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Studium fyziologických funkcí Panbo-RPCH u *Porcellio scaber* (stínka obecná)

Magisterská diplomová práce

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Zralá J., 2010: Studium fyziologických funkcí Panbo-RPCH u *Porcellio scaber* (stínka obecná) [A novel function of red pigment-concentrating hormone in crustaceans: *Porcellio scaber* (Isopoda) as a model species] – 25p., Faculty of Biological Sciences, The University of South Bohemia, České Budějovice, Czech Republic, Mgr. thesis in English.

Annotation: The HPLC and LC/MS analyses of the CNS from isopod crustacean the woodlouse, *Porcellio scaber* revealed a presence of the red pigment-concentrating hormone (Panbo-RPCH) in this species. It has been shown that this neuropeptide plays a role in mobilization of energy stores: topical treatments of *P. scaber* individuals by Panbo-RPCH in a concentration 20 pmol/µl increased the level of glucose in haemolymph about 4 times. Glucose was the main carbohydrate mobilized by the Panbo-RPCH treatment. Despite the demonstration of hyperglycaemic activity of Panbo-RPCH, no stimulatory effect of this hormone on the locomotory activity of *P. scaber* was observed. The present study is the first discovery of an occurrence of Panbo-RPCH and its hyperglycaemic activity in the representative of the isopod crustacean.

Keywords: crustacea, carbohydrates, metabolism, hormone, locomotion, HPLC, LC/MS, GS

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Jana Zralá

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OBSAH

ÚVOD

Endokrinní soustava korýšů

Endokrinní soustava je funkčně a v mnoha případech i anatomicky úzce spjatá s nervovou soustavou a její produkty – hormony - regulují biochemické a fyziologické procesy a ovlivňují tak řadu biologických vlastností organismu. Ze všech členovců je nejlépe prozkoumána endokrinní soustava korýšů a hmyzu. Produkuje převážně neurohormony, tj. látky, které jsou vylučovány neurosekretorickými buňkami nervové soustavy, uvolňovány do hemolymfy a tou roznášeny na místo určení.

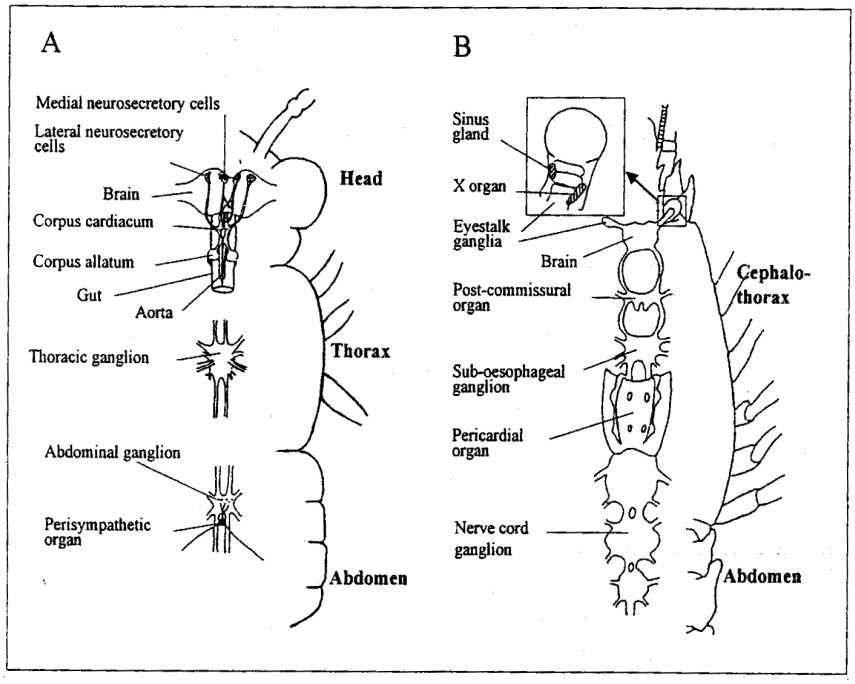
Mnoho neurohormonů vyskytujících se u korýšů bylo popsáno také u hmyzu a naopak. Jsou to například peptidy odvozené od tachykininu (ovlivňují svalovou kontrakci), natriuretické peptidy nebo neurohormony tzv. AKH/RPCH rodiny (**A**dipo**k**inetic **H**ormone**/R**ed **P**igment **C**oncentrating **H**ormone family).

> *U hmyzu rozlišujeme dvě hormonální soustavy fungující v těsné závislosti a několik dalších skupin buněk s endokrinní funkcí. Centrem endokrinní soustavy hmyzu je sekretorický komplex, který zahrnuje neurosekretorické buňky v centrální nervové soustavě mozku produkující řadu neurohormonů, z nichž nejznámější je PTTH (prothoracikotropní hormon) řídící produkci ekdysteroidů. V blízkosti mozku se nacházejí žlázy corpora cardiaca (CC) a corpora allata (CA), na něž jsou napojené některé neurosektetorické buňky mozkových hemisfér. Hlavním endokrinním produktem CC jsou adipokinetické hormony (AKH), CA zase vylučují juvenilní hormony. Druhým významným hmyzím endokrinním centrem jsou prothorakální žlázy, zpravidla párové orgány produkující ekdysteroidy. Endokrinní soustava hmyzu je ještě doplněna několika dalšími žlázami jako jsou například neurosekretorické buňky ostatních ganglií v břišní nervové pásce, endokrinní buňky střeva, epitracheální buňky a gonády (viz **obr. 1A**).*

U korýšů jsou nejznámější a nejlépe probádanou třídou rakovci (Malacostraca) (viz **tab.1**). Téměř veškeré znalosti o endokrinologii korýšů (viz **obr. 1B**) pocházejí

z řádu desetinožců (Decapoda) a jsou někdy zobecňovány pro celý podkmen korýšů.

Sídlem endokrinního centra korýšů je oční stvol a jím vylučovaný hormonální koktejl kontroluje řadu fyziologických funkcí: např. metabolismus glycidů, svlékání, rozmnožování a epiteliální pigmentaci. Neuroendokrinní komplex očního stvolu se skládá především z tzv. X-orgánu (XO), což je shluk obrovských neuronů v medulla terminalis, kde se syntetizují neuropeptidy. Komplex dále obsahuje sinusovou žlázu, neurohemální orgán, který je tvořen konci axonů z neuronů XO, a který slouží ke skladování a k vylučování neuropeptidů do hemolymfy. Jedna skupina hormonů ovlivňujících pigmentaci patří do tzv. PDH/PDF rodiny (**P**igment **D**ispersing **H**ormone/**P**igment **D**ispersing **F**actor family), α-PDH a β-PDH. Ovlivňují pigmentovou dispersi u všech typu chromatophorů (Gäde a Marco, 2006).



Obrázek 1: Endokrinní soustava: A) Insecta a B) Crustacea (Gäde a Marco, 2006)

Mezi další hormony vylučovanými očním stvolem patří hormony tzv. cHH rodiny. Tato rodina obsahuje mnoho hormonů, které se zpravidla třídí do několika skupin. Jejich hlavními funkcemi je řízení metabolismu, svlékání a reprodukce u samic.

