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Ecdysteroids in female shore crabs *Carcinus maenas* during the moulting cycle and oocyte development

BJARNE STYRISHAVE, TORBEN LUND AND OLE ANDERSEN
Department of Science, Systems and Models, University of Roskilde, PO Box 260, 4000 Roskilde, Denmark

The ecdysteroids ecdysone (E), 20-hydroxyecdysone (20E) and ponasterone A (PoA) were measured in the haemolymph, hepatopancreas and oocytes of female shore crabs Carcinus maenas *during the moulting cycle and during oocyte maturation using HPLC-MS. In the haemolymph, ecdysteroid titres varied over the moulting cycle with high levels during premoult and low levels during postmoult and intermoult, however, no significant change in haemolymph ecdysteroid titres was observed in relation to oocyte development. In the hepatopancreas, PoA levels were high during premoult but low during postmoult and intermoult. This is in contrast to E and 20E where levels remained high from early intermoult (C_1) until late premoult (D_3) and only decreased during postmoult. In the oocytes, ecdysteroid levels were low during postmoult and for 20E and PoA also during late premoult D_2 and D_3. In contrast, all three ecdysteroids were observed to increase in the oocytes during oocyte development, in particular E and PoA. The present study demonstrates that changes in haemolymph ecdysteroid titres relate to changes in moulting status and not to changes in oocyte development. Also, the study indicates that the hepatopancreas is involved in the metabolism of ecdysteroids related to the moulting cycle but may also be involved in ecdysteroid metabolism during oocyte development. Furthermore, the pronounced increase in oocyte ecdysteroids during oocyte development during periods where haemolymph ecdysteroids titre is low, indicates that the oocytes are capable of* de novo *synthesis of the three ecdysteroids investigated.*

Keywords: *Carcinus maenas*; ecdysone; growth; 20-hydroxyecdysone; ponasterone A; reproduction; shore crab

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INTRODUCTION

In female shore crabs, *Carcinus maenas*, moulting development and egg preparation is tightly coupled. In the Isefjord, most females spawn in early July, when most of the females are in intermoult or early premoult. Mating usually occurs in August just after the female has moulted when it is still soft-shelled, as this is the only time in which the oviducts are open and can be penetrated by the male.

In crustaceans, the moulting hormones, ecdysteroids, are heavily involved in the regulation and physiological control of moulting and evidence is mounting that ecdysteroids are also vital in gamete production and maturation (review by Subramonian, 2000). The major ecdysteroids involved in shore crab growth and reproduction appear to be ecdysone (E), 20-hydroxyecdysone (20E) and ponasterone A (PoA). At least two ecdysteroids, presumably synthesized from cholesterol, 25-deoxyecdysone and ecdysone are excreted into the haemolymph by the Y-organ (Chang *et al.*, 1976; Soumoff & Skinner, 1988; Lachaise *et al.*, 1989). These compounds are hydroxylated in peripheral tissues to produce PoA and the active moulting hormone 20E. In insects, all ecdysteroid intermediates produced from 5ß-ketodiol to 20E are known and it has been firmly established that a group of cytochrome P450 (CYP) enzymes, the so-called 'Halloween enzymes' are responsible for each hydroxylation step (Warren *et al.*, 2004). In crustaceans, however, the production of ecdysteroids from cholesterol and the enzymes performing the necessary hydroxylations are entirely unknown and not a single Halloween gene or enzyme has been identified. Furthermore, PoA is not found in insects and so far appears to be unique to crustaceans (Lachaise *et al.*, 1989). During premoult, PoA is found in shore crab haemolymph in higher concentrations than any other ecdysteroids (Styrishave *et al.*, 2004), but its involvement in growth and reproduction is unknown. Ecdysone 20-monooxygenation is known to occur in several tissues of crabs (Chang *et al.*, 1976; James & Shiverick, 1984; Soumoff & Skinner, 1988) but the specific enzymes and the tissues involved in the *de novo* synthesis of ecdysteroids are unknown. It is presently unclear as to whether the ecdysteroids needed for oogenesis, vitellogenesis and embryogenic development after spawning are synthesized by the eggs themselves during development or ecdysteroids are being synthesized by other tissues such as the hepatopancreas and then transported to the developing eggs by the haemolymph.

