

Crustacean red pigment-concentrating hormone Panbo-RPCH affects lipid mobilization and walking activity in a flightless bug *Pyrhocris apterus* (Heteroptera) similarly to its own AKH-peptides

RADOMÍR SOCHA¹, DALIBOR KODRÍK^{1,2} and ROSTISLAV ZEMEK¹

¹Biology Centre ASCR, Institute of Entomology, Branišovská 31, CZ-370 05 České Budějovice, Czech Republic;
e-mail: socha@entu.cas.cz

²Faculty of Biological Sciences, South Bohemian University, Branišovská 31, České Budějovice, Czech Republic

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Abstract. In the present study we tested whether the walking activity of macropterous females of the flightless wing-polymorphic bug *Pyrhocris apterus* (L.) can be stimulated by its native adipokinetic hormone Peram-CAH-II and the crustacean red pigment-concentrating hormone (Panbo-RPCH), and the effectiveness of the latter hormone in a lipid mobilization assay. Two different doses (10 or 40 pmol) of Peram-CAH-II or Panbo-RPCH were injected into 10-day-old macropterous females of *P. apterus* to evaluate their effects on the walking activity of treated females. The results obtained showed a significant stimulation of walking activity only with the lower dose (10 pmol) of either hormone Peram-CAH-II or Panbo-RPCH. On the contrary, the walking activity of the same-aged females of macropterous morph treated with the higher dose (40 pmol) of these hormones was decreased. The energy substrates mobilized in Panbo-RPCH-treated macropterous females were lipids. The question of whether the stimulation of locomotion by Panbo-RPCH is limited only to *P. apterus* or if it might also represent an important function of this hormone in other insects or even in crustaceans is discussed.

INTRODUCTION

An intensive investigation of insect endocrine and nervous systems in the last few decades has revealed structures of hundreds of neuropeptides that control various physiological aspects of insect life (Gäde et al., 1997). Metabolism, and especially that related to generation of energy, is controlled by adipokinetic hormones (AKHs), which represent structurally-related neuropeptides of 8–10 amino acids and which are grouped together with a crustacean chromatophorotropin – the red pigment-concentrating hormone (Panbo-RPCH), isolated and characterized from the eyestalk of the crustacean, *Pandalus borealis* (Fernelund & Josefsson, 1972) – into the AKH/RPCH family. Up to the present time more than 40 different AKHs have been isolated and characterized from representatives of many insect orders (Gäde et al., 1997; Gäde & Goldsworthy, 2003), including Heteroptera (Kodrík et al., 2000, 2002b; Gäde et al., 2003, 2004, 2006). The majority of AKHs are synthesized, stored and released by neurosecretory cells from the corpora cardiaca (CC), a neuroendocrine gland connected with the insect brain. However, the brain of some insects also contains AKH-like material as was shown immunohistochemically and by radioimmunoassay (Schooneveld et al., 1985; Bray et al., 1993; Kodrík et al., 2003), or by using HPLC and amino acid sequence analyses (Moshitzky et al., 1987a,b). The AKHs regulate various aspects of insect intermediary metabolism and operate as typical stress hormones: they stimulate catabolic reactions (mobilize lipids, carbohydrates and/or certain amino acids),

making energy more available while inhibiting synthetic reactions (Van der Horst et al., 2001).

In spite of the fact that AKHs, like true multifunctional and pleiotropic hormones, exert a wide range of actions, the primary function of these neuropeptides is undoubtedly the control of energy metabolism during insect locomotion. Originally, it was supposed that the AKHs, especially Locmi-AKH-I from the migratory locust *Locusta migratoria*, mobilize energy reserves necessary for long-distance insect flight (Goldsworthy, 1983) by elevating haemolymph diacylglycerol levels, as well as affecting flight speed (Goldsworthy et al., 1979). However, discovery of the stimulatory effect of injections of Locmi-AKH-I on the walking activity of flightless macropterous females of the firebug, *Pyrhocris apterus*, indicated that AKHs can also influence other types of insect movement (Socha et al., 1999). Subsequent studies showed that injection of the native hormone of this bug, denoted as Pyrap-AKH (Kodrík et al., 2000), also significantly increased the walking activity in macropterous females of this bug. The stimulatory effect of Pyrap-AKH injection was positively correlated with its effect on lipid mobilization, which is consistent with a metabolic mechanism for AKH effects on locomotion (Maxová et al., 2001). Moreover, diel rhythm in *P. apterus* locomotory activity was also positively correlated with the diel rhythm of AKH content in the central nervous system (CNS) of this bug (Kodrík et al., 2003). The suggestion that AKHs exert a more general stimulatory effect on insect locomotion (Socha et al., 1999) was confirmed by

