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Flight substrates in blister beetles (Coleoptera: Meloidae) and their regulation by neuropeptides of the AKH/RPCH family

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Abstract. We measured energy substrates in haemolymph and flight muscles of the large blister beetle *Mylabris oculata* at rest and after tethered, lift-generating flight. Flight of 1 min duration at an ambient temperature of 38–42°C did not effect a change in the concentration of lipids in the haemolymph, whereas a small, significant decrease in the concentrations of carbohydrates and a 3-fold larger one in the levels of proline were noted, as well as a concomitant increase in alanine. In the flight muscles, glycogen and proline concentrations were diminished slightly but significantly upon flight, whereas alanine levels were increased. Two hours of rest after a flight of 1 min completely reversed the metabolic situation in haemolymph and flight muscles to pre-flight levels. We could isolate two neuropeptides from the corpora cardiaca of *M. oculata*, which by retention time and mass analyses are characterised as the decapeptide Del-CC (pGlu-Leu-Asn-Phe-Ser-Pro-Asn-Trp-Gly-Asn-NH_2) and the octapeptide Tem-HrTH (pGlu-Leu-Asn-Phe-Ser-Pro-Asn-Trp-NH_2) previously fully identified from the corpora cardiaca of the blister beetle, *Decapotoma lunata* (Gäde, 1995). Subsequently, it was unequivocally demonstrated that low doses of Del-CC and Tem-HrTH elicited increases in the concentration of proline and carbohydrates in the haemolymph of *D. lunata* and *M. oculata*, but did not change the concentration of lipids in both species. In conclusion, the two endogenous peptides are hypertrehalosaemic and hyperprolinaemic, thus very likely regulating the mobilisation of the two important flight substrates of blister beetles, namely carbohydrates and proline.

INTRODUCTION

It is well known that insect flight is the most energy-demanding process in nature and, hence, that for the aerobic work performance of such flight muscles, a large quantity of stored fuel is needed for mobilisation. This latter process is apparently achieved by the action of small neuropeptides synthesised and stored in the corpora cardiaca (see Gäde, 1996, 1997a). Fuels that are utilised during flight can be carbohydrates, lipids and proline or combinations thereof (see Gäde, 1992; Gäde & Auerswald, 1998).

Our ongoing research studies flight metabolism of various beetle families. Our previous investigations have indicated that certain dung beetles may exclusively use the amino acid proline (Gäde 1997b,c) as it was suggested for tsetse flies (see Bursell, 1981), whereas the fruit beetle, *Pachnoda sinuata* (Zebe & Gäde, 1993; Auerswald et al., 1998a), the blister beetle, *Decapotoma lunata* (Auerswald & Gäde, 1995), and the Colorado potato beetle, *Leptinotarsa decemlineata* (Weeda et al., 1979; Gäde, 1999) oxidise carbohydrates simultaneously with proline.

In the present study we give evidence that another member of the meloid family, *Mylabris oculata*, also powers its flight by the oxidation of carbohydrates and proline. Moreover, we isolated its endogenous neuropeptides from the corpora cardiaca and demonstrate that low concentrations of these peptides are able to regulate the flight substrates carbohydrates and proline in *M. oculata* and in *D. lunata* which had recently been analysed in respect of its flight metabolites; however, no mobilisation of substrates by the endogenous neuropeptides of the AKH/RPCH family had previously been shown in the latter species (Auerswald & Gäde, 1995; Gäde, 1995).

MATERIAL AND METHODS

Animals

Blister beetles of both sexes of *Decapotoma lunata* Pallas were caught on the slopes of Table Mountain, Cape Town, South Africa during the austral summer, while those of *Mylabris oculata* Thunberg were caught in the Karoo between Laingsburg and Beaufort West, South Africa. Animals of both sexes were used for experimentation. The average weight of *D. lunata* was 203 ± 82 mg (n = 29), while that of *M. oculata* was 1006 ± 216 mg (n = 36).

