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Distinct Sequences of AKH/RPCH Family Members in Beetle (*Scarabaeus*-Species) Corpus Cardiacum Contain Three Aromatic Amino Acid Residues

Gerd Gäde[1]
Zoology Department, University of Cape Town, Rondebosch 7700, South Africa

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Two forms of AKH/RPCH family peptides have been identified from the corpus cardiacum of various dung beetle species of the genus *Scarabaeus* and the related genus *Gareta* by using RP-HPLC. The primary structures were established in two species by automated Edman degradation and mass spectral analysis as blocked octapeptides containing three aromatic amino acids (at positions 2, 4 and 8): peptide I: pGlu-Phe-Asn-Tyr-Ser-Pro-Asp-Trp-NH_2; peptide II: pGlu-Phe-Asn-Tyr-Ser-Pro-Val-Trp-NH_2. The peptides were not active in the heterologous adipokinetic bioassay in locusts, but increased the concentration of proline in the haemolymph of *Scarabaeus* beetles. Since proline is also used in these species to provide the energy for contraction of the flight muscles during flight, it is proposed that the distinct peptides characterized here are responsible for the hormonal control of proline homeostasis.

The adipokinetic hormones (AKHs) produced in the neurosecretory corpus cardiacum of insects are among the best studied insect neuropeptides and are structurally related to the crustacean red pigment-concentrating hormone (RPCH). These neuropeptides are grouped together into the AKH/RPCH-family of peptides, which contains octa-, nona- and decapeptide members characterized by an N-terminal pyroglutamyl residue, a C-terminal amide and aromatic residues at position 4 (Phe or Tyr) and 8 (Trp) [1,2]. All but two members occurring in certain Diptera (flies) [3,4] and Coleoptera (beetles) [5,6] are uncharged. From a functional point AKHs are involved in the regulation of substrate mobilization from the storage organ (the fat body) during flight [7,8]. Whereas such peptides regulate lipid mobilization during long-distance flight in locusts, our recent results with various beetle species have shown that the amino acid proline is the major fuel to power flight muscle metabolism [9,10]. Therefore, the objectives of the present study were two-fold: (1) to separate, purify and identify the putative AKH members from the corpus cardiacum of beetles of the genus *Scarabaeus* which comprises of many excellent flyers - these beetles have to fly rapidly to dung pats of herbivores in order to exploit this food source efficiently, and (2) to analyze the flight substrates of *Scarabaeus* species and identify the substrates mobilized by injection of the endogenous AKH peptides.

[1] Fax: +2721 6503301. E-mail: ggade@botzoo.uct.ac.za.

Abbreviations used: AKH, adipokinetic hormone; RPCH, red pigment-concentrating hormone; RP-HPLC, reversed-phase high performance liquid chromatography; Fmoc, N-(9-fluorenyl)methoxycarbonyl.

MATERIALS AND METHODS

Experimental animals and bioassays. All insects used in this study were adult specimens. The various *Scarabaeus* species were collected during the last five years either on the west coast in the Western Cape Province (*S. proboscideus, S. rugosus* and *S. aesculapius*), or in the Kalahari Gemsbok National Park in the Northern Cape Province (*S. deludens*), or in the Umfolozi Game Reserve in KwaZulu/Natal (*Gareta nitens*) or near the Brandberg in Namibia (*S. satyrus*). After capture, beetles were kept in cages with cattle dung in the laboratory until bioassays or flight experiments were performed. Corpora cardiaca were dissected from the insects as quickly as possible after collection. For heterologous bioassays adult (15-25-day old) male migratory locusts, *Locusta migratoria,* from our own colony were used [11]. Haemolymph sampling, injection of material, and the hyperlipaemic bioassay in locusts were executed as outlined earlier [12]. For bioassays, the beetles were kept separately, without food and at room temperature (± 22°C) in small plastic boxes the night before experimentation. At time zero and 90 min after injection of the appropriate test solution, 1 μl of haemolymph was taken by carefully puncturing the neck membrane. Determination of total carbohydrates and lipids in the haemolymph was performed as for the cockroach and lipid bioassay, respectively [12]. The amino acids proline and alanine were determined after derivatization and subsequent HPLC separation as outlined in [9].

