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Search for hepatopancreatic ecdysteroid-responsive genes during the crayfish molt cycle: from a single gene to multigenicity

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Summary

The expression of the vitellogenin gene of the red-claw crayfish *Cherax quadricarinatus* (*CqVg*) was previously demonstrated in male crayfish during an endocrinologically induced molt cycle. The hypothesis that this expression is under the direct control of ecdysteroids was tested in this study both *in vivo* and *in vitro*. Unlike vitellogenin of insects, *CqVg* was not found to be ecdysteroid-responsive. Thus, a multigenic approach was employed for the identification of other hepatopancreatic ecdysteroid-responsive genes by a cDNA microarray. For the purposes of this study, a multi-parametric molt-staging technique, based on X-ray detection of gastrolith growth, was developed. To identify ecdysteroid-responsive genes during premolt, the molt cycle was induced by two manipulations, 20-hydroxyecdysone administration and X-organ–sinus gland complex removal; both resulted in significant elevation of ecdysteroids. Two clusters of affected genes (129 and 122 genes, respectively) were revealed by the microarray. It is suggested that only genes belonging to similarly responsive (up- or downregulated) gene clusters in both manipulations (102 genes) could be considered putative ecdysteroid-responsive genes. Some of these ecdysteroid-responsive genes showed homology to genes controlling chitin metabolism, proteases and other cellular activities, while 56.8% were unknown. The majority of the genes were downregulated, presumably by an energetic shift of the hepatopancreas prior to ecdysis. The effect of 20-hydroxyecdysone on representative genes from this group was confirmed *in vitro* using a hepatopancreas tissue culture. This approach for ecdysteroid-responsive gene identification could also be implemented in other tissues for the elucidation of ecdysteroid-specific signaling pathways during the crustacean molt cycle.

Key words: *Cherax quadricarinatus*, Decapoda, Crustacea, molt staging, gastrolith X-ray imaging, ecdysteroids, X-organ–sinus gland complex, cDNA microarray, hepatopancreas.

Introduction

A possible linkage between the reproductive and molt cycles in the red-claw crayfish *Cherax quadricarinatus* was demonstrated in our previous work on induced molt cycle. In that study we showed differential expression in males of the vitellogenin gene *CqVg*, a female reproductive-related gene (Shechter et al., 2005). A putative explanation put forward for the regulatory pattern of expression of this gene was the premolt surge in ecdysteroids – a group of molecules comprising the molting hormone family, which includes ecdysone and 20-hydroxyecdysone (20E). To date, only a few ecdysteroid-responsive genes have been isolated and studied in crustacean species (El Haj et al., 1997; Kim et al., 2005; Rewitz et al., 2003; Testeniere et al., 2002; Tsutsui et al., 1999), although the action of ecdysteroids during the molt cycle and their effect on ecdysteroid-responsive genes has been studied extensively in insects (Cherbas, 1993; Margam et al., 2006; Thummel, 2002). It has previously been shown that in male insects vitellogenin synthesis can be induced by exposure to 20E (Deitsch et al., 1995; Deloof et al., 1981; Lamy, 1984), which may suggest that the vitellogenin gene in crustaceans, as in insects, is an ecdysteroid-responsive gene. However, in insects, ecdysteroids control the molt during the larval and pupal stages, whereas in adults (which do not molt) they play a role in reproduction. In our crustacean model – *C. quadricarinatus* – molting occurs throughout both the juvenile and adult phases, and there is a clear separation between the molt and reproductive processes (Barki et al., 1997). These differences between insects and crustaceans call for the identification of ecdysteroid-responsive genes responsible for molt-related events in crustaceans.

The crustacean molt cycle is divided into four distinct stages: intermolt, premolt, ecdysis (shedding of the old cuticle) and

postmolt (Aiken and Waddy, 1992; Chang, 1991; Chang, 1993; Skinner, 1985). The premolt stage comprises the preparatory events required for ecdysis, such as an increase in circulating ecdysteroids, separation of the old cuticle from the underlying hypodermis (apolysis), and, in some crustacean species, the accumulation of calcium in the gastroliths, paired disk-like calcium-storage organs enveloped by epithelial cells, which are situated in the cardiac stomach wall (McWhinnie, 1962; Travis, 1960; Ueno, 1980). The surge in circulating ecdysteroids triggers a programmed sequence of expression of different genes and synthesis of proteins in a variety of tissues, including the hypodermis and the hepatopancreas (Stringfellow and Skinner, 1988; Traub et al., 1987). This increase in circulating ecdysteroids is followed by a decrease in the levels of circulating ecdysteroids just before ecdysis, a step that is essential for the shedding of the cuticle; with ecdysis being delayed in the absence of theses changes in the ecdysteroids levels (Chang, 1993). Although a number of ecdysteroid metabolites, including ponasterone A and ecdysone (Snyder and Chang, 1991), have been isolated from crustacean hemolymph, it is currently accepted that 20E is the major active circulating ecdysteroid (Traub et al., 1987). In crustaceans, ecdysteroids are synthesized in steroidogenic glands, the Y-organs, found in the anterior thorax (Lachaise et al., 1993). Y-organ activity itself is under multi-factorial control (Lachaise et al., 1993), which is not completely understood at present, although it is generally accepted that the Y-organ is under the hormonal control of the neurosecretory X-organ–sinus gland (XO–SG) complex, located in the crustacean eyestalk (Keller, 1992).

Endocrine manipulations of the crustacean molt cycle have, for many years, been used by researchers to study the molt process. One of the most common induction methods is the surgical removal of the XO–SG complex (Aiken and Waddy, 1992; Shechter et al., 2005). The removal of the complex results in a decrease in neurosecretion, leading to accelerated synthesis of ecdysteroids, the onset of premolt and ecdysis. The administration of exogenous ecdysteroids has also been used in many crustacean studies for endocrine manipulation of the molt cycle. However, this type of manipulation has yielded mixed results, ranging from acceleration of premolt, death at ecdysis or viable ecdysis (Aiken and Waddy, 1992; Dall and Barclay, 1977).

The ability to accurately determine the molt stage is essential for any study of molt-cycle-related physiological changes. In the current study, we used three complementary methods. The first was based on the scheme suggested by Drach (Drach, 1939), in which the crustacean molt cycle is modeled as a complex sequential series of well-defined physiological changes, manifested as the morphological changes that occur in the cuticle during the molt cycle, such as apolysis and the synthesis of the new cuticle from the underlying hypodermis (Aiken and Waddy, 1992; Burton and Mitchell, 1987). The second method for accurate molt staging involved the changes in circulating ecdysteroid levels along the molt cycle, which have already been elucidated in a number of crustacean species (Snyder and Chang, 1991). The third molt staging marker was the size of the gastroliths (Pavey and Fielder, 1990). During the premolt stage in lobsters, several land crabs (Luquet and Marin, 2004) and crayfish, including *C. quadricarinatus,* calcium is mobilized and accumulated in the gastroliths. At ecdysis, the gastroliths collapse into the stomach, where they are digested, and calcium is mobilized to the new hardening cuticle. Gastroliths thus reach their maximum size at ecdysis and decrease in size during the postmolt stage (Travis, 1960). Gastrolith size along the molt cycle was determined radiographically in a continuous non-invasive manner, as has been done in a number of studies (McWhinnie, 1962; Nakatsuji et al., 2000; Rao et al., 1977).

In insects, such as *Drosophila melanogaster*, for which complete genomic sequence information is available, molecular high-throughput techniques, such as DNA microarrays, have been used to identify ecdysteroid-responsive genes during different developmental stages (Arbeitman et al., 2002; White et al., 1999). In crustacean species for which even partial genomic information is not available, the use of high-throughput cDNA microarrays for this purpose is yet to be achieved. Several studies using cDNA microarrays have, however, been performed for the identification of pathological viral-responsive genes and ecological markers in crustaceans (Chapman et al., 2006; Dhar et al., 2003; Soetaert et al., 2006).