Flight experiments

Experiments with *M. oculata* were performed at 38–42°C in direct sunlight. The beetles were attached at the prothorax to a bent insect pin by dental wax. These experiments were performed using a method which assured flight with lift-generation as previously described in detail in Auerswald et al. (1998b). Beetles which did not generate lift were excluded from the experiment. Samples for determination of metabolites in haemolymph and flight muscles were taken and stored as described for *Pachnoda sinuata* in Auerswald et al. (1998b).

Bioassays

The night before experimentation, individuals of *D. lunata* and *M. oculata* were kept separately in small plastic containers (approximately 50 ml) at room temperature (22 ± 2°C). The animals had access to their food plant and water. Before injection of the appropriate test solution and at different periods post-injection, a 1 μl haemolymph sample was taken from the neck membrane of the same individual and used for the determination of free amino acids, lipids and carbohydrates.

Preparation of samples and metabolite determination

Haemolymph. Samples of 1 µl of haemolymph were either blown immediately into 100 µl of concentrated H_2SO_4 for the determination of total lipids (Zöllner & Kirsch, 1962) or carbohydrates (Spik & Montreuil, 1964), or pipetted into 100 µl of 80% acetonitrile for amino acid analysis (see below).

Tissue samples. Perchloric acid extracts from frozen flight muscles were made according to Zebe & Gäde (1993).

Glycogen determination. Glycogen was extracted as previously described in Zebe & Gäde (1993) and analysed by the modified anthrone method (Spik & Montreuil, 1964) with glucose as a standard.

Proline and alanine determination. Derivatisation of extracts with dansyl chloride and determination of proline and alanine levels by HPLC were carried out as previously described (Zebe & Gäde, 1993).

Isolation and identification of peptides from corpora cardiaca

Glands were dissected into 80% methanol and crude extracts were prepared as described previously (Gäde et al., 1984). Application of the extract onto a Nucleosil C-18 column and development of that column were as outlined previously (Gäde, 1985; see also Fig. 3). Two purified fractions were used to generate spectra of mass data using a Voyager Elite (PerSeptive Biosystems, Framingham, Massachusetts, USA) matrix-assisted laser desorption/ionisation instrument. Samples were prepared in α-cyano-4-hydroxycinnamic acid, and spectra acquired in positive, linear mode.

Synthetic peptides

The neuropeptides of blister beetles, code-named Del-CC and Tem-HrTH, were synthesised employing Fmoc chemistry as outlined previously (Gäde, 1995).

RESULTS

Flight experiments with *M. oculata*

After 1 min of flight, proline concentration in the haemolymph was decreased significantly from an initial 27.6 to 18.7 µmol·ml^{-1}, while alanine concentration was increased from 4.5 to 11.6 µmol·ml^{-1} (Fig. 1). Resting levels of the two amino acids were re-established during 2 h of rest following flight.

Carbohydrate concentration dropped significantly from 17.3 to 14.2 mg·ml^{-1} during 1 min of flight. The resting level was reached after 2 h of subsequent rest (Fig. 1). No changes occurred in the lipid concentration during flight and subsequent rest (Fig. 1).

The concentration of proline in the flight muscles was diminished significantly from an initial 20.6 to 16.0 µmol·g^{-1} during 1 min of flight and was re-established within 2 h thereafter (Fig. 2). Alanine concentration rose significantly from 1.6 to 5.4 µmol·g^{-1} during flight. After 2 h of subsequent rest the concentration reached pre-flight levels (Fig. 2).

The initial glycogen concentration in the flight muscles of 21.0 µmol glucose equivalents g^{-1} was significantly diminished to 17.4 µmol·g^{-1} during 1 min of flight. The pre-flight levels were reached after 2 h of rest following flight (Fig. 2).