Purification and isolation of corpus cardiacum peptides. Methanolic extracts from corpora cardiaca were prepared as described previously

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[13] and applied to a Nucleosil 100 C-18 column (i.d. 4.6 mm × 250mm length) for RP-HPLC using equipment described before [14]. Fractions displaying distinct UV (at 214 nm) and/or fluorescence (excitation at 276 nm, emission at 350 nm) peaks were collected manually and used for bioassays (see above). Once biologically active fractions had been identified, sufficient material of these fractions was collected in subsequent HPLC runs and used for structure identification.

Sequence determination and mass spectrometry. Aliquots of the HPLC-purified materials were used for cleavage with oxoprolylpeptidase [15]. The deblocked peptides were separated from the native undigested peptides by HPLC [5], manually collected and subjected to automated Edman degradation on a model 477 A instrument of Applied Biosystems (Foster City, CA, U.S.A.) or a home-made instrument in the Department of Biochemistry of the University of Cape Town. The collected native peptides were used for electrospray mass spectrometry (VG Quattro ES-MS, VG Organic, Altrincham, England). The synthetic adipokinetic hormone, Lom-AKH-I, was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.), whereas the peptides of this study were synthesized by standard solid-phase techniques employing Fmoc chemistry, and their identity verified by amino acid analysis and mass spectrometry.

Flight experiments. Adult specimens of *S. rugosus* were allowed to fly and generate lift at about 22°C. In the first series of experiments, haemolymph samples were taken as described above (see bioassays) from individual beetles before flight, after one min of flight and at 60 min rest after a flight of 1 min duration. In a second series of experiments, flight muscles were removed from beetles at rest, after 1 min of flight and after 60 min rest following a flight of 1 min duration. The muscles were frozen immediately in liquid nitrogen. Preparation of perchloric acid extracts and determination of the levels of glycogen and the amino acids, alanine and proline, were performed as described previously [9].

RESULTS

Biological activity. Surprisingly, a crude methanolic extract of 0.5 gland equivalents from *Scarabaeus rugosus* or from *Gareta nitens* was not able to increase haemolymph lipids in the migratory locust; control injections of water increased the lipid levels by 1.2 ± 2.0 mg/ml, 0.5 gland equivalents from *S. rugosus* by 1.7 ± 3.6 mg/ml, 0.5 gland equivalents from *G. nitens* by 2.8 ± 2.7 mg/ml and 10 pmol synthetic Lom-AKH-I by 47.9 ± 20.1 mg/ml; n=7 in all cases. Haemolymph levels of total carbohydrates and lipids in *S. deludens* were very low and did not increase upon injection of a crude corpus cardiacum extract (Table 1). The proline concentration, however, which is high in non-injected beetles, showed a significant increase upon injection of a conspecific corpus cardiacum extract, wheras the alanine level significantly decreased (Table 1).

Peptide purification, sequence and mass spectral analyses. Fig. 1A and C depict the UV absorbance elution profiles of methanolic extracts of corpora cardiaca from *S. deludens* and *G. nitens* using an analytical C-18 column for separation. Only a few absorbance peaks at 214 nm can be seen, two of which, numbered 1 and 2, also gave a fluorescence signal (Fig. 1B and D) under the conditions employed, targetting for the presence of Trp which is conserved in the AKH/RPCH family of peptides [2]. Both peaks, numbered 1 and 2, occur in both extracts and have retention times of 12.0 min and 18.2 min. Peak material was not active in the lipid bioassay using migratory locusts (data not shown), but gave significant increases in the haemolymph concentration of proline when conspecifically injected (Table 2). Crude methanolic extracts of related

TABLE 1

Conspecific Assay for Adipokinetic, Hypertrehalosaemic and Hyperprolinaemic Activity of a Crude Methanolic Extract of Corpora Cardiaca from *S. deludens*

		Treatment	
Haemolymph metabolite		Control (10 μl of distilled water)	Beetle extract (1 gland equivalent)
Lipids (mg/ml)	n	7	9
	0 min	1.4 ± 0.3	2.5 ± 1.2
	90 min	1.3 ± 0.8	2.0 ± 1.0
	Difference	0.1 ± 0.7	−0.5 ± 0.3
Carbohydrates (mg/ml)	n	7	9
	0 min	0.3 ± 0.4	0.3 ± 0.5
	90 min	0.4 ± 0.8	1.4 ± 1.0
	Difference	0.1 ± 0.9	1.1 ± 1.0
Proline (μmol/ml)	n	6	9
	0 min	56.1 ± 7.0	76.9 ± 11.2
	90 min	59.7 ± 6.2	90.9 ± 15.3
	Difference	3.6 ± 5.4	14.0 ± 7.1**
Alanine (μmol/ml)	n	6	9
	0 min	4.3 ± 1.6	5.8 ± 3.0
	90 min	4.9 ± 1.9	2.8 ± 2.2
	Difference	0.6 ± 2.5	−3.0 ± 3.5*

Values are means ± S.D.
Significant difference: *$p < 0.05$; **$p < 0.01$ compared with water injections using Student's *t* test.

