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Variations in ecdysteroid levels and Cytochrome p_{450} expression during moult and reproduction in male shore crabs *Carcinus maenas*

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ABSTRACT: Ecdysteroid levels were investigated by HPLC-MS over the moult cycle and in relation to reproduction in male shore crabs *Carcinus maenas*. Ecdysone (E), 20-hydroxyecdysone (20E) and Ponasterone A (PoA) were quantified in the haemolymph, hepatopancreas and testis. Also, the expression of 2 recently discovered Cytochrome p_{450} genes (CYP330A1 and CYP4C39) inducible by ecdysteroids was studied in the hepatopancreas by Northern blot hybridisation analysis. In the haemolymph and hepatopancreas, all 3 ecdysteroids varied over the moult cycle with high levels in premoult and low levels in postmoult and intermoult. In the testis, 20E and E were present at high levels except in Postmoult Stage A, where low levels were observed. PoA was never observed in the testis. Ecdysteroids were quantified in the red and green colour forms of late intermoult C_4 crabs. In both phenotypes, 20E was the dominating ecdysteroid in late intermoult. In the haemolymph, 20E levels did not vary between the 2 phenotypes, but haemolymph 20E levels were negatively related to size. Also, haemolymph 20E levels varied with season in late-intermoult crabs, with higher levels during spring and autumn than during summer. Green crabs had significantly higher testicular E levels than red crabs. Ecdysteroid levels were negatively related to *CYP330A1* and *CYP4C39* gene expression. CYP330A1 and CYP4C39 mRNA levels were low during intermoult and premoult but high during postmoult. The results suggest that E and 20E are involved in both growth and reproduction whereas PoA is involved in moulting but not in reproduction and that the testis of male shore crab may be a possible source of ecdysone production in addition to the Y-organ. The results also support the concept that the probability of male shore crabs entering a new moult cycle decreases with increasing size.

KEY WORDS: Shore crab · *Carcinus maenas* · Moult · Reproduction · Ecdysteroids · 20-hydroxyecdysone · Ecdysone · Ponasterone A · Cytochrome p_{450} · CYP330A1 · CYP4C39 · HPLC-MS

INTRODUCTION

The dominating life cycle trait of male shore crabs *Carcinus maenas* in Scandinavian waters is rapid growth, with multiple moultings in juveniles decreasing to a single ecdysis in early summer in adults, followed by extensive somatic and testicular growth during the next 2 to 3 mo in intermoult, leading to reproduction in those males large enough to successfully compete with other males for receptive females during the mating season in late summer. Shore crabs spend most of their lives in late intermoult (Crothers 1967), in the Isefjord about 70% or more in Stages C_3 to C_4 and 50% or more in C_4 alone. During late intermoult, shore crabs grow within their integument, gradually replacing water with organic matter such as lipids and proteins (Crothers 1967, Heath & Barnes 1970) to prepare for the next moult and/or mating. Consequently, great changes occur in the crabs during these stages, and the physiological status of late intermoult male shore crabs varies extensively, depending on size and intermoult duration.

The molecular physiological mechanisms governing growth and reproduction are not known, but certainly involve ecdysteroids (reviews by Chang 1989 and Sub-

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ramonian 2000). Ecdysteroids are moulting hormones present in all arthropods examined so far. The active moulting hormone 20-hydroxyecdysone (20E) is formed in peripheral tissues by a single-step hydroxylation of ecdysone (E) which is produced and excreted from the Y-organ into the haemolymph (Chang & O'Connor 1977, Soumoff & Skinner 1988, Snyder & Chang 1991). 20E affects kinase activity and protein synthesis in epidermal tissues (Christ & Sedlmeier 1987, Traub et al. 1987). Putatively cytochrome p_{450} enzyme (CYP enzyme)- catalysed ecdysone 20-monooxygenation (hydroxylation of E to 20E) occurs in a number of crustaceans, but the specific CYP enzyme(s) responsible remains unknown. High ecdysone 20-monooxygenase activity has been reported in the green gland of the spiny lobster *Panulirus argus* (James & Shiverick 1984) and in the hepatopancreas of the crab *Gecarcinus lateralis* (Soumoff & Skinner 1988). In the crab *Pachygrapsus crassipes*, high ecdysone monooxygenase activity was also observed in the testis (Chang et al. 1976), indicating that E and 20E might conceivably be involved in spermatophore maturation of crabs. CYP enzymes are also involved in the inactivation of ecdysteroids, presumably by the irreversible hydroxylation of 20E to 20,26-dihydroxyecdysone (20,26-OH-E) (Williams et al. 2000).

In contrast to insects, crustaceans have a third major ecdysteroid in their circulating haemolymph, Ponasterone A (PoA) (McCarthy 1979, Lachaise et al. 1981, Lachaise & Lafont 1984). The precursor 25-deoxyecdysone is excreted by the Y-organ and hydroxylated at C-20 in peripheral tissues to produce PoA. Accordingly, the Y-organ excretes at least 2 ecdysteroids, ecdysone and 25-deoxyecdysone, which after peripheral tissue C-20 hydroxylation yield the 2 major haemolymph ecdysteroids 20E and PoA (Lachaise et al. 1986, 1988, 1989). The function of PoA is unknown, but PoA may potentially be converted into the active moulting hormone 20E by C-25 hydroxylation, even though such a mechanism has not yet been reported.

Individual ecdysteroids levels in crustaceans vary considerably between species and tissues. It is generally agreed that haemolymph and tissue ecdysteroid levels are higher during premoult than during postmoult and intermoult (Soumoff & Skinner 1988, Lachaise et al. 1989, Snyder 1998). PoA and 20E are the dominant ecdysteroids in shore crab haemolymph, whereas E occurs at much lower levels, probably due to a rapid transformation of E into 20E in peripheral tissues (James & Shiverick 1984, Soumoff & Skinner 1988).