The assembly of a crustacean microarray chip by our group previously enabled us to identify hepatopancreatic ecdysteroid-responsive, molt-related genes (Yudkovski et al., 2007). In the present study, we identified ecdysteroid-responsive genes in the *C. quadricarinatus* hepatopancreas by applying a polygenic cDNA microarray technology combined with the accurate multi-parametric non-invasive molt staging method described above. The identification of the ecdysteroid-responsive genes was achieved by two ecdysteroid-mediated endocrine manipulations: direct administration of 20E and an indirect triggering of ecdysteroid elevation. It is posited here that only genes belonging to similarly responsive gene clusters in both manipulations may be considered as putative ecdysteroid-responsive genes.

Materials and methods

Animals

Cherax quadricarinatus von Martens males were grown in artificial ponds at Ben-Gurion University of the Negev, Beer-Sheva, Israel. Food comprising shrimp pellets (Rangen Inc., Buhl, ID, USA, 30% protein) and wheat grains was supplied *ad libitum* three times a week. The temperature of the ponds was held at 27±2°C, and a 14 h:10 h L:D photoperiod was applied. Water quality was assured by circulating the entire volume of water through a biofilter. During the experiment, the pH of the water was 8.3±0.5, nitrite concentration was less than 0.1 p.p.m., nitrate concentration was less than 50 p.p.m., ammonium levels were negligible, and oxygen exceeded 5 mg l^{-1}.

Continuous multi-parametric molt staging

Gastrolith growth during the molt cycle was evaluated in terms of the molt mineralization index (MMI) (gastrolith width/crayfish carapace length), which was used as a molt stage marker. Gastrolith width was measured by X-ray digital imaging (Instrument Imaging, Focus model; and USB CDR Size 2, Schick Technologies, Long Island City, NY, USA); a metal grid of 10 mm diameter was used for size calibration.

Upon sacrifice of the animals at the end of the experiment,

additional molt staging was performed by the Drach (Drach, 1939) staging method, i.e. the maxillar exopodite was surgically removed, and setal development was evaluated under a light microscope (Fig. 1C).

For evaluation of circulating ecdysteroid levels, a 100 μl sample of hemolymph was obtained by syringe puncture of the ventral abdominal arthrodial membranes. The hemolymph sample was mixed with 300 μl of methanol, centrifuged at 10 000 ***g*** for 5 min to remove precipitated proteins, and stored at –20°C for later analysis. Ecdysteroid concentration in the supernatant was quantified in duplicate by radioimmunoassay, as previously described (Chang and O'Connor, 1979). Briefly, 10–100 μl samples were lyophilized and dissolved in 100 μl of a ^{3}H-ecdysone solution (6000 c.p.m. per 100 μl of borate buffer, pH 8.4, made up of 0.1 mol l^{-1} boric acid, 0.1 mol l^{-1} sodium tetraborate, and 0.075 mol l^{-1} NaCl). The actual amount of sample assayed depended upon the molt cycle stage of the donor crayfish (smaller volumes were assayed for premolt animals). Polyclonal ecdysteroid antiserum (Horn et al., 1976) (100 μl, 1:1000 dilution in 6% normal rabbit serum in borate buffer) was added to each tube, and the tubes were incubated overnight at 4°C. After incubation, antibodies were precipitated with 200 μl of saturated (100%) ammonium sulfate in borate buffer, and the tubes were centrifuged at 4800 ***g*** at 4°C for 15 min. The pellets were washed with 400 μl of 50% saturated ammonium sulfate, centrifuged at 4 800 ***g*** at 4°C for 15 min, dissolved in 25 μl of deionized water, and mixed with 600 μl of CytoScint scintillation cocktail (ICN Biomedicals, Inc., Irvine, CA, USA). Radioactivity was quantified with a liquid scintillation spectrometer.

Endocrine induction of the molt cycle

Intermolt crayfish were endocrinologically induced to enter premolt as described below. The crayfish were held in individual cages, and the progression of the molt cycle was monitored daily by measuring gastrolith MMI. Two alternative molt induction methods were applied: (1) Removal of the XO–SG complex by a bilateral surgical removal of the eyestalk with a surgical blade; or (2) administration of 20E, by repeated injections into the sinus at the base of the fifth walking leg. Different concentrations of 20E (from a stock solution: 1 μg μl^{-1} of a saline buffer containing 10% ethanol) were injected twice a day, 1000 pg μl^{-1} was calculated as the maximum physiological level (Fig. 1B), assuming that hemolymph volume was 30% of the wet mass of the crayfish. Injections of 20E, at the maximal concentration, were continued until an MMI value of 0.1 was obtained. Control animals were injected with comparable amounts of 10% ethanol in saline.

In vivo effect of 20E on CqVg expression

In the *in vivo* experiment, 20 intermolt males (mean wet mass of 30±2 g) were divided into three groups (of seven, seven and six). In the first group, the XO–SG complexes were surgically removed from seven males, and four of these were repeatedly injected with 20E to a maximum concentration of 1000 pg μl^{-1}. The other three crayfish were sham-injected with carrier. The crayfish were sacrificed 1 day after ecdysis, and hepatopancreatic RNA was extracted. In the second group, four of the intermolt males were repeatedly injected with 20E as described above, and three were repeatedly sham-injected with carrier. All the animals in this group were sacrificed 1 day after the ecdysis of the 20E-injected animals (sham-injected animals did not molt), and hepatopancreatic RNA was extracted. The third group of six intact males was not treated (control group), and hepatopancreatic RNA was extracted 6 days after the start of the experiment.

Primary hepatocyte cell culture

Primary cell culture from intermolt male *C. quadricarinatus* hepatopancreatic tissue was obtained by a non-enzymatic method using 1 mmol l^{-1} EDTA in crustacean saline (200 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 1.2 mmol l^{-1} KH_2PO_4, 33 mmol l^{-1} $NaHCO_3$, adjusted to pH 7.5) and protease inhibitor (Aprotinin; Sigma-Aldrich, St Louis, MO, USA). Pooled hepatocytes from 12 individuals were seeded in 24-well plates, pre-coated with coagulated hemolymph of *C. quadricarinatus* diluted 1:1 with adjusted M199 medium (Gibco, Invitrogene Corporation, Grand Island, NY, USA). The cells were cultured in a M199 medium including penicillin and streptomycin (Biological Industries, Kibbutz Beit Haemek, Israel) and 10% heat-inactivated fetal bovine serum (Biological Industries, Israel) adjusted to 420 mOsm with crustacean saline.

The culture was incubated at 28°C with 5% CO_2 for 24 h. After 24 h RNA was extracted from the cells using EZ-RNA (Biological Industries, Israel). Culture conditions were elaborated and optimized for *C. quadricarinatus* hepatopancreas cells enabling up to 7 days cell viability and *CqVg* expression.

Hepatopancreas organ culture

The hepatopancreas of an intermolt *C. quadricarinatus* male was dissected out and divided into 15 pieces, each weighing approximately 150 mg. The pieces were washed together three times with gentle shaking for 10 min each time with saline–EDTA and aprotinin at room temperature. Each organ piece was then transferred to the well of a non-coated 24-well plate containing 0.5 ml of supplemented medium. Each piece was incubated with the same conditions as those described for primary hepatocytes.

Exposure to 20E in vitro

Primary cell cultures (in quadruplicate) or organ cultures (in triplicate) were incubated with 1, 10, 100 or 1000 pg μl^{-1} 20E, dissolved in 10% ethanol in saline (such that the ethanol concentration was less than 0.1% in the culture medium). Cells and organs in the control wells were exposed to the carrier solution.

Relative quantitative real-time RT–PCR

RNA was extracted from each sample with an EZ-RNA kit (Biological Industries, Israel). First-strand cDNA was generated by reverse transcription using random hexamer primers (Reverse-iT^{TM}-1st Strand Synthesis Kit, ABgene®, Rochester, NY, USA). Relative quantification of *CqVg*, *trypsin* and *digestive cysteine proteinase 1* expressions was performed with the following primers: *CqVg* primers: QPCR-F 5′-GCTTC-CCGGTGGTTAATCCT-3′ and QPCR-R 5′-GGGCGGCAT-GACACACATCT-3′; *Trypsin* primers: F2 5′-TGCAAA-

AGGTGACTATCCCTACC-3′ and R2 5′-CTTGCAACCAT-TGGTCCTCC-3′; *Digestive cysteine proteinase 1* primers: QF 5′-ATATCGCAGAAGCGTCTTCGA-3′ and QR 5′-ACTT-GTTCATGGCCAGAGTGAA-3′. Real-time reactions were performed using a SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with an ABI Prism 7000 Sequence Detection System (Applied Biosystems; one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min). 18S rRNA, used as the normalizing agent, was also evaluated by real-time RT-PCR at similar conditions using the following primers: 18s Cherax R 5′-CCGGAATCGAACCCTGATT-3′ and 18s Cherax F 5′-GGCGCTGTGTCTTTCAAGTG-3′. All real-time experiments were conducted with N=4±1 for each group.