recent studies showing an increase in walking activity after AKH treatment in the cricket *Gryllus bimaculatus* (Lorenz et al., 2004) and the American cockroach *Periplaneta americana* (Wicher et al., 2006). The stimulatory effect of AKH on walking activity was also demonstrated by means of a genetically-modified fruit fly, *Drosophila melanogaster*, with ablated neurosecretory cells in the CC (Isabel et al., 2004; Lee & Park, 2004).

Two native AKHs (Pyrp-AKH and Peram-CAH-II) have been identified in the bug *P. apterus* (Kodrík et al., 2000, 2002a, b). It is not known whether, like Pyrap-AKH, the latter hormone is able to stimulate walking activity in this heteropteran. It has not been excluded, however, that both AKHs of *P. apterus*, as well as some other neuropeptides from the AKH/RPCH family, primarily or secondarily acquired a role in stimulation of locomotion in insects which are not able to fly or which have secondarily evolved flightlessness and disperse by walking only (Socha & Zemek, 2003). Moreover, it appears that the line between the insects and crustaceans is not so distinct from the point of view of evolution of the neuropeptides of the AKH/RPCH family, since it was recently found that Panbo-RPCH is not limited only to crustaceans, but also occurs in the stinkbug, *Nezara viridula* (Gäde et al., 2003), and some other heteropterans (Kodrík et al., unpubl. data).

The aim of the present study was, therefore, to test the possibility that the second native neuropeptide of *P. apterus*, Peram-CAH-II, and Panbo-RPCH can stimulate the walking activity in this bug. The results are interesting especially from the point of view of the role and function of the neuropeptides of the AKH/RPCH family.

MATERIAL AND METHODS

Experimental animals

The firebug *Pyrrhocoris apterus* (L.) is a palaearctic species from the family Pyrrhocoridae (Heteroptera), with a core distribution in the Mediterranean area and eastern and central Asia. It is characterized by a non-functional wing-polymorphism (Socha, 1993) since both the long-winged (macropterous) and short-winged (brachypterous) specimens of this bug are flightless (Socha & Zemek, 2000a), in spite of the fact that the indirect flight muscles of macropterous adults are well developed (Socha & Šula, 2006). In central Europe, the bug mostly lives at the foot of lime trees (*Tilia cordata* Miller, *T. platyphyllos* Scopoli) whose seeds are the basic component of its food (Socha, 1993). A laboratory stock culture of *P. apterus* originating from a wild population collected at České Budějovice, Czech Republic (48°59'N, 14°28'E) was used in the present study. All stages from egg to adult were reared under a long-day (18L : 6D) photoperiod and a constant temperature of $26 \pm 1^\circ\text{C}$, allowing continuous breeding of the bugs. Larvae and adults were kept in glass jars (0.5 l) in mass culture (approximately 40 specimens per jar) and supplied with linden seeds and water ad libitum, which were replenished twice a week. The water was supplied in small glass tubes plugged with cotton wool. More details on this culture are described elsewhere (Socha & Šula 1996; Socha et al., 1997, 1998). Freshly ecdysed adult females of macropterous morph were transferred in groups of 10–20 specimens to small glass jars (250 ml) and kept under the same photoperiodic and temperature regimes in which they had developed. They were supplied with linden seeds and water and, after

reaching the required age, were used in lipid mobilization assays or for measurement of locomotory activity.