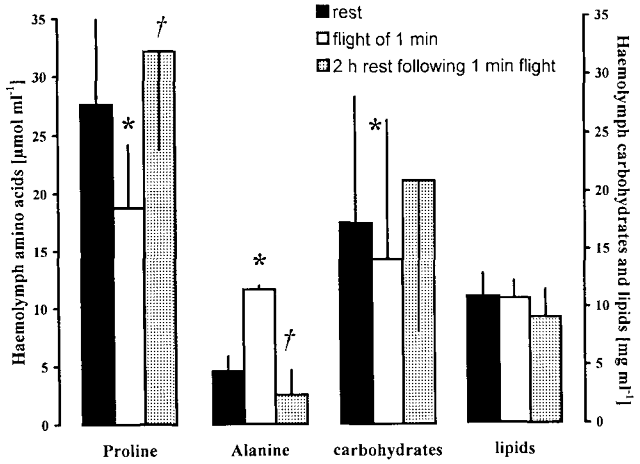

Fig. 1. Changes in metabolite concentrations in the haemolymph of *M. oculata* during flight and subsequent rest. Values are means ± S.D. (n = 5). Significance of changes compared with resting value * – $p < 0.05$; compared with flight value † – $p < 0.02$ using paired t-test.

Purification of neuropeptides

The UV elution profile of a methanolic extract of corpora cardiaca from *M. oculata* (17 gland equivalents; Fig. 3A) on an analytical RP-C-18 column shows two distinct absorbance peaks at 10.4 (peak 1) and 13.4 min (peak 2), which correspond to fluorescent peaks when monitored for the characteristics of tryptophan, a conserved residue at position 8 in the sequence of all known peptides from the AKH/RPCH family (Fig. 3B). Co-injection of the peptides Del-CC and Tem-HrTH previously isolated from the corpora cardiaca of the blister beetle, *D. lunata* (Gäde, 1995), resulted in co-elution (results not shown). When peaks 1 and 2 material from *M. oculata* was analysed by MALDI mass spectrometry, mass peaks at *m/z* 1180 and 1196 were detected for peak 1 and mass peaks at *m/z* 1009 and 1025 for peak 2. These mass peaks are easily interpreted as the sodiated ($[M+Na]^+$) and potassiated ($[M+K]^+$) ionic forms of Del-CC (M = 1157 Da) and Tem-HrTH (M = 986 Da). Identical mass data were found

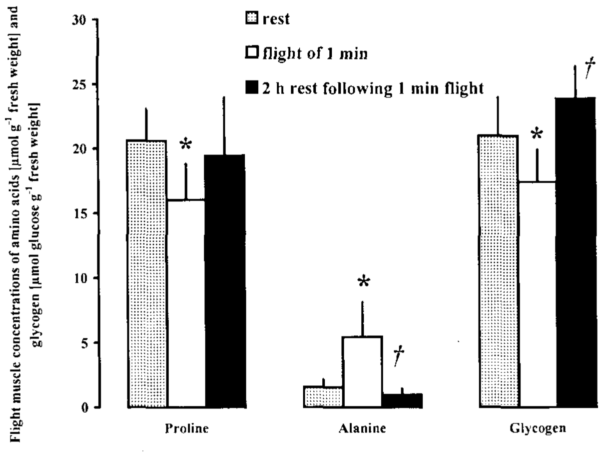

Fig. 2. Changes in metabolite concentrations in the flight muscles of *M. oculata* during flight and subsequent rest. Values are means ± S.D. (n = 5). Significance of changes compared with resting value * – $p < 0.05$; compared with flight value † – $p < 0.01$ using paired t-test.