Statistical analysis

Real-time RT-PCR data were expressed as means ± s.e.m. One-way analysis of variance (ANOVA) was performed using Statistica 6.1 software (StaSoft Inc., Tulsa, USA). P values <0.05 were considered statistically significant.

Identification of ecdysteroid-responsive genes

Ecdysteroid-responsive genes were identified through the analysis of hepatopancreatic expression patterns using a 4800-clone cDNA microarray constructed by our group (Yudkovski et al., 2007). The endocrine manipulations described above were applied to induce premolt in six intermolt crayfish with no apparent gastroliths (MMI values of 0), three males for each treatment.

The control group comprised nine intermolt males and divided into three RNA pools. Ecdysteroid levels were determined prior to sacrifice of the animals. Crayfish of both treatment groups were sacrificed at premolt stage D_1–D_2 (with MMI values of 0.09–0.1), whereas the control intact crayfish were sacrificed at stage C, which represents the intermolt stage (MMI value of 0) (Table 1). The entire hepatopancreas was removed from each crayfish and snap frozen in liquid nitrogen. mRNA was extracted, and cDNA was generated and labeled in preparation for the hybridization with the microarray slide (see Yudkovski et al., 2007). Dye swap was performed for statistical purposes. Data analysis was conducted using the Limma (linear models for microarray data analysis) package. GenePix files were imported into Limma; spots with quality flags ⩽50 were marked as unreliable, and the Cy3 and Cy5 intensities within each chip were normalized using the print-tip Loess method. A linear model was then fitted to the treatment and control $\log_2$ ratio for each clone, and Limma's empirical Bayes method was used to moderate the standard errors of the estimated log ratios. To make full use of the duplicate spots on each array, Limma's pooled inter-duplicate correlation method was used at the linear modeling step. Spotted clones were considered differentially expressed by any of the treatments if they fulfilled the following criteria: average $\log_2$ absolute intensity across all channels and all arrays (A) >8.5 and estimated treatment and control $\log_2$ ratios (M) >1 or <–1, with a P value <0.05. Hierarchical clustering was performed on the M values of 2,053 clones whose A values were >8.5. Euclidean distance was used as a dissimilarity measure, and cluster merging was performed using complete linkage. A subset of the spotted array clones, including most of the differentially expressed clones, were sequenced, assembled using CAP3, and further annotated by sequence similarity to Swiss-Prot proteins. Only BLAST hits with E values $<10^{-5}$ were considered for the annotation. The microarray platform was deposited in GenBank GEO database (platform number GPL4786).

Results

Endocrine induction of the molt cycle

Continuous non-invasive monitoring by X-ray imaging of gastrolith growth in a male *C. quadricarinatus* crayfish was performed throughout a molt cycle induced by XO–SG removal (Fig. 1A). Changes in gastrolith size, represented by the MMI index, showed the induced molt cycle comprised four distinct phases (Fig. 1B): From day –14 to day –10 in relation to ecdysis, there was no change in gastrolith size. From day –9 to day –2 there was a sharp increase in gastrolith size (MMI reached a value of 0.145). From day –2 until ecdysis the MMI remained constant. Upon ecdysis, the gastrolith collapsed into the stomach, where it was digested, and a sharp decrease in the MMI was observed. During this induced molt cycle, circulating ecdysteroid levels increased gradually (0–75 pg μl^{-1}) from day –14 to day –7 and then sharply (200–1 100 pg μl^{-1}) from day –6 to day –3. The peak on day –3 was followed by a sharp drop to the basal level on the day of ecdysis, and the levels remained low during the postmolt stage.

Fig. 1C illustrates the adaptation, using the maxillar exopodite, of the Drach molt staging method for *C. quadricarinatus* (Drach, 1939). During intermolt (stage C) and prior to the endocrine induction, no apparent new cuticle was observed. Following the endocrine induction and initiation of premolt (stage D_0), the old cuticle separated from the hypodermis. Formation of a new cuticle, as indicated by the appearance of new setae, was observed at stage D_1, and stage D_2 was characterized by 'folded-glove' shaped new setae. Identical staging was observed for both natural and 20E-induced molt cycles.

The molt cycle of *C. quadricarinatus* was also successfully induced by repeated injections of 20E. The effect of injections of 20E (and of the carrier alone) on gastrolith growth and progression of the molt cycle was followed by digital X-ray imaging (Fig. 2). In the 20E-injected animals, the radiographs did not show any gastroliths on day –13, but on days –6 and –4 gastroliths were visible. Gastrolith growth in the 20E-injected crayfish was characterized by three distinct phases (Fig. 2B): from day –14 to day –12 there were no apparent gastroliths; from day –11 to day –1 gastrolith growth was rapid (to an MMI value of 0.125); and on day –1 gastrolith growth was arrested and remained so until ecdysis. The postmolt stage, which begins immediately after ecdysis, was characterized by a rapid decrease in gastrolith size (due to their collapse into the stomach, followed by digestion). Gastroliths were not detected in the carrier-injected controls.

CqVg expression following exposure to ecdysteroids

To test the effect of 20E on *CqVg* expression, intact and XO–SG-extirpated males were injected daily with either 20E or carrier until ecdysis (Fig. 3A). The intact group injected with carrier and the intact control group that was not injected did not