The process of moulting preparation in females occurs simultaneously with the maturation of the ovaries. Consequently, ecdysteroid production and levels in haemolymph and tissues such as hepatopancreas and ovaries that are involved in these processes can, at any given time, be affected by and contribute to, both developmental schemes. To study the influence of ecdysteroid changes on the moulting cycle and oocyte development, we investigated the distribution of the three major crustacean ecdysteroids E, 20E and PoA in haemolymph,

Corresponding author:
B. Styrishave
Email: styris@ruc.dk

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hepatopancreas and eggs during the moulting cycle and during egg development from early oogenesis to early embryogenesis.

MATERIALS AND METHODS

From mid-April until late September, female shore crabs, *Carcinus maenas*, were caught with approximately two weeks interval in un-baited eel seines in the Isefjord at Rørvig, Zealand, Denmark. Haemolymph samples were collected from the 4th pereiopod and immediately frozen in liquid nitrogen, the crabs were then dissected and hepatopancreas and eggs were collected and frozen. Only intact individuals were included in the study since the loss of a single or several periopods may affect ecdysteroid levels and moult cycle patterns. Moult stages were assessed according to Aiken (1973) and O'Halloran & O'Dor (1988). Oocyte development was separated into five stages on the basis of three morphological characteristics, oocyte diameter, oocyte colour and reproductive index (Lachaise & Hoffmann, 1977; Gunamalai *et al.*, 2004); RI is the total amount of oocytes in wet weight as percentage of crab fresh weight. In oocyte developmental Stage 1 the ovaries contain primary oocytes with a diameter of less than 100 μm. The oocytes are translucent or white in colour and with a reproductive index (RI) $>1\%$. Stage 2 is early vitellogenesis with oocytes with diameters of 100–200 μm, white/yellow/orange in colour, and RI of 1–4%. Vitellogenesis occurs during Stage 3 with oocytes of 200–300 μm in diameter, yellow/orange in colour and $4 < \mathrm{RI} < 6.8\%$. Stage 4 was eggs with an oocyte diameter greater than 300 μm, they were all orange in colour and the RI was higher than 6.8%. In this stage of late vitellogenesis, most oocytes are mature. Stage 5 corresponds to early embryogenesis. In this stage, eggs were attached to the abdomen of the female and egg diameters were up to 346 μm.

Ecdysteroids from haemolymph, hepatopancreas and gonads/eggs were extracted and analysed according to Styrishave *et al.* (2004). In total 403 samples from 139 individuals were analysed. In ten individuals, gonads were too small to be recovered during dissection. Haemolymph (100–300 μl) or tissue samples (50–150 mg, wet weight) and makisterone A (MaA) (1 ml of 0.1 to 0.3 μg ml^{-1} in methanol) added as internal standard were homogenized in a 10 ml mixture of water and chloroform (1:1) and centrifuged for 10 minutes at 4000 rpm. The supernatant (aqueous phase) was collected, another 5 ml water was added and the sample was mixed and centrifuged again. This procedure was performed four times in all. The water extracts were then eluted on a reverse-phase cartridge (Water Oasis™ HLB) preconditioned with 2 ml water and 5 ml methanol. To elute the ecdysteroids from the cartridge, 5 ml 60% methanol in water was then added. The extract was then reduced to dryness by nitrogen and re-dissolved in 100 μl methanol and diluted with 200 μl water and transferred to a vial.

Samples were analysed using high performance liquid chromatography coupled to mass spectrometry (HPLC-MS). The HPLC instrument was a TSP Spectra system with an AS3000 autosampler, a P4000 gradient pump and a vacuum degasser. The mass detector was a LCQ-Deca ion-trap instrument (Thermo-Finnigan) fitted with an atmospheric pressure chemical-ionization (APCI) interface running in negative mode; metal needle potential: −5 kV; discharge current: 4.5 μA; capillary voltage: −26V; heated capillary temperature: 200°C; vaporizer temperature: 350°C; sheet gas: N_2 (31 arbitrary units); auxiliary gas: N_2 (8 arbitrary units). The analytical column was a 50 mm Waters Xterra MS reverse phase C18 column with an inside diameter of 2.1 mm equipped with a 10 mm guard column (Xterra RP C18). Twenty μl aliquots of samples containing ecdysteroids were fractionized using a linear gradient of 35% (v/v) methanol in water to 60% (v/v) in water over 20 minutes at a flow rate of 0.2 ml min^{-1}. The MS detector was run in selective ion monitoring (SIM). Specific mass to charge intervals (m/z) were 464.9–465.9 $[E\text{-}H]^-$ and $[PoA\text{-}H]^-$, 479.5–480.5 $[20E\text{-}H]^-$, 488.8–489.8 $[PoA + MeOH\text{-}H]^-$, and 492.8–493.8 $[MaA\text{-}H]^-$. The chromatographic and mass spectrometric analyses were controlled by the LCQ software Xcalibur 1.2.