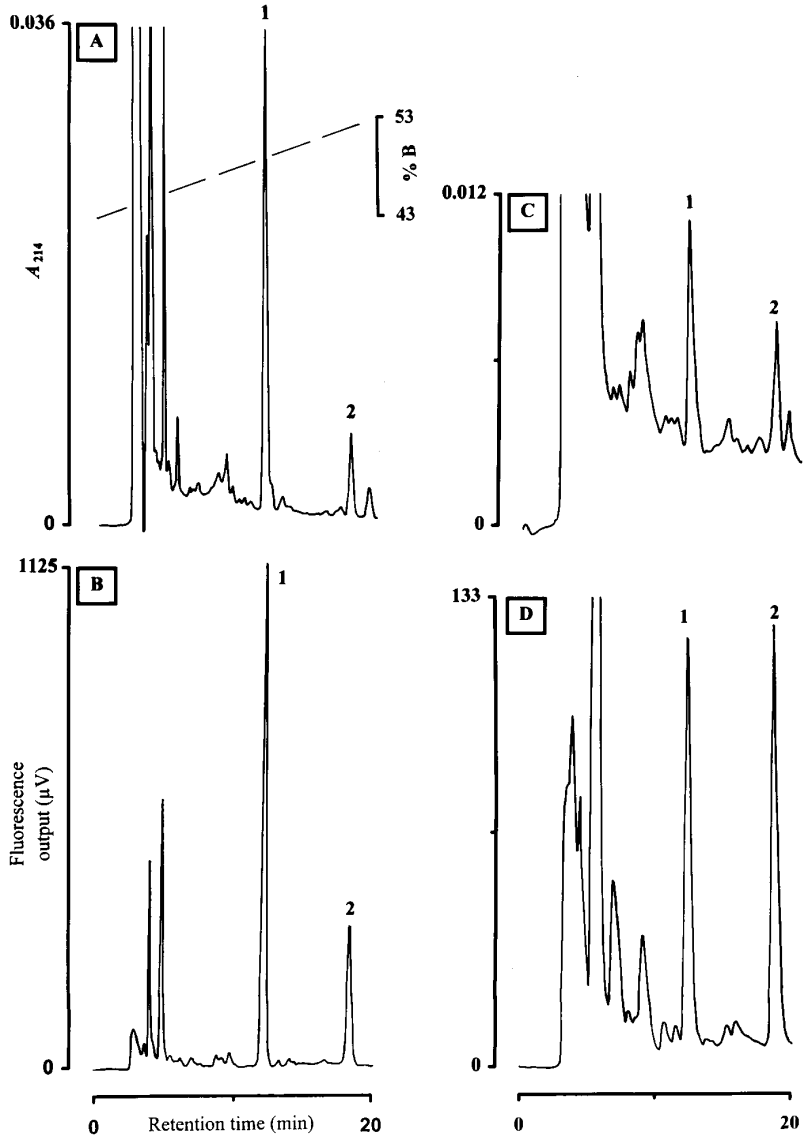

FIG. 1. Purification of dung beetle peptides by HPLC. A methanolic extract of 5 corpora cardiaca from *Scarabaeus deludens* (A,B) and 4 glands from *Gareta nitens* (C,D) was applied. Analyses were performed on a Nucleosil C-18 column under conditions as described in the text. The elution was monitored either with a UV detector at 214 nm (A,C) or with a fluorimeter at 276 nm (excitation)/350 nm (emission) (B,C).

Scarabaeus species, *S. aesculapius, S. satyrus* and *S. proboscideus,* all resulted in the same two peaks with retention times of 12.0 min and 18.2 min when chromatographed under identical conditions (data not shown).