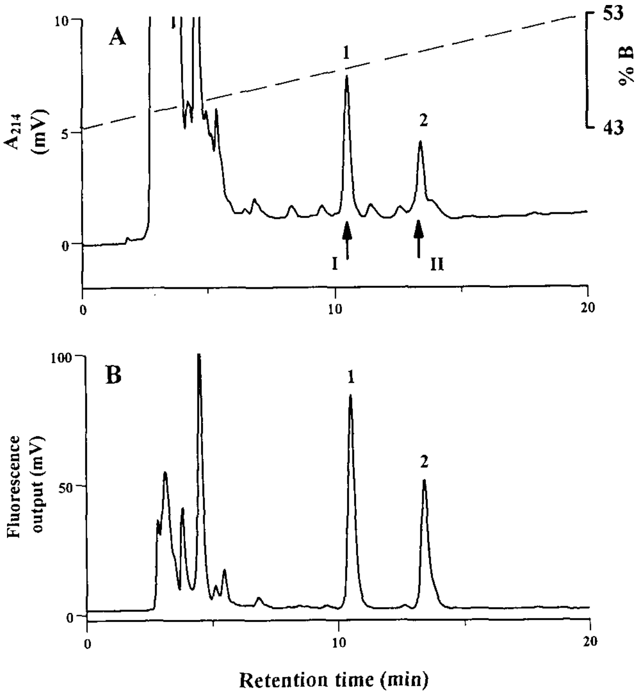

Fig. 3. Separation of a crude methanolic extract of 17 pairs of corpora cardiaca from *M. oculata* on RP-HPLC. Chromatographic conditions: A Nucleosil C-18 column (i.d. 4.6 × 250 mm length) plus guard column of the same material was eluted with a linear gradient of 0.11% trifluoroacetic acid (TFA; solvent A) and 0.1% TFA in 60% acetonitrile (solvent B). The gradient ran from 43% B to 53% B within 20 min at a flow rate of 1 ml·min^{-1}. The elution was monitored with a UV detector at 214 nm (A) and a fluorescent detector at 276 nm (excitation) and 350 nm (emission, B). Retention times of synthetic peptides are indicated by arrows (I = Del-CC; II = Tem-HrTH).

when 5 pmol of synthetic Del-CC and Tem-HrTH were analysed on the same day under identical conditions.

Bioassays with *M. oculata*

The levels of proline in the haemolymph were significantly elevated and those of alanine decreased upon injection of a crude CC-extract of *M. oculata*, while control injections had no effect (Table 1). Small but significant increases of proline were also monitored after injection of 5 and 10 pmol of the endogenous peptides, Tem-HrTH and Del-CC. At the lower dose the decapeptide Del-CC was significantly ($p < 0.01$) more effective than the octapeptide.

Injection of a crude CC-extract caused also a very small but significant elevation of the carbohydrate levels in the haemolymph (Table 1). Equally small but significant changes were found after injection of Del-CC and Tem-HrTH. Low doses (5 pmol) were sufficient to establish such a hypertrehalosaemic effect (Table 1).

The lipid concentration was not influenced by injection of CC-extract or synthetic peptides (Table 1).

Bioassays with *D. lunata*

Injection of a crude CC-extract as well as various doses of Del-CC and Tem-HrTH were able to significantly elevate the proline concentration in the haemolymph of *D. lunata*. Concomitantly, the alanine levels were decreased (Table 2). The effect can be achieved with low doses (2 pmol) of the two synthetic peptides, however, it appears that the effect of the decapeptide was stronger.

Carbohydrate levels in the haemolymph of *D. lunata* were also significantly increased by injection of CC-extract and the two endogenous peptides but the effect was small. Low doses (2 pmol) were sufficient to achieve this elevation (Table 2).

Neither the injection of crude own CC-extract nor of the neuropeptides Del-CC and Tem-HrTH caused a change in the lipid concentration in the haemolymph of *D. lunata* (Table 2).

DISCUSSION

The objectives of the present study were three-fold: To analyse another member of the relatively small (about 3,000 species; Gillot, 1995) family of Meloidae with respect to its main fuel source during flight, to identify the

TABLE 1. Levels of metabolites in the haemolymph of *M. oculata* before and after injection of synthetic endogenous neuropeptides and crude CC-extract.

