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Putative involvement of crustacean hyperglycemic hormone isoforms in the neuroendocrine mediation of osmoregulation in the crayfish *Astacus leptodactylus*

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Summary

This study investigates the involvement of eyestalk neuroendocrine factors on osmoregulation in the crayfish *Astacus leptodactylus* maintained in freshwater. Eyestalk removal was followed by a significant decrease in hemolymph osmolality and Na^+ concentration and by a 50% increase in mass after one molting cycle. Several neurohormones have been isolated from the sinus gland through high-performance liquid chromatography (HPLC), and different crustacean hyperglycemic hormone (CHH)-related peptides, including stereoisomers (L-CHH and D-Phe^3 CHH), have been identified by direct enzyme-linked immunosorbent assay (ELISA). A glucose quantification bioassay demonstrated a strong hyperglycemic activity following injection of the immunoreactive chromatographic fractions and showed that the D-Phe^3 CHH was the most efficient. Destalked crayfish were then injected with purified CHH HPLC fractions. The D-Phe^3 CHH fraction significantly increased the hemolymph osmolality and Na^+ content 24 h after injection. Two other CHH-related peptides caused a smaller increase in Na^+ concentration. No significant variation was observed in hemolymph Cl^- concentration following injection of any of the CHH isoforms. These results constitute the first observation of the effects of a CHH isoform, specifically the D-Phe^3 CHH, on osmoregulatory parameters in a freshwater crustacean. The effects of eyestalk ablation and CHH injection on osmoregulation and the identification of different CHH-related peptides and isoforms in crustaceans are discussed.

Key words: Crustacea, crayfish, *Astacus leptodactylus*, eyestalk, crustacean hyperglycemic hormone, osmoregulation, neuropeptide, CHH, isoform, D-Phe^3 CHH.

Introduction

Most crustaceans live in saline water, where they may be exposed to a wide range of salinities. According to the salinity of the medium, they are submitted to osmotic water exchanges and diffusive ion movements between their hemolymph and the external medium. In order to maintain their hydromineral balance, euryhaline crustaceans are able to regulate their body fluid through a decrease in the tegument permeability and an active uptake or excretion of ions (reviewed by Mantel and Farmer, 1983; Lucu, 1990; Péqueux, 1995). Few decapod crustaceans (mainly crayfish, along with Potamoidea crabs and a few Caridea shrimps) are fully adapted to freshwater (FW), where they spend their entire life cycle. These FW decapods, whose ancestors have supposedly originated from seawater, face a constant influx of water and loss of ions.

Studies on crayfish osmoregulation have demonstrated that these crustaceans hyperosmoregulate in FW, and thus maintain a high hemolymph osmolality and ion content, through three main physiological mechanisms: (1) low permeability of the chitinoproteic cuticle to prevent water invasion and ion loss; (2) active uptake of ions (essentially Na^+ and Cl^-) by specialized cells, or ionocytes, located in the epithelia of the branchial chambers and (3) production of hypotonic urine through the excretory antennal glands (reviewed by Potts and Parry, 1964; Mantel and Farmer, 1983; Péqueux, 1995; Wheatly and Gannon, 1995).

Since the early work of Scudamore (1947), numerous experiments have established the existence of neuroendocrine control of hydromineral metabolism in decapod crustaceans, mainly in marine species. The presence of active factors has been suggested in neuroendocrine centers located in the eyestalks, the pericardial organs, the cerebroid ganglia, the thoracic ganglionic mass and the ventral nervous system (reviewed by Kamemoto, 1976, 1991; Kleinholz, 1976; Mantel, 1985; Muramoto, 1988; Morris, 2001). Generally,

eyestalk ablation or ligation performed on crustaceans acclimated to dilute media results in a decrease in hemolymph osmolality, ion content and ionic (Na^+ and Cl^-) influx and an increase in water content (reviewed by Kamemoto, 1976; Charmantier et al., 1984; Mantel, 1985; Muramoto, 1988). Implantation of eyestalk tissue or injection of eyestalk extracts restores or enhances ionic and osmotic regulation in eyestalkless crustaceans (Kamemoto, 1976; Charmantier et al., 1984; Mantel, 1985; Charmantier-Daures et al., 1988; Freire and McNamara, 1992). These results have suggested the involvement of an eyestalk neuroendocrine factor(s) in the control of osmoregulatory processes.

In decapod crustaceans, each eyestalk hosts, within the medulla terminalis, a group of neurosecretory cells, called the X-organ, which synthesize neurohormones that are subsequently stored in and released from a neurohemal organ, the sinus gland (SG). Studies on the neuroendocrine control of osmoregulation point to the involvement of a factor(s) from the SG. For instance, injection of total extracts of SG into destalked juvenile lobsters increases the hemolymph osmolality in dilute media in a dose- and time-dependent manner (Charmantier-Daures et al., 1988). Studies on the hyper–hypo-regulating crab *Pachygrapsus marmoratus* have demonstrated that SG extracts perfused through isolated posterior gills stimulate ionic regulation mechanisms (Pierrot et al., 1994; Eckhardt et al., 1995).