Zástupce **C**rustacean **h**yperglycaemic **h**ormone (cHH) byl prvně strukturálně charakterizován u kraba pobřežního *Carcinus maenas* (Kegel a kol., 1989). Skládá se z 72 aminokyselin a je na obou koncích blokován: na N-konci zbytkem kyseliny pyroglutamové a C-konci amidem. Obsahuje 6 cysteinových zbytků, které formují 3 intramolekulární disulfidické můstky (Kegel a kol., 1989). Hraje důležitou roli v mobilizaci glukózy. Dalšími zástupci této rodiny jsou **m**oult-**i**nhibiting **h**ormony (MIH), které prodlužují intervaly mezi svlékáním tím, že inhibují činnost Y-orgánu (viz dále). Svlékání je tedy u korýšů řízeno negativní zpětnou vazbou (Gäde a Marco, 2006). Mezi další zástupce cHH rodiny je počítán také **v**itellogenesis-**i**nhibiting **h**ormon (VIH), který má negativní vliv na reprodukci. Kromě zmíněných hormonů patří do cHH rodiny i méně prozkoumané hormony – např. mandibular organ-inhibiting peptide. U korýšů byly popsány také allatostatiny – velká skupina hormonů (několik desítek zástupců), která je dobře známá i u hmyzu, a která – jak již plyne z jejich názvu – inhibuje produkci juvenilních hormonů nebo jejich prekurzorů, které jsou vylučovány mandibulárním orgánem. Dále u korýšů nacházíme velmi početnou skupinu neuropeptidů ovlivňujících svalovou aktivitu, patří sem např. crustacean cardioactive peptide a mnoho dalších.

Jako další zdroj neuropeptidů u korýšů byl identifikován perikardiální orgán, neurohemální orgán ležící v osrdečníku a vylučující látky, které ovlivňují tep. Další endokrinní strukturou je suboesophageální–postkomisurální orgán, kde jsou hormony neurokrinních buněk suboesophageálního ganglia vedeny do poskomisurální žlázy, která slouží jako neurohemální orgán.

V hlavě korýšů je ještě umístěn párový Y-orgán, který funguje jako epiteliální endokrinní žláza analogická prothorakálním žlázám hmyzu. Vylučuje tedy ekdysteroidy jako například 20-hydroxyekdyson. Ten hraje důležitou roli při svlékání.

Velmi dobře popsaným korýším hormonem je již dříve zmíněný RPCH, významný zástupce AKH/RPCH rodiny. Jeho biochemické a fyziologické vlastnosti a význam pro tuto diplomovou práci jsou podrobně popsány v následující kapitole.

Zajímavým řádem třídy rakovců, která je i u nás hojně zastoupena, jsou stejnonožci (Isopoda). Nejběžnějšími zástupci jsou *Oniscus asellus* (stínka zední) a *Porcellio scaber* (stínka obecná). Jejich mozek obsahuje protocerebrum, zmenšené deutocerebrum a prodloužené tritocerebrum. Prodloužené štíhlé laloky protocerebra zahrnují optické laloky s medula externa, interna a terminalis – typické pro

Malacostraca, a cibulovité sinusové žlázy, které následují po optickém laloku (Nussbaum a Dircksen, 1995). Zástupce této třídy – *P. scaber* – sloužil jako modelový organismus pro studium funkcí RPCH, které byly předmětem této diplomové práce (viz dále).

Tabulka 1: Stručný systém korýšů

CRUSTACEA (KORÝŠI)

Remipedia
Cephalocarida
Branchiopoda=Phyllopoda(lupenonožci)
- Anostraca(žábronožky)
- Notostraca (listonošky)
- Conchostraca (škeblovky)
- Cladocera (perloočky)

Maxillopoda
- Mystacocarida
- Ostracoda (lasturnatky)
- Copepoda (klanonožci)
- Cirripedia =thecostraca (svijonožci)
- Tantulocarida
- Branchiura (kapřivci)
- Pestastomida = Linguatulida (jazyčnatky)

Malacostraca (rakovci)
- Nebaliacea = Leptostraca (nebálie)
- Stomatopoda (ústonožci)
- Bathynellacea (bezkrunýřky)
- Mysidacea (vidlonožci)
- **Isopoda (stejnonožci)**
- Amphipoda (různonožci)
- Decapoda (desetinožci)

Neurohormony AKH/RPCH rodiny

Intenzivní výzkum endokrinního a nervového systému u hmyzu a korýšů v poslední době odhalil strukturu mnoha desítek až několik stovek látek, které kontrolují v podstatě všechny aspekty jejich života (ekdyse, metamorfóza, změna barvy, metabolismus, rozmnožování). Mezi nejznámější a nejvíce prozkoumané hormony u korýšů a hmyzu patří neurohormony AKH/RPCH rodiny. Jméno je odvozeno od poprvé charakterizovaných členů této rodiny: chromatotropinu (RPCH) z korýše *Pandalus*

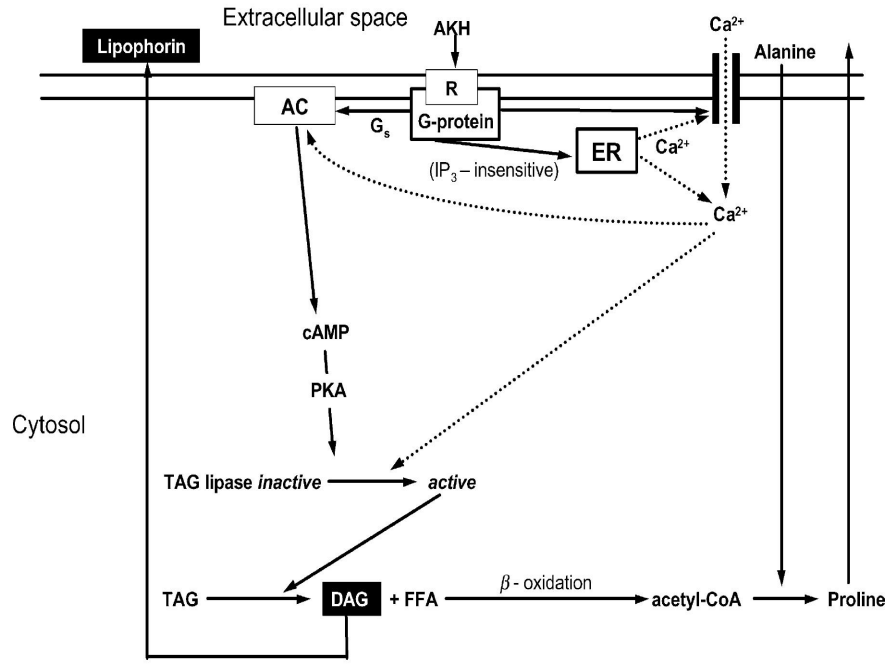
borealis (Fernlund a Jossefson, 1972) a adipokinetického hormonu (Locmi-AHK-I) ze saranče *Locusta migratoria* (Stone a kol., 1976).

Adipokinetické peptidy jsou octa-, nona- nebo decapeptidy na obou koncích molekuly blokované (Gäde a kol., 1997), na N-konci zbytkem kyseliny pyroglutamové a na C-konci amidem. Aminokyseliny v pozici 8 a 9 (když jsou přítomné) jsou Trp a Gly. V molekule jsou nejméně dvě aromatické kyseliny - v pozici 4 hlavně Phe (ale někdy Tyr) a v pozici 8 Trp; několik peptidů má třetinu aromatických kyselin – další v pozici 2 Tyr nebo Phe nebo v pozici 7 Trp (Gäde a kol., 1997; Gäde a Marco, 2006). Neobvyklý AKH byl objeven u motýla *Vannessa cardui* (Köllish a kol., 2000). Tento AKH obsahuje 11 aminokyselin, předpokládá se však, že se jedná pouze o nedokonale upravený prohormon, který je na C-konci prodloužený. Další zajímavostí je fosforylovaný člen AHK/RPCH rodiny, který byl identifikován u brouka *Trichostetha fascicularis* reprezentující jedinečnou translační modifikaci u této rodiny (Gäde a kol., 2006).