			Proline (μmol·ml^{-1})			Alanine (μmol·ml^{-1})			Carbohydrates (mg·ml^{-1})			Lipids (mg·ml^{-1})		
			0 min	90 min	Δ	0 min	90 min	Δ	0 min	90 min	Δ	0 min	90 min	Δ
Control: distilled water		mean	24.9	24.8	–0.1	4.0	4.1	0.1	16.4	16.5	0.1	9.7	9.6	–0.1
		S.D.	6.2	6.1	0.7	1.5	1.6	0.4	2.5	2.4	0.3	1.1	1.1	0.0
Tem-HrTH	5 pmol	mean	25.5	27.5	2.0*	4.3	1.8	–2.5*	16.2	17.7	1.5*			
		S.D.	4.1	3.0	1.7	1.9	0.7	1.5††	2.4	2.2	0.7††			
	10 pmol	mean	25.1	31.2	6.1*	3.9	1.4	–2.5*	16.7	19.1	2.4*	9.9	10.0	0.1
		S.D.	5.6	3.4	2.8††	1.8	0.5	1.6††	3.3	3.4	0.9††	1.5	1.5	0.4
Del-CC	5 pmol	mean	26.9	31.6	4.7**	4.8	1.7	–3.1*	15.5	17.0	1.5*			
		S.D.	3.6	3.4	1.4††	2.4	0.5	2.5††	4.3	4.1	1.2†			
	10 pmol	mean	25.1	31.8	6.7*	4.5	1.2	–3.3**	16.2	18.9	2.7*	9.8	9.6	–0.2
		S.D.	5.5	3.5	2.6††	0.4	0.4	0.6††	3.5	2.5	1.2††	1.4	1.3	0.3
1 pair CC equivalent		mean	26.6	37.3	10.7**	4.1	1.1	–3.0*	16.0	19.3	3.3**	10.2	10.1	–0.1
		S.D.	4.4	3.2	2.2††	1.6	0.4	1.5††	2.0	2.2	0.4††	1.8	1.8	0.3

Data are presented as means ± S.D. (n = 6–11). Significance of change: * – $p < 0.05$; ** – $p < 0.001$, using paired t-test. Significantly different compared with change of control group († – $p < 0.02$; †† – $p < 0.001$) using Student's t-test.

TABLE 2. Levels of metabolites in the haemolymph of *D. lunata* before and after injection of synthetic endogenous neuropeptides and crude CC-extract.

			Proline ($\mu mol \cdot ml^{-1}$)			Alanine ($\mu mol \cdot ml^{-1}$)			Carbohydrates ($mg \cdot ml^{-1}$)			Lipids ($mg \cdot ml^{-1}$)		
			0 min	90 min	Δ	0 min	90 min	Δ	0 min	90 min	Δ	0 min	90 min	Δ
Distilled water		mean	29.5	29.0	–0.5	3.1	3.1	0.0	11.6	11.3	–0.3	8.8	8.5	–0.3
		S.D.	4.9	4.6	1.8	0.8	1.0	0.5	1.5	1.6	0.5	2.4	3.4	0.6
Del-CC	2 pmol	mean	29.4	32.0	2.6*	4.1	1.1	–3.0*	11.7	13.1	1.6*			
		S.D.	3.1	2.0	2.0†	0.9	0.4	1.1††	1.6	1.8	0.7†			
	5 pmol	mean	31.0	33.2	2.2*	2.8	1.2	–1.6*	11.8	14.2	2.4*			
		S.D.	8.2	8.2	1.2†	1.3	0.9	1.1†	1.9	2.0	1.1†			
	10 pmol	mean	30.5	43.3	12.8**	3.9	0.9	–3.0*	12.9	16.4	3.5*	8.2	8.1	–0.1
		S.D.	8.7	6.4	3.8††	3.2	1.0	2.5†	2.2	1.8	2.3†	2.6	2.3	0.4
Tem-HrTH	2 pmol	mean	30.6	32.8	2.2	3.8	1.4	–2.4*	11.8	13.0	1.2**			
		S.D.	8.1	6.5	2.2†	0.8	0.6	1.4†	1.6	1.7	0.1††			
	5 pmol	mean	31.1	32.6	1.5	2.9	1.8	–1.1*	12.1	14.6	2.5*			
		S.D.	6.3	8.1	2.1	1.7	1.5	0.5†	1.3	1.8	1.5†			
	10 pmol	mean	35.2	46.7	11.5**	3.8	1.0	–2.8*	13.0	16.1	3.1**	7.9	7.4	–0.5
		S.D.	7.5	5.1	5.5††	2.5	0.6	2.3†	0.6	1.1	1.1††	2.5	2.8	0.7
1 pair CC equivalent		mean	28.4	45.2	16.8*	2.4	0.8	–1.6*	11.5	13.7	2.2*	7.6	7.5	–0.1
		S.D.	6.2	10.9	7.2††	1.2	0.9	1.1†	2.1	1.1	1.2†	2.5	2.7	0.6