Moult and reproduction are controlled by the metabolic kinetics of the ecdysteroids, hence studying relationships between individual ecdysteroids is important for understanding the basic ecological strategy of the shore crab, i.e. interactions between growth and reproduction. Radio-immunoassay, the method most often employed in ecdysteroid studies, has the disadvantage of responding differently to the relative levels of different ecdysteroids due to cross-reactivity (Lachaise et al. 1988). Hence, results may be misleading. The method employed in the present study is simpler, cheaper and faster, is not susceptible to similar methodological problems, and quantifies individual ecdysteroids levels in several tissues and compartments, even in those moult stages where the levels are lowest.

The present article reports tissue level variations of the 3 major crustacean ecdysteroids, E, 20E, and PoA, in male shore crabs *Carcinus maenas* in relation to moulting and reproduction. We recently reported 2 *CYP* genes, *CYP330A1* and *CYP4C39*, in this species and demonstrated inducibility of CYP330A1 by ecdysteroids and exogenous compounds (Rewitz et al. 2003). Accordingly, the moult cycle variations in the expression of these genes in relation to ecdysteroid moult cycle variations were also investigated.

MATERIALS AND METHODS

Male shore crabs *Carcinus maenas* sampled in the Isefjord at Rørvig, Zealand, Denmark, were scored according to size (carapace width, CW) and coloration of the abdomen (green or red). After haemolymph sampling (approximately 0.5 ml) from the arthrodial membrane of the 4th pereiopod, the crabs were sacrificed and tissue samples (hepatopancreas, testis) were briefly blot-dried on paper towels to allow surplus haemolymph to drain before the tissue was frozen in liquid nitrogen. Moult stages were determined according to Drach (1939), Aiken (1973) and O'Halloran & O'Dor (1988) as follows: postmoult = A to B, early intermoult = C_1 to C_2, late intermoult = C_3 to C_4, terminal anecdysis = C_4T, early premoult = D_0 to D_1, late premoult = D_2 to D_3. At the peak of the male moulting season (early June) (Rasmussen 1973) large male crabs in late intermoult stage C_4 were caught. These crabs were generally red in appearance and showed a high degree of 'wear and tear' (epibionts, integument and chelae injuries) and would probably have maintained their old exoskeletons and remained in C_4 throughout the moulting season. These crabs were allocated to Stage C_4T, i.e. terminal anecdysis.

To obtain sufficient samples from all moult stages, some crabs were caught in spring and maintained in the laboratory in 10 l polystyrene aquaria (4 individuals in each aquarium) with aerated seawater (20‰) and fed *ad libitum* twice a week with fish. Light regimes were 12 h light:12 h dark, and temperatures were 15 ± 1°C. These crabs were sacrificed at different pre- and postmoult stages.

Ecdysteroid measurements. Ecdysteroids from haemolymph and tissue samples were extracted and analysed by methods modified from Lafont et al. (1982) and Wainwright et al. (1997). Haemolymph (approximately 0.5 ml) and tissue samples (50 to 200 mg wet weight) were mixed with 8 ml water and 8 ml chloroform. An internal standard, Makisterone A (MaA) (1 ml of 0.1 to 0.3 µg ml^{-1} in methanol) was added, and the samples were homogenised and then centrifuged for 10 min at 4000 rpm. The supernatant was then recovered, another 8 ml of water was added and the sample was once again mixed and centrifuged. This procedure was carried out 4 times in all, resulting in an approximately 100% recovery of E, 20E and MaA and a 92 to 97% recovery of PoA. The combined water extracts were eluted on a reverse-phase cartridge (Waters Oasis™ HLB extraction cartridges) preconditioned with 2 ml methanol and 5 ml water. Ecdysteroids were eluted from the cartridge by the addition of 5 ml 60% methanol in water. The aqueous methanol extracts were then reduced to dryness at 40°C by a gentle flow of nitrogen, redissolved in 100 µl methanol and diluted with 200 µl water. Quantification of the extracted ecdysteroids was performed by high-performance liquid chromatography coupled to mass spectrometry (HPLC–MS).

The HPLC instrument (TSP Spectra system) was composed of an AS3000 auto sampler, P4000 gradient pump, and a vacuum degasser. The fractionation of a 20 µl aliquot of an ecdysteroid extract was performed using a linear gradient of 35% (v/v) methanol in water to 60% (v/v) methanol in water over 22 min at a flow rate of 0.2 ml min^{-1}. The analytical column was a 50 mm Xterra MS RP C18 column from Waters with an inside diameter of 2.1 mm fitted with a 10 mm guard column (Xterra RP C18). The mass detector was a LCQ-Deca ion-trap instrument from Thermo-Finnigan, equipped with an atmospheric pressure chemical ionisation interface (APCI) running in negative mode. A negative potential (–5 kV) was applied to the metal needle, with a discharge current of 4.5 µA, capillary voltage of –26 V, heated capillary temperature of 200°C, vaporiser temperature of 350°C, and sheet gas (N_2) and auxiliary gas (N_2) of 31 and 8 arbitrary units, respectively. The MS detector was run in selective ion monitoring (SIM) at the specific mass to charge intervals (m/z) of 464.9 to 465.9 $[E\text{-}H]^-$ and $[PoA\text{-}H]^-$, 479.5 to 480.5 $[20E\text{-}H]^-$, 488.8 to 489.8 [PoA + MeOH $-H]^-$ and 492.8 to 493.8 $[MaA\text{-}H]^-$. The LCQ software Xcalibur 1.2 controlled the chromatographic and mass spectrometric analyses.

Response factors for E, 20E and PoA relative to that of MaA were calculated from authentic standards (purchased from Sigma and GE Scientific). Response factors relative to MaA were E = 0.92 ± 0.04, 20E = 1.02 ± 0.02, and PoA = 1.06 ± 0.03.