Response factors were calculated from authentic standards purchased from Sigma (E and 20E) and G.E. Scientific (PoA) (>95% purity). Response factors relative to MaA were $E = 0.90 \pm 0.07$, $20E = 1.03 \pm 0.05$, $PoA = 1.06 \pm 0.05$. Detection limits were <100 pg g^{-1} for E and PoA and <10 pg g^{-1} for 20E.

One-way analysis of variance and Student's *t*-test were used to analyse data that were normally distributed. Data that were not normally distributed were log transformed after which a normal distribution could be obtained. Levene's test was employed to test for homogeneity of variances.

RESULTS

Shore crabs, *Carcinus maenas*, were caught throughout the growth season from April until September. The numbers of intact individuals caught each month from which gonads could be recovered were: April: 16; May: 19; June: 17; July: 21; August: 34; and September: 22. Table 1 demonstrates that female shore crabs prepare for both moult and reproduction simultaneously and go through all moult stages and oocyte developmental stages during this period. In April, when shore crabs return to shallow waters, most females that were caught were in intermoult Stages C_2–C_4, a few (less than 10%) were in either C_1 or D_0. All these females were in oocyte developmental Stages 2–4. In May, the female moult stages were similar to that of females caught in April, but some of the females had eggs attached to their periopods (Stage 5). In June, most females were in intermoult (C_2–C_4) but several females (29% of the catch) had now propagated into premoult D_0 and D_1 and a single female was in postmoult B. All oocytes from these females were in Stages 2–4, or they had eggs attached to their periopods. In July, all moult stages from C_2–D_2 were observed with 43% being in intermoult C_2–C_4 and 57% being in premoult D_0–D_2. All oocyte

Table 1. *Carcinus maenas*: range in moult stage and oocyte developmental stage of females caught each month during the growth season from April until September. Moult stages in parentheses are moult stages in which less than 10% of the crabs caught were in. *, only a single individual in this stage was caught; **, two individuals in this stage were caught.

Month	Moult Stage	Egg Development Stage
April	$C_2 \rightarrow C_4$ (C_1, D_0)	$2 \rightarrow 4$
May	$C_2 \rightarrow C_4$ (C_1, D_0)	$2 \rightarrow 5$
June	$C_2 \rightarrow D_1$ (B*)	$2 \rightarrow 5$
July	$C_2 \rightarrow D_2$	$3 \rightarrow 1$
August	$C_2 \rightarrow C_1$	1 (4**)
September	$C_1 \rightarrow C_4$	$1 \rightarrow 2$

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stages from Stage 3 to Stage 1 were observed. Females with oocytes in Stage 1 had presumably spawned and entered a new ovarian cycle with very small creamy white oocytes. In the present study, August was the only month in which females of all moult stages could be caught. With the exception of two females with oocytes in Stage 4, all females caught possessed oocytes in Stage 1. In September, all females caught were in intermoult C_1–C_4 and the oocytes were in either Stage 1 or Stage 2.

Figure 1 shows the percentage of female shore crabs in each oocyte developmental stage in relation to moult stage. The majority of the egg development occurs when the crabs are in intermoult C_1–C_4 with the majority of females carrying eggs in vitellogenesis and late vitellogenesis. Female shore crabs with eggs attached to their periopods are all in late intermoult C_3–C_4 or in early premoult D_0. Just prior to moulting (moult Stages D_2 & D_3) and after moulting when the female is soft-shelled (moult Stages A & B) all eggs have been released and the new eggs formed in the ovaries are primary oocytes in Stage 1.