Data are presented as means ± S.D. (n = 5–11). Significance of change: * – $p < 0.05$; ** – $p < 0.001$, using paired t-test. Significantly different compared with change of control group († – $p < 0.05$; †† – $p < 0.001$) using Student's t-test.

endogenous AKH-family peptides of that species, and to clarify the possible physiological function of these neuropeptides.

The data provided here answer all the above questions in a satisfactory manner.

Fuel sources in *Mylabris oculata* during flight

Contrary to our previous study on the blister beetle, *D. lunata* (Auerswald & Gäde, 1995), when beetles were fixed with a thin thread for performing putative free flight, the present experiments with *M. oculata* used a tethered flight method which guaranteed lift-generation (Auerswald et al., 1998b). It had been shown that power output of flight muscles during this lift-generating flight was significantly superior to previously used tethered, flight mill experiments (Auerswald et al., 1998a). This was corroborated in the present study: One min of flight was sufficient to show significant changes in the concentration of proline, alanine and total carbohydrates, but not lipids in the haemolymph despite high fluctuations of the actual concentrations in individuals. No changes in the substrate concentrations of the haemolymph were recorded even after 2 min of flight in our previous study (Auerswald & Gäde, 1995) suggesting that the present method is more energy-demanding.

The fact that proline and carbohydrates both contribute to power the contraction of the flight muscles was supported by direct measurements in the flight muscles themselves. Again, a 1 min flight was sufficient to show significant changes in proline, alanine and glycogen concentrations. Hence, in accordance to previous data on the blister beetle, *D. lunata* (Auerswald & Gäde, 1995), the potato beetle, *L. decemlineata* (Weeda et al., 1979; Gäde, 1999), and the fruit beetle, *P. sinuata* (Zebe & Gäde, 1993; Lopata & Gäde, 1994; Auerswald et al., 1998a), *M. oculata* oxidises both, proline and carbohydrates, to fuel the contraction of flight muscles. As in the other cases, proline oxidation is only partial, generating alanine as a "temporary" end product (see Bursell, 1981).

Energy-mobilising peptides in *M. oculata*

A minor objective of this study was the identification of the members of the AKH-family in *M. oculata*. Because elution behaviour on RP-column and, additionally, fluorescence properties and mass analysis gave identical results to those of other species of blister beetles in which a family-specific occurrence had been postulated (Gäde, 1995), we were satisfied by these criteria and conclude that also the corpus cardiacum of *M. oculata*, produces the neuropeptides Del-CC and Tem-HrTH.

Physiological functions of AKH-peptides in *M. oculata* and *D. lunata*

A major, novel objective of this study was to find out whether the identified neuropeptides are used for substrate mobilisation. Hence, we tested low doses of Del-CC and Tem-HrTH in both species of blister beetles, *M. oculata* and *D. lunata*. Indeed, injection of physiological doses of both peptides were sufficient to increase the haemolymph concentrations of proline and carbohydrates significantly in both beetles. Similar results have recently been obtained for the potato beetle (Gäde, 1999) and the fruit beetle (Auerswald & Gäde, 1999).

It can, therefore, be concluded that this study unequivocally demonstrates the usage of carbohydrates and proline as fuels for flight and that both of these substrates can be mobilised by the action of the endogenous neuropeptide members of the AKH-family, Del-CC and Tem-HrTH. This dual action of a neuropeptide, Mem-CC in this case, was also postulated for the fruit beetle, *P. sinuata* (Auerswald & Gäde, 1999). A major future task will be to analyse in detail the mode of action of that substrate mobilisation. Specifically, the following question has to be addressed: Is the same second messenger involved in signal transduction regulating carbohydrate and proline mobilisation or, alternatively, do we encounter two different second messenger systems?

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