Gene expression analysis. Total RNA for Northern blot analysis was isolated from the hepatopancreas, testis and vas deferens of red and green shore crabs using the QIAamp RNA kit (QIAGEN) according to the manufacturer's instructions. The quality of the RNA was checked by gel electrophoresis, quantified spectrophotometrically at 260/280 nm, and stored at –80°C until use.

Specific CYP330A1 (Accession No. AY328466), CYP4C39 (Accession No. AY328467) and actin partial cDNA probes were synthesised by PCR (polymerase chain reaction) using 1st-strands cDNA as template and specific primers generating cDNA fragments of approximately 400, 450 and 600 bp, respectively. Primer sequences were according to Rewitz et al. (2003). PCR conditions were: 94°C, 2 min; 94°C, 15 s; 60°C, 30 s; 72°C, 2 min for 40 cycles followed by a 7 min extension period at 72°C. The PCR products were loaded onto a 1% ethidiumbromide-stained low-melt agarose gel, allowing the excised fragments to be directly used in the *redi*prime™II random prime-labelling system (Amersham Pharmacia). The cDNA probes were randomly labelled with α-[^{32}P]-dCTP (250 U Ci/25 µl). Removal of unincorporated nucleotides was performed using 'Push Column Beta Shield Device' and 'NucTrap Probe purification columns' (STRATAGENE).

Total RNA (20 µg $lane^{-1}$) from the hepatopancreas, testis and vas deferens of individual shore crabs were denatured and electrophoresed on a 1% agarose gel containing 0.2 M formaldehyde, and then transferred to a nylon membrane (S & S Nytran SuPerCharge, Schleicher & Schuell). Hybridisation with the ^{32}P-labelled probe was carried out at 65°C for 1 h 30 min in Rapid hybridisation buffer (Amersham Pharmacia) containing 10 mg ml^{-1} sheared salmon sperm DNA (Gibco BRL) to facilitate the formation of DNA-RNA hybrids. The membrane was then washed in 200 ml 2 × saline sodium citrate (SSC)/0.1% sodium dodecylsulphate (SDS) at room temperature for 2 × 15 min and subsequently in 200 ml 0.1 × SSC/0.1% SDS at 60°C for 30 min. The membrane was stripped in 200 ml 1% glycerol/0.1% SDS, pre-heated to near-boiling temperature for 30 min before re-hybridisation. The blots were scanned using a phosphoimager (Storm 840, Pharmacia Biotech), and band intensities were determined (software: ImageMaster TotalLab Version 1.11). CYP mRNA levels were calculated using actin on the same blot as reference.

Data analysis. Levene's test was used to examine homogenity of variances. A Student's *t*-test and a 1-way ANOVA were used to analyse those data revealed to be normally distributed. If data were not normally distributed, even after log-transformation, a Mann-Whitney *U*-test was used to detect significant differences.

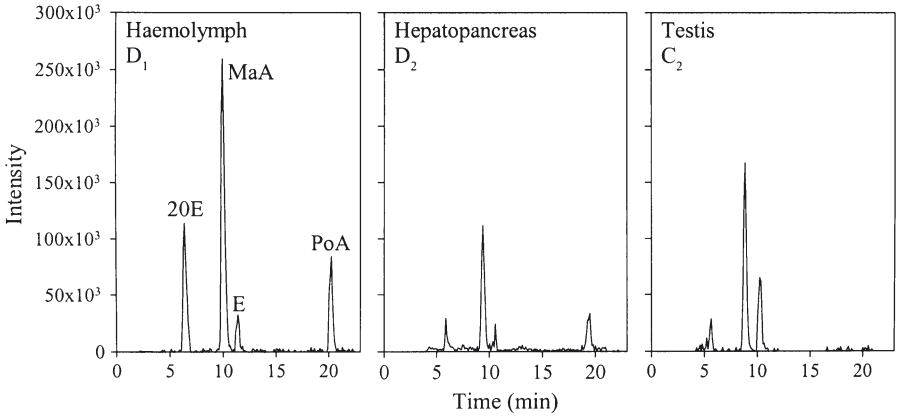

Fig. 1. *Carcinus maenas*. HPLC-MS chromatograms of haemolymph (400 µl), hepatopancreas (122 mg) and testis (80 mg) in 3 different moult stages. Internal standard = Makisterone A (MaA). 20E: 20-hydroxyecdysone; E: ecdysone; PoA: Ponasterone A

RESULTS

Ecdysteroid HPLC-MS chromatograms

Fig. 1 shows 3 examples of chromatograms obtained from the HPLC-MS ecdysteroid analysis. The chromatograms are from the haemolymph, hepatopancreas and testis, respectively, for 3 different moult stages (D_1, D_2 and C_2). All 3 major ecdysteroids were detected in the haemolymph and hepatopancreas, but PoA was never detected in the testis. MaA is suitable as an internal standard since it has chromatographic properties immediately between that of 20E and E, and is not endogenous in shore crabs. With the method employed in the present study, retention times were approximately 6.40 min for 20E, 9.50 min for MaA, 11.40 min for E and 20.00 min for PoA.