Several 8–9.5 kDa neuropeptides, forming the so-called crustacean hyperglycemic hormone family, have been isolated from the X-organ–SG complex: the molt inhibiting hormone (MIH) involved in molting, the vitellogenesis inhibiting hormone (VIH) involved in reproduction, the mandibular organ inhibiting hormone (MOIH) involved in reproduction and development, and the crustacean hyperglycemic hormone (CHH) involved in the regulation of hemolymph glucose level (reviewed by Keller, 1992; Van Herp, 1998). CHH has been extensively studied and has been purified from the SG of numerous species (reviewed by Soyez, 1997; Lacombe et al., 1999). This neuropeptide appears to be an important multifunctional hormone: primarily involved in carbohydrate metabolism, it also controls other physiological activities including secretion of digestive enzymes (Keller and Sedlmeier, 1988) and lipid metabolism (Santos et al., 1997). In addition, CHH can exhibit MIH, VIH and/or MOIH activities (reviewed by Van Herp, 1998). Interestingly, Charmantier-Daures et al. (1994) have demonstrated that one of the CHH isoforms from the SG of *Homarus americanus* can restore the osmoregulatory capacity in eyestalkless adult lobsters acclimated to low salinities. A recent study has shown that CHH purified from the SG of the crab *P. marmoratus* increases the Na^+ influx and the transepithelial potential difference in perfused posterior gills from crabs acclimated to diluted seawater (Spanings-Pierrot et al., 2000). CHH thus seems to be involved in the control of osmoregulation in marine crustaceans.

However, very little information is available on the neuroendocrine factors involved in osmoregulation in FW crustaceans. The main objective of the present study was to examine the potential involvement of CHH isoforms in the neuroendocrine mediation of osmoregulation in a FW species, the crayfish *Astacus leptodactylus*. First, the effect of eyestalk ablation on the hemolymph osmolality and ion content was reported, suggesting a neuroendocrine control of osmoregulation. Then, CHH was purified, isolated and characterized by high-performance liquid chromatographic (HPLC) fractionation of SG extracts together with immunochemical tests and bioassays of hemolymph glucose concentration. Finally, the effect of the neuropeptide on osmotic regulation was determined following injection of different CHH isoforms into eyestalkless crayfish.

Materials and methods

Animals

Adult crayfish (*Astacus leptodactylus* Escholtz 1823) imported from Russia and Turkey were obtained from a commercial retailer (Petit Verdus, Saint Guilhem-le-Désert, Hérault, France). In the laboratory, the animals were first kept in 3 m^3 FW tanks then transferred to individual boxes provided with recirculated, dechlorinated, filtered (Eheim system) and aerated tapwater. Temperature was kept at 19±1°C, and photoperiod was held constant at 12 h:12 h L:D. Animals were fed with fragments of mussels three times per week. As several hemolymph physiological parameters change over the molting cycle, and as feeding is known to affect glycemia, only specimens in intermolt stage C_4, established by microscopic observation of an abdominal pleopod (Drach and Tchernigovtzeff, 1967), that had starved for 2 days were retained for the experiments.

Isolation and characterization of the crustacean hyperglycemic hormone

Sinus gland extraction and RP-HPLC

Sinus glands (SG) from freshly excised eyestalks were isolated with a minimum of surrounding tissue and were ground in the incubation medium (ice-cold 10% acetic acid) with a Potter (glass–glass) microhomogenizer. The homogenates were incubated in a water bath at 80°C for 5 min and were then pooled and stored at –20°C.

A pool of frozen SG homogenate was centrifuged at 12 000 ***g*** for 20 min. The supernatant was again centrifuged for 30 min in a centrifugal evaporator. The pellet was re-extracted twice with 200 µl of 10% acetic acid and then centrifuged at 12 000 ***g*** for 20 min. The pooled supernatants were injected onto a reverse-phase HPLC (RP-HPLC) column (250 mm length × 4.6 mm i.d.) filled with Nucleosil C-18 (5 µm particle size) and eluted using a gradient of solvent B [0.1% trifluoroacetic acid (TFA) in 100% acetonitrile] in solvent A (0.1% TFA in water) at a flow rate of 0.75 ml min^{-1}. UV absorbance was monitored at 220 nm with an LDC-Milton Roy Spectromonitor 3000 spectrophotometer. Fractions were collected every 30 s or 60 s.

For hyperglycemia and osmoregulation bioassays, pools of

50 SG equivalents (SGequiv) were extracted and subjected to HPLC. Fractions with a retention time of 45–48 min (see Fig. 4A) were pooled in three different zones (Z1, Z2 and Z3), lyophilized and stored at –20°C before use.

Identification of the hyperglycemic hormone: localization of CHH by ELISA of RP-HPLC fractions

Direct enzyme-linked immunosorbent assay (ELISA) tests with three different antibodies were performed on RP-HPLC fractions. 5 µl of each fraction was deposited into the wells of three microtitration plates and dried under vacuum before addition of 100 µl of sodium carbonate buffer (0.1 mol l^{-1}, pH 9.6) to each well. The plates were incubated at 37°C for 2 h then at 4°C for 12 h and washed three times with 150 µl of phosphate-buffered saline (PBS)–tween–azide (0.2 mol l^{-1} PBS, pH 7.2, containing 0.1% Tween 20 and 0.02% Na azide); 100 µl of rabbit specific antisera, diluted 1:500 in PBS–tween–azide, were then added to each well. The antisera used in this study were anti-*Astacus* CHH antiserum and two hapten-specific antisera discriminating between the amino-terminal of CHH stereoisomers (anti-octapeptide antisera). These antisera were raised against two synthetic octapeptides with a sequence identical to the amino-terminal part of the isoforms of the lobster *Homarus americanus* CHH: pGlu-Val-Phe-Asp-Gln-Ala-Cys-Lys for anti-L antiserum and pGlu-Val-D-Phe-Asp-Gln-Ala-Cys-Lys for anti-D antiserum. The production and characterization of the two antisera have been described by Soyez et al. (1998, 2000). The plates were incubated at 37°C for 1.5 h and were then washed three times with 150 µl of PBS–tween–azide. Incubation with 100 µl of a goat anti-rabbit antiserum conjugated with alkaline phosphatase and diluted 1:50 in PBS–tween–azide was performed at 37°C for 1.5 h. The plates were washed three times with PBS–tween. A volume of 100 µl of substrate (two tablets of *p*-nitrophenyl phosphate dissolved in 30 ml of 0.1 mol l^{-1} carbonate buffer, pH 9.6) was added to each well to visualize the alkaline phosphatase activity. The absorbance was determined at 405 nm using a Titertek Multiskan Plus reader.