Adipokinetické hormony jsou syntetizovány v drsném endoplazmatickém retikulu (ER), transportovány do vesikulů Golgiho aparátu, které se přesunují k povrchu buňky, kde fúzují s membránou a uvolňují svůj obsah exocytózou do hemolymfy. U hmyzu jsou produkovány syntetickými buňkami CC a u korýšů buňkami X-orgánu. Každý hormon AKH/RPCH rodiny má svou specifickou RNA.

Uvolněné hormony putují hemolymfou, kterou se dostávají do svého hlavního cílového orgánu, tukového tělesa, kde mohou aktivovat dva rozdílné, druhově specifické mechanismy působení. AKH jsou hormony peptidické povahy a tudíž nemohou samovolně procházet přes buněčnou membránu. Jejich přeměna na vnitrobuněčný signál je zprostředkována membránovým receptorem (Park a kol., 2002; Staubli a kol., 2002; Hansen a kol., 2006; Kaufmann a Brown, 2006). Receptor, který 7krát prochází skrz buněčnou membránu, je spřažen s G-proteinem. Proces mobilizace lipidů (viz **obr. 2**) spuštěný prostřednictvím AKH byl podrobně popsán na vnitrobuněčné úrovni u saranči. Po navázání AKH na receptor zapne aktivovaný G-protein enzym adenylátcyklázu produkující cAMP (druhý posel vznikající z ATP). Ten v přítomnosti Ca^{2+} iontů spouští proteinkinázovou kaskádu, která vede k aktivaci lipázy štěpící triacylglycerol na diacylglycerol (DAG). Do místa syntézy energie, tedy nejčastěji do pracujícího svalu, se DAG dostává prostřednictvím komplikovaného kyvadlového systému přepravy zajištěné specializovanými jednotkami - lipoforiny. Na

membráně svalové buňky je DAG hydrolyzován na glycerol a mastné kyseliny pomocí lipázy, za uvolnění lipoforinového nosiče. Konečným zdrojem energie jsou pak produkty enzymatického odbourávání mastných kyselin. U některých druhů hmyzu (někteří brouci a dvoukřídlí) AKH zajišťuje mobilizaci prolinu – jako energetického zdroje (viz **obr. 2** dráha vpravo dole).

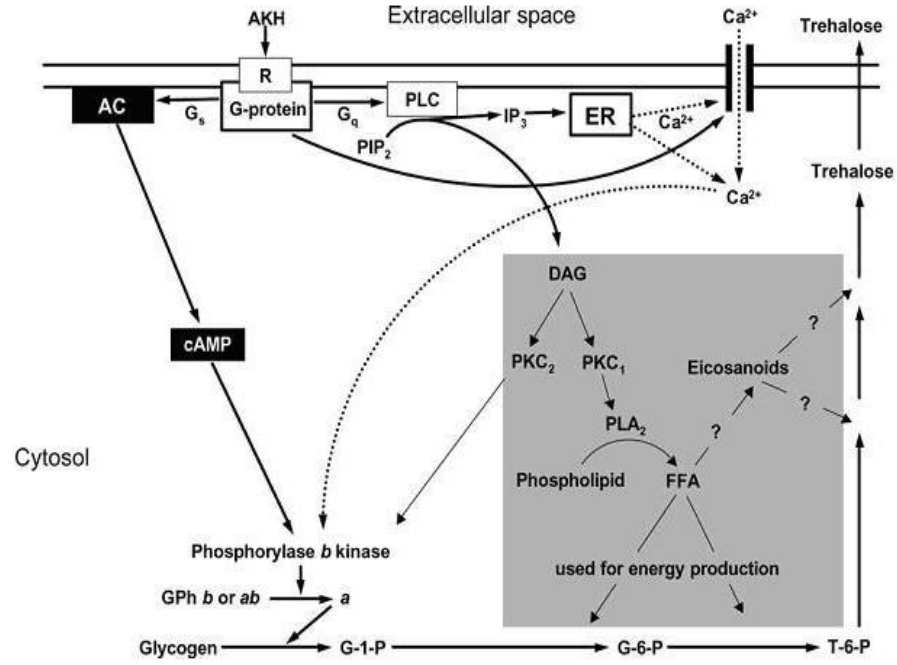


Vysvětlivky k obrázku: ***AC*** *– adenylátcykláza,* ***cAMP*** *– cyklický adenozinmonofosfát,* ***DAG*** *– diacylglycerol,* ***ER*** *– endoplazmatické retikulum,* ***FFA*** *– volné mastné kyseliny,* ***PKA*** *– proteinkináza A,* ***R*** *– receptor,* ***TGA*** *– triacylglycerol*

Obrázek 2: Mechanismus působení AKH vedoucí k mobilizaci lipidů (Gäde a Auerswald, 2003)

U jiných druhů hmyzu (např. švábi a někteří brouci) funguje odlišný mechanismus, který vede k mobilizaci glycidů (viz **obr. 3**). Po aktivaci G-proteinu dojde ke stimulaci fosfolipázy C (PLC), která štěpí inositolový fosfolipid na inositol trifosfát (IP_3) a DAG. IP_3 uvolňuje Ca^{2+} ionty z ER, které spolu s DAG spouští proteinkinázovou kaskádu. Jejím hlavním úkolem je aktivovat glykogen fosforylázu,

která štěpí glykogen na glukózu. Ta se přeměňuje na trehalózu (D-glukopyranosyl-D-glukopyranosid), která se transportuje hemolymfou na místo své potřeby a to většinou do pracujícího svalu (nosiče zde není třeba), kde se přeměňuje opět na glukózu, která je dále štěpena za vzniku potřebné energie. U některých druhů hmyzu (např. *L. migratoria*) je do procesu mobilizace trehalózy zapojena také adenylátcyklázová dráha (viz **obr. 3** černé obdélníky vlevo nahoře).



Vysvětlivky k obrázku:* AC** *-adenylátcykláza,* **cAMP** *– cyklický adenozinmonofosfát,* **ER** *– endoplazmatické retikulim,* **G-1-P** *– glukóza-1-fosfát,* **G-6-P** *– glukóza-6-fosfát,* **GPh** *– glykogenfosforyláza,* **IP_3** *– inositol trifosfát,* **PIP_2** *– fosfatidylinositol difosfát,* **PLC- fosfolipáza C,* **R** *– receptor,* **T-6-P** *– trehalóza-6-fosfát*

Obrázek 3: Mechanismus působení AKH vedoucí k mobilizaci glycidů (Gäde a Auerswald, 2003)

AKH jsou typické stresové hormony – jejich primární funkcí je mobilizace energetických zásob za účelem eliminace nebo alespoň zmírnění dopadu stresu na organismus. Na druhé straně AKH inhibují ty metabolické, biochemické nebo

fyziologické děje, které jsou momentálně nedůležité, a které by mohly odčerpat energii mobilizovanou na řízení stresové situace – mají tedy pleiotropní účinek. Kromě jejich výše popsané funkce v mobilizaci lipidů (Spencer a Candy, 1976; Vroenen a kol., 1997), glycidů (Van Marrewijk a kol., 1980) a aminokyseliny prolinu (Gäde, 1997), AKH dále aktivují antioxidační mechanismy za účelem odstranění oxidačního stresu (Kodrík a kol., 2007), inhibují syntézu lipidů (Gokuldas a kol., 1988), proteinů (Carlisle a Loughton, 1979) a RNA (Kodrík a Goldsworthy, 1995). Na fyziologické úrovni pak stimulují lokomoční aktivitu (Socha a kol., 1999), ovlivňují srdeční rytmus (Scarborough a kol., 1984), zvyšují svalový tonus (O´Shea a kol., 1984), zlepšují imunitní odpověď (Goldsworthy a kol., 2002, 2003) a inhibují zrání vajíček (Lorenz, 2003).