Hormonal treatments

Two neuropeptides, Peram-CAH-II and Panbo-RPCH, were tested in the present study for their effects on the level of haemolymph lipids and walking activity of *P. apterus* females. The cockroach neuropeptide Peram-CAH-II was custom-synthesized by Polypeptide Laboratories s.r.o. (Prague, Czech Republic). The crustacean red pigment-concentrating hormone of *P. borealis*, code-named Panbo-RPCH, was purchased from Bachem (Switzerland).

Peptides used in this study were dissolved in 20% methanol in Ringer saline to give the desired content per 2 µl solution. The test samples were injected using a 10 µl syringe (Hamilton Co., Reno, Nevada, USA) through the metathoracic-abdominal inter-segmental membrane into the thorax of 10-day-old macropterous females of *P. apterus* – the reasons for this choice of age are explained elsewhere (Socha & Kodrík, 1999). Control females were injected with 2 µl of 20% methanol in Ringer saline in the same way. To avoid the possible effects of rhythmic changes, all treatments of macropterous females with Peram-CAH-II and RPCH were performed at 9:00–10:00, i.e. 2–3 h after the light was switched on. This time was chosen according to the results of previous studies that showed that the highest walking activity (Socha & Zemek, 2000b), the content of Pyrap-AKH in CNS (Kodrík et al., 2003, 2005) and the intensity of adipokinetic response (Maxová et al., 2001) in *P. apterus* adults occurred during the photophase, irrespective of wing morph.

Lipid mobilization assay

The lipid content of the haemolymph samples was determined by the assay described previously (Kodrík et al., 2000) that is based on the sulpho-phosphovanilin test (Zöllner & Kirsch, 1962; modified by Holwerda et al., 1977 and Van Marrewijk et al., 1984). The haemolymph samples were taken from the cut end of an antenna: a drop of haemolymph was leaked onto a piece of parafilm M and 0.5 µl taken up by a micropipette (Eppendorf Varipipette 4810); the samples were collected just before and 90 min after the hormonal injection and used for the determination of lipids. The optical densities at 546 nm measured in a spectrophotometer (UV 1601 Shimadzu) were converted to mg lipids per ml haemolymph with the aid of a calibration graph based on known amounts of oleic acid. Results are expressed as a mean of haemolymph lipid elevation for 6 to 12 observations (difference of lipid levels after and before injection) \pm SEM.

Measurement of locomotor activity

A computerized multichannel data acquisition system was used for the measurements of the walking activity in hormone- and solution-treated macropterous females. It consisted of 30 monitoring units and an HP 6942A Multiprogrammer equipped with FET Scanner and A/D converter cards. The Multiprogrammer was connected to an IBM-compatible PC running a program written in HP Basic. For a detailed description of the system see Kodrík et al. (2000).

Females injected with either 10 or 40 pmol of Peram CAH-II or Panbo-RPCH and those treated with saline only (controls) were immediately transferred individually into the monitoring units where they had access to water and food (linden seed). The doses of both adipokinetic peptides used were chosen according to the results of the lipid mobilization assays. The higher dose (40 pmol) of the hormones exhibited the most active adipokinetic response, while the lower one (10 pmol) was used to compare its effect with that of Pyrap-AKH (Kodrík et al., 2000).