Injection of purified fractions

Preparation of samples. Lyophilized HPLC fractions (Z1, Z2 and Z3) were resuspended in a Van Harreveld (1936) saline solution (205.33 mmol l^{-1} NaCl; 5.36 mmol l^{-1} KCl; 2.46 mmol l^{-1} $MgCl_2.6H_2O$; 15.3 mmol l^{-1} $CaCl_2.2H_2O$; 5 mmol l^{-1} maleic acid; 5 mmol l^{-1} Tris; pH 7.4) adjusted to the mean hemolymph osmotic pressure of the injected animals (approximately 375 mosmol kg^{-1}). Aliquots of 10 SGequiv in 50 µl saline solution were prepared. Control animals were injected with 50 µl of Van Harreveld saline. Before injection, samples were vortexed, sonicated for 5 min and then briefly microcentrifuged.

Injection of samples. Injections were performed into the blood sinus at the base of one cheliped using a heat-sharpened glass micropipette (Drummond microcaps) connected *via* a polyethylene tubing to a 50 µl calibrated Hamilton syringe.

Measured parameters

The crayfish abdomen was dried with absorbent paper. Hemolymph was sampled from the abdominal blood sinus using a hypodermic needle mounted on a syringe and was then deposited on Parafilm.

Osmolality

The osmolality of 50 µl hemolymph samples was measured on a Roebling micro-osmometer.

Ionic concentration

Hemolymph Na^+ was titrated with an Eppendorf flame photometer following appropriate dilution (3 µl of hemolymph in 2 ml deionised water). Hemolymph Cl^- titration was measured with an amperometric Aminco-Cotlove chloridimeter (10 µl hemolymph sample diluted in 0.5 ml deionised water and 3 ml acetic–nitric reagent).

Hemolymph glucose quantification

Each 50 µl hemolymph sample was mixed with 50 µl of 0.66 mol l^{-1} perchloric acid (PCA), which precipitates proteins, then vortexed and centrifuged at 13 000 ***g*** for 20 min. Hemolymph glucose concentration was quantified using the glucose oxidase method (Peridochrom Glucose/GOD–PAP; Fisher-Osi Biolabo, Fismes, France). A standard curve was established by twofold serial dilutions ranging from 0 mg ml^{-1} to 2 mg ml^{-1} glucose solution in 0.66 mol l^{-1} PCA. Into wells of a microtitration plate, 20 µl of 0.66 mol l^{-1} PCA (blank), 20 µl of each standard dilution and 20 µl of hemolymph samples were deposited in duplicate before the addition of 200 µl of glucose oxidase peroxidase 4-amino-phenazone (GOD–PAP) reagent. The plate was incubated at 37°C for 1 h. The absorbance of each well was measured at 490 nm by an Elx800 Bio-Tek Instruments reader.

Mass

Animals were blotted with absorbent paper to remove peripheric water and branchial chambers water and were then weighed on an electronic balance (±0.1 g).

Statistical analysis

Analysis of variance (ANOVA), Fisher's multiple-range least significant difference (LSD) *post hoc* test and Student's *t*-test were used for multiple and pairwise statistical comparisons of mean values, respectively, after appropriate checks of normal distribution and equality of variance (Scherrer, 1984). Indicated values represent means ± S.E.M.

Results

Effects of eyestalk ablation on osmoregulation

Hemolymph osmolality, Na^+ and Cl^- concentrations and mass were measured over a period of 43 days in 20 eyestalkless crayfish and in 20 intact animals (controls). All eyestalkless crayfish molted after 22–23 days. Among control animals, eight had molted 31–36 days after the beginning of the

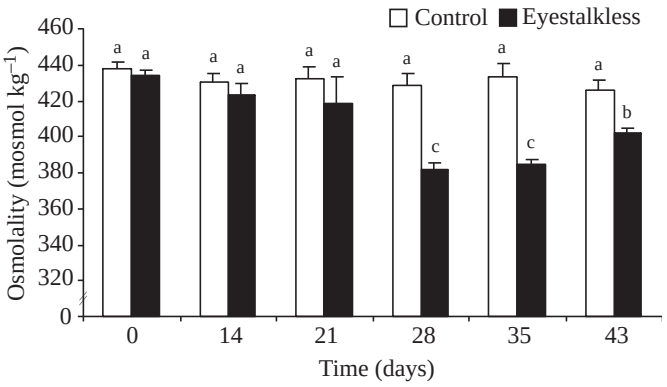

Fig. 1. Time course of variations in hemolymph osmolality in control (*N*=7–8) and eyestalkless (*N*=6–11) *Astacus leptodactylus*. Among the measurements taken from the beginning of the experiment, only values of control crayfish that had molted before 35 days are considered. All eyestalkless crayfish molted within 22–23 days of the start of the experiment and only six crayfish were still alive after 43 days. Each column represents the mean ± S.E.M. Different letters indicate statistical differences: a–b, $P<0.01$; a–c, $P<0.001$; b–c, $P<0.05$.

experiment, and only measurements from these control animals, which had reached the following intermolt stage C at *t*=43 days, were used. The experiment was stopped at 43 days due to the 100% mortality of eyestalkless crayfish during the second post-operative molt in the next days.