Variations in 20E, E and PoA over the moulting cycle for haemolymph, hepatopancreas and oocytes are shown in Figure 2. In the haemolymph, all three ecdysteroids are found in the highest concentrations in premoult, peaking in D_2 with PoA found in the highest concentrations (186 ng ml^{-1}). However, all three ecdysteroids decrease just prior to moult, and when the shore crabs enter postmoult Stage A, ecdysteroids are at their lowest level, 3.1 ng ml^{-1} for 20E and less than 0.1 ng ml^{-1} for and E and PoA which is close to the detection limits. During postmoult and intermoult C_1–C_4, 20E increase steadily to 38 ng ml^{-1} in early postmoult Stage D_o, and this level is maintained during Stage D_1, increasing to its maximum in D_2. E increases during postmoult Stage B to ~1 ng ml^{-1} and this level is maintained throughout intermoult and early premoult until D_2. PoA remains low from A to D_1, increasing to its maximum in D_2. For 20E and E, moult Stage A is significantly ($P < 0.05$) lower than all other moult stages. Also, the moult Stages D_2 and D_3 are significantly higher than the remaining stages ($P < 0.05$). For PoA, the premoult Stages D_2 and D_3 are significantly different for Stage A (D_2: $P < 0.001$; D_3: $P < 0.01$).

In the hepatopancreas, a similar pattern can be observed for PoA. In this tissue, levels are also high during the postmoult Stages D_1–D_3, peaking in D_2 (348 ng g^{-1}, dw). In the remaining stages, PoA levels approach detection limits. Moult stages D_1, D_2 and D_3 are significantly higher than the remaining stages (D_1: $P < 0.05$; D_2: $P < 0.001$; D_3: $P < 0.01$). For 20E and E, levels are high in all intermoult and premoult stages but decrease shortly during postmoult (20E: Stage A; E: Stages A & B). The 20E levels decrease just after moulting in postmoult Stage A but increase in late postmoult Stage B. 20E are found in significantly lower levels in Stage A than in all other stages ($P < 0.05$) with the exception of Stage D_o. There is no significant difference in 20E levels between the remaining stages. Ecdysone levels are approaching detection limits in both postmoult stages (A & B). Also, E levels decrease during premoult and are significantly higher in D_o than in D_3 ($P < 0.05$). There are no significant differences between the remaining stages.

The variations in oocyte 20E levels were very similar to that observed for hepatopancreas. The 20E level was low in postmoult Stage A, but higher in all the remaining stages with no significant differences observed between these stages. The 20E level of Stage A was significantly lower than the Stages D_1, D_2, D_3 and B ($P < 0.05$). For E, levels decreased during premoult and in D_2 and D_3 E levels were on the limits of detection. E levels increased during both postmoult stages and this level was maintained during intermoult and early premoult. Stage A (12 ng g^{-1}) was significantly ($P < 0.05$) lower than the Stages B (53 ng g^{-1}) and C_3–C_4 (44 ng g^{-1}). For PoA, very low levels around the detection limit were observed from late premoult (D_2) to late postmoult (B). In the remaining stages, levels around 20–30 ng g^{-1} were observed with no significant differences.

Variations in the three ecdysteroids for haemolymph, hepatopancreas and gonads during oocyte development are shown in Figure 3. The haemolymph ecdysteroid levels are largely unaffected by the developing oocytes. For all three ecdysteroids, levels decrease from oocyte developmental Stage 1–5, but in none of the cases is the decrease significant. This is also the case for 20E and E in the hepatopancreas. In contrast, the hepatopancreas PoA levels decrease from oocyte developmental Stage 1 (431 ng g^{-1}) to Stage 5 (7 ng g^{-1}). At Stage 1, the PoA level is significantly higher than all the remaining stages ($P < 0.001$). Also, oocyte developmental Stage 5 is significantly lower than Stage 2 ($P < 0.01$).

All three ecdysteroids were observed to increase in concentration in the oocytes during oocyte development. The oocyte 20E levels increased significantly ($P < 0.05$) from 108 ng g^{-1} at Stage 1 to 226 ng g^{-1} in Stage 4. The 20E level of Stage 5 was 9 ng g^{-1} which was significantly lower than the remaining stages (Stages 1–3: $P < 0.05$; Stage 4: $P < 0.01$). Oocyte E levels also increase during oocyte development, from 6 ng g^{-1} at Stage 1 to 62 ng g^{-1} at Stage 4. The E levels of Stages 3–4 were significantly higher than that of Stage 1 and Stage 2 ($P < 0.01$). This was also significantly ($P < 0.01$) higher than that observed for Stage 5 (5 ng g^{-1}). Also, the oocyte PoA levels increased significantly during oocyte development from 47 ng g^{-1} at Stage 1 to 199 ng g^{-1} at Stage 4 ($P < 0.01$). Furthermore, the PoA level of Stage 5 (21 ng g^{-1}) was significantly lower than the remaining stages (Stage 1: $P < 0.05$; Stages 2–4: $P < 0.01$).