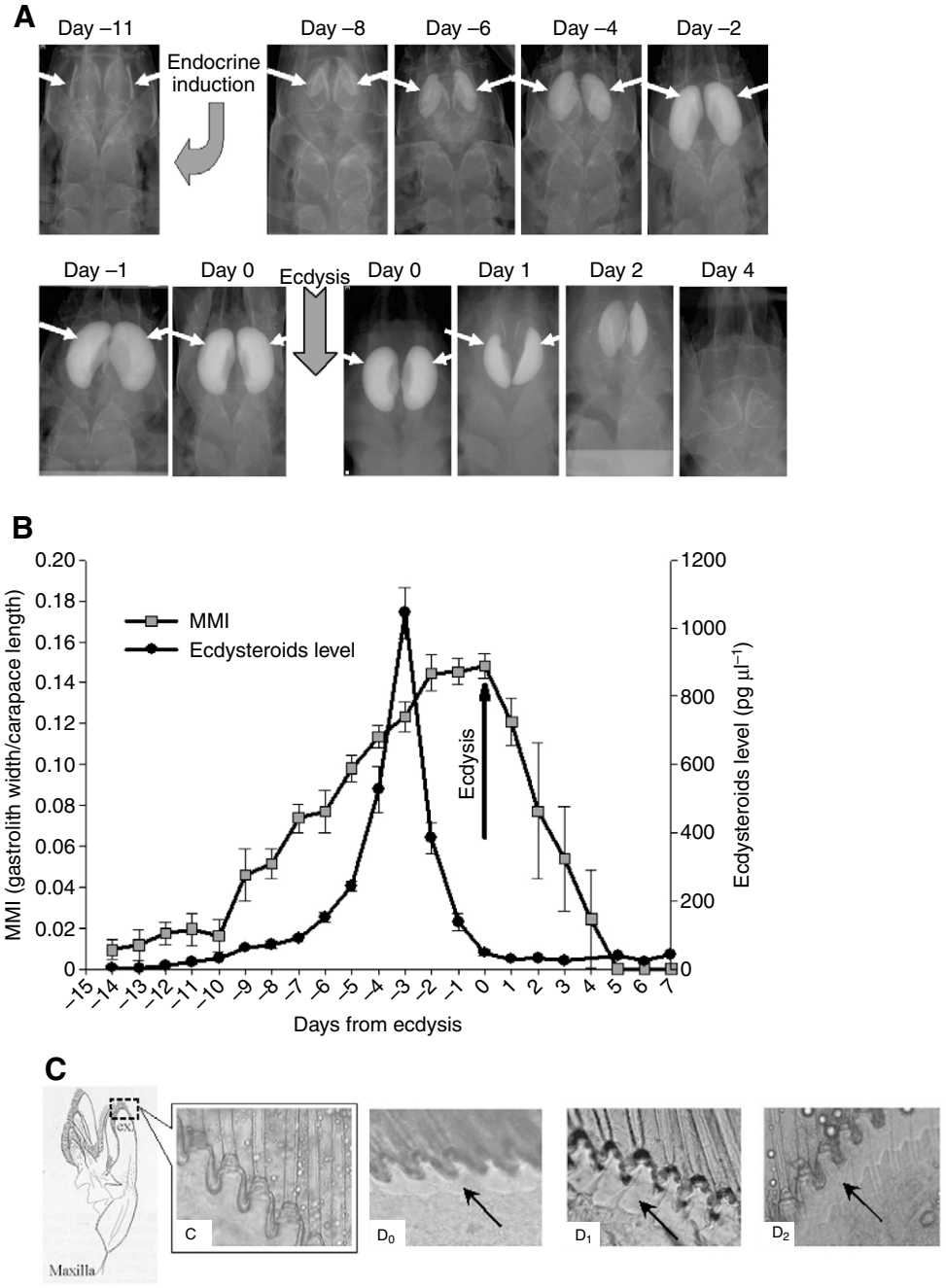

Fig. 1. Changes in gastrolith size, ecdysteroids levels and development of setae during an endocrinologically induced molt cycle in an XO–SG-extirpated male *C. quadricarinatus*. (A) Digital X-ray imaging of gastrolith growth during a representative endocrinologically induced molt cycle of XO–SG-extirpated male. White arrows point to the gastroliths. Times of endocrine induction and ecdysis are shown by gray arrows. (B) Changes in gastrolith size (squares) determined by MMI (molt mineralization index: gastrolith width as detected by X-ray imaging/carapace length), and circulating ecdysteroids levels (pg μl^{-1}; circles). The *x* axis is normalized to days from ecdysis. (C) Diagram and light microscope sections of the maxillar exopodite during intermolt (stage C, broken square) and three stages of premolt (D_0, D_1 and D_2). Arrows indicate apolysis regions. Magnification ×20.

reach ecdysis within the framework of this study (data not shown). Real-time RT-PCR results showed basal levels of *CqVg* expression in intact males injected with 20E or carrier, the levels were similar to those in the control intact group (Fig. 3A). By contrast, *CqVg* expression levels were significantly higher in XO–SG-extirpated males, with levels in the injected group being slightly lower than those in the non-injected group.

CqVg expression in a 24 h primary cell culture of intermolt male hepatocytes was not affected by 20E at any of the four applied concentrations (1, 10, 100 and 1000 pg μl^{-1}) (Fig. 3B). The control culture (subjected solely to carrier) showed no expression, as expected, whereas a primary cell culture of female hepatocytes (positive control) showed significantly higher *CqVg* expression.

Ecdysteroid-responsive genes – the multigenic approach

The chip, which contained ~4800 clones, represented polygenic expression patterns in the hepatopancreas during the premolt stage with respect to the treatment, i.e. endocrinological induction of premolt by either administration of 20E or surgical removal of the XO–SG complex (Fig. 4). The crayfish of both treatment groups were sacrificed at premolt stage D_1–D_2 (with MMI values of 0.09–0.1), whereas the control intact crayfish were sacrificed at stage C, i.e. intermolt (MMI value of 0). High circulating ecdysteroid levels were recorded for both treatments, although the levels in the injected crayfish were twice those the XO–SG-extirpated crayfish. The intact intermolt crayfish exhibited basal levels of circulating ecdysteroids (Table 1).

Fig. 4A demonstrates the hybridization design of the

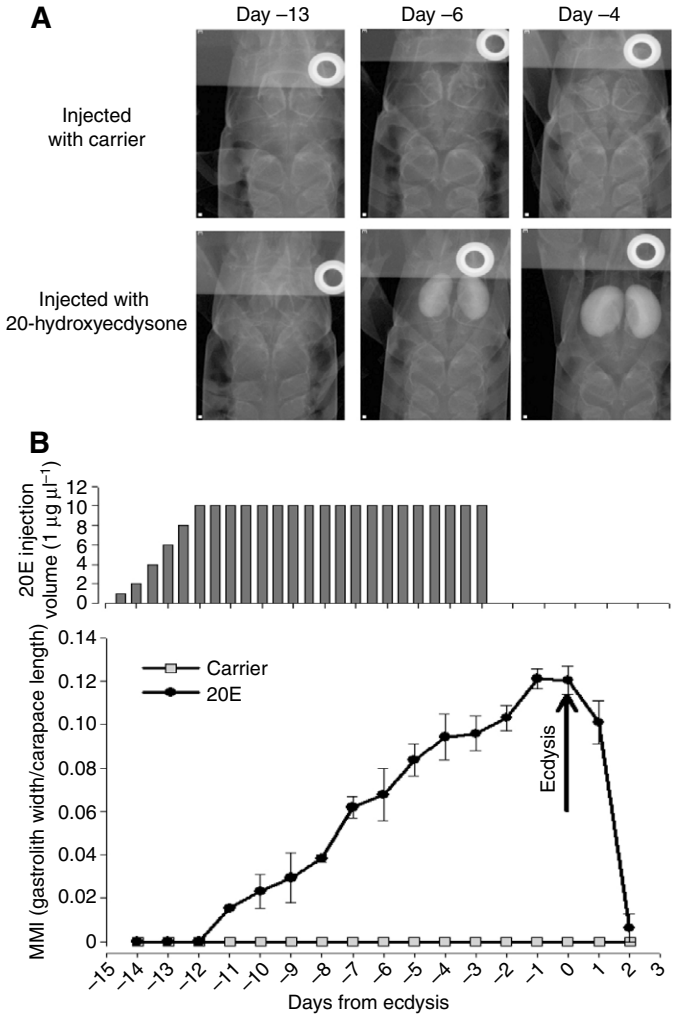

Fig. 2. Changes in gastrolith size during an induced molt cycle in a 20E-injected male *C. quadricarinatus.* (A) Digital X-ray imaging of the gastrolith in two representative specimens, one injected with 20E (bottom) and the other with carrier (top), which served as control. Images were obtained in the premolt stage on days –13, –6 and –4 relative to the anticipated time of ecdysis; a round metal grid was used for size calibration (10 mm diameter). (B) Upper graph shows the injection regime of 20E, (from a stock solution: 1 μg μl^{-1} of a saline buffer containing 10% ethanol) injected twice a day; 1000 pg μl^{-1} was calculated as the maximum physiological level. The injection regime and volume of the carrier were identical in the control group and 20E-injected groups. The *x* axis is normalized to days from ecdysis. Lower graph shows changes in MMI during the induced molt cycle by repetitive injections of 20E. The *x* axis is normalized to days from ecdysis. Squares, 20E-injected animal; circles, carrier-injected control.