Hemolymph osmolality

No significant variation in osmolality was observed among control crayfish during the 43 days of the experiment (Fig. 1). During the first 21 days following the operation, no significant difference in osmolality was noted in eyestalk-ablated crayfish. However, a significant decrease of 52±4 mosmol kg^{-1} ($P<0.001$, $N=20$) in hemolymph osmolality occurred in eyestalkless crayfish after 28 days (Fig. 1), i.e. several days after the molt of all eyestalkless crayfish. The osmolality remained significantly lower until the end of the experiment at day 43, even though a higher osmolality was then noticed compared with that on days 28 and 35.

Statistical comparison of the two groups of crayfish at the same molting stage C was conducted. At the beginning of the experiment, before any molt had occurred (*t*=0 days and *t*=14 days), no significant difference was observed between control and eyestalkless crayfish. On the contrary, after all the crayfish had molted, a significant difference ($P<0.001$) was observed between intermolt controls in stage C at *t*=43 days and eyestalkless crayfish in stage C at *t*=35 days (Fig. 1).

Mass

Fourteen days after the beginning of the experiment, the increase in mass was 2.5±0.5% in controls, which was significantly different from 5.3±1.6% in eyestalkless animals (Fig. 2). In the latter group, a sharp increase of 48.9±2.7% was observed 43 days after eyestalk ablation. This increase was

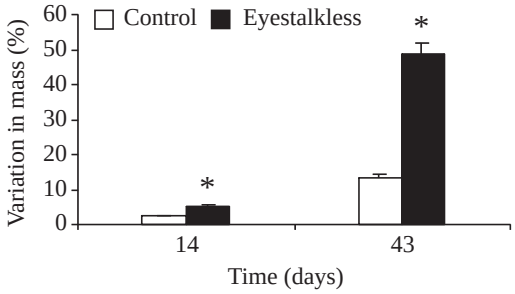

Fig. 2. Variations of mass in control (*N*=8) and eyestalkless (*N*=6–11) *Astacus leptodactylus* before and after molting of all animals at 14 days and 43 days, respectively. Only values of control crayfish that had molted before 35 days are considered. Each column represents the mean ± S.E.M. percentage increase compared with the initial mass (preceding the operation). Statistical comparisons with controls: *$P<0.001$.

significantly higher ($P<0.001$) than the increase in controls (13.4±1.2%) after the molt (Fig. 2).

Na^+ and Cl^- concentrations

Hemolymph Na^+ (Fig. 3A) and Cl^- (Fig. 3B) concentrations were not significantly different between control and eyestalk-ablated crayfish 14 days after the beginning of the experiment. After 43 days, hemolymph Na^+ concentration was significantly

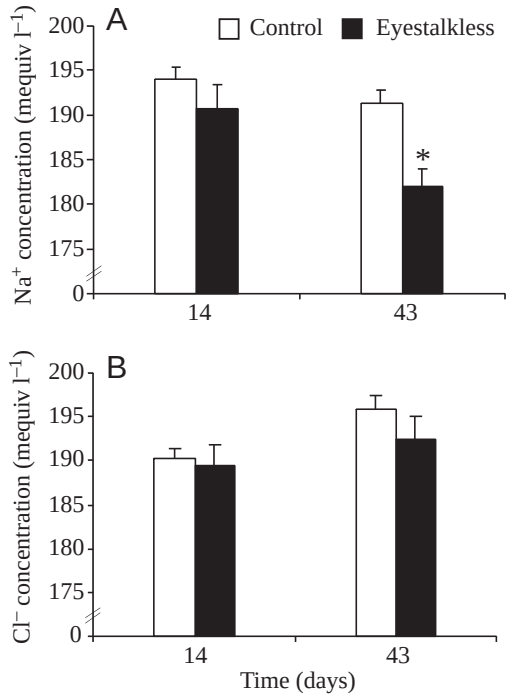

Fig. 3. Hemolymph Na^+ (A) and Cl^- (B) concentrations in control (*N*=8) and eyestalkless (*N*=6–11) *Astacus leptodactylus* before and after molting of all animals at 14 days and 43 days, respectively. Only values of control crayfish that had molted before 35 days are considered. Each column represents the mean ± S.E.M. Statistical comparisons with controls: *$P<0.005$.

lower in eyestalkless animals compared with controls (P<0.005), while no difference was observed in hemolymph Cl^- concentration.

Identification and characterization of CHH

RP-HPLC fractionation of 50 SGequiv of *A. leptodactylus* resulted in several peaks on the chromatogram (Fig. 4A). A major absorbance zone with two high peaks was observed between 45 min and 48 min. The first peak was called P1, and the second peak comprised two peaks, P2 and P3, the latter corresponding to a small peak on the descending edge of the higher P2 peak (see Fig. 4A); P2 is approximately two-thirds and P3 approximately one-third of the total surface of the second high peak. Three minor peaks with lower absorbance were eluted earlier in the chromatogram (retention times, 20 min, 29 min and 37 min), and a fourth peak was observed at a retention time of 53 min. This analysis was repeated several times with similar results.