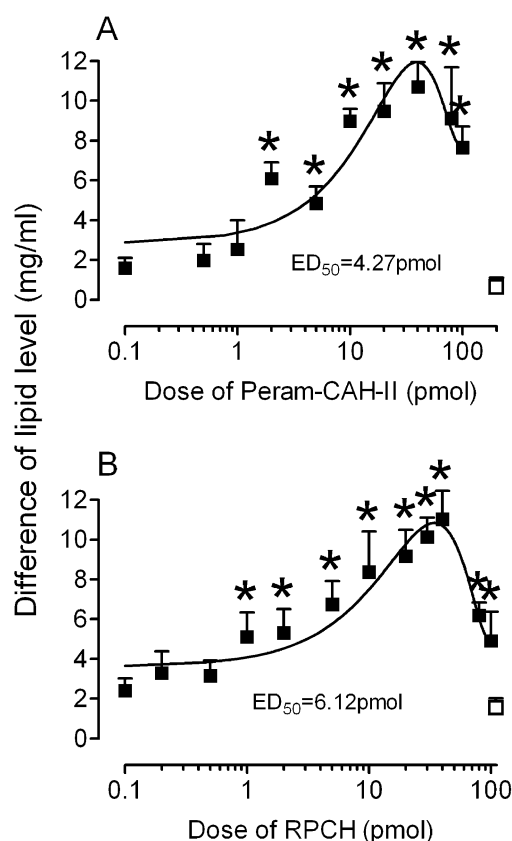


Fig. 1. The effect of increasing doses of injected Peram-CAH-II (A) and Panbo-RPCH (B) on the elevation of haemolymph lipid level in 10-day-old macropterous females of *P. apterus*. Control females were treated with 20% methanol in Ringer saline. Statistically significant differences at the 5% level (experimental vs. control) are indicated by *. Hormone-treated females (■); control females (□); vertical lines indicate SEM; $n = 10-20$.

Moreover, the lower dose of tested hormones tested was close to the physiological range of the AKH levels in *P. apterus* (Kodrik et al., 2000).

The locomotory activity of each of the tested females that were allowed to move freely in glass Petri dishes was monitored individually for 15 h and the results expressed as the number of infrared beam interruptions per hour. To control the possible differences between groups of saline- and hormone-treated females, the females treated with saline only were tested alongside bugs treated with either Peram CAH-II or Panbo-RPCH. The duration of the experiment was restricted by the length of the light phase, as the activity of *P. apterus* in scotophases is generally very low (Socha & Zemek, 2000b). Locomotory activity was measured in at least 31 bugs in both the experimental and control groups.

Data presentation and statistical analyses

Statistics were computed and figures, including the effects of the tested neuropeptides on mobilization of lipids and walking activity of macropterous females were prepared by means of the software GraphPad Prism, version 4.0 (San Diego, CA, USA). Data on locomotory activity of hormone-treated females were compared statistically with the corresponding control group for each dose used, using a repeated measures ANOVA. The computations were done by means of the PROC GLM procedure in SAS/STAT package (SAS Inst., 2000).

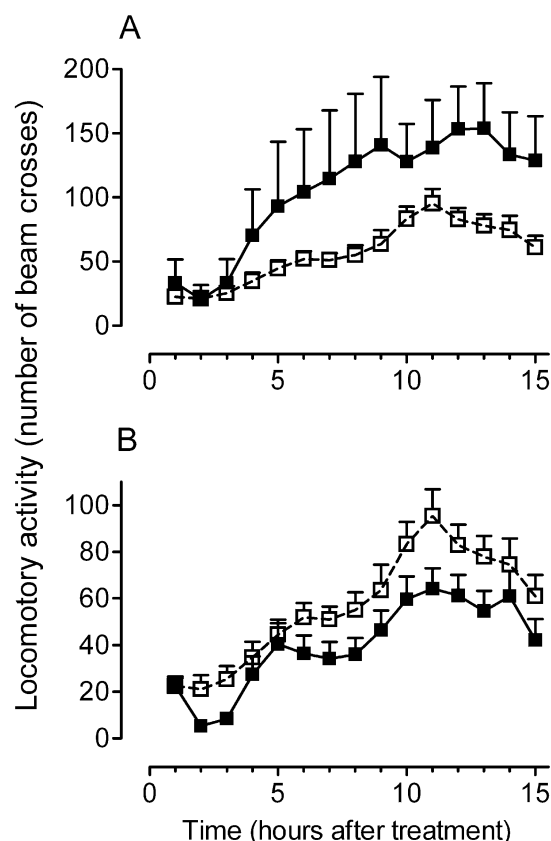


Fig. 2. The locomotory activity (mean number of infrared beam crosses per female per hour) of 10-day-old macropterous females of *P. apterus* injected either with 10 pmol (A) or 40 pmol (B) of Peram-CAH-II. Macropterous females of the same age treated with 20% methanol in Ringer saline were used as controls. For statistics see the text. Peram-CAH-II-treated females (—■—); control females (—□—); vertical lines indicate SEM; $n = 31-105$.

