking the Edman degradation and mass data together, the three peptides were identified as two well-known octapeptides, viz. Schgr-AKH-II and Peram-CAH-II, respectively and one decapeptide, viz. Phyle-CC (see Table 2 for primary sequences). Further confirmation came from HPLC experiments demonstrating that native and synthetic peptides elute at the same retention time as indicated in Fig. 1C. Schgr-AKH-II and Peram-CAH-II were first discovered in the desert locust and the American cockroach, respectively (Scarborough et al., 1984; Witten et al., 1984; Siegert et al., 1985; Gäde et al., 1986). Schgr-AKH-II has since been found in a number of grasshoppers, but also in ancestral Ensifera and in Hymenoptera (Gäde, 1992, 2006; Gäde & Kellner, 1995; Gäde et al., 1996, 2003b; Gäde & Auerswald, 1998; Lorenz et al., 2001; Taub-Montemayor et al., 2002). Peram-CAH-II is one of the typical AKH duos in blattid cockroaches (Predel & Gäde, 2005), but has also been elucidated as the AKH in one species of Coleoptera (family Chrysomelidae), one species of Hemiptera (family Pyrrhocoridae) and one species of pyrgomorphid grasshopper (Gäde & Kellner, 1989; Kodrík et al., 2002; Gäde, 2006). The third peptide, Phyle-CC, a decapeptide, has only been found in the CC of the pyrgomorphid grasshopper *Phymateus leprosus* to date (Gäde & Kellner, 1995).

Corpora cardiaca of a few larvae of *B. unicolor* were only analysed by MALDI-TOF MS and the expected sodiated and potassiated mass ion peaks for the three peptides Phyle-CC, Peram-CAH-II and Schgr-AKH-II were observed (data not shown).

Peptide complement

The bladder grasshopper is the eigth species that expresses 3 AKH peptides. Whereas the ancestral insect taxa, such as Odonata and Ephemeroptera, apparently produce only one AKH peptide in their CC (Gäde & Marco, 2005), later during evolution duplication of the AKH gene takes place in members of more advanced taxa, including blattid Blattaria, caeliferan Orthoptera, some Coleoptera, Lepidoptera and Diptera (Gäde & Marco, 2006). However, the synthesis of 3 AKH peptides has only been documented in a few species of Caelifera, viz. *L. migratoria* (Oudejans et al., 1991), *P. morbillosus* and *D. spumans* (Siegert et al., 2000), *Z. elegans* and *L. sparrmani* (Gäde, 2006), and in *P. leprosus* and *Locustana pardalina* (G. Gäde, unpubl. results), while in other

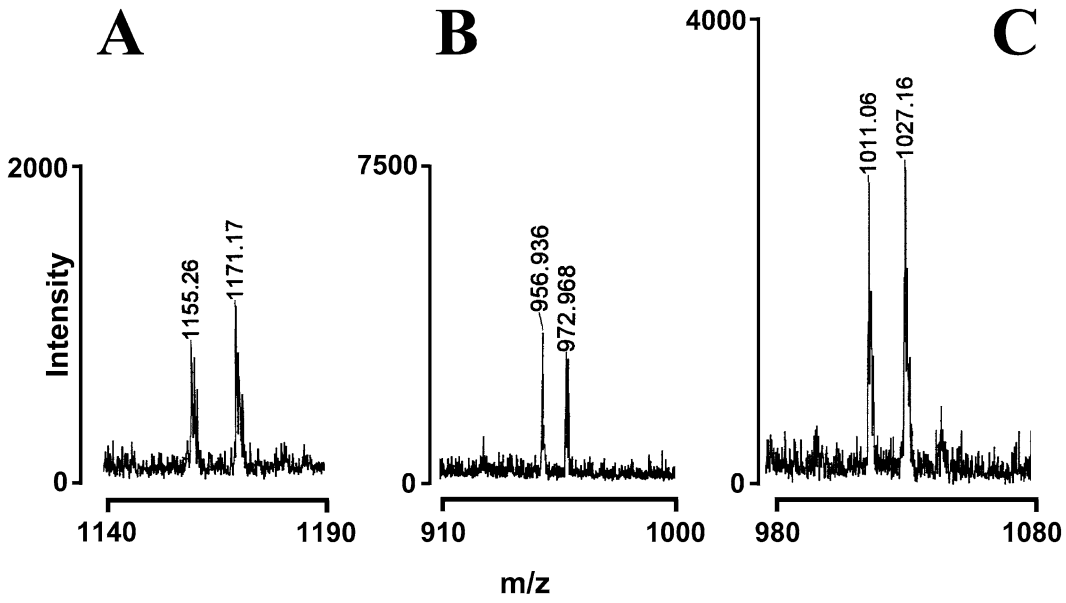

Fig. 3. MALDI-TOF mass spectra of fractions I (Phyle-CC), II (Schgr-AKH-II) and III (Peram-CAH-II). Less than one gland pair was used. Monoisotopic masses of adducts $[M+Na]^+$ and $[M+K]^+$ for each AKH peptide are labelled.

TABLE 2. Various caeliferan families with three AKH peptides.