U korýšů byl donedávna znám pouze jediný hormon AKH/RPCH rodiny, oktapeptid Panbo-RPCH: **pGlu – Leu – Asn – Phe – Ser – Pro – Gly – Trp** amid. Prvně byl izolován z očního stvolu korýše *P. borealis* a později prokázán u mnoha dalších druhů (Gäde a Marco, 2006). Řídí zde barevné změny těla nahromaděním pigmentových granulí ve specifických buňkách v schránce korýše a přesuny pigmentů ve složených očích (Gäde a kol., 2003). Účinky tohoto a jiných chromoropních faktorů na změnu barvy těla anebo pohyb očních pigmentů byly námětem četných studií u několika zástupců Decapoda a i Isopoda (Stahl, 1938 a, b; Kleinholz, 1937, 1961; Fingerman 1969, 1970; Castrucci a Mendes, 1975; Rao a kol., 1985). Předpokládalo se také, že Panbo-RPCH má pouze zmíněné chromotropní účinky bez jakéhokoliv vlivu na energetický metabolismus, a že jeho existence je omezena pouze na korýše. To bylo zajímavé z evolučního hlediska, protože u hmyzu je dnes známo téměř 50 různých zástupců AKH/RPCH rodiny.

V posledních několika málo letech se však situace dramaticky změnila:

1) U ploštice *Nezara viridula* (Heteroptera: Pentatomidae) (Gäde a kol., 2003) se podařilo prokázat RPCH jako u prvního hmyzího zástupce a později byl RPCH dokonce popsán u všech zkoumaných zástupců čeledi Pentatomidae (Kodrík a kol., 2010).

2) Dále byl u korýše *Daphnia pulex* identifikován nový zástupce AKH/RPCH rodiny u korýšů: Dappu-RPCH. Tento oktapeptid o složení: **pGlu – Val – Asn - Phe – Ser – Thr – Ser - Trp amid** se liší od Panbo-RPCH ve třech pozicích: Val^2 – Leu^2, Thr^6 - Pro^6, Ser^7 - Gly^7 (Marco a Gäde, 2010). Hormon Dappu-RPCH byl injikován do

krevety *Palaemon pacificus* a ploštice *N. viridula* a bylo zjištěno, že tento zástupce AKH/RPCH rodiny není aktivní při agregaci pigmentu u *P. pacificus* a dokonce nehraje ani roli při mobilizaci lipidů u ploštice *N. viridula* (Marco a Gäde, 2010).

3) Nakonec byl u korýše *P. scaber* z řádu Isopoda izolován a charakterizován hormon strukturálně identický s Panbo-RPCH, u kterého byla prokázaná role v energetickém metabolismu spočívající v mobilizaci glycidů (sacharidů) - Zralá a kol., 2010. Tato publikace je předmětem předkládané magisterské práce.

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A novel function of red pigment-concentrating hormone in crustaceans: *Porcellio scaber* (Isopoda) as a model species

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ABSTRACT

The RP HPLC and LC/MS QTOF analyses of the methanolic CNS extract from isopod crustacean the woodlouse, *Porcellio scaber* revealed a presence of the red pigment-concentrating hormone (Panbo-RPCH) in this species. It has been shown that this neuropeptide plays a role in mobilization of energy stores: topical treatments of *P. scaber* individuals by Panbo-RPCH in a concentration 20 pmol/μl increased the level of glucose in haemolymph about 4 times, while the level of trehalose was only doubled. The results demonstrated that glucose was the main carbohydrate mobilized by the Panbo-RPCH treatment: glucose was responsible for about 97% of total carbohydrate increasing. Despite the demonstration of hyperglycaemic activity of Panbo-RPCH, no stimulatory effect of this hormone on the locomotory activity of *P. scaber* was observed. The present study is the first discovery of an occurrence of Panbo-RPCH and its hyperglycaemic activity in the representative of the isopod crustaceans. The relationship of the function of Panbo-RPCH in *P. scaber* to the role of this neuropeptide and adipokinetic hormones in insects is discussed.

1. Introduction

The first invertebrate neuropeptide that was fully structurally characterized was the red pigment-concentrating hormone (Panbo-RPCH) (Fernlund and Josefsson, 1972) from the eyestalk of a decapod crustacean, *Pandalus borealis*. Since the time the same peptide has been found in a number of representatives of Decapoda (Rao, 2001; Marco et al., 2002). After discovery of many structurally related adipokinetic hormones (AKHs) in insects, the Panbo-RPCH was grouped together with them into the AKH/RPCH family (for review see Gäde et al., 1997). These peptides are functionally diverse in insects, but generally they behave as typical stress hormones – they stimulate catabolic reactions (mobilize lipids, carbohydrates and/or certain amino acids), making energy more available, while inhibiting synthetic reactions. The X-organ-sinus gland complex in the crustacean eyestalk, the source of the crustacean Panbo-RPCH, as well as the neurosecretory cells from the corpora cardiaca, a neuroendocrine gland connected with the insect brain, where the insect AKHs are synthesized, are with some simplification analogous to the well known hypothalamo-hypophyseal system in vertebrates (Scharrer and Scharrer, 1944).

Although the Panbo-RPCH has primarily chromatophorotropic activity in crustaceans (Fernlund and Josefsson, 1972) and the AKHs play a crucial role in insect energy metabolism, there may be a unifying potency for the neuropetides that spans both arthropod groups, crustaceans and insects. It is known that the Panbo-RPCH has a neuromodulatory effect in a crab and in a spiny lobster, and the same effect has been recorded for AKHs in various insects (Nässel, 2002). These findings well support the theory that insects and crustaceans are phylogenetically very closely related groups. The crustacean-hexapod clade is supported by a number of molecular data and morphological evidence (Giribert et al., 2001).

However, it was believed for many years that presence of the Panbo-RPCH is strictly limited to crustaceans, but several years ago it was found that this statement is not truthful. The Panbo-RPCH was found also in the bug *Nezara viridula* (Gäde et al., 2003) and also in some other heteropteran species, representatives of Pentatomidae and Tessaratomidae families (Kodrík et al., unpublished results). While the Panbo-RPCH in crustaceans is involved in change of their body colouration by aggregation of pigment granules in specific cells in the integument and in the regulation of retina sensitivity, the classical function of AKHs in insects is the control of energy metabolism. It is supposed that the Panbo-RPCH has no true metabolic function in crustaceans (Gäde and Marco, 2006), but all data are according to our knowledge limited to decapod crustaceans. It was also supposed for many years that Panbo-RPCH could be only one crustacean representative of the AKH/

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RPCH family. But recently it has been shown that some cladoceran crustaceans (*Daphnia*) possess a peptide differing in several amino acids from the classical Panbo-RPCH (Christie et al., 2008). Situation in other crustacean groups is entirely unclear.