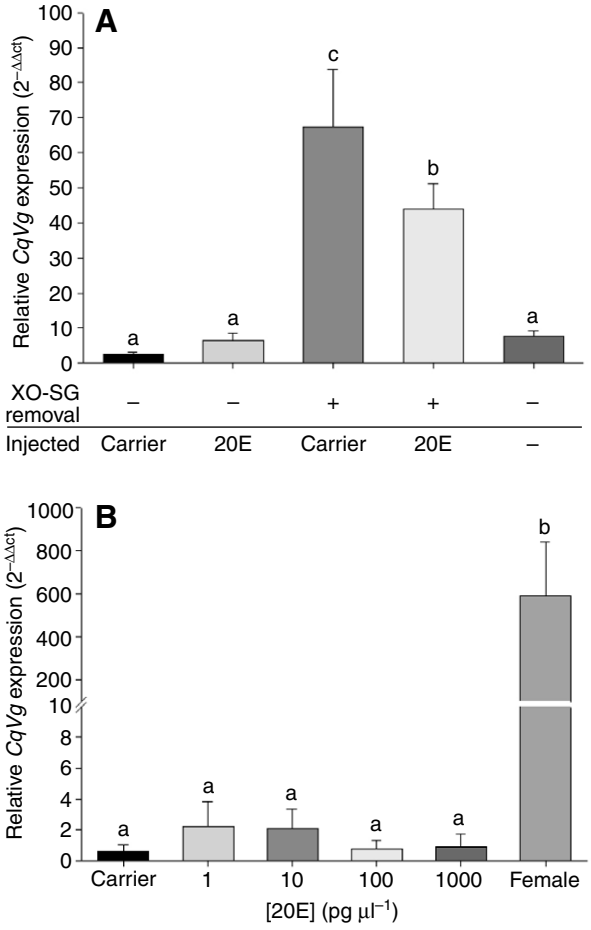

Fig. 3. Relative quantification of the effect of 20E on *CqVg* expression *in vivo* and in primary hepatocyte cell culture. (A) Intact and XO–SG-extirpated crayfish were injected daily with either 20E or carrier until they reached ecdysis. They were then sacrificed, and RNA was extracted for evaluation of *CqVg* expression levels. The control intact crayfish was not subjected to any injection. Different letters indicate statistical significance ($P<0.05$). (B) A 24 h primary hepatocyte cell culture from an intermolt male was subjected to four different concentrations of 20E: 1, 10, 100 and 1000 pg μl^{-1}. In the carrier sample no 20E was added. The positive control was a 24 h female primary hepatocytes cell culture with no addition of 20E (Female). Different letters indicate statistical significance ($P<0.05$).

multigenic experiment; it shows three biological replicates in each endocrine manipulation, hybridized against reference intermolt crayfish, with dye-swap between the replicates. The different colors of the circles in each treatment group represent different individuals, whereas in the reference group the same color represents pooling of the samples. Clustering analysis of the log ratios (*M*) of all the array clones except those with low absolute expression in all crayfish (*A*), illustrated in Fig. 4B, demonstrates the expression patterns in the different treatments. Variability in expression was evident not only between the two endocrine manipulations but also between animals within the same treatment. Clustering revealed that expression of most of the genes in the endocrinologically manipulated males was not significantly different from that in the reference intermolt intact crayfish (Fig. 4B). However, there were some genes that were found to be significantly different from the control reference by specific statistical criteria (described in Materials and methods); these were considered as 'differentially expressed genes'. Our working hypothesis is that genes that were differentially expressed in both treatments in the same direction, i.e. either up- or downregulated, may be considered putative ecdysteroid-responsive genes. Our hypothesis is based on the fact that both manipulations involved significant

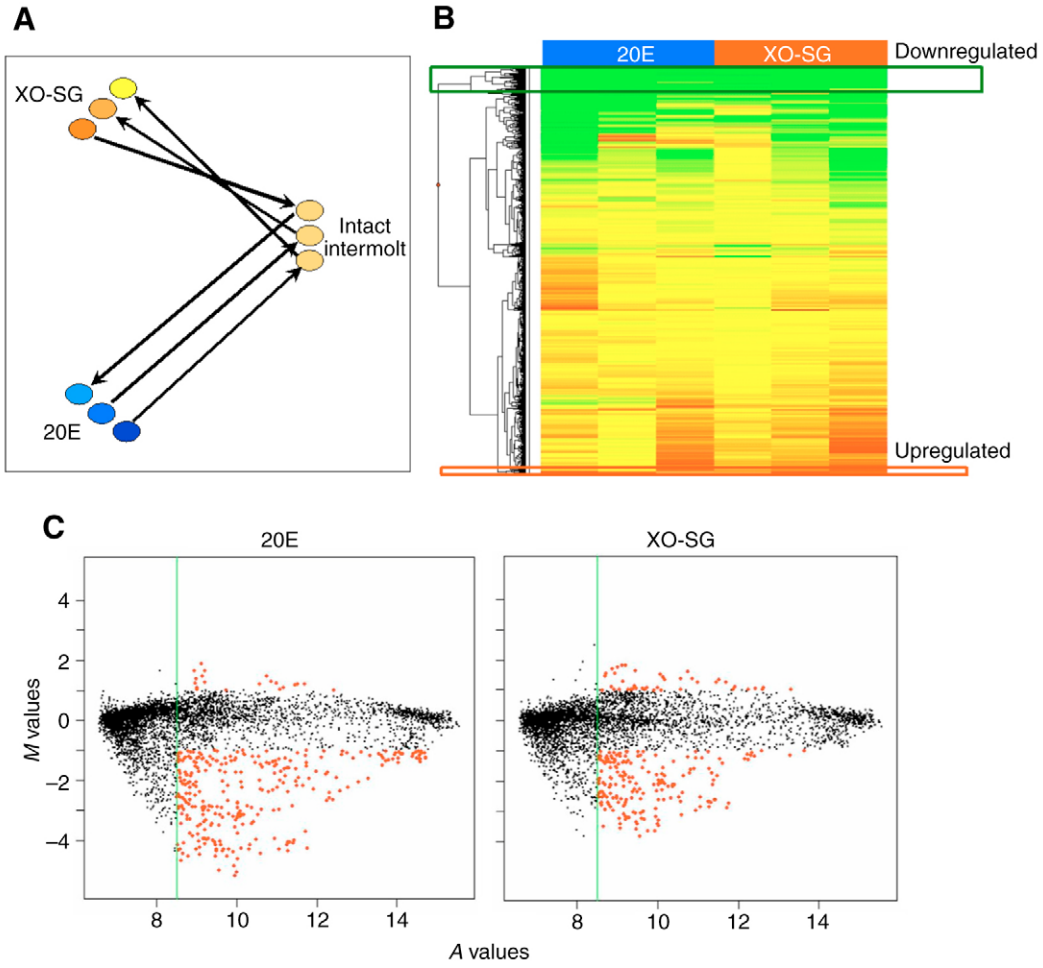

Fig. 4. Assembly of the microarray chip and multigene expression patterns related to elevation of ecdysteroids by two methods of endocrine molt induction. (A) Schematic representation of the hybridization design of the experiment showing each of the biological replicates. Arrowhead represents Cy3 labeling, and tail represents Cy5 labeling. XO–SG, premolt crayfish induced by XO–SG removal; 20E, premolt crayfish induced by repetitive 20E injections. The different colors of the circles in each treatment group represent different individuals, whereas in the reference group the same color represents pooling of the samples. (B) Overview of all the clustered genes according to treatments in the experiment. Red, treatment higher than reference; green, reference higher than treatment; yellow, equal expression. Two representative clusters of the ecdysteroid-responsive genes are indicated by boxes: the green box marks the downregulated cluster and the red box the upregulated cluster. (C) Expression scatter plots of all the genes represented on the chip in the two treatments. *M*, $\log_2$-fold change of normalized emission intensity between the treatment and the control; *A*, average signal in all treatments; green line, the minimum threshold of *A* at 8.5; red spots, genes with $P<0.05$, *M* value >1 or <–1, and *A* value >8.5.

elevation of ecdysteroids, which induced premolt. Two representative clusters of the ecdysteroid-responsive genes are indicated in two boxes (Fig. 4B), the green box marks the downregulated cluster and the red box marks the upregulated cluster. The cluster at the top of the figure represents genes that were downregulated in both treatments, whereas the cluster at the bottom of the figure represents genes that were upregulated in both manipulations.

An illustration of the effect of the two manipulations on all spotted clones is presented in Fig. 4C. For each manipulation, the figure shows a scatter plot of the estimated *M versus A* values. Each spot represents an average of all three biological replicates of the presented treatment. In the 20E-injected crayfish, 129 affected genes were identified during premolt, whereas in the XO–SG-extirpated crayfish 122 affected genes were identified. In both treatments, it can be seen that the majority of the genes were downregulated. Clones with *A* values <8.5 (green line) were excluded from further analysis, since their expression was close to the background level. Of the remaining clones, those with an *M* value of >1 or <–1, and with a *P* value of <0.05, were termed ‘differentially expressed genes’. The differentially expressed genes with *A* values >8.5 are represented by red dots.