RESULTS

Lipid mobilization

The adipokinetic activities of different doses of the two synthetic neuropeptides tested (Peram-CAH-II and Panbo-RPCH) were determined. Data obtained from the lipid mobilization bioassays showed that injection of either Peram-CAH-II (Fig. 1A) or Panbo-RPCH (Fig. 1B) induced dose-dependent hyperlipemia. The maximal response required at least 40 pmol for both hormones ($ED_{50} = 4.27$ pmol for Peram-CAH-II, $ED_{50} = 6.12$ pmol for Panbo-RPCH). Higher doses of these hormones appeared to be less active, as was the case for females treated with higher doses of Pyrap-AKH (Kodrik et al., 2000). The results showed that lipids are mobilized in *P. apterus* not only by its native hormone Peram-CAH-II, but also by the crustacean red pigment-concentrating hormone Panbo-RPCH.

Locomotory activity

The results of the effects of Peram-CAH-II and Panbo-RPCH on the walking activity of 10-day-old macropterous females of *P. apterus* are presented in Figs 2 and 3, respectively. Comparison of the temporal curves of

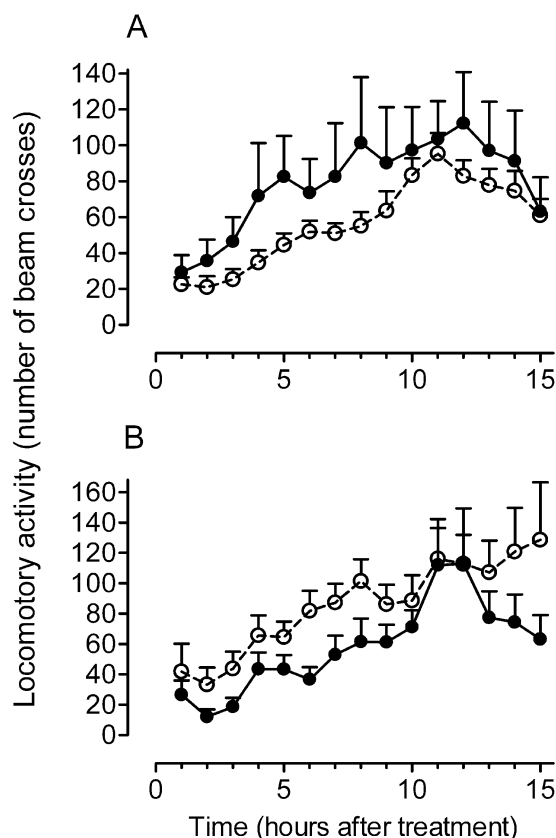


Fig. 3. The locomotory activity (mean number of infrared beam crosses per female per hour) of 10-day-old macropterous females of *P. apterus* injected either with 10 pmol (A) or 40 pmol (B) of Panbo-RPCH. Macropterous females of the same age treated with 20% methanol in Ringer saline were used as controls. For statistics see the text. Panbo-RPCH-treated females –●–; control females –○–; vertical lines indicate SEM; $n = 42\text{--}105$.

walking activities of macropterous females injected with 10 pmol of Peram-CAH-II (Fig. 2A) and those injected with Ringer saline showed significantly (ANOVA, $F_{1,134} = 5.90$, $P = 0.016$) increased walking activity in hormone-treated macropterous females, with a peak of activity after twelve hours. Nevertheless, a significant stimulatory effect of 10 pmol of Peram-CAH-II on walking activity ($P < 0.001$) occurred as early as the 3rd hour after application of this peptide. Surprisingly, injection of a higher dose (40 pmol) of Peram-CAH-II (Fig. 2B) showed a weak inhibition of walking activity compared to control bugs, although the overall difference was not significant (ANOVA, $F_{1,139} = 2.78$, $P = 0.098$).