A question arises whether Panbo-RPCH or its possible other crustacean modifications have, contrary to decapods, a function in metabolism of other crustacean groups. It is interesting that Panbo-RPCH exhibits the metabolic function in insects. An injection of the Panbo-RPCH into the firebug, *Pyrrhocoris apterus* body resulted in an increase of haemolymph lipids and stimulation of locomotory activity (Socha et al., 2007), similarly as described for its own AKHs, the Pyrap-AKH and Peram-CAH-II (Kodrík et al., 2000, 2002). It was also shown that the level of native *P. apterus* AKHs was not affected after the Panbo-RPCH injection that indicated direct hyperlipaemic reaction of the Panbo-RPCH (Socha et al., 2007). It is well known that the insect AKHs are truly multifunctional and have pleiotropic tasks, the situation in crustaceans is not so clear.

However, it is known that the mobilization of glucose from glycogen storage pools is a fundamental physiological process in decapod crustaceans. The crustacean hyperglycaemic hormones (CHHs) – neurohormones synthesized and released from the X-organ sinus gland complex, are primarily involved in carbohydrate metabolism and control of haemolymph glucose concentration (for reviews see Chang, 2001; Lorenzon, 2005). The CHHs are neuropeptides of relatively high molecular mass (7–8 kDa), which are synthesized by a group of large perikarya in X-organ of medulla terminalis and also in several parts of nervous system. In crustaceans increasing of CHH titre and subsequent hyperglycaemia are reported to occur following exposure to several environmental and physiological stress conditions (Lorenzon et al., 1997, 2002; Durand et al., 2000; Santos et al., 2001). Moreover, the glucose level in the haemolymph of decapod crustaceans reveals a day/night rhythmicity characterized by a low basal level during the light period and a peak glucose level appear during several hours after the onset of darkness (Hamann, 1974; Reddy et al., 1981). Biogenic amines (e.g. serotonin) and peptidergic neuroregulators are known to modulate the release of the CHHs and hence the level of haemolymph glucose (Sathyanandam et al., 2008).

Considering all the above data a model species – the woodlouse *Porcellio scaber* – was used in the present study with the main aim (1) to isolate and characterize the woodlouse AKH/RPCH family representative to prove whether the Panbo-RPCH occurs also within Isopoda, and to show (2) whether the peptide has a function in mobilization of energy reserves, and (3) whether it is capable to stimulate the locomotory activity in this crustacean species.

2. Materials and methods

2.1. Experimental animals

The stock culture of the woodlouse, *Porcellio scaber*, originating from wild populations collected at Strakonice and České Budějovice (Czech Republic, 49°N), was used in the present study. All stages from egg to adult were kept in small glass jars filled by a tree bark with pieces of carrot and cabbage, and water *ad libitum* at constant temperature of 26 ± 1 °C and under long-day conditions (LD 18:6 h). Adults of unidentified age and sex were used in the experiments.

2.2. Panbo-RPCH identification

2.2.1. Extraction of Panbo-RPCH from P. scaber CNS

The CNS including the sinus glands was dissected from the adults using a dissecting microscope. Subsequently, the organs were extracted in 80% methanol with sonication. The supernatant was then evaporated to dryness and the residue used either for HPLC or for LC/MS analyses.

2.2.2. HPLC analysis

HPLC analysis of the CNS extract was performed on a Waters chromatography system using HPLC Clarity ver. 2.4.01.043 software (DataApex Ltd., Praha), at a flow rate of 1 ml/min and fluorometric monitoring at λ_{ex} = 280 nm and λ_{em} = 348 nm. The sample was fractionated on the LiChrospher WP-300 RP-18 (5 μm) column (Merck) 250 × 4 mm with gradient: 0–2 min 10% B, 2–32 min 10–100% B (A = 0.11% TFA in water, B = 0.1% TFA in 60% acetonitrile). Fractions of retention times 6.5–30 min were then taken for the ELISA competitive tests (see below). Retention time of the synthetic Panbo-RPCH is 18.72 min in this system.

2.2.3. ELISA

The presence of Panbo-RPCH in fractions of HPLC fractionated CNS methanolic extract was determined using a competitive ELISA described earlier (Goldsworthy et al., 2002). Rabbit antibodies against Cys^1-Pyrap-AKH partially recognizing also the Panbo-RPCH (IgG dilutions 1:5000), and a biotinylated probe prepared from Cys^1-Pyrap-AKH using Biotin Long Arm Maleimide (BLAM) were used.

2.2.4. LC/MS

The analysis has been done by the Waters Company as a demonstration of the QTOF Premier and nanoACQUITY System. The *P. scaber* methanolic extract from 10 individuals was processed under the following conditions: NanoAcquity UPLC configuration – trap column: nanoAcquity™ BEH C_{18} 1.7 μm, 180 μm × 20 mm, analytical column nanoAcquity™ BEH C_{18} 1.7 μm, 75 μm × 150 mm, mobile phase A: 0.1% formic acid in water, B: 0.1% formic acid in acetonitrile, nanoAcquity UPLC gradient: 0–15 min 10–60% B, 15–16 min 60–95% B, 16–17 min isocratic conditions 95% B, 17–18 min 95–10% B, 18–30 min isocratic conditions 10% B. The instrument parameters were as follows: acquisition mode MS^E, m/z range 350–1500, V optics, scan time 0.5 s, cone voltage 40 V, Lockmass [Glu^1]-Fibrinopeptide B (M + 2H) 785.8426.

2.3. Hormonal treatment

The Panbo-RPCH was applied topically by a dipping of the experimental animals into a methanolic solution (20% methanol in Ringer saline) of the hormone in concentrations from 5 to 40 pmol/μl. It has been estimated that about 11 μl of the solution adhered to the body surface.

2.4. Lipid and carbohydrate mobilization assays

The effect of Panbo-RPCH on mobilization of carbohydrate from fat body into haemolymph was evaluated by phenol test (Montgomery, 1957; modified by Socha et al., 2004). The effect of increasing doses of Panbo-RPCH (dipping in the solutions 5–40 pmol/μl) was measured in 1 μl haemolymph taken from the cut end of an antenna 2 h after the treatment. The same amount of haemolymph was taken for evaluation of lipid level by the phosphovaniline method (Zöllner and Kirsch, 1962; modified by Kodrík et al., 2000).