Sufficient material of peak 1 and 2 from corpora cardiaca of *S. deludens* and *G. nitens* was collected, digested separately with pyroglutamyl aminopeptidase, and the resulting fragments yielded the following sequences after Edman degradation (pmoles in brackets); *S. deludens* peptide 1: Phe (197)-Asn (40)-Tyr (110)-Ser (38)-Pro (37)-Asp (14)-Trp (11); *S. deludens* peptide 2: Phe (44)-Asn (11)-Tyr (32)-Ser (19)-Pro (3)-Val (8)-Trp (1); *G. nitens* peptide 1: Phe (62)-Asn (37)-Tyr (52)-Ser (22)-Pro (20)-Asp (18)-Trp (3); *G. nitens* peptide 2: Phe (22)-Asn (13)-Tyr (17)-Ser (7)-Pro (5)-Val (4)-Trp (1).

The two octapeptides with a pyroglutamyl residue at the N-terminus and a Trp-amide at the C-terminus were synthesized. These synthetic peptides were eluted at the same retention times as the native peptides under the conditions employed on RP-HPLC (data not shown). Mass spectral analyses on the native peak material from *S. deludens* further confirmed assignment of the correct peptides. The positive ion spectrum

TABLE 2

Conspecific Hyperprolinaemic Activity of HPLC Fractions as Well as Synthetic Peptides in *S. deludens*

Treatment	Changes in			
	n	Proline (μmol/ml)	n	Alanine (μmol/ml)
Control (10 μl of distilled water)	5	2.4 ± 4.4	5	−2.8 ± 3.4
Peak numbered 1 (Fig. 1A; 0.5 gland equivalents)	6	14.3 ± 5.1**	6	−3.1 ± 2.7
Peak numbered 2 (Fig. 1A; 0.5 gland equivalents)	7	10.6 ± 7.6*	7	−5.1 ± 5.7
Scd-CC-I (5 pmol)	6	12.2 ± 5.1*	6	−3.5 ± 2.8
Scd-CC-II (5 pmol)	6	11.8 ± 6.8*	6	−3.0 ± 2.6

The change of metabolite concentration for alanine and proline in the haemolymph is given between the pre- and post-injection value. Values are means ± S.D.

Significant difference: *$p < 0.01$, **$p < 0.05$ compared with water injections using Student's *t* test.

showed distinct peaks at *m/z* 1060 and 1076 for peptide 1 and at *m/z* 1044 and 1060 for peptide 2 corresponding to the sodium $[M + Na]^+$ and potassium $[M + K]^+$ cationized molecules, respectively. Thus, the combined data are in keeping with the following primary structures for the two peptides: peptide 1 is pGlu-Phe-Asn-Tyr-Ser-Pro-Asp-Trp-NH_2 and peptide 2 is pGlu-Phe-Asn-Tyr-Ser-Pro-Val-Trp-NH_2. When a low dose of each peptide was injected separately into *S. deludens*, a significant increase of the haemolymph proline concentration was elicited (Table 2).

Physiological studies. Insect flight muscles are metabolically the most active tissues known in nature. We therefore investigated the type of fuel mobilized during free flight in the dung beetle *S. rugosus*. Whereas the low concentrations of total lipids and carbohydrates in the haemolymph did not change during a 1 min flight period or rest after flight, the amino acids, proline and alanine, in the haemolymph significantly decreased and increased, respectively, upon flight (Fig. 2A). These changes were reversed during rest after flight (Fig. 2A). Glycogen, the storage form of carbohydrates which occurred in very low concentrations in the flight muscles, was not diminished by flight (Fig. 2B). In contrast, there was a significant decrease in the concentration of proline in the flight muscles from 43.7 to 26.2 μmol/g fresh weight with a concomitant increase of the alanine concentration from 2.5 to 22.5 μmol/g fresh weight (Fig. 2B). Sixty min of rest after flight were sufficient to almost reverse the changes completely (Fig. 2B).

DISCUSSION

The corpus cardiacum of *Scarabaeus deludens* and *Gareta nitens* each contains the same two forms of AKH/RPCH-family members with unique amino acid sequences. According to the elution pattern in HPLC other *Scarabaeus* species contain the same peptides. Both peptides are almost identical; the only difference between peptide1, designated here Scd-CC-I (*Scarabaeus deludens* corpus cardiacum peptide I) and peptide 2 (designated Scd-CC-II) is the negatively charged amino acid Asp^7 in Scd-CC-I and the neutral residue Val^7 in Scd-CC-II. Thus, a polar amino acid (Asp^7) is replaced by a nonpolar residue (Val^7) which is, of course, the reason that Scd-CC-I is eluted at 12.0 min under the HPLC conditions employed, while Scd-CC-II is eluted at 18.2 min (Fig. 1). The presence of the two peptides in one species offers the possibility that they arose by gene duplication. A change in only one nucleotide base (GAU to GUU or GAC to GUC) is required to transform the Asp to a Val codon. Scd-CC-I is also remarkably similar in structure to another beetle peptide, Mem-CC, occurring in the corpus cardiacum of cockchafers and fruit beetles [2] (Table 3). Here, the aromatic amino acid Phe^2 of Scd-CC-I is exchanged to the nonaromatic Leu^2 residue; both residues are hydrophobic and therefore retention times are fairly similar. Both peptides of this study are unique in containing three aromatic residues in the molecule. Only recently a similar pattern was discovered in two peptides isolated from dung beetles of the genus *Onitis*, codenamed Ona-CC-I and II (see Table 3) in which a pep-