	Pyrgomorphidae *Phymateus morbillosus Dictyophorus spumans*	*Phymateus leprosus*	*Zonocerus elegans*	Pamphagidae *Lamarckiana sparrmani*	Pneumoridae *Bullacris discolor*	Acrididae *Locusta migratoria Locustana pardalina*
Peptide 1: decapeptide	pELNFTPNWGSa (Phymo-AKH)	pEL**T**FTPNWGSa (Phyle-CC)	pEL**T**FTPNWa (Peram-CAH-II = Phyle-CC minus GS)	pELNFTPNWa (Pyrap-AKH = Phymo or Locmi-AKH-I minus GS or GT)	pEL**T**FTPNWGSa (Phyle-CC)	pELNFTPNWG**T**a (Locmi-AKH-I)
Peptide 2: octapeptide	pELNFSTGWa (Schgr-AKH-II)	pELNFSTGWa (Schgr-AKH-II)	pELNFSTGWa (Schgr-AKH-II)	pE**V**NFSTGWa (Grybi-AKH)	pELNFSTGWa (Schgr-AKH-II)	pELNFS**A**GWa (Locmi-AKH-II)
Peptide 3: octapeptide	pEINFTPWWa (Phymo-AKH-III)	pEINFTPWWa (Phymo-AKH-III)	pEINFTPWWa (Phymo-AKH-III)	pEINFTPWWa (Phymo-AKH-III)	pE**LT**FTP**N**Wa (Peram-CAH-II)	pE**L**NFTPWWa (Locmi-AKH-III)

locusts and grasshoppers, such as *S. gregaria* (Oudejans et al., 1991), *Anacridium aegypticum*, *Nomadacris septemfasciata* and *Acanthacris ruficornis* (G. Gäde, unpubl. results) only two AKH peptides occur.

Most grasshoppers and locust species in which three AKH peptides occur, including the bladder grasshopper of the current study, produce one decapeptide and two octapeptide AKHs, while the complement of three octapeptides as found in *Z. elegans* and *L. sparrmani* (Gäde, 2006), seems rather to be the exception than the rule. It is quite revealing to take a closer look at the three peptides found in the various species of Caelifera (Table 2): One could argue that all have one decapeptide (Phymo-AKH, Phyle-AKH, Locmi-AKH) which only differs by one amino acid and represents a point mutation, and that those two species with a third octapeptide have acquired this by deletion from a decapeptide. Thus, the Peram-CAH-II in *Z. elegans* may be derived from Phyle-CC (missing the two C-terminal residues GS) and the Pyrap-AKH of *L. sparrmani* may be derived from Locmi-AKH-I (missing the N-terminal residues GT, etc; see Table 2). Octapeptide 1 in all species is Schgr-AKH-II with two exceptions: Grybi-AKH occurs in *L. sparrmani* and Locmi-AKH-II in *L. migratoria*; again the peptides differ only in one residue (Table 2). Octapeptide 2 in all species is Phymo-AKH-III with the following two exceptions: Locmi-AKH-III in *L. migratoria* which differs in one residue and Peram-CAH-II in *B. discolor* which differs in three positions from Phymo-AKH-III (Table 2).

Lastly, our data on AKH sequences neither refute nor support the hypothesis that Pyrgomorphidae are closest placed to Pneumoridae, whereas Pamphagidae are closely grouped to acridid subfamilies when caeliferan phylogeny was reconstructed using mitochondrial and nuclear ribosomal RNA gene sequences (Rowell & Flook, 1998).

Functional considerations

It is interesting to note that injection of the conspecific CC extract, or one of the endogenous peptides, Schgr-AKH-II, in its synthetic form, had no effect to increase either the concentrations of carbohydrates or lipids in the haemolymph of *B. discolor*. It appears that most, if not all, grasshoppers fall into such a category of insects that do not react with hyperlipaemia, although their CC contain peptides of the AKH family (see, for example, Spring & Gäde, 1987; Ziegler et al., 1988; Gäde, 2002, 2006). The "typical" adipokinetic response seems to be developed only, or at least preferentially, in locust; thus, in those Caelifera that can occur in gregarious forms to build huge swarms and undertake sustained flights, such as in *Locusta migratoria*, *Schistocerca gregaria* (Goldsworthy, 1983) and *Locustana pardalina* (G. Gäde, unpubl. results) but, as discussed above, not in grasshoppers. This fact is surprising, because in other orders, for example in terrestrial and aquatic Hemiptera, a number of species respond with hyperlipaemia upon conspecific injection of CC extract and is has been demonstrated that those hemipterans use lipid oxidation for muscle contraction (Gäde et al., 2004, 2006, 2007a, b), even though none of these species are particularly well-known for long-distance flights.

This brings us to the question of what the function of AKH peptides may be in the bladder grasshopper. As argued before (Gäde, 2006), the higher concentration of carbohydrate in the haemolymph appears to suggest that these may play a role during locomotion. As shown previously in a romaleid (*Romalea microptera*; Gäde & Spring, 1989), an acridid (*Barytettix psolus*; Ziegler et al., 1988) and a pyrgomorphid (*Phymateus leprosus*; G. Gäde, unpubl. results) grasshopper species, glycogen phosphorylase of the fat body can be stimulated by low doses of endogenous AKH peptide or conspecific CC extract without observing any overt effect on the concentration of carbohydrates in the haemolymph. But is the bladder grasshopper prone to a lot of activity during its life cycle? It is generally believed that only adult males with macropterous wings and inflated abdomen can fly, albeit for short distances only; non-inflated males have vestigial wings and females are mostly micropterous; both, therefore, cannot fly (Moira van Staaden, personal communication). Locomotory activity is reduced to hopping then (as in nymphs) and main muscular activities may be stridulation in males which results in calls which can be heard kilometres away. It has been reported that AKH peptides are involved in stimulation of locomotory activity in *P. apterus* and *Gryllus bimaculatus* (Socha et al., 1999; Lorenz et al., 2004). This may be true for the bladder grasshopper as well, with carbohydrates fueling the activity. There is, however, no report to date that demonstrates a direct causative link between stridulation, AKH release and substrate mobilisation. Better researched, but not exhaustively, is the physiological action of AKH with respect to reproduction (Gäde, 2004;

Lorenz, 2003). It may be in this context that AKH plays a role in the bladder grasshopper. Speculativly, one could also envisage a function of AKH as control agent of energy provision during a moult cycle.

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