2.5. GC (gas chromatography) analysis of carbohydrates

Samples of haemolymph (5 μl) from *P. scaber* were collected. The carbohydrates were extracted from the haemolymph with 80 μl of 75% EtOH, 1 μg of internal standard (10 μl of xylitol in methanol solution) was added. The derivatization, analytical and

quantification schemes were similar as described earlier in Košťál and Šimek (1995, 1996). Briefly, the haemolymph extract was purified from lipid fraction by extraction with 80 µl of hexane, evaporated to dryness under a stream of nitrogen, oximated by dimethylformamide and *O*-methylhydroxylamine treatment (at 80 °C for 30 min), silylated by dimethylformamide and trimethylsilylimidazol (at 80 °C for 30 min) and extracted by 150 µl of isooctane. Aliquots of 0.5 µl isooctane layer were injected using AOC 20 s autosampler and AOC 20i injector into the gas chromatograph Shimadzu GC-2014 equipped with flame ionization detector, all controlled by a GC Solution software from Shimadzu (Japan). The capillary column used was DB1 (26 m × 0.25 mm, 0.25 µm film thickness) from J&W (Rancho Cordova, USA, CA). Hydrogen flow rate was kept constant at 1.18 ml/min. The injector and detector temperature were 270 and 320 °C, respectively. The following oven temperature program was used: initial temperature 110 °C, the rise of 10 °C/min to 300 °C, held for 3 min. Carbohydrates were quantified using the calibration curve prepared at six different concentrations (0.1, 0.5, 1, 5, 10 and 50 µg injected). All standards used were p.a. grade, purchased from Sigma–Aldrich (St. Louis, USA).

2.6. Locomotory assay

The computerized multichannel data acquisition system was used for the measurements of the walking activity in Panbo-RPCH-treated bugs. It consisted of 18 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).

The woodlouses dipped in the Panbo-RPCH solution (20 pmol/µl) or in 20% methanol in the saline only (control) were immediately individually transferred into the monitoring units. The locomotory activity of each of tested animals that were allowed to move freely in glass Petri dishes littered by filter paper was monitored individually for 7 h and the results expressed as number of infrared beam interruptions per hour. To control the possible differences between batches of control and experimental woodlouses, the woodlouses treated with saline only were tested alongside the woodlouses treated with Panbo-RPCH. Duration of the experiment was restricted by 7-h long period because afterwards significant decreasing of locomotory activity has been recorded. The locomotory activity was measured in 20–27 individuals in both the experimental and control groups.

2.7. Data presentation and statistical analysis

The obtained results were plotted using the graphic program Prism (GraphPad Software, version 5.0, San Diego, CA, USA). The bar and linear graphs represent the mean of measurement ± SD (n = 16–20 for Fig. 3 and n = 4–5 for Fig. 5) and/or ± SE (n = 27 for Fig. 6). Significances of the results were evaluated by the Student's t-test on 5% significance level (Figs. 3 and 5).

3. Results

In the first step of the experimental series an attempt to isolate and characterize putative AKH/RPCH family member(s) in the woodlouse was made. The RP HPLC analysis of the methanolic CNS extract from *P. scaber* generated a number of fluorescent peaks (Fig. 1A). As there were certain immunochemical indications of presence of the Panbo-RPCH in the CNS of related isopod species *Oniscus asellus* (Nussbaum and Dircksen, 1995), the fractions from the area where the AKH/RPCH family members are expected to elute were tested in the competitive ELISA test using the antibody recognizing the Panbo-RPCH (Fig. 1B). The results indicated the highest activity in the fraction of RT = 18.0–19.6 min; the quantification that was estimated by use of a synthetic Panbo-RPCH standard curve generated by a reaction of various Panbo-RPCH doses ranging from 1 fmol to 25 pmol with the antibody revealed a presence of about 300 fmol of the hormone per CNS. The most active fraction accorded with the RT of the synthetic Panbo-RPCH run on the used column under the same HPLC conditions (Fig. 1A). This suggested a presence of the Panbo-RPCH in the *P. scaber* CNS. The data were confirmed by LC/MS QTOF analysis of the whole CNS methanolic extract (Fig. 2A). The Panbo-RPCH was identified there as a main peak eluting from the system at RT = 14.72 min, which was verified by a synthetic standard (Fig. 2B). The results also showed that the presence of other members of the AKH/RPCH family is highly improbable (data not shown).

In the next steps a capability of the Panbo-RPCH to mobilize energy stores in the *P. scaber* body was tested. The Panbo-RPCH

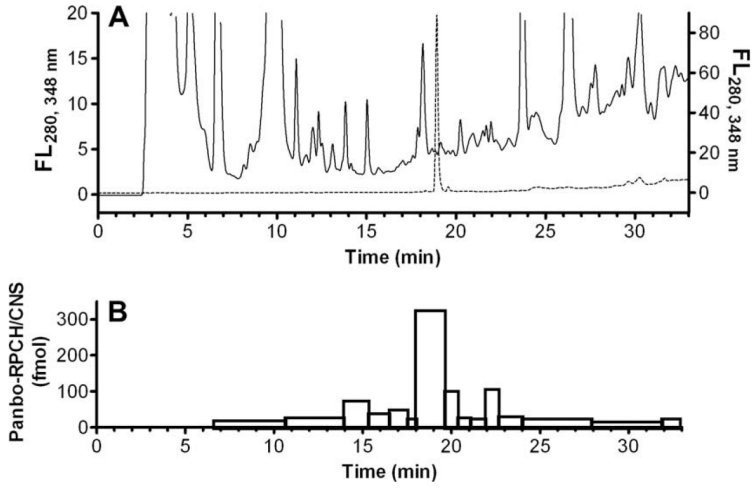

Fig. 1. (A) The HPLC elution profile of an extract of 50 CNS complexes from *P. scaber* (solid line, left *y* axis). The elution profile of synthetic Panbo-RPCH standard (130 pmol) is marked by dashed line (right *y* axis) (RT Panbo-RPCH = 18.72 min). (B) Activity of corresponding fractions of the 50 CNS complexes in the ELISA test (for details see Section 2). The highest activity was recorded in the fraction of RT = 18.0–19.6 min.

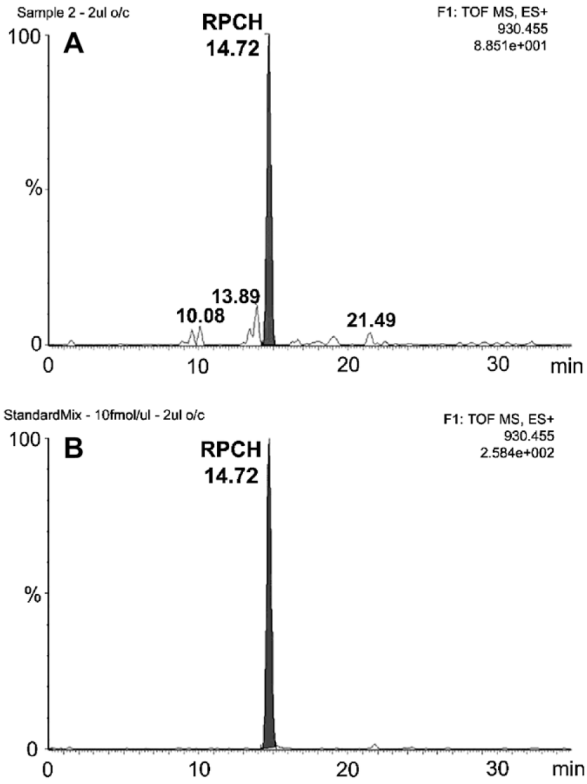

Fig. 2. Structural MS analysis of the methanolic extract of *P. scaber* CNS (A) using QTOF Premier and nanoACQUITY System (for details see Section 2). The results were confirmed by contemporary analysis of synthetic standard (Panbo-RPCH) (B).

was prepared in increasing concentrations ranging from 5 to 40 pmol/μl and the experimental woodlouses were dipped into the solutions for a period of about 1 s. Controls were dipped into the solvent only. The treatment revealed no significant increase of lipids in haemolymph in the classical phosphovaniline test (data not shown) after the hormonal treatment. On the other hand, the treatment increased the level of haemolymph carbohydrates (Fig. 3): the most potent solution – concentration 20 pmol/μl – increased the level about 3 times. Lower and also higher (see also Section 4) concentrations were not so potent.