Variations in ecdysteroid levels in relation to moult stage

Haemolymph and hepatopancreas levels of all 3 major ecdysteroids were elevated during premoult, with 20E and PoA reaching maximum in Stage D_1, whereas maximum E levels were observed in D_2 (Fig. 2). Ecdysteroids were only observed in trace amounts in postmoult crabs (Stages A,B) and early intermoult crabs (C_1 to C_2). PoA was never observed in late intermoult (C_3 to C_4), in either the haemolymph or in the hepatopancreas. In late intermoult, the 20E level increased in the haemolymph and hepatopancreas, while the E level increased only slightly above detection limit in haemolymph. Ecdysteroids were never observed in C_4T crabs. During premoult, PoA levels were 5- to 8-fold higher than the

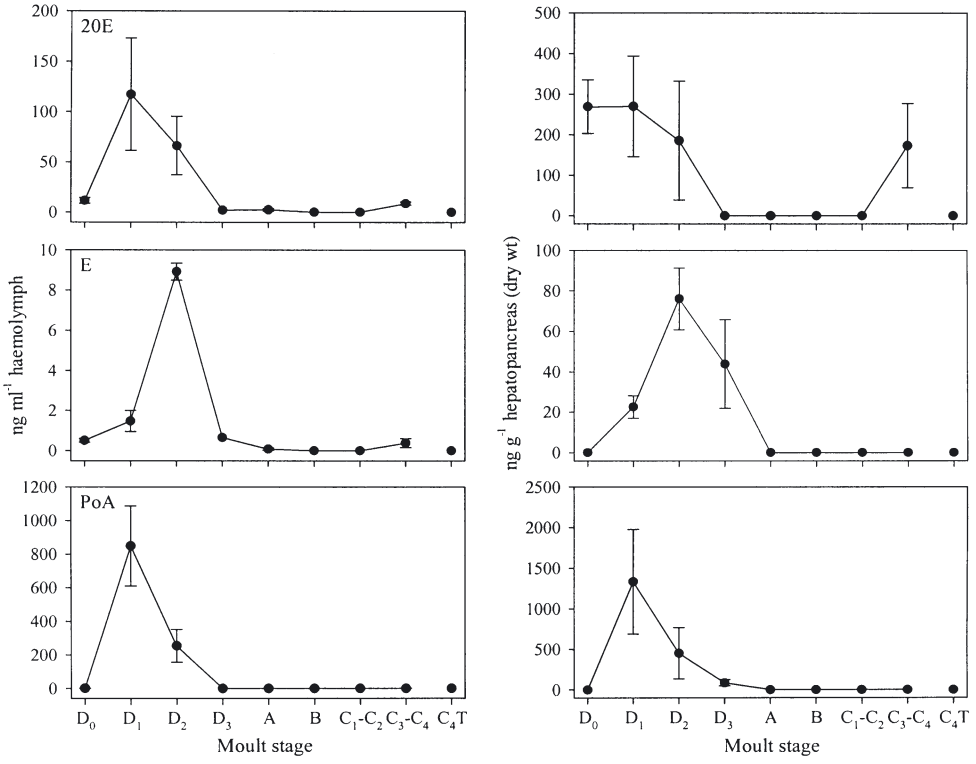

Fig. 2. *Carcinus maenas*. Variations in ecdysteroid concentrations in haemolymph and hepatopancreas over moult cycle (mean ± SD, 8 to 12 individuals in each group). 20E: 20-hydroxyecdysone; E: ecdysone; PoA: Ponasterone A. Note differences in ordinate scales

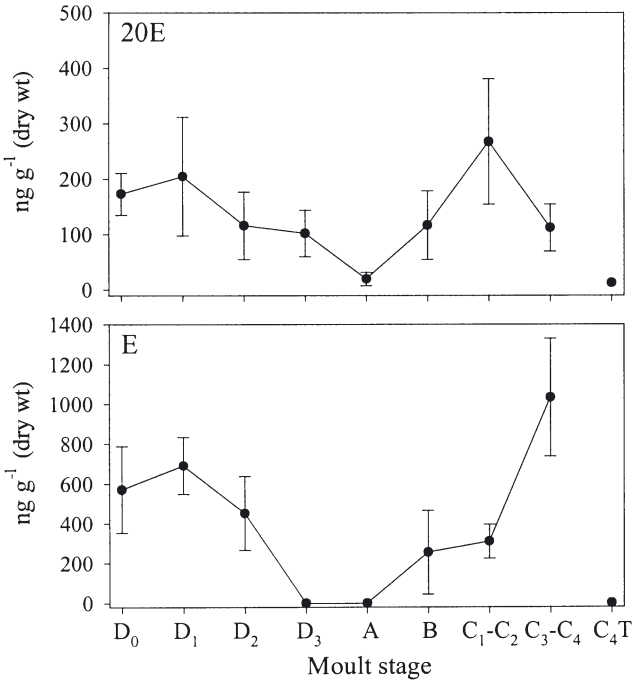

Fig. 3. *Carcinus maenas*. Variations in ecdysteroid concentrations in testis over moult cycle (mean ± SD, 5 to 10 individuals in each group). 20E: 20-hydroxyecdysone; E: ecdysone

20E levels in both haemolymph and hepatopancreas and 100- and 20-fold higher than E in the haemolymph and hepatopancreas, respectively. This makes PoA by far the most abundant ecdysteroid in premoult crabs.

PoA was never observed in the testis of male shore crabs, whereas E and 20E content varied over the moult cycle (Fig. 3). In the testis, 20E was found in all moult stages; however, in A-stage crabs, 20E levels were significantly lower ($p < 0.01$) than in any other stage (19.6 ng g^{-1} dry wt). No significant differences were observed between the other moulting stages, levels ranging from 102 to 267 ng g^{-1} dry wt. In the testis of shore crabs in C$_4$T, 20E was only observed in a single individual (12.4 ng g^{-1} dry wt). Ecdysone levels also varied over the moult cycle, with high levels (452 to 1004 ng g^{-1} dry wt) in the late intermoult (C$_3$ to C$_4$) and premoult (D$_0$ to D$_2$), and low levels in stages from late premoult (D$_3$) to early intermoult (C$_1$ to C$_2$). E levels in the testis during late intermoult and premoult were therefore 10-fold or higher than in hepatopancreas in the same individuals.