Comparison of the temporal curves of walking activities of macropterous females injected with 10 pmol of Panbo-RPCH (Fig. 3A) and those injected with Ringer saline showed significantly increased walking activity of hormone-treated bugs during the first eight hours after treatment (ANOVA, $F_{1,145} = 4.76$, $P = 0.031$). When the whole temporal curve of walking activity of macropterous females injected with 40 pmol of Panbo-RPCH (Fig. 3B) was compared with that of controls, non-significant (ANOVA, $F_{1,70} = 3.23$, $P = 0.077$) inhibition in hormone-

treated females was demonstrated; the inhibitory effect of 40 pmol of Panbo-RPCH on the walking activity of these bugs was statistically significant ($P < 0.001$) only for the first eight hours after the hormonal treatment.

The results obtained indicate that not only the native hormone of *P. apterus*, but also Panbo-RPCH, is able to stimulate walking activity in this insect species when applied in appropriate doses.

DISCUSSION

Adipokinetic activity of Peram-CAH-II and Panbo-RPCH in *P. apterus*

The results of the present study showed that both hormones tested – Peram-CAH-II and Panbo-RPCH – are active in increasing lipid levels in haemolymph and stimulation of walking activity in macropterous females of *P. apterus*. It is the first proof of the stimulatory action of Panbo-RPCH on locomotion. Co-ordinated enhancement of both these phenomena is in accordance with the metabolic pathway of the hypothetical model describing the AKH stimulatory effect on locomotion via mobilization of lipids (Socha et al., 1999) and with the fact that diel rhythm of locomotory activity in macropterous females of *P. apterus* is positively correlated with the diel rhythm of AKH content in the CNS (Kodrík et al., 2003). Similarly, as in *P. apterus*, injections of synthetic Panbo-RPCH elicited an increase of haemolymph lipids in the stinkbug *Nezara viridula* (Gäde et al., 2003), which also belongs to the insect order Hemiptera, suborder Heteroptera. The question arises whether the stimulatory effects of Panbo-RPCH on the level of haemolymph lipids and walking activity in macropterous females of *P. apterus* is triggered by this peptide or is mediated through its positive influence on the release of bug's own native AKHs from the brain and CC into the haemolymph. However, our preliminary results obtained with the use of the competitive ELISA (Goldsworthy et al., 2002) suggest that the hyperlipemic effect and stimulation of walking activity in macropterous females by 10 pmol Panbo-RPCH was not mediated through the release of bug's own native AKHs, because the levels of the bug's native AKHs in both CNS and haemolymph were not significantly ($P < 0.05$) increased after application of Panbo-RPCH. It should be noted, however, that while the Peram-CAH-II activates the lipid metabolic pathway in the bug *P. apterus* (present results), in the cockroaches *P. americana* and *Blatta orientalis* this hormone increases trehalose levels in the haemolymph (Witten et al., 1984; Scarborough et al., 1984). These facts support our previous findings that the type of AKH-mobilized energy substrates in insects depends rather on the recipient species than on the structure of the AKH (Socha et al., 2004).

It must be noted, however, that the hyperlipemic effect and stimulation of walking activity in AKH-treated insects did not result from the long-acting effect of AKH, because half-lives of those hormones in haemolymph are relatively short and range from minutes to dozens of minutes. For example, the half-life of of Pyrap-AKH is about 18 min (Goldsworthy et al., 2002). In locusts, rates of deg-

radation of the endogenous AKHs are different for each particular hormone and are influenced by locomotory activity; half-lives of Locmi-AKH-I, -II and -III at rest are 51, 40 and 5 min, and 35, 37 and 3 min, respectively, during flight (Oudejans et al., 1996; Van der Horst et al., 1999). Considering these facts, it is probable that the applied AKHs only trigger the cascade pathways leading to mobilization of lipid from the fat body and to increased locomotory activity. Anyway, in *P. apterus* injected with 10 pmol Peram-CAH-II or Panbo-RPCH the haemolymph lipid levels were significantly higher in hormone-treated females than in saline-treated controls for the first 4 h after application of the hormones (10.88 ± 2.32 mg/ml ($n = 7$) vs. -0.11 ± 2.01 mg/ml ($n = 12$), $P < 0.0001$ for Peram-CAH-II and 14.34 ± 6.89 mg/ml ($n = 6$) vs. -0.11 ± 2.01 mg/ml ($n = 12$), $P < 0.0001$ for Panbo-RPCH), which indicates the relationship between increased lipid level in haemolymph and walking activity, at least during this period. Nevertheless, the subsequently higher walking activity of macropterous females at 8 and 12 h after application of 10 pmol Peram-CAH-II or Panbo-RPCH represents most probably either a delayed or an indirect effect of these hormones mediated through their endocrinological actions, because no significant differences (at $P < 0.05$) in the lipid levels between hormone-treated and control bugs were recorded during those periods of time.