A subset of the microarray clones, including most of the differentially expressed clones, were sequenced, clustered and annotated. The resulting 1427 expressed sequence tags (ESTs) were further clustered, resulting in 562 unique sequences (Yudkovski et al., 2007). Of these putative genes, only 176 were differentially expressed, and their mode of regulation (up or down) by each of the treatments is shown in Fig. 5A. Differentially expressed genes that responded in the same manner to the two treatments were taken to be ecdysteroid-

Table 1. *Multiparametric molt staging of the crayfish in the experiment*

Treatment	*N*	Mean carapace length (mm)	Mean MMI	Drach index*	Circulating ecdysteroids (pg μl^{-1})	Molt stage
Control	9	35.6	0	C	4.8±1.05	Intermolt
20E injection	3	36.4	0.09	D_2	1076±79	Premolt
XO–SG removal	3	33.8	0.09	D_1–D_2	534±25	Premolt

N, number of crayfish; MMI, molt mineralization index.
*Stage of molt; see text and Fig. 1C.

responsive genes. The expression of 32 unique sequences was significantly affected only by XO–SG removal; 26 of them were upregulated and six were downregulated. The expression of 42 unique sequences was significantly affected solely by injection of 20E; five of them were upregulated and 37 were downregulated. The expression of 102 unique sequences was significantly affected by both manipulations in the same direction, i.e. downregulated (92 genes) or upregulated (10 genes); these 102 genes thus constituted the ecdysteroid-responsive group.

The annotation results of the 102 ecdysteroid-responsive genes classified into functional groups is shown in Fig. 5B. 58 of the 102 (56.8%) genes did not show homology to any known protein in the database. Annotated genes belonged mainly to four large groups encoding the following: transport proteins such as hemocyanin (5%), chitin and carbohydrate metabolism proteins such as chitinase (10%), proteases such as cathepsin (10%), and control of cellular mechanisms such as cell division and protein synthesis (14%). Table 2 presents a complete list of the annotated genes from the ecdysteroid-responsive gene group, their expression trend (up or down *vs* intermolt) and their Gene Ontology (GO) terms. The table also indicates the genes for which expression patterns were validated by real-time RT-PCR in a companion study (Yudkovski et al., 2007).

The expression of two representative genes of the ecdysteroid-responsive group, *trypsin* and *digestive cysteine proteinase 1*, was further evaluated in a 24 h *in vitro* hepatopancreatic tissue culture subjected to four different physiological doses of 20E (Fig. 6). A significant decrease in *trypsin* expression was detected for a concentration of 100 pg μl^{-1} of 20E, whereas concentrations of 1, 10 and 1000 pg μl^{-1} did not have a significant effect on gene expression in reference to the control exposed solely to the carrier (Fig. 6A). *Digestive cysteine proteinase 1* expression showed a significant decrease in tissue exposed to 10 pg μl^{-1} of 20E, whereas the other concentrations did not show a significant effect in reference to the control exposed solely to the carrier (Fig. 6B).

Discussion

The considerable fluctuations in ecdysteroid titers that occur during the crustacean molt cycle suggest that ecdysteroid-responsive genes may be expressed differentially along the process. Indeed, in a previous study we showed that *CqVg* is differentially expressed in males during an endocrinologically induced molt cycle (Shechter et al., 2005). This finding, together with studies in insects showing that vitellogenin can

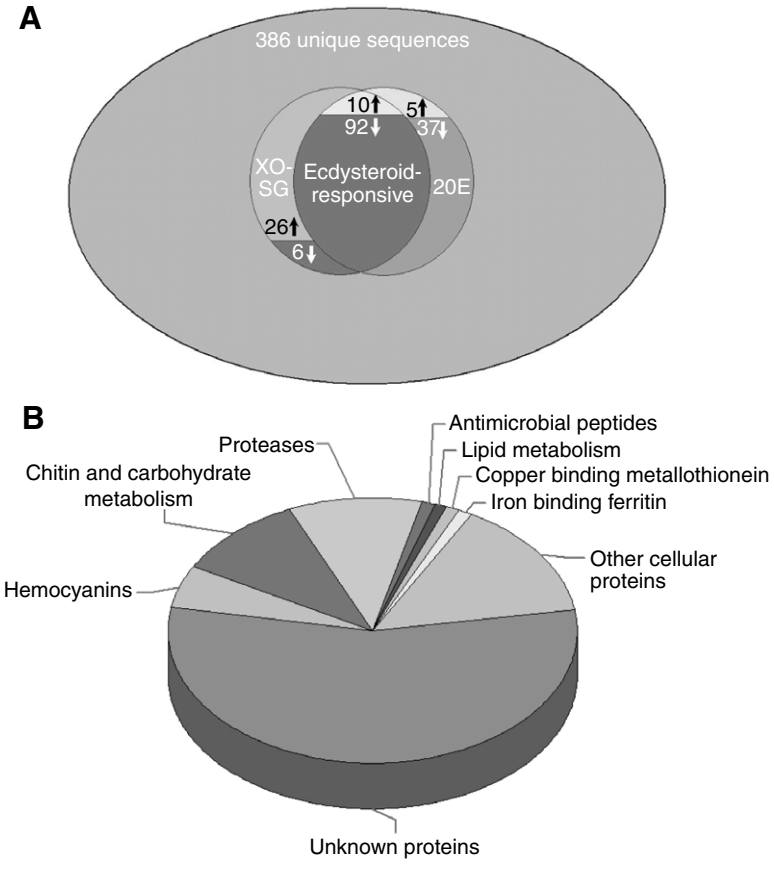

Fig. 5. Gene expression patterns resulting from the two endocrine manipulations out of all the sequenced and assembled genes from the chip, and the gene ontology (GO) pie graph of the putative ecdysteroid-responsive genes. (A) Schematic overview of the experiment showing the overlap between the two manipulations (XO–SG and 20E), which forms the ecdysteroid-responsive group. Upward-pointing arrows indicate upregulated genes; downward-pointing arrows indicate downregulated genes; 'unique sequences' are all the sequenced and clustered genes from the chip. In cases where different clones (spots) from the same gene belonged to different sections of the diagram, these genes were counted multiple times. (B) Pie graph of GO of the putative ecdysteroid-responsive genes. These genes showed a significant response during premolt when ecdysteroid titers were elevated in both treatments.