Variations in ecdysteroid levels in C$_4$ crabs in relation to colour, size and season

The increased haemolymph and hepatopancreas ecdysteroid levels in C$_4$ individuals indicate preparation for premoult and ultimately the onset of a new moult cycle. 20E was the dominant ecdysteroid in male shore crabs in Moult Stage C$_4$. Fig. 4 shows the haemolymph levels of 20E at Stage 4 in relation to coloration, size and season. On average, green C$_4$ shore crabs had 8.1 ng ml^{-1} 20E in the haemolymph, whereas red C$_4$ shore crabs had 4.5 ng ml^{-1} 20E, but this difference was not significant (Fig. 4A).

Fig. 4B demonstrates a significant ($p < 0.001$) negative correlation between the haemolymph 20E level and size over a size range of 37 to 77 mm CW. Haemolymph 20E concentrations exceeding 5 ng ml^{-1} were

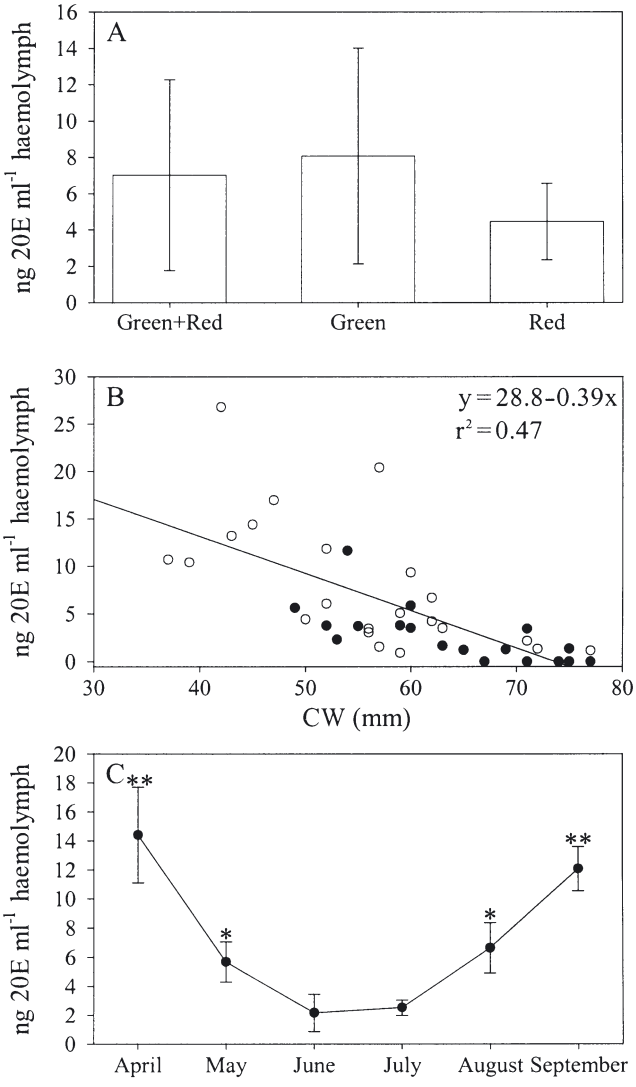

Fig. 4. *Carcinus maenas*. 20-hydroxyecdysone (20E) concentrations in haemolymph of Late Intermoult Stage C$_4$ crabs. (A) Colour variations (mean ± SD, 17 to 23 individuals in each group). (B) Variations in carapace width (CW): (○) green crabs; (●) red crabs. (C) Seasonal variation (mean ± SD, 7 to 12 individuals in each group). *,**: significant differences from June sample at $p < 0.05$ and $p < 0.01$, respectively

never observed in shore crabs with CW >62 mm, and only a single red individual had 20E levels higher than 5 ng ml^{-1} haemolymph.