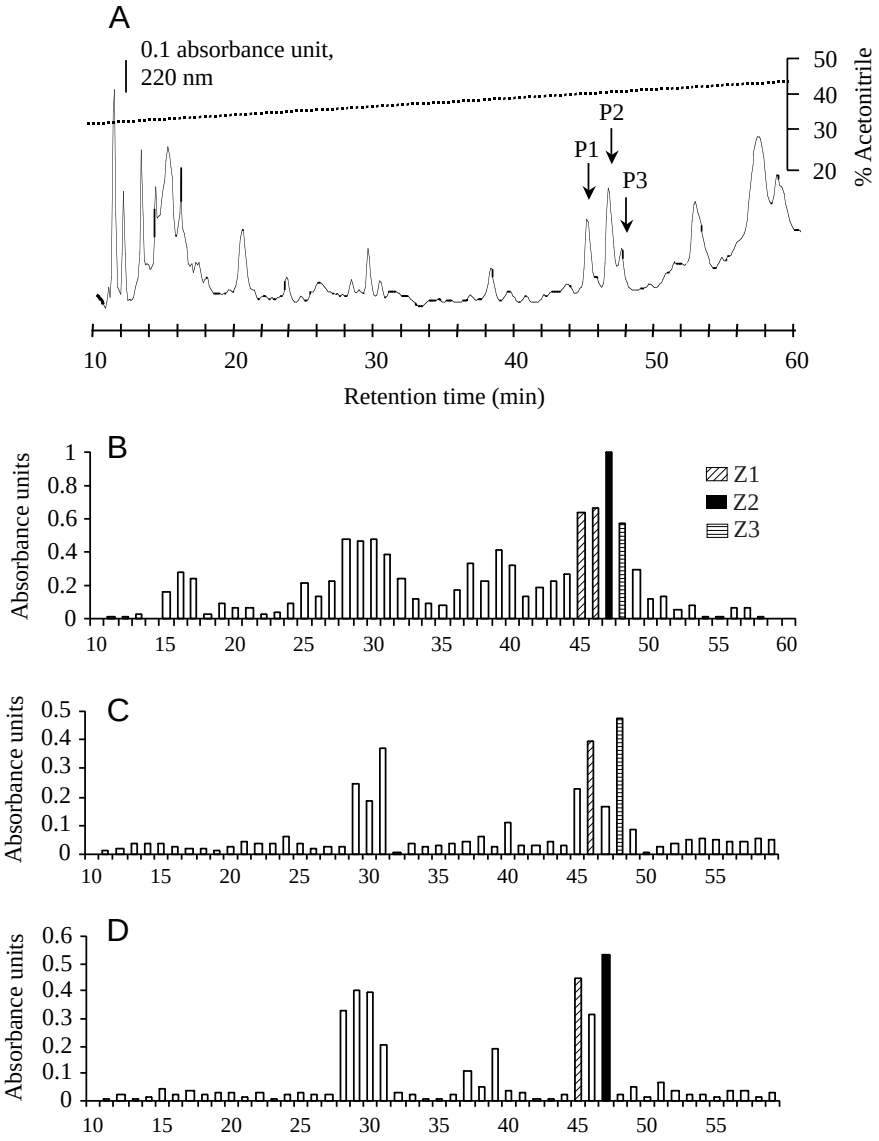

Fig. 4. (A) High-performance liquid chromatography (HPLC) fractionation of an acetic acid extract from 50 SGequiv of *Astacus leptodactylus*. A reverse-phase column (Nucleosil C-18; 250 mm length × 4.6 mm i.d.; 5 µm particle size) was used. Eluant A: 0.1% trifluoroacetic acid (TFA) in water; eluant B: 0.1% TFA in 100% acetonitrile. The elution gradient of eluant B is indicated by the dotted line. Flow rate was 750 µl min^{-1} and fraction duration was 1 min. UV detection was set at 220 nm. Arrows P1, P2 and P3 show the major immunoreactive peaks. Fractions 45–48 were pooled in zones Z1 (fractions 45+46), Z2 (fraction 47) and Z3 (fraction 48) for bioassay. (B–D) Results of the enzyme-linked immunosorbent assay (ELISA) tests performed on 5 µl of each HPLC fraction with different antisera: (B) anti-*Astacus* crustacean hyperglycemic hormone (CHH); (C) anti-D antiserum; (D) anti-L antiserum. The columns represent the absorbance determined at 405 nm after 120 min.

RP-HPLC fraction samples were subjected to three different direct ELISA tests in order to localize CHH and related peptides (Fig. 4B–D). Maximal immunoreactivity with the anti-*Astacus* CHH antiserum coincided with the major absorbance double peak of the chromatogram (retention times 45–48 min; Fig. 4B). The earlier minor immunoreactive peaks are generally considered to be oxidized or degraded forms of CHH. The anti-octapeptide antisera revealed that the most immunoreactive fractions had retention times of 46 min and 48 min for the anti-D antiserum (Fig. 4C) and 45 min and 47 min for the anti-L antiserum (Fig. 4D).

Based on the HPLC fractionation and the results of the three direct ELISA tests, fractions between 45 min and 48 min retention times were pooled in three zones in order to study the effects of CHH isoforms on specific osmoregulatory parameters: Z1 (fractions 45 and 46), Z2 (fraction 47) and Z3 (fraction 48).

Effects of the injection of RP-HPLC fractions on glucose concentration and osmoregulation

This experiment was conducted on 30 eyestalkless crayfish in early stage C, 28 days after eyestalk ablation. Three groups of eight animals were injected with aliquots of 10 SGequiv of zones Z1–Z3. The controls (N=6) were injected with Van Harreveld saline. As loss of material or degradation could occur during the different steps of sample preparation (extraction, HPLC, etc.), hyperglycemic activity was measured after injection of RP-HPLC fractions to assess the presence of bioactive material in the preparations.