Table 2. *Annotation of the ecdysteroid-responsive gene group*

Organism	E-value	Annotation	GO terms	Validation	Regulation trend	Accession number
Homarus americanus	0	(P13277) Digestive cysteine proteinase 1 precursor		–	Down	DQ847948
Homarus americanus	0	(P25784) Digestive cysteine proteinase 3 precursor		–	Down	DQ847745
Homarus americanus	0	(Q6KF81) Pseudohemocyanin-2 precursor	Non-respiratory pseudo-hemocyanin	–	Down	DQ847704
Homarus americanus	0	(Q6KF82) Pseudohemocyanin-1 precursor		+	Down	DQ847706
Cherax destructor	0	(Q6TXR6) NADH-ubiquinone oxidoreductase chain 1	Electron transport	–	Down	DQ847754
Pacifastacus leniusculus	0	(Q6Y0Z1) Hemocyanin 2	Oxygen transporter activity	–	Down	DQ847739
Penaeus vannamei	0	(Q8ITU3) Chitinase	Chitin binding, chitinase activity, carbohydrate metabolism	–	Down	DQ847638
Callinectes sapidus	0	(Q9NGL5) Hemocyanin subunit	Oxygen transporter activity	–	Down	DQ847695
Drosophila melanogaster	0	(Q9V3K2) CG8439-PA, isoform A	ATP binding	–	Up	DQ847956
Penaeus vannamei	5.00E-41	(O62562) Trypsin	Proteolysis	–	Down	DQ847600
Penaeus monodon	1.00E-39	(Q95V28) Hemocyanin	Oxygen transporter activity	–	Down	DQ847586
Panulirus japonicus	2.00E-36	(Q8HD83) NADH dehydrogenase subunit 6	Mitochondrial electron transport	–	Down	DQ847649
Homarus americanus	6.00E-33	(P13277) Digestive cysteine proteinase 1 precursor	Peptidase	+	Down	DQ847749
Penaeus japonicus	1.00E-32	(Q26042) Chitinase	Chitin binding, chitinase activity, carbohydrate metabolism	+	Down	DQ847818
Penaeus vannamei	4.00E-32	(Q26180) Hemocyanin precursor	Oxygen transporter activity	–	Down	DQ847626
Antheraea yamamai	9.00E-32	(Q6I6G6) Ribosomal protein S18	Ribosome	–	Down	DQ847951
Drosophila pseudoobscura	4.00E-30	(Q28XD6) GA14025-PA	Carbohydrate metabolism	–	Down	DQ847601
Anopheles gambiae	2.00E-29	(Q7PF90) ENSANGP00000009410	ATP binding	–	Down	DQ847734
Corbicula fluminea	8.00E-26	(Q8I9P8) Alpha-amylase	Carbohydrate metabolism	–	Down	DQ847632
Nephrops norvegicus	1.00E-24	(Q6LBE7) Cathepsin l	Proteolysis	–	Up	DQ847755
Drosophila melanogaster	7.00E-24	(Q8WTI6) PFTAIRE-interacting factor 2	Protein binding	–	Up	DQ847689
Leptinotarsa decemlineata	1.00E-22	(Q3I414) Cytochrome P450	Metal ion binding, electron transport	–	Down	DQ847736
Fenneropenaeus chinensis	6.00E-17	(Q2TJP7) Antimicrobial peptide	Putative antilipopolysaccharide factor	–	Down	DQ847746
Anopheles gambiae	2.00E-15	(Q7QJV2) ENSANGP00000020729	Lipid metabolism	–	Down	DQ847724
Tenebrio molitor	3.00E-15	(Q86FS6) Cathepsin L-like cysteine proteinase	Proteolysis	–	Down	DQ847633
Tetraodon nigroviridis	1.00E-14	(Q4RXZ0) Chromosome 11 SCAF14979	S-adenosylmethionine-dependent methyltransferase	–	Down	DQ847619
Callinectes sapidus	4.00E-13	(Q9U620) Copper-specific metallothionein-2	Copper-specific metallothionein	–	Down	DQ847612
Homo sapiens	8.00E-13	(Q3LID0) Alpha-L-fucosidase	Carbohydrate metabolism	–	Down	DQ847682
Trichoplusia ni	4.00E-12	(Q3B9L9) Peritrophic membrane chitin binding protein	Carbohydrate metabolism	–	Down	DQ847692
Macaca mulatta	1.00E-11	(Q3YAN9) Cathepsin C	Proteolysis	–	Down	DQ847584
Caenorhabditis briggsae	9.00E-11	(Q60U71) Hypothetical protein CBG20141	Monooxygenase	–	Down	DQ847885
Penaeus vannamei	2.00E-09	(Q9U0F9) Amylase I	Alpha-amylase	–	Down	DQ847892
Penaeus stylirostris	1.00E-08	(Q8MTB8) Elongation factor 1-alpha	Translation elongation factor	–	Down	DQ847534
Penaeus vannamei	7.00E-08	(Q20AS9) ENSANGP00000021035-like	Chitin metabolism	–	Down	DQ847757
Xenopus tropicalis	1.00E-07	(Q5BKJ5) Clca1 protein	Chloride transport	–	Down	DQ847958
Penaeus monodon	6.00E-07	(Q9Y0D4) Chitinase 1	Chitin binding, chitinase activity, carbohydrate metabolism	–	Down	DQ847618
Plasmodium falciparum	1.00E-06	(Q6UDW6) Erythrocyte membrane protein 1	Pathogenesis, receptor activity, glycosaminoglycan binding	–	Down	DQ847679
Trypanosoma cruzi	3.00E-06	(Q4DLB6) Mucin-associated surface protein (MASP)		–	Down	DQ847959
Cherax destructor	4.00E-06	(P83172) Hemocyanin C chain (Fragment)	Oxygen transport	–	Down	DQ847639
Penaeus vannamei	1.00E-05	(Q58FL0) Ferritin	Ferric iron binding, iron ion transport	–	Down	DQ847590
Cryptosporidium hominis	2.00E-05	(Q5CKD2) T13C2.5		–	Down	DQ847957
Cherax quadricarinatus	4.00E-05	(Q9Y0W2) Beta 1,4-endoglucanase	Carbohydrate metabolism	–	Down	DQ847628

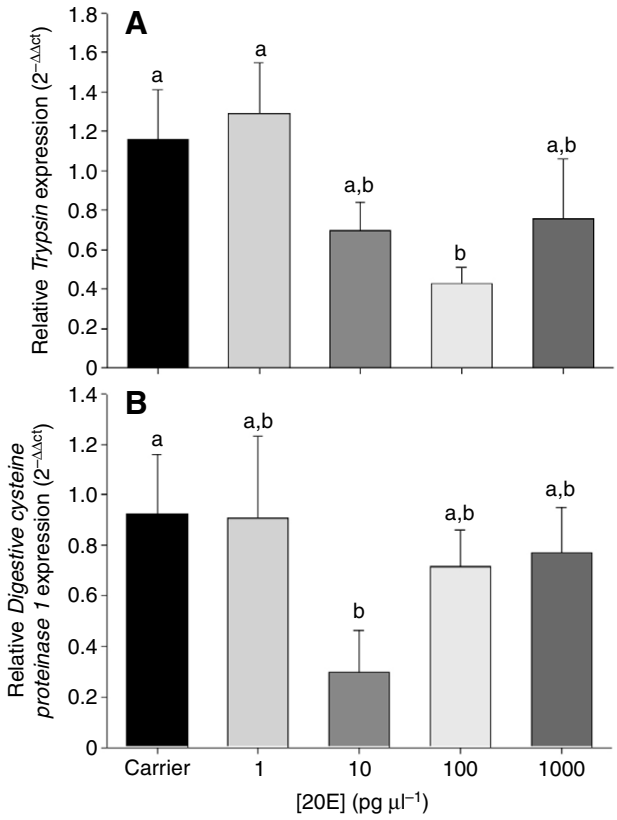

Fig. 6. *In vitro* confirmation of the effects of 20E on ecdysteroid-responsive genes. Real-time RT-PCR relative quantification of two putative ecdysteroid-responsive genes, *digestive cysteine proteinase 1* (A) and *trypsin* (B) in a 24 h hepatopancreatic tissue culture subjected to four different concentrations of 20E (1, 10, 100 and 1000 pg μl^{-1}). Different letters indicate statistical significance ($P<0.05$).

be induced by exposure to ecdysteroids (Huybrechts and Deloof, 1977; Martin et al., 2001), led us to suspect that *CqVg* might be an ecdysteroid-responsive gene. This hypothesis was tested here by exposing the hepatopancreas of *C. quadricarinatus* to 20E both *in vivo* and *in vitro*. However, the results of the present study imply that, unlike insect vitellogenin, *CqVg* does not seem to be an ecdysteroid-responsive gene or, at least, that it is not induced directly by 20E in our male crayfish model. The differential expression of *CqVg* may be explained by an indirect effect of ecdysteroids via other factors or a non-ecdysteroid mechanism.

The above finding led us to shift our focus towards a search for other ecdysteroid-responsive genes in the hepatopancreas. The hepatopancreas is a major metabolic and storage organ. During the course of the molt cycle, changes in proteins, lipids and carbohydrates have been observed in the hepatopancreas of several crustaceans (Chang, 1995). In our search for other ecdysteroid-responsive genes, we used a multigenic approach in the form of a cDNA microarray. The use of high-throughput methods, such as microarrays, for the identification of molt-related genes has already been performed in insects (Arbeitman et al., 2002; Goodisman et al., 2005; Ote et al., 2004; Tomancak et al., 2002; White et al., 1999). Since complete genomic information is available for some of the studied insects, the aim of those studies was to identify expression of gene clusters induced by 20E as well as developmental pathways and their regulating transcription factors. However, in crustacean species, for which such genomic information is not available, the use of microarray studies is still in its infancy. The only studies that have been performed are those on pathological viral-responsive genes in the shrimp (Dhar et al., 2003; Khadijah et al., 2003; Wang et al., 2006) and research aiming to find molecular ecological markers in *Daphnia magna* (Soetaert et al., 2006). Our *C. quadricarinatus* cDNA microarray was assembled with ESTs from the hepatopancreas, epidermis and Y-organs of premolt males (Yudkovski et al., 2007).