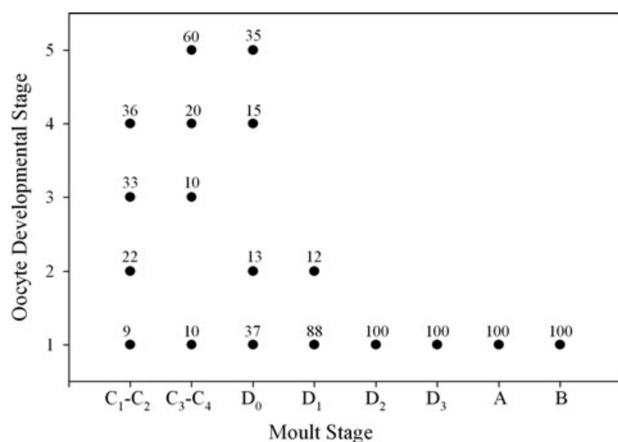

Fig. 1. *Carcinus maenas*: distribution of females in different oocyte development stages (oocyte Stages I–V) in relation to moult stage (•). The value indicates the proportion (%) of females in each oocyte developmental stage for each moult stage. N = 129.

DISCUSSION

In the present study, haemolymph ecdysteroid titres vary with a factor of up to a 100 over the moulting cycle but no

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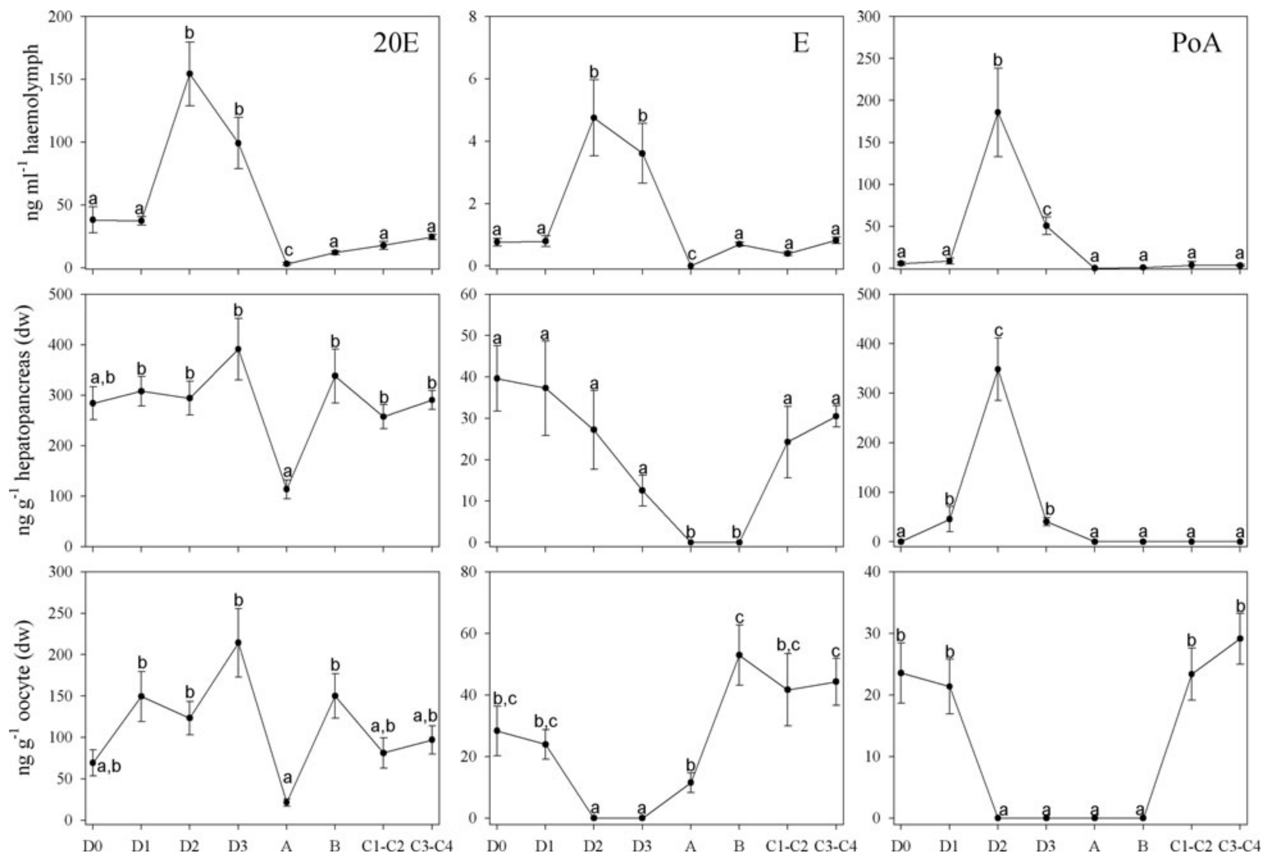