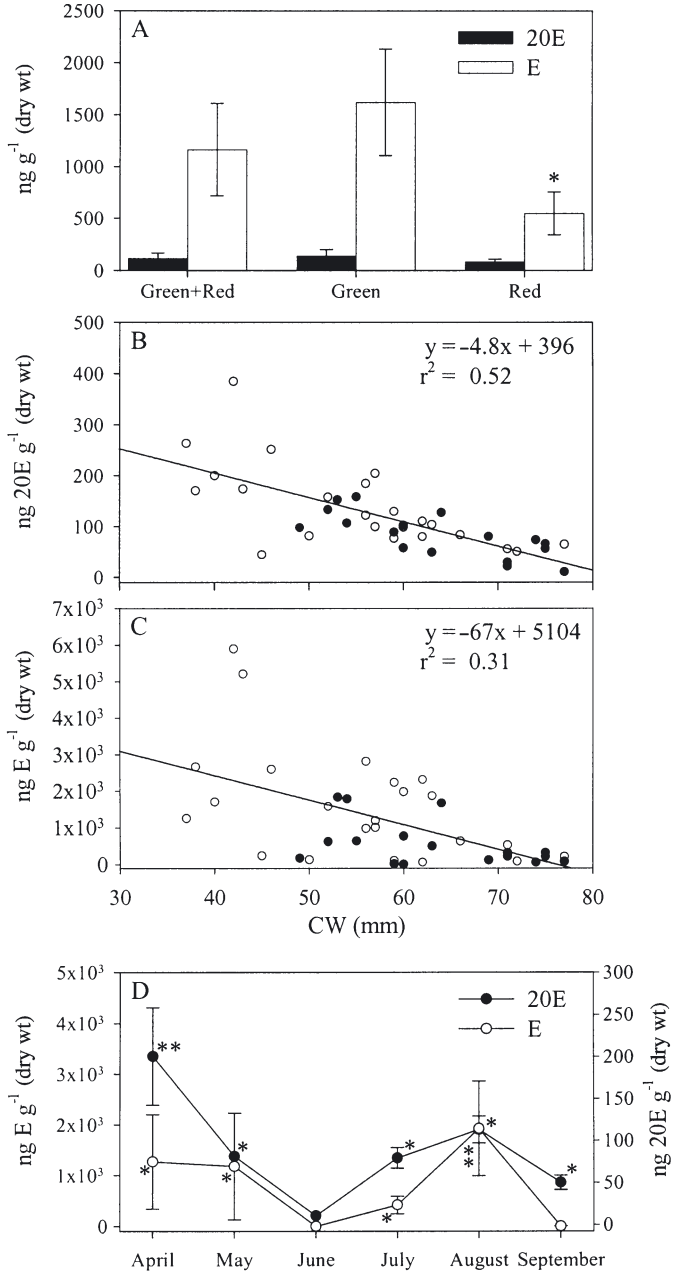

Fig. 5. *Carcinus maenas*. Ecdysone (E) and 20-hydroxyecdysone (20E) concentrations in testis of Intermoult C_4 crabs. (A) Colour variations (mean ± SD, 17 to 23 individuals); open bars: ecdysone (*: significant difference between red and green crabs at $p < 0.05$); black bars: 20-hydroxyecdysone. (B,C) Variations in carapace width (CW): (○) green crabs; (●) red crabs. (D) Seasonal variations: (○) ecdysone; (●) 20-hydroxyecdysone (mean ± SD, 7 to 11 individuals); *,**: significant differences from June sample at $p < 0.05$ and $p < 0.01$, respectively

Haemolymph 20E levels in C_4 crabs varied during the growth season (Fig. 4C). In late April and September, levels were significantly ($p < 0.05$) higher (14.4 and 12.1 ng ml^{-1}, respectively) than in the remaining samples. Haemolymph 20E levels decreased during late spring and were lowest (approx. 2 ng ml^{-1}) in early June, which is the peak of the male moulting season, and in late July, which is the onset of the mating season. In late August, after moulting and mating, haemolymph 20E levels increased to approximately 7 ng ml^{-1}, significantly ($p < 0.05$) higher than levels in June and July. No shore crabs in the C_4 moult stage with a CW lower than 62 mm were caught in June; only crabs in premoult, postmoult or early intermoult (C_1 to C_2) were found in this size class. Accordingly, the June sample is biased towards large crabs, but no significant size difference was observed between the remaining groups.

Testicular E levels in Stage C_4 crabs were much higher than levels in the haemolymph and particularly in the hepatopancreas, where only traces of E were found in this moult stage. Fig. 5 shows variations in testis levels of E and 20E in C_4 crabs in relation to colour, size and season. On average, green crabs contained 1619 ng E g^{-1} dry wt, which is significantly ($p < 0.01$) more than the 547 ng E g^{-1} dry wt observed in red crabs. Green and red crabs contained 83 and 44 ng 20E g^{-1} dry wt, respectively, but this difference was not significant (Fig. 5A). The high ecdysteroid concentrations observed in the testis of C_4 crabs implies that the majority of ecdysteroids found were located in the testis even though testis is a minor organ in terms of gonad/somatic index.

For both 20E and E, testis ecdysteroid levels were significantly negatively correlated ($p < 0.001$) to size (Fig. 5B,C). Even though E levels as high as 5892 ng E g^{-1} dry wt were observed in the testis of C_4 shore crabs, E levels above 1000 ng E g^{-1} dry wt were only observed once in red shore crabs with a CW >59 mm.

Similar to haemolymph levels, the testicular E and 20E levels decreased from April to June, and increased again throughout the mating season towards August (Fig. 5D). In contrast to the haemolymph, however, testis ecdysteroid levels decreased again after the mating season. Testicular E levels in crabs collected in June and September were significantly ($p < 0.05$) lower than in crabs from other samples. Also, testicular E levels were significantly ($p < 0.05$) lower in crabs collected in July than in crabs collected in August. No significant differences were found between the remaining groups. 20E levels were significantly ($p < 0.05$) lower in June than in the remaining samples. Also, 20E levels were significantly ($p < 0.05$) higher in April than in July and September.

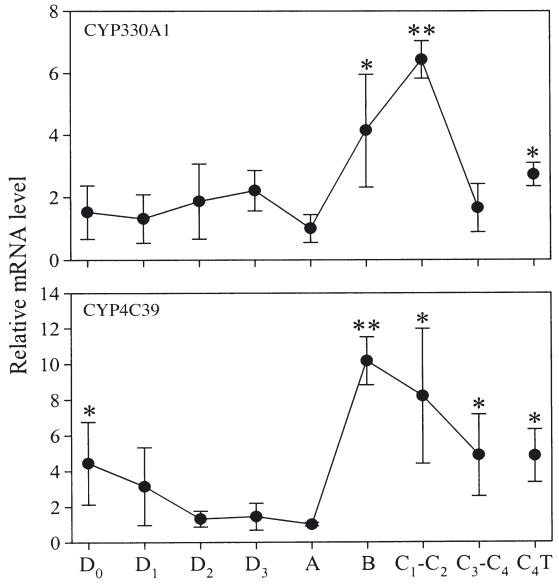

Fig. 6. *Carcinus maenas*. Moult cycle variations in hepatopancreas CYP330A1 and CYP4C39 mRNA levels (mean ± SD, n = 5 to 8). *,**: significant differences from Moult Stage A at $p < 0.05$ and $p < 0.01$, respectively

Moult cycle variations in *CYP330A1* and *CYP4C39* gene expression

The relative hepatopancreas CYP330A1 and CYP4C39 mRNA levels varied over the moult cycle, with low levels during premoult (D_0 to D_3) and early postmoult (A), when crabs are soft-shelled (Fig. 6). The mRNA levels of CYP330A1 and CYP4C39 increased significantly ($p < 0.05$) from early postmoult (A) to late postmoult (B) and early intermoult (C_1 to C_2). Maximum CYP330A1 and CYP4C39 mRNA levels were observed in C_1 to C_2 and B stages, respectively. From A to C_1 to C_2, CYP330A1 mRNA levels increased 6.4-fold and were significantly ($p < 0.01$) higher in early intermoult (C_1 to C_2) than in late intermoult (C_3 to C_4/C_4T), premoult ($p < 0.05$) or early postmoult (A).