The ability to accurately determine the molt stage (using a multi-parametric method described here) and to endocrinologically induce the molt cycle is essential for the investigation of differential expression of ecdysteroid-responsive genes. Although each of the molt-stage parameters, i.e. morphological changes in the cuticle (Drach, 1939), gastrolith size (McWhinnie, 1962; Nakatsuji et al., 2000; Rao et al., 1977) and ecdysteroid titers (Snyder and Chang, 1991), has been evaluated separately in the past for molt stage determination, in the present study the combination of all three parameters enabled molt staging with a high degree of accuracy.

Induction of the molt cycle in this study was performed by two different methods – either direct administration of 20E or removal of the XO–SG complex – both resulting in elevation of ecdysteroid levels and subsequently in viable ecdysis. Although the direct administration of 20E has previously been used in many crustacean studies, some reported high mortality rates during premolt (Krishnakumaran and Schneideman, 1968; Skinner, 1985), whereas others reported successful viable ecdysis (Dall and Barclay, 1977; Gilgan and Burns, 1977; Webster, 1983). The failure to obtain viable ecdysis may be attributed to incorrect dosage of 20E – too high or too low – needed in each specific molt stage. Our ability to accurately determine the molt stage in a non-invasive, continuous manner enabled repeated injections of the exact required amounts of 20E, which eventually culminated in viable ecdysis.

The removal of the XO–SG complex in crustaceans to manipulate both the reproductive cycle and the molt cycle (Brown and Cunningham, 1939; Kyer, 1942; Meade and Watts, 2001; Okumura and Aida, 2001; Warner and Stevenson, 1972) has been widely used since the early 1940s (Smith, 1940). In our previous work, removal of the XO–SG complex in males not only induced an accelerated molt cycle but also resulted in differential expression of *CqVg* along the molt cycle, suggesting a possible involvement of ecdysteroids in *CqVg* expression (Shechter et al., 2005). Since XO–SG removal, as a means of endocrine intervention, leads to a wide array of neurohormonal changes – in addition to ecdysteroid elevation – its outcome was compared to that of direct administration of exogenous ecdysteroids to specifically sort ecdysteroid-responsive genes. We hypothesized that only those genes belonging to similarly responsive gene clusters in both manipulations could be considered putative ecdysteroid-responsive genes. Analysis of the expression patterns in this experiment revealed that most of the hepatopancreas genes that were affected by one manipulation were also affected by the other manipulation and were expressed in the same manner (i.e. up- or downregulated).

This analysis thus suggests that the effect of XO–SG removal in hepatopancreatic gene expression is mostly under the direct regulation of ecdysteroids. Dose dependency and the issue of secondary effects were not evaluated.

Previous studies in crustaceans have reported that ecdysteroid administration stimulated hepatopancreatic protein and mRNA synthesis (Gorell and Gilbert, 1969; Skinner, 1985), whereas our findings showed that the majority of the ecdysteroid-responsive genes were downregulated. We believe that this downregulation in response to ecdysteroids may be a result of an energetic shift of the hepatopancreas with the progress of premolt. Since the hepatopancreas is a major metabolic organ, it is not surprising that most of the genes found in the ecdysteroid-responsive group were typical metabolic genes, such as those encoding for digestive enzymes and those controlling transport and carbohydrate metabolism. Metabolic studies in penaeid shrimps have revealed that digestive enzymes produced in the hepatopancreas, such as trypsin, cathepsin-L and chitinase, are differentially expressed during the molt cycle, with a possible involvement of ecdysteroids in their expression (Klein et al., 1996; LeBoulay et al., 1996; Tan et al., 2000). These studies reported dramatically high *trypsin* and *cathepsin L* expression during D_1, followed by a drop in expression during D_2 and D_3, whereas the expression of *chitinase* in the hepatopancreas was elevated during D_2 and dropped during D_3 (Tan et al., 2000). All three genes in the above reports in penaeids were identified in our ecdysteroid-responsive group of the crayfish, but their expression patterns in our study stand in contrast to some of the above findings. We found that in *C. quadricarinatus* all three genes were downregulated during D_1–D_2, with *trypsin* also being found to be directly downregulated by 20E *in vitro*. These dissimilarities in expression patterns during the molt cycle may be explained by differences in hepatopancreas metabolism between penaeid shrimp and crayfish.

The downregulation of the above metabolic enzymes obtained in our study could be the result of a prolonged period of arrested feeding prior to ecdysis in *C. quadricarinatus*: all crustaceans cease feeding several days prior to ecdysis (Dall et al., 1990) with this period varying between species according to their activity and metabolism. The stress caused by the endocrine manipulations should also be considered as a factor that may also have prolonged the period in which the animals stopped feeding and hence caused an additional effect on the metabolism.

Another problem that may impede studies of ecdysteroid-related gene expressions by determining the molt stage lies in the high variability, in each molt stage, of ecdysteroid titers determined by the classical method (Drach and Tchernigovtzeff, 1967), as was the case in the above-mentioned studies (Klein et al., 1996; LeBoulay et al., 1996; Tan et al., 2000). This variability can raise difficulties in comparisons of differential molt-related gene expressions. This problem emphasizes the need for an accurate multi-parametric molt staging technique, such as that used in the present study, to facilitate meaningful comparisons between different studies.

Another group of genes found to be downregulated in the ecdysteroid-responsive group were those of transport proteins such as *hemocyanin*. The hepatopancreas is the major synthesis site of hemocyanin (Gellissen et al., 1991), which is secreted into the hemolymph and has been reported to account for 95% of the hemolymph protein (Sellos et al., 1997). Concentrations of hemolymphatic hemocyanin in the crayfish *Astacus leptodactylus* during the molt cycle were reported to follow a biphasic profile, with the highest values being found in the late intermolt (C_4) and early premolt (D_1) periods (Spindler et al., 1992), whereas in juveniles of the lobster species *Homarus gammarus*, hemolymph hemocyanin concentrations peaked gradually during D_1–D_4 (Hagerman, 1983). By contrast, the current study revealed a decrease in the expression of a number of *hemocyanins* during the late premolt (D_2) period. It should be remembered, however, that we studied hepatopancreatic gene expression, whereas the above studies reported hemolymph protein concentrations.

Interestingly, genes that were affected exclusively by XO–SG removal were mostly upregulated, probably because these genes are under the direct or indirect control of XO–SG inhibitory neuropeptides. The absence of such neuropeptides could thus have been the reason for this over-expression, as was found previously for *CqVg* (Shechter et al., 2005). This heterogenic group of genes that were upregulated included those with diverse functions, ranging from control of cell division to encoding for kinases and proteases. However, the majority of the genes in this group were unknown. Only a few genes were found to be downregulated, all of which encoded the same digestive enzymes as those in the ecdysteroid-responsive group. Future studies testing the effect of sinus gland extracts on the expression of these genes in the hepatopancreas *in vitro* would shed light on this regulation. Genes that were affected exclusively by the 20E injections were mostly unknown and downregulated, as were most of the genes in the ecdysteroid-responsive group, probably as a result of the high ecdysteroids titers or of the repetitive stress caused by the daily injections. Although it is possible that some of the genes from this group could also be referred to as putative ecdysteroid-responsive genes, our hypothesis suggests that this effect may result from the manipulation itself rather than by the direct effect of ecdysteroids. Therefore, only genes that are responsive in the same manner in both manipulations could be considered putative ecdysteroid-responsive genes.

For understanding the molt cycle in crustaceans, it is essential to identify the genes controlling it; some of these genes are known from other biological processes, but others are yet to be identified. Our search for ecdysteroid-responsive genes, particularly in the hepatopancreas, revealed mostly differentially expressed genes encoding metabolic and transport enzymes, as was to be expected from to the function of this tissue. Other target tissues, such as the epidermis, the Y-organs and gastrolith-forming tissue, could serve as promising candidates for such a search in future studies. The use of both direct and indirect manipulations together with our multiparametric method for molt staging and the high throughput cDNA microarray constitute a platform for future investigations of genes and patterns important for the understanding of this complex process.

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