TABLE 3

Primary Structures of Structurally Closely Related Peptides of Beetles and Grasshoppers

Group	Peptide codename	Sequence
Scarabaeus- dung beetles	Scd-CC-I	pQFNYSPDWamide
and onitine dung beetles	Ona-CC-II	pQFNYSPDWamide
Scarabaeus- dung beetles	Scd-CC-II	pQFNYSP**V**Wamide
fruit beetles	Mem-CC	pQ**L**NYSPDWamide
Onitine dung beetles	Ona-CC-I	pQ**Y**NFSTGWamide
Short- and long-horned grasshoppers	Scg-AKH-II	pQ**L**NFSTGWamide

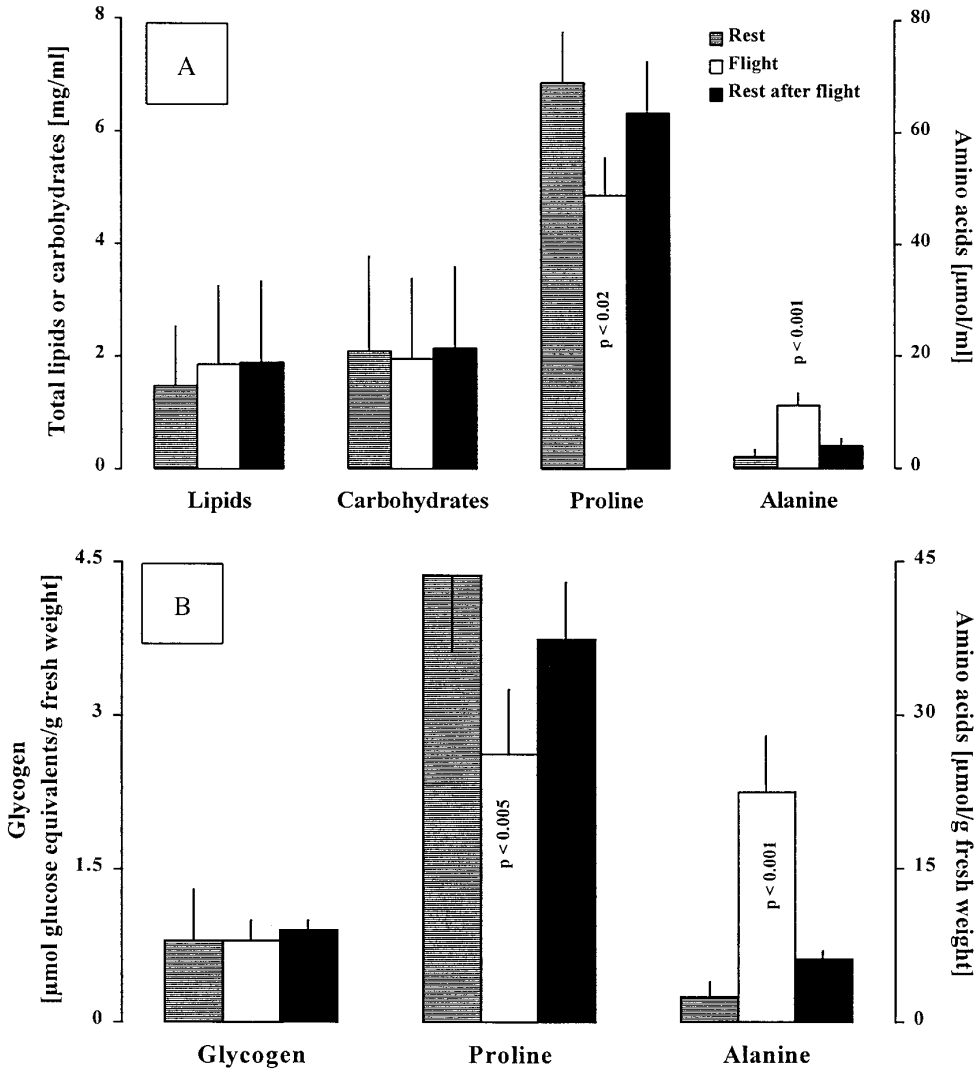

FIG. 2. Changes of the major substrates during flight of *Scarabaeus rugosus*. (A) Concentrations of total lipids, total carbohydrates and of the amino acids alanine and proline in the haemolymph are given as means ± S.D. during rest, after 1 min of flight and after 60 min of rest after a 1 min period of flight. (B) Concentrations of glycogen, alanine and proline in the flight muscles are given as means ± S.D.

tide identical to Scd-CC-I was found (Gäde, in preparation). Examination of the structures of fruit- and dung beetles' AKHs in the context of evolutionary trends suggests that Scd-CC-I and -II, Ona-CC-II and Mem-CC are very closely related and differ from each other by a point mutation at position 7 or 2, whereas Ona-CC-I is closely related to a peptide code-named Scg-AKH-II (Table 3), which contains a Leu at position 2 instead of the Tyr in Ona-CC-I and is found in certain long- and short-horned grasshoppers [2]. A change in two nucleotide bases is required to transform the Tyr to a Leu codon. However, amino acid substitutions of this type are rare [16].

It is interesting to note that both novel peptides of this study, Scd-CC-I and II, did not result in hyperlipaemia in locusts. As mentioned earlier, mainly because of the Tyr residue at position 4, Mem-CC apparently does not fit into the locust fat body receptor [17]. This then explains the failure of Scd-CC-I and II to interact in the locust system. These peptides also failed to induce hyperlipaemia or hypertrehalosaemia in beetles itself. However, high levels