Between Stages A and B, CYP4C39 mRNA levels increased 10.5-fold and CYP4C39 mRNA levels were significantly lower during late premoult (D_2 to D_3) and early postmoult (A) than during late postmoult (B) ($p < 0.01$), intermoult (C_1 to C_4) ($p < 0.05$) and early premoult (D_0 to D_1) ($p < 0.05$). Also, the CYP4C39 mRNA level in C_4T was significantly higher than in Stages D_2, D_3 and A ($p < 0.05$).

The *CYP330A1* gene expression was detectable in all moult stages, whereas CYP4C39 was almost undetectable in the D_2, D_3 and A stages. CYP330A1 and CYP4C39 mRNA levels were not detectable in the testis and vas deferens by Northern blot analysis. Accordingly, moult cycle variations could not be studied in these organs.

DISCUSSION

The present study confirms previous findings on ecdysteroid levels variations during the moult cycle in male shore crabs, and adds significant information regarding their ecophysiological relevance: E and 20E levels in the haemolymph and hepatopancreas are strongly correlated. The haemolymph E level is tightly correlated to E production in the Y-organ in relation to moulting (Lachaise et al. 1989). In peripheral tissues including the hepatopancreas, E is converted into 20E, which is further metabolised into inactive forms such as 20,26-OH-E, excreted primarily in urine (Snyder & Chang 1991, Kayser et al. 1997, Williams et al. 2000). The low ecdysteroid levels in the tissues just prior to and after moulting indicate that all active ecdysteroids must be removed in order to allow chitinisation of the new exoskeleton (Freeman 1980).

The E and 20E levels in the testis vary independently of this system. In crustaceans, the testis has a high 20-monooxygenase activity, converting E into 20E (James & Shiverick 1984). Except in Early Postmoult Stage A, testis levels of 20E are high and largely constant throughout the moult cycle. The testicular E level approaches zero only during Late Premoult D_3 and Early Postmoult A. These 2 stages last only 3 to 6 d in all, i.e. about 1 to 2 % of the total adult moult cycle. In the remaining stages, lasting about 1 yr, the crabs maintain high testicular E and 20E levels even during stages where other tissue levels approach zero. This suggests that the crabs testis is a possible source of ecdysone production in addition to the Y-organ. Whether conversion of E into 20E within the testis is important for spermatophore maturation is presently unknown.

The hepatopancreas and haemolymph PoA levels are strongly correlated during the moult cycle. The high accumulation of PoA in the hepatopancreas during premoult indicates that further conversion or inactivation of PoA may occur in the hepatopancreas; however, the mechanism as well as the biochemical role of PoA is presently not understood.

PoA is present in the haemolymph and hepatopancreas but not in the testis. Accordingly, PoA is likely to be involved in growth but not in reproduction, and the metabolic pathways of ecdysteroids must therefore be tissue-specific. In female shore crabs, PoA levels in ovaries increase during ovarian maturation (Lachaise et al. 1981, B. Styrishave et al. unpubl.) and also in eggs during embryonic development (McCarthy 1979, McCarthy & Skinner 1979, Lachaise & Hoffmann 1982, Goudeau & Lachaise 1983). To our knowledge, a role of PoA in spermatophore maturation has not been described, however, Lachaise & Lafont (1984) demonstrated that the testes of *Carcinus maenas* are capable

of transforming radiolabelled PoA into 20E and inokosterone by C-25 and C-26 hydroxylation, respectively. This indicates that PoA may have quite different roles in male and female shore crabs. PoA might be essential for gonad and/or embryo development or, alternatively, PoA may be stored in apolar forms (Chan 1995) to be released and used in embryo development until the Y-organs develop and become active in the embryo.

The negative correlation between C_4 haemolymph 20E levels and size must have a physiological explanation. In shore crabs, males compete aggressively for receptive females during the mating season, and males must therefore moult to a certain size to achieve mating success. The new exoskeleton is bright green in appearance. As the exoskeleton becomes older, however, it gradually changes to a darker red colour (Reid et al. 1997). The mechanism for this colour change is unknown, but it has been suggested that the exoskeleton pigment astaxanthin turns red during photo-denaturation (Jencks & Buten 1964, Lee 1977). Reid et al. (1994) demonstrated that the mating success of male shore crabs increased dramatically in size classes above 61 mm CW. All crabs above this size caught during the male moulting season had little or no 20E in the haemolymph in the present study, and consequently large crabs may completely suspend moulting and enter terminal anecdysis, C_4T. This leads to an increase in the proportion of red males with increasing size (McGaw et al. 1992, Abelló et al. 1994, Aagaard et al. 1995). Obviously, male shore crabs already grown to a size allowing access to receptive females have no need to invest resources into growth, and more energy may be allocated into the development of the reproductive apparatus. The low haemolymph ecdysteroid levels in large male shore crabs in Stage C_4 are likely to reflect the lack of a need to enter premoult.