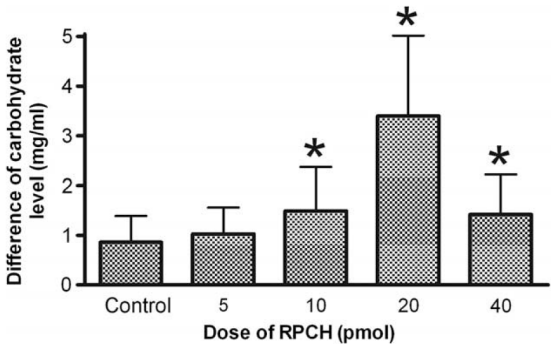

Fig. 3. Dose response of the Panbo-RPCH – dipping of the *P. scaber* into the solution of referred concentrations (evaluation in 2 h). Statistically significant differences at the 5% level (experimental vs. control) are indicated by asterisks. Vertical lines indicate SD.

A question arose which species of carbohydrates are responsible for the increasing. The GC analysis of haemolymph ethanolic extract revealed a presence of four main carbohydrates (Fig. 4) – fructose, glucose, myo-inositol and trehalose, identified using their commercially available standards. It appears that this quaternion is responsible for the majority of the changes; levels of other carbohydrates were either negligible and/or their standards were not at disposal (see Fig. 4 RT = 16.5 min). The quantification data revealed that unambiguously major role is played by glucose (Fig. 5). Glucose was the main *P. scaber* carbohydrate and also changes of its level after the hormonal treatment were by far the biggest. The level of glucose increased about 4 times after the dip-

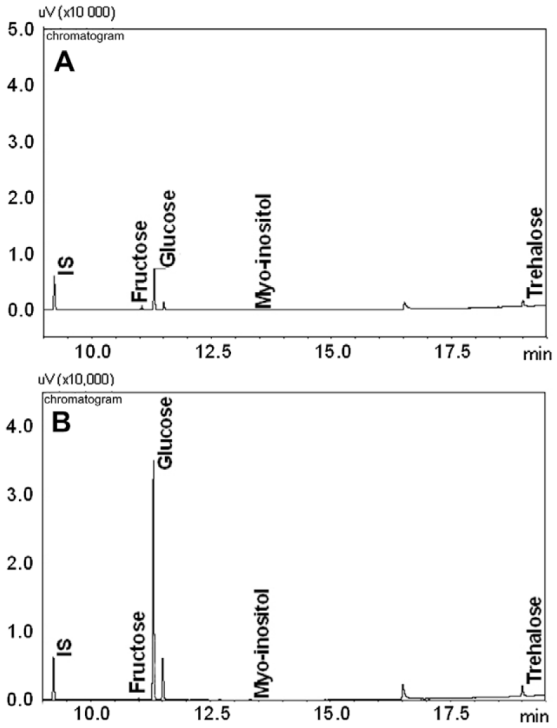

Fig. 4. GC chromatograms of silylated carbohydrates contained in control (A) and Panbo-RPCH treated haemolymph (B).

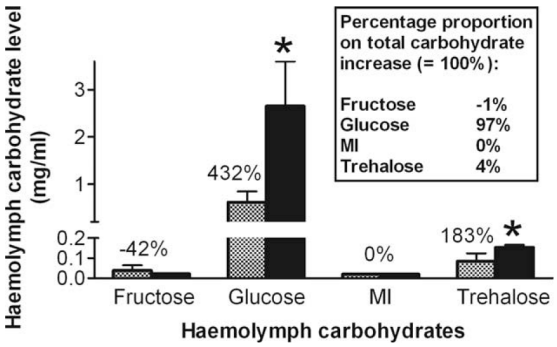

Fig. 5. GC analysis of mobilized carbohydrates after the Panbo-RPCH treatment – dipping in the Panbo-RPCH solution (concentration 20 pmol/μl). The numbers represent percentage increase of the referred carbohydrates. Statistically significant differences at the 5% level (experimental vs. control) are indicated by asterisks. Hatched bars, control *P. scaber*; black bars, Panbo-RPCH treated *P. scaber*; MI, myo-inositol. Vertical lines indicate SD.

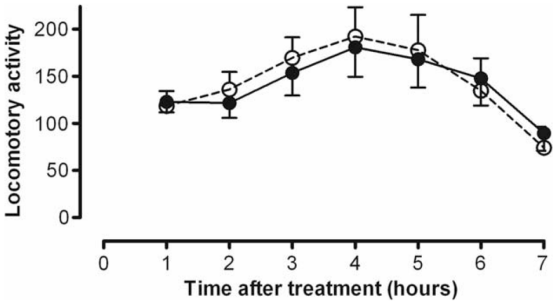

Fig. 6. The locomotory activity (mean number of infrared beam crosses per animal per hour) of experimental *P. scaber* treated with Panbo-RPCH solution (concentration 20 pmol/μl) (solid circles). Controls were treated with 20% methanol in Ringer saline only (open circles). Vertical lines indicate SEM.

ping in the hormonal solution (again concentration of Panbo-RPCH 20 pmol/μl), while the level of trehalose just approximately doubled. Level of myo-inositol showed no changes and level of fructose even dropped to almost one half. The exclusive position of glucose in *P. scaber* carbohydrate metabolism was confirmed also by expression of the results as percentage proportion of individual carbohydrates on their total increasing after the Panbo-RPCH treatment (Fig. 5): the glucose was responsible for about 97% of total increasing, while trehalose for only 4%; the equilibration was reached by 1% fall of the fructose level – but probably ordinary physiological fluctuation can be responsible for the reduction.

It is generally accepted now that the AKH/RPCH family members stimulate in certain insect species locomotory activities (for review see Kodrík, 2008). That was the reason why we decided to verify whether this is true also for the crustacean *P. scaber*. The results revealed (Fig. 6) that there was apparently no positive effect of the Panbo-RPCH treatment on the woodlouse locomotory activity.

4. Discussion

4.1. Identification of Panbo-RPCH in P. scaber CNS

It is well known that the hormone firstly identified from the eyestalks of *P. borealis* is highly conserved among decapod crustaceans (Rao, 2001). The Panbo-RPCH with identical sequence was isolated from *Cancer magister*, *Carcinus maenas*, *Orconectes limosus* (Gaus et al., 1990), *Penaeus japonicus* (Yang et al., 1999), *Callinectes sapidus* (Klein et al., 1995), *Liocarcinus puber*, *Nephrops norvegica*, *Pacifastacus leniusculus*, *Procambarus clarkii*, *Palaemon squilla*, *Palaemonetes pugio*, *Homarus americanus*, *Cardiosoma carnifer* (Gaus et al., 1990; Rodríguez-Sosa et al., 1994) and several other decapod crustaceans (Gäde and Marco, 2006). Our present study with the RP HPLC and LC/MS QTOF analyses proved a presence of the Panbo-RPCH also in the brain of *P. scaber*. Thus, this is the first report of an occurrence of Panbo-RPCH in the representative of the isopod group of crustaceans. The situation in other crustacean groups is practically unknown; nevertheless, recently an occurrence of another representative of the AKH/RPCH family was predicted in cladoceran crustaceans (Brachiopoda) from the genomic database of the water flea *Daphnia pulex* and *D. magna* (www.wfleabase.org; Christie et al., 2008). This octapeptide – the Dappu-RPCH (pQVNFSTSWamide) – is structurally more similar to insect AKHs than to Panbo-RPCH. If Dappu-RPCH controls the pigment transport in the *Daphnia* as is common for Panbo-RPCH in decapod crustaceans, or if it has some other (metabolic?) functions has not been published yet. However, it was found that in isopodas *Ligia occidentalis* and *L. exotica* the RPCH did not induce any pigment migration in their tegument chromatophores (Rao and Riehm, 1988; Tuma et al., 1993) which indicated that this hormone is probably involved in some other functions. This suggestion is well supported by the results of our present study.