of proline are found in haemolymph and flight muscles of these beetles. In conjunction with the effect of crude gland extract and of low doses of the synthetic peptides to increase the proline concentration in the haemolymph as well as the usage of proline as a substrate during flight, it is suggested that the two peptides characterized in this paper are responsible for a hormonal control of proline metabolism.

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REFERENCES

1. Gäde, G., Reynolds, S. E., and Beeching, J. R. (1994) *in* Perspectives in Comparative Endocrinology (Davey, K. G., Peter, R. E., and Tobe, S. S., Eds.), pp. 119–128, National Research Council of Canada, Ottawa.
2. Gäde, G. (1996) *Z. Naturforsch.*, in press.
3. Gäde, G., Wilps, H., and Kellner, R. (1990) *Biochem. J.* **269,** 309–313.
4. Schaffer, M. H., Noyes, B. E., Slaughter, C. A., Thorne, G. C., and Gaskell, S. J. (1990) *Biochem. J.* **269,** 315–320.
5. Gäde, G. (1991) *Biochem. J.* **275,** 671–677.
6. Gäde, G., Lopata, A., Kellner, R., and Rinehart, K. L. (1992) *Biol. Chem. Hoppe-Seyler* **373,** 133–142.
7. Goldsworthy, G. J. (1990) *in* Biology of Grasshoppers (Chapman, R. F., and Joern, A., Eds.), pp. 205–225, Wiley, New York.
8. Gäde, G. (1992) *Zool. Jb. Physiol.* **96,** 211–225.
9. Zebe, E., and Gäde, G. (1993) *J. Comp. Physiol. B* **163,** 107–112.
10. Auerswald, L., and Gade, G. (1995) *J. Exp. Biol.* **198,** 1423–1431.
11. Gäde, G. (1992) *Biol. Chem. Hoppe-Seyler* **373,** 1169–1178.
12. Gäde, G. (1980) *J. Insect Physiol.* **26,** 351–360.
13. Gade, G., Goldsworthy, G. J., Kegel, G., and Keller, R. (1984) *Hoppe-Seyler's Z. Physiol. Chem.* **365,** 393–398.
14. Gäde, G. (1985) *Biol. Chem. Hoppe-Seyler* **366,** 195–199.
15. Gäde, G., Hilbich, C., Beyreuther, K., and Rinehart, K. L. (1988) *Peptides* **9,** 681–688.
16. Schultz, G. E., and Schirmer, R. H. (1979) *in* Principles of Protein Structure, pp. 171–172, Springer, New York.
17. Gäde, G. (1993) *J. Insect Physiol.* **39,** 375–383.