B. Styrishave (unpubl.) found that the amount of reproductive tissue in shore crabs in late June, after moulting but prior to mating, can be described as follows: W_T = 4E-09 $CW^{4.6}$, where CW is the carapace width (in mm) and W_T is the wet weight (in g) of reproductive tissues (testis + vas deferens). Consequently, the relative size of the reproductive tissues increases when the crabs grow. If ecdysteroids are essential for spermatozoa maturation, testis ecdysteroid levels would be expected to increase with increasing size. In the present study, this was clearly not the case. In fact, testis ecdysteroid levels decreased significantly with increasing size and green crabs had much higher ecdysteroid levels in the testes than red crabs. Testis levels of E and 20E were high throughout the growth season, except during the male moulting season in early to midsummer. A similar variation was observed in the haemolymph for 20E. This indicates that both somatic tissue growth and spermatozoa production occur in crabs with CW < 62 mm, only being briefly suspended when the crabs are actually moulting. In the crab *Metopograpsus messor*, spermatogenesis is influenced by moult stage (Suganthi & Anilkumar 1999). During intermoult, a uniform proportion between proliferative and maturing sperm cells exists. The onset of premoult, however, results in a reduction of maturing cells but an increase in spermatogonial proliferation. A similar pattern is observed in the spider crab *Libinia emarginata*, which is known to exhibit terminal anecdysis. In this species, the highest amounts of testis ecdysteroids are found in non-reproductive males, whereas testis ecdysteroid levels are low in actively mating males (Laufer et al. 1993). The data presented here are in accordance with these studies and indicate that ecdysteroids may play different roles in germ proliferation and maturation and that this process is affected by moult stage and reproductive status. In view of this, a study of germ cell proliferation and maturation in shore crabs is necessary. The fact that ecdysone is largely undetectable in the testes of shore crabs after the mating season (September), which also concludes the growth season, indicates a seasonal diapause in male shore crab gamete development, which further adds to the complexity of shore crab growth and reproduction.

Considering the importance of ecdysteroids in moulting, and presumably reproduction, surprisingly little is known about their metabolic pathways and the enzymes involved. Ecdysteroids are activated and inactivated by CYP enzyme-mediated hydroxylations. Only recently have the CYP enzymes responsible for the last 2 steps of the biosynthesis of E (Warren et al. 2002) and the hydroxylation to 20E been discovered in a single species of insect (Petryk et al. 2003). The enzyme inactivating 20E by converting it into 20,26-OH-E has not yet been characterised. In marine invertebrates none of these enzymes are known. The only CYP enzymes reported in any crab species so far are the CYP330A1 and CYP4C39 from *Carcinus maenas* (Rewitz et al. 2003). Injection of E or PoA into the haemolymph of male late intermoult shore crabs (C_3 to C_4) increased the *CYP330A1* gene expression significantly by 5.8-fold in the hepatopancreas, which is known to be the primary tissue for ecdysteroid catabolism (Rewitz et al. 2003), suggesting that *C. maenas* CYP330A1 may be involved in the catabolism of ecdysteroids in association with growth, development or reproduction. The significant increase in hepatopancreas *CYP330A1* gene expression in postmoult and early intermoult crabs in the present study supports these findings. If CYP330A1 is involved in the catabolism of ecdysteroids, it seems reasonable that this enzyme is induced in the postmoult, when ecdysteroids are apparently being removed. The fact that *CYP330A1* gene expression is high in the hepatopan-

creas but undetectable in the testis and vas deferens suggests different roles of CYP330A1 in ecdysteroid catabolism during moulting and reproduction.

The CYP4C39 expression also varies over the moult cycle and is also largely undetectable in the testes of male shore crabs. However, CYP4C39 differs from CYP330A1 in 2 important aspects: CYP4C39 is not inducible by ecdysteroids and certain xenobiotica (Rewitz et al. 2003), and it is expressed at high levels in the gills, hepatopancreas, heart, eye-stalk and intestine (in descending order) (in particular during Intermoult Stage C), but not in the testis, vas deferens, muscle or Y-organ (K. Rewitz et al. unpubl.). This is in contrast to CYP330A1, which is expressed only in the hepatopancreas, and indicates that CYP4C39 has a much broader physiological role than CYP330A1, which is not directly related to ecdysteroid metabolism. Vertebrate CYP4 enzymes are involved in fatty acid metabolism (Simpson 1997), and CYP4C1 from fat bodies of insects (54% amino acid homology to CYP4C39) is controlled by the hyperglycemic hormone and most probably catalyses fatty acid oxidation (Bradfield et al. 1991). The crustacean hyperglycemic hormone (CHH) controls lipids and fatty acid levels in the haemolymph of *Chasmagnathus granulata* and *Carcinus maenas*, respectively. Eyestalk ablation terminates CHH production, decreases fatty acid levels, and frequently induces ecdysis (Santos et al. 1997), during which the CYP4C39 mRNA level is lowest. Based on analogy, we therefore speculate that *C. maenas* CYP4C39 may be involved in fatty acid metabolism and could be under hormonal control of CHH; however, further investigations are necessary.

In the present study, the *CYP330A1* and *CYP4C39* gene expressions were inversely related to ecdysteroid levels. A relationship between CYP enzyme levels and tolerance to certain xenobiotics may exist. The concentration of polycyclic aromatic hydrocarbons (PAHs) in the hepatopancreas was approximately 3 times higher in newly moulted Stage A blue crab *Callinectes sapidus* than in intermoult Stage C crabs (Mothershead & Hale 1992). Mothershead & Hale (1992) suggested increased water uptake and exoskeleton permeability during ecdysis and decreased PAH metabolism to be the explanation. We have observed higher mortalities during exposure of late premoult (D_2 to D_3) crabs to pyrene compared to late intermoult (C_3 to C_4) crabs (unpubl. result). Also, Cytochrome p_{450} enzyme-mediated BaP-hydroxylase activity was lowered in *C. sapidus* during premoult and at ecdysis and then increased in postmoult and intermoult (Singer & Lee 1977). Therefore, a decrease in CYP enzyme activity around ecdysis may at least partly explain why crabs accumulate high PAH concentrations and suffer higher mortalities around ecdysis.

The present study provides evidence that ecdysteroids are involved in both growth and reproduction in the shore crab *Carcinus maenas*. The moult cycle variations in *CYP330A1* and *CYP4C39* gene expressions indicate that these enzymes may be involved in growth, CYP330A1 possibly through ecdysteroid metabolism. The data presented here also support the concept that large male shore crabs may delay moulting or may enter a state of terminal anecdysis. This appears to influence the involvement of ecdysteroids in spermatogenesis; however, a detailed study on germ cell proliferation and maturation is essential to understand the relationships between growth and reproduction.

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