Fig. 2. *Carcinus maenas*: moult cycle variations in ecdysteroid concentrations in haemolymph, hepatopancreas and oocytes (mean ± standard error for 5–21 individuals in each group). 20E, 20-hydroxyecdysone; E, ecdysone; PoA, ponasterone A. Note differences in ordinate scales. Different letters indicate significant differences between moult stages ($P < 0.05$).

significant change in haemolymph ecdysteroid titres can be observed during oocyte development and haemolymph ecdysteroid titres are high when there is little or no egg development (Figure 1). This indicates, that haemolymph ecdysteroids reflect changes in moulting status rather than changes in oocyte development. Figure 1 demonstrates that the decrease in haemolymph and hepatopancreas PoA observed in Figure 3 during oocyte development actually relates to the moulting cycle and not to the reproductive cycle. In oocyte developmental Stage 1, which is the oogenesis, female shore crabs of all moult stages are present, including crabs in premoult with high ecdysteroids titre. This results in a higher average haemolymph and hepatopancreas ecdysteroids titre and greater standard deviations than the remaining oocyte stages. Haemolymph and hepatopancreas PoA levels for intermoult crabs in oocyte developmental Stage 1 are not significantly different from that of the remaining oocyte stages, and consequently the hepatopancreas PoA levels result from changes in moult stages and not changes in oocyte development.

In the hepatopancreas, E and 20E levels remain relatively high and uniform during oocyte development whereas they vary significantly over the moulting cycle. The fact that hepatopancreas ecdysteroid levels are high both during premoult when crabs prepare for moult and during intermoult when egg development occurs indicates that the hepatopancreas is involved in the hormonal control of both growth and reproduction.

In the semi-terrestrial crab, *Gecarcinus lateralis*, and in the spiny lobster, *Panulirus argus*, the hepatopancreas is the primary site for ecdysone 20-monooxygenase (Chang *et al.*, 1976; Soumoff & Skinner, 1988). These studies and the high hepatopancreas E levels in premoult shore crabs (Styrishave *et al.*, 2004; present study) indicate that the hepatopancreas is important in the production of the active moulting hormone 20E. It should be mentioned, however, that James & Shiverick (1984) reported low ecdysone 20-monooxygenase activity in the hepatopancreas of the crab *Pachygrapsus crassipes*. Evidence is mounting that the hepatopancreas is also involved in the inactivation of 20E. In the tobacco hornworm, *Manduca sexta*, the hepatopancreas performs the irreversible hydroxylation of 20E to 20, 26-dihydroxyecdysone (20, 26E) (Williams *et al.*, 2000) and 20, 26E has been reported in the hepatopancreas of lobsters *Homarus americanus* (Snyder & Chang, 1992) and shore crabs (Lachaise & Lafont, 1984).