It is not so surprising that Panbo-RPCH occurs also in isopod crustaceans: it looks like its appearance within Arthropoda might be more widespread then it was supposed until recently. This neuropeptide was found to occur also in some heteropteran insects, firstly in the bug *Nezara viridula* (Gäde et al., 2003) and later also in some other species of Pentatomomorpha, such as the bugs *Graphosoma lineatum*, *Eurydema oleraceum oleraceum*, *E. ornatum*, *Rhaphigaster nebulosa* and *Coenomorpha* sp., all from the family Pentatomidae, as well as in the *Encosternum delegorguei* from the family Tessaratomidae (Kodrík et al., unpublished results).

4.2. Metabolic function of Panbo-RPCH in P. scaber

As regards the knowledge about the control of energy metabolism in crustaceans, the information is limited to crustacean hyperglycaemic hormones (CHHs), because it was supposed for many years that the Panbo-RPCH has no true metabolic function in crustaceans (Gäde and Marco, 2006). However, our present study showed this statement not to be truthful. We found 4- and 2-fold increase in the glucose and trehalose levels in haemolymph of *P. scaber* individuals, respectively, after their topical treatments with Panbo-RPCH (concentration 20 pmol/μl). The changes in the levels of other carbohydrates were much lower, because the glucose was responsible for about 97% of total increase of haemolymph carbohydrates in Panbo-RPCH-treated *P. scaber*. Thus, the glucose plays the most important role in carbohydrate metabolism and mobilization of energy reserves in this isopod crustacean species.

The maximal carbohydrate mobilization has been recorded for the optimal Panbo-RPCH dose (dipping in concentration 20 pmol/μl). Higher doses appeared to be less effective: this characteristic of the hormonal response is known phenomenon and has been recorded also for the AKHs in insects (Kodrík et al., 2000).

It is generally accepted that crustacean carbohydrate metabolism is controlled predominantly by the CHHs. However, the CHHs are reported to be polyfunctional, since they are involved also in different functions such as stress response, reproduction, moulting, hydromineral regulation and lipid mobilization (for review see Lorenzon, 2005). However, their main function is the regulation of glucose level in haemolymph through the classical mechanisms of glycogen mobilization. But in the blue crab, *Callinectes sapidus*, it is the trehalose that seems to be a major sugar implicated in energy metabolism and physiological adaptation, because it shows higher levels that the glucose in haemolymph and haemocytes of this crustacean species (Chung, 2008). Multifunctionality of the CHHs resembles the AKHs in insects, where the neuropeptides control not only energy metabolism, but also a number of actions attached to the metabolic role (for review see Gäde et al., 1997; Kodrík, 2008).

4.3. Panbo-RPCH and locomotion of P. scaber

In insects, a best part of known AKH functions is related to a movement. Originally, it was supposed that AKH – energy reserves mobilization machinery is activated predominantly during a long-distance insect flight, as was documented for the Locmi-AKH-I from the migratory locust *Locusta migratoria* (Goldsworthy, 1983). However, the studies on the firebug *P. apterus* showed that Locmi-AKH-I and as well its own AKHs (Pyrap-AKH and Peram-CAH-II) influence also other types of insect movements, e.g. the walking activity (Socha et al., 1999; Kodrík et al., 2000, 2002). The stimulatory effects of these AKHs on the walking were posi-

tively correlated with their effects on lipid mobilization (Maxová et al., 2001). Additionally, diel rhythm in the locomotory activity was positively correlated with the diel rhythm of AKH content in CNS (Kodrík et al., 2003). An increase of walking activity after AKH treatment was later observed also in the cricket *Gryllus bimaculatus* (Lorenz et al., 2004) and cockroach *Periplaneta americana* (Wicher et al., 2006), and also in *Drosophila melanogaster* (Lee and Park, 2004; Isabel et al., 2005) where gene manipulation techniques had the same effect.

All these studies demonstrate that AKHs exert more general stimulatory effect on insect locomotion. Similar effect was also recorded even when the Panbo-RPCH was applied to the *P. apterus* (Socha et al., 2007). The treatment resulted not only in stimulation of locomotory activity, but also in increase of the bug haemolymph lipids. These findings together with a failure of the Panbo-RPCH to stimulate *P. scaber* locomotion (this paper) clearly confirm the conclusion that a role of the AKH/RPCH family members is determined by features of the recipient species rather than by the structure and origin of this hormone (Socha et al., 2004). On the other hand, it is possible that a failure to find stimulation of locomotion in *P. scaber* is coupled with specific life strategy of this crustacean involving a rather slow locomotory activity. One can speculate, therefore, that in crustaceans with a considerably higher locomotory activity the situation might be different.

In summary, it has been shown in this paper that presence of the Panbo-RPCH is not limited to Decapoda, but it occurs also at least in one another malacostracan group – in Isopoda. It has been proven for the first time that this neurohormone plays a role in crustacean energy metabolism as is common for the members of the AKH/RPCH representatives in insects. It appears that Panbo-RPCH stimulates predominantly mobilization of glucose as the main energy substrate of isopod crustaceans. No effect of this neurohormone on locomotion of *P. scaber* has been observed.

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ZÁVĚR

1) V methanolovém extraktu centrální nervové soustavy (CNS) *P. scaber*, který byl purifikován pomocí RP HPLC, byla pomocí protilátky proti Pyrap-AKH prokázána přítomnost imunoreaktivního materiálu. Nejvyšší aktivitu vykazovala frakce s retenčním časem odpovídajícím retenčnímu času syntetického RPCH.

2) LC/MS analýza potvrdila přítomnost RPCH v této frakci a tedy i v CNS *P. scaber.* Jedná se o první důkaz výskytu RPCH u korýšů mimo řád Decapoda.

3) Po ošetření hormonem Panbo-RPCH nebyl pozorován u *P. scaber* významný nárůst hladiny lipidů v hemolymfě, byla však prokázána zvýšená hladina glycidů. Nejvyšší hodnota mobilizace glycidů byla zaznamenána při dávce 20 pmol, kdy se zvýšila přibližně třikrát. Jedná se o první důkaz metabolické funkce RPCH u korýšů.

4) V ethanolovém extraktu hemolymfy *P. scaber* odhalila GC analýza přítomnost čtyř hlavních glycidů – fruktózy, glukózy, myo-inositolu a trehalózy. Ukázalo se také, že Panbo-RPCH stimuluje převážně glukózu jako hlavní energetický substrát.

5) Po ošetření hormonem Panbo-RPCH nebyla u *P. scaber* pozorována žádná stimulace lokomoční aktivity.