Hemolymph glucose level

The hemolymph glucose level was markedly elevated (P<0.001) 3 h after the

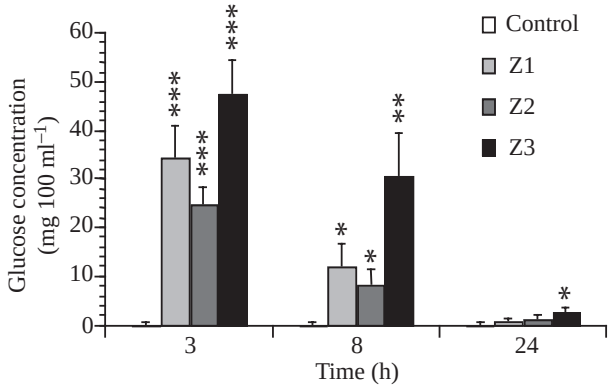

Fig. 5. Changes in glycemia of eyestalkless *Astacus leptodactylus* 3 h, 8 h and 24 h following injection of high-performance liquid chromatography (HPLC) fractions (10 SGequiv) corresponding to zones Z1 (N=8), Z2 (N=8) and Z3 (N=8) (see Fig. 4A). Each column represents the mean ± S.E.M. of the increase in hemolymph glucose concentration compared with the control. Statistical comparison with controls injected with a Van Harreveld saline (N=6): *P<0.05; **P<0.01; ***P<0.001.

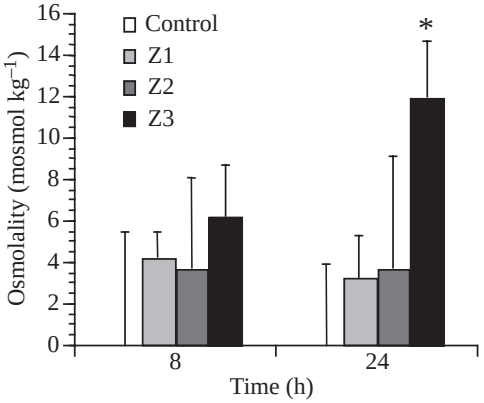

Fig. 6. Changes in osmolality of eyestalkless *Astacus leptodactylus* 8 h and 24 h following injection of high-performance liquid chromatography (HPLC) fractions (10 SGequiv) corresponding to zones Z1 (N=8), Z2 (N=8) and Z3 (N=8) (see Fig. 4A). Each column represents the mean ± S.E.M. of the increase in hemolymph osmolality compared with the control. Statistical comparison with controls injected with a Van Harreveld saline (N=6): *P<0.05.

crayfish received an injection of each of zones Z1, Z2 or Z3 (Fig. 5). At 8 h, the effect, still significant, declined, the increase in glycemia remaining higher in Z3 (P<0.01) compared with the other two zones (P<0.05). At 24 h, only Z3 maintained a very low, but significant, hyperglycemic response. Statistical comparisons indicated a significant difference in hyperglycemia between zones Z2 and Z3, with a higher effect in Z3, 3 h (P<0.01) and 8 h (P<0.05) after injection.

Osmolality

Hemolymph osmolality in eyestalkless crayfish was

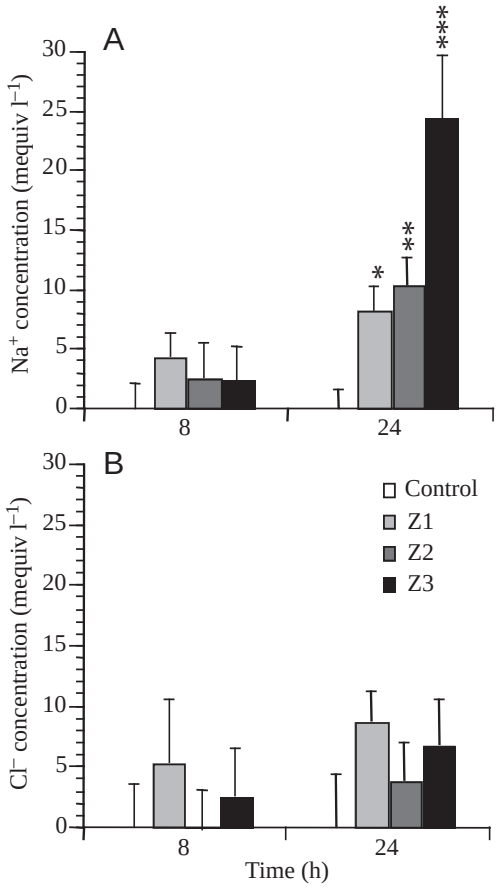

Fig. 7. Changes in hemolymph Na^+ (A) and Cl^- (B) concentrations of eyestalkless *Astacus leptodactylus* 8 h and 24 h following injection of high-performance liquid chromatography (HPLC) fractions (10 SGequiv) corresponding to zones Z1 (N=8), Z2 (N=8) and Z3 (N=8) (see Fig. 4A). Each column represents the mean ± S.E.M. of the increase in ion concentration compared with the control. Statistical comparison with controls injected with a Van Harreveld saline (N=6): *P<0.05; **P<0.01; ***P<0.005.

370±2 mosmol kg^{-1} immediately before injection (t=0). Eight hours after injection, the osmolality was not significantly different among all injected groups. A significant increase in hemolymph osmolality (P<0.05) was observed 24 h after injection of Z3. Injection of Z1 and Z2 did not evoke a significant increase in hemolymph osmolality (Fig. 6).