The hepatopancreas may also potentially be involved in the metabolism of highly polar ecdysteroids such as 20-hydroxyecdysonoic acid produced from 20E and 20, 26E and that of 25-deoxy-20-hydroxyecdysonoic acid produced from PoA excreted primarily in the urine (up to 95%) but also in the faeces (Lachaise & Lafont, 1984; Snyder & Chang, 1992). Furthermore, crustaceans appear to possess an additional route for excreting ecdysteroids by conjugating ecdysteroids into apolar metabolites destined for excretion in the faeces (Snyder & Chang, 1991a, b, 1992). In insects, these apolar ecdysteroids are long-chain fatty acid esters. These apolar conjugates have not been identified in crustaceans; however, in lobsters

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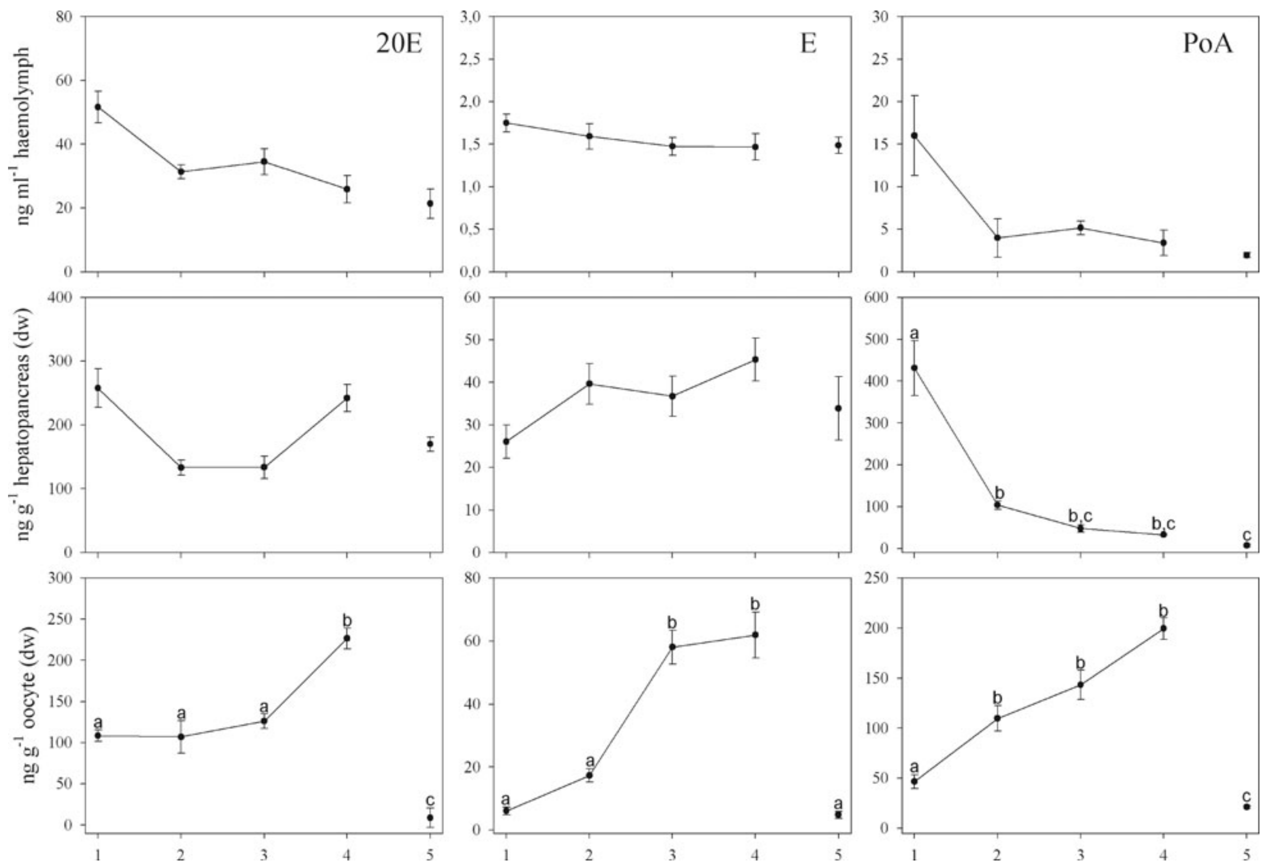

Fig. 3. *Carcinus maenas*: variations in ecdysteroid concentrations in haemolymph, hepatopancreas and oocytes during oocyte development (mean ± standard error for 5–22 individuals in each group). Different letters indicate significant differences between oocytes developmental stages ($P < 0.05$).

H. americanus, there is good evidence, that the hepatopancreas is involved in the production of these apolar ecdysteroids as the hepatopancreas is the only tissue with considerable amounts of these conjugates (Snyder & Chang, 1991a, b). Snyder & Chang (1992) reported increased amounts of apolar conjugates in the hepatopancreas of lobsters during late premoult D_3, and early postmoult A. This is well in agreement with the present study as a decrease in free ecdysteroids in the *Carcinus* hepatopancreas was observed during these stages.