In our hypothetical model (Socha et al., 1999) we presented a dual role for the probable involvement of AKH action (neuromodulatory and metabolic) in the stimulation of walking activity. The model was constructed with the assumption that the metabolic role controls energy metabolism and ensures the supply of fuels necessary for locomotion. A neuromodulatory role could involve increased excitation of the motor nervous system (or removal of inhibitors) or direct neuromuscular effects in the legs, similarly to that of myostimulatory peptides affecting the contractile activity of either visceral and/or skeletal muscles (Gäde et al., 1997). However, considering the results of our present study, it can be hypothesized that the kind of neuromodulatory role in *P. apterus* might depend upon the dose of applied AKHs. While the lower dose of applied AKHs had stimulatory effects on walking activity of macropterous females of *P. apterus*, higher doses of these hormones showed no or even slightly inhibitory effects on the locomotion of this bug, despite their positive effects on the mobilization of lipids. The presumption that AKH might play an important role as a neuromodulator is supported by findings that CC extracts, neurohormone D, and AKH can exhibit, in a manner similar to octopamine, a strong modulatory effect on the CNS of some insects (Milburn & Roeder, 1962; Milde et al., 1995; Wicher et al., 1994). In addition, the presence of AKH in CNS neurones (Schooneveld et al., 1985) or even in axons (Kodrík et al., 2003) was demonstrated by immunohistochemistry, but, as mentioned earlier, a direct explanation of its physiological role is not yet available.

The possible role of Panbo-RPCH in insects and AKHs in crustaceans

Recent studies showed that Panbo-RPCH is not exclusively present in crustaceans, but also occurs, at least, in some heteropteran insects (Gäde et al., 2003; Kodrík et al., unpubl. data). These findings support the theory that insects and crustaceans are phylogenetically very closely related groups. However, while the insect AKHs are truly multifunctional and have pleiotropic tasks, the function corroborated for Panbo-RPCH is more narrow and involves a modulation of the crustacean photoreceptor cells, stimulation of the release of methyl farnesoate from mandibular organs and modulation of the rhythms of certain parts of crustacean stomatogastric and swimmeret systems (Gäde & Marco, 2006). Unfortunately, no data are so far available as to whether the Panbo-RPCH has any true metabolic effect in crustaceans and, conversely, if AKH/RPCH family members play any role in the colour change of insects (Gäde & Marco, 2006). Nevertheless, there may be a unifying activity for Panbo-RPCH that spans both arthropod groups, since Panbo-RPCH has a neuromodulatory effect in a crab and in a spiny lobster and AKHs also have neuromodulatory effects in various insects (Nässel, 2002). Our present study is the first report of the stimulatory action of Panbo-RPCH on insect locomotion. Since the primary structures of Panbo-RPCH (pELNFSFGW-NH₂) and Locmi-AKH-I (pELNFTPNWGT-NH₂) and the structure of Pyrap-AKH (pELNFTPNW-NH₂) are similar, this might be the main reason why they are able to mobilize lipids from the fat body and to stimulate walking activity in macropterous females of *P. apterus*.

Considering the possible effects of insect AKHs applied on crustaceans, one could expect that AKHs will stimulate functions characteristic for crustaceans because, as mentioned above, the AKH function is determined by features of the recipient species rather than of the structure and origin of the AKH tested (Socha et al., 2004). Whether the neuropeptides from AKH/RPCH family, including Panbo-RPCH, can mobilize energy substrates and also stimulate locomotory activity in some crustaceans is a matter of speculation, but it cannot be excluded.

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