Na^+ and Cl^- concentrations

Hemolymph ionic concentrations in eyestalkless crayfish were 168±1 mequiv Na^+ l^{-1} and 184±2 mequiv Cl^- l^{-1} immediately before injection (t=0). These ionic concentrations were not significantly different 8 h after injection. After 24 h, hemolymph Na^+ concentration significantly increased in animals injected with the three different zones (Fig. 7A). The effect resulting from the injection of Z3 was significantly higher compared with injection of Z1 (P<0.01) and Z2 (P<0.05). No significant increase was noted in hemolymph Cl^- concentration after the injections (Fig. 7B).

Discussion

Effects of eyestalk ablation on osmoregulatory parameters

Bilateral eyestalk ablation in *A. leptodactylus* did not affect hemolymph osmolality and Na^+ and Cl^- concentrations until the animals had molted. Then, osmolality and Na^+ concentration significantly dropped, mass increased and Cl^- concentration did not change.

These results are in agreement with eyestalk-ablation experiments performed on another Astacidae, *Homarus americanus*, which also show an effect only after molting (Charmantier et al., 1984; Charmantier-Daures et al., 1994). Moreover, *in vivo* experiments conducted on different species of shrimps (Nagabhushanam and Jyoti, 1977; McNamara et al., 1990), crayfishes (Kamemoto et al., 1966; Kamemoto and Ono, 1969; Kamemoto and Tullis, 1972) and crabs (Kamemoto et al., 1966; Kamemoto and Ono, 1969; Kato and Kamemoto, 1969; Kamemoto and Tullis, 1972; Heit and Fingerman, 1975; Davis and Hagadorn, 1982) have shown that eyestalk ligation or ablation increased water influx and decreased hemolymph osmolality and/or ions (Na^+, Cl^-) concentration in animals acclimated to low salinities. In eyestalkless crustaceans, the decrease in osmolality and hemolymph ion concentrations could be due to: (1) a higher influx of water resulting from an alteration in the permeability of the teguments, (2) a greater loss of ions by an increase of permeability and/or a modification of the active ion uptake mechanisms and/or an increase in urine production, (3) a modification of the drinking rate and/or (4) a modification of the function of aquaporins (if they exist in crustaceans) caused by the absence of an eyestalk factor. In our study, the apparent increase in volume and the cuticle softness of the eyestalk-ablated crayfish following the molt suggested that the increase in mass is due to an important influx of water. In decapod crustaceans, eyestalk ablation is known to affect water metabolism, which leads to an increase in water content after a molt (Muramoto, 1988). The effects of eyestalk ablation are generally compensated for by the injection of eyestalk or SG extracts (reviewed by Kamemoto, 1976, 1991; Mantel and Farmer, 1983; Mantel, 1985; Muramoto, 1988). The results of these previous studies suggested the presence of a neuroendocrine factor(s) in the eyestalk that can influence osmoregulatory processes.

In *A. leptodactylus*, our results suggest that osmoregulatory mechanisms might be permanently activated to maintain a high hemolymph osmolality during the intermolt stage. At ecdysis, to compensate for water influx and/or loss of ions, factors are probably released from the eyestalks to stimulate osmotic processes. In this species, a significant increase in the nucleus volume of the X-organ neuroendocrine cells was measured in postmolt stage A, suggesting an intensive synthesis of mRNA immediately after ecdysis (Kallen, 1985). Moreover, Chung et al. (1999) have observed that a hormonal factor regulating water and ion movements is precisely time-released during exuviation in a crab species. Furthermore, the activities of both Na^+/K^+-ATPase and V-ATPase double in early postmolt in the crayfish *Cherax destructor* (Zare and Greenaway, 1998). It has been demonstrated that the activity of these transporters is stimulated by eyestalk or SG extracts (Eckhardt et al., 1995; Onken et al., 2000). These observations corroborate our hypothesis that the operated *A. leptodactylus* devoid of eyestalk neuroendocrine factor(s) might not be able to prevent the drop of osmolality following molting.

Involvement of CHH in the control of osmoregulation

Identification and characterization of CHH

SG peptides of *A. leptodactylus* have been separated by RP-HPLC. Based on the ELISA performed with an anti-*Astacus* CHH antiserum and on the bioassay for hyperglycemic activity, two main UV-absorbing peaks corresponding to the CHH of *A. leptodactylus* were eluted at retention times ranging from 45 min to 48 min. Using anti-octapeptide antisera, different molecular forms of CHH have been identified, including stereoisomers L-CHH and D-Phe^3 CHH (P2 and P3, respectively). P1 may correspond to another minor CHH form that differs in amino acid residue sequence, as was demonstrated in the lobster *H. americanus* (Tensen et al., 1989; Soyez et al., 1990). CHH polymorphism resulting from isomerization of one amino acid residue in position 3 of the amino-terminal fragment from the L- to the D-configuration has been reported in other crayfish species, such as *Orconectes limosus* (Soyez et al., 1998), *Procambarus bouvieri* (Aguilar et al., 1995) and *Procambarus clarkii* (Yasuda et al., 1994), and in *H. americanus* (Soyez et al., 1994). A D-amino acid in the amino-terminal part of a peptide may increase its resistance to proteolytic enzymes (Kreil, 1997). Injection of D-Phe^3 CHH into *A. leptodactylus* (this study) and *O. limosus* (Keller et al., 1999) thus significantly extends the hyperglycemic response compared with the injection of L-CHH. Furthermore, changes in the secondary structure of the peptide may modify the affinity with its receptor, which might account for the difference observed in the biological activity following injection of the different isoforms: the D-Phe^3 CHH isoform is particularly effective on blood glucose regulation and osmoregulatory parameters in *A. leptodactylus* (this study), as well as in *O. limosus*, where the D-Phe^3 CHH has a hyperglycemic activity ten times higher than the L-CHH isoform 1 h after injection (Keller et al., 1999).