Studies indicate that the hepatopancreas is also involved in the production of vitellogenin and its corresponding egg yolk protein vitellin. Paulus & Laufer (1982) demonstrated that both the hepatopancreas and the ovaries of female shore crabs produce vitellogenin during vitellogenesis. The hepatopancreas vitellogenin production reached a maximum at oocyte developmental Stages 3–4 (egg diameter: 200–400 μm), whereas the maximum oocyte vitellogenin production was observed in larger eggs during embryogenesis. It is not clear whether or not ecdysteroids stimulate vitellogenesis, and conflicting results have been obtained. In shore crabs, a study by Lye *et al.* (2005) indicated a relationship between ecdysteroids and hepatopancreas vitellogenin and Lachaise *et al.* (1981) observed a stimulatory effect of ecdysteroids on vitellogenesis in the same species. However, ecdysteroids have also been observed to exert an inhibiting effect on vitellogenesis (Chang, 1993). Furthermore, the effect of ecdysteroids on vitellogenesis appear to vary with species (Loeb, 1993) and in some species such as the blue crab *Callinectes sapidus*, studies indicate that only the ovaries are involved in vitellogenesis (Lee *et al.*, 1996).

The present study demonstrates an increase in ecdysteroids in gametes from oogenesis and early vitellogenesis to the end of vitellogenesis, in particular for E and PoA. The significance of this ecdysteroid accumulation is not known and it is not clear whether or not oocytes are capable of *de novo* synthesis of the three major ecdysteroids or whether they are accumulated from the haemolymph as hypothesized by Okazaki & Chang (1991). The present study demonstrates, however, that vitellogenesis occurs when haemolymph ecdysteroid titres are low. If ecdysteroids are produced in the Y-organ and transported to the oocytes, it is reasonable to expect a relationship between haemolymph and oocyte ecdysteroids during oocyte development. Such a relationship was not observed in the present study. Also, a study by Hetru *et al.* (1978) indicated the presence of ecdysone precursors such as 2-deoxyecdysone in *Carcinus maenas* ovaries. In female shore crabs, ecdysteroids are necessary for the initial stages of oocyte development (Arvy *et al.*, 1954) but the secondary vitellogenesis can continue after surgical removal of the Y-organ, and also in the absence of ecdysteroids (Demeusy, 1962). These studies indicate that the ovaries are capable of the *de novo* synthesis of ecdysteroids and that the function of the ecdysteroids may change during oocyte maturation.

There is a pronounced decrease in ecdysteroid titres of newly laid eggs compared to that of late vitellogenesis. The

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values obtained in the present study are, however, lower than those obtained in other studies on early embryonic development of crustaceans (Lachaise *et al.*, 1981; Okazaki & Chang, 1991). This apparent discrepancy may result from the majority of ecdysteroids in early embryogenesis of *C. maenas* being present as conjugates, as demonstrated by Lachaise & Hoffmann (1982). It is presently unclear at what stage in embryonic development the Y-organ becomes active. It is presumed, however, that the marked increase in embryonic ecdysteroids from the nauplius stage and onwards results from the Y-organ producing ecdysteroids. These aspects of crustacean development await further investigations.

In shore crabs, moult preparation and gamete maturation occurs simultaneously and depend on the same hormones. Also, several tissues are involved in the coordination of these processes which significantly add to the complexity of the system. To further understand how these processes of growth and reproduction are controlled and inter-related it is important to identify the biochemical pathways of the ecdysteroids in the tissues involved and to identify and characterize the enzymes responsible for the metabolism of the relevant ecdysteroids. In the fruit fly, *Drosophila melanogaster*, the enzymes responsible for the last four sequential steps in the production of 20E are now known (Warren *et al.*, 2004). These enzymes are all cytochrome P450 enzymes, belonging to the Halloween group. Identifying the crustacean equivalents to the insect Halloween enzymes and studying their function and tissue distribution in relation to moulting and reproduction will be a major step forward in understanding these vital processes.

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Correspondence should be addressed to:
Bjarne Styrishave
Department of Science, Systems and Models
University of Roskilde
PO Box 260, 4000 Roskilde
Denmark
email: styris@ruc.dk

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