CHH and ionic movements

In the present study, hemolymph osmolality and Na^+ concentration were significantly higher in *A. leptodactylus* injected with the HPLC-purified D-Phe^3 isoform of CHH (Z3) compared with control animals after 24 h. The two other CHH forms (Z1 and Z2) did not induce a significant effect on osmolality but increased the hemolymph Na^+ concentration. However, these increases are lower than the one observed with Z3. These results indicate that the eyestalk hyperglycemic hormone stimulates osmoregulatory parameters in an FW crustacean.

Our observations are in agreement with a few results reporting effects of CHH-like peptides on osmoregulation in marine species. Charmantier-Daures et al. (1994) have

demonstrated that one CHH isoform stimulated hyperosmoregulation in *H. americanus* acclimated to low salinities. Chiral analysis performed on HPLC fractions of *H. americanus* SGs has identified this peptide as a D-Phe^3 CHH isoform (Soyez et al., 1994). Chung et al. (1999) have also observed a direct implication of a gut CHH-like peptide in osmoregulating mechanisms of the crab *Carcinus maenas*. In addition, Spanings-Pierrot et al. (2000) have demonstrated that purified CHH from the SG of the crab *P. marmoratus* increases Na^+ influx in perfused posterior gills. Interestingly, another neurohormone involved in osmoregulatory processes, the ion transport peptide (ITP), has been isolated from the corpora cardiaca of different orthopteran insects (Audsley et al., 1992a,b; Macins et al., 1999); showing high structural homology with the hormones of the CHH family (Soyez, 1997), ITP, which has thus been integrated into the CHH family as the first non-crustacean member, stimulates ileal reabsorption of Na^+, Cl^-, K^+ and fluid (reviewed by Phillips et al., 1998).

In our study, injection of Z3 containing 10 SGequiv induced a significant increase in hemolymph osmolality and Na^+ concentration. However, the corresponding amount of CHH is unknown, as part of the bioactive material can be lost or degraded during purification, lyophilization and preparative steps of injected samples (Spanings-Pierrot et al., 2000). As discussed by Lin et al. (1998), data from the literature show that, although 0.1–2 endocrine organ equivalents are enough to induce a significant hyperglycemic response in several species of crustaceans, most of the *in vivo* or *in vitro* experiments reporting a positive effect of endocrine organ extracts on osmoregulation have been conducted with 2–10 organ equivalents (Kamemoto and Ono, 1969; Heit and Fingerman, 1975; Kamemoto and Oyama, 1985; Charmantier-Daures et al., 1988; Kamemoto, 1991; Pierrot et al., 1994; Spanings-Pierrot et al., 2000).

In *A. leptodactylus*, our results indicate that fraction Z3 increased both hemolymph osmolality and Na^+ concentration, while Z1 and Z2 increased only hemolymph Na^+ concentration, with a lower response than Z3. This implies more than a single target system and a complex message/receptor for the different isoforms. The activation of D-Phe^3 CHH receptors, which may be more frequent in osmoregulating organs such as branchial chambers, antennal glands and gut, could modify the hydromineral balance. Furthermore, as we know that a 3:1 mixture of L-CHH:D-CHH isoforms is produced by the crayfish X-organ–SG complex (Soyez et al., 1998; this study), we can also postulate that the affinity with the receptors located on osmoregulating target organs is higher for the D-Phe^3 CHH than for the L-CHH. The mode of action of CHH on osmoregulation could also be indirect. As suggested by Spanings-Pierrot et al. (2000), CHH might increase the availability of metabolizable energy to the ion-exchange pumps through increased glycogenolysis. As a matter of fact, osmoregulation is an important energy-demanding process, especially in FW crustaceans constantly submitted to osmotic water influx and diffusive ion loss.

Other storage organs for CHH or other (neuro)endocrine factors located outside the eyestalk might also be involved in osmoregulation in *A. leptodactylus*. Several authors have reported the effects of thoracic ganglion or pericardial organ extracts and catecholamines on water and ion movements (Kamemoto and Oyama, 1985; Sommer and Mantel, 1988; Kamemoto, 1991; Mo et al., 1998). As CHH-related molecules have been recently described in these organs (Dircksen and Heyn, 1998; Chang et al., 1999; Dircksen et al., 2001; H. Dircksen and D. Soyez, unpublished data), or very transiently in other endocrine cells (Webster et al., 2000), these CHH-like peptides could also contribute to the control of hydromineral regulation. Interestingly, in the crab *C. maenas*, the pericardial organ CHH-like peptide appears to regulate neither hemolymph glucose nor ecdysteroid synthesis (Dircksen et al., 2001) but seems to be involved in another physiological regulation such as osmoregulation (Townsend et al., 2001). Moreover, during exuviation of this species, an important surge of a CHH-like peptide, synthesized in specialized gut endocrine cells, regulates water and ion uptake but induces only a moderate increase of the blood glucose level (Chung et al., 1999).

The present study shows for the first time in an FW crustacean the effects of CHH isoforms on osmoregulatory parameters, particularly hemolymph osmolality and Na^+ concentration. Nevertheless, future studies appear necessary to identify the mode of action of CHH and the cellular mechanisms in target organs involved in the control of salt and water movements in FW crayfish. However, a differential effect has been demonstrated between the different CHH isoforms, with a higher activity of the modified enantiomer (D-Phe^3 CHH). This suggests that the post-translational isomerization of one amino acid leads to a higher functional diversity of molecules, generating peptides with different specialized functions from one gene (Soyez et al., 2000).

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