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Gonad maturation, morphological and physiological changes during the first reproductive cycle of the crayfish *Cherax quadricarinatus* female

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Summary

Concurrent morphological, anatomical and physiological changes took place during the first reproductive cycle in the Australian red-claw crayfish *Cherax quadricarinatus,* which prepared the female for spawning and holding of the newly deposited eggs. The endopod became longer and wider than the exopod and developed a mixture of plumose and long thin simple (ovigerous) setae. Small oocytes (0.24±0.05 mm) were present in the immature ovary. The growing ovary contained two distinct oocyte populations: one consisted of small (0.55±0.07 mm), barely growing oocytes, while the other consisted of large oocytes, which increased in size continuously (0.73 to 2.55 mm) until egg laying took place. A gradual change in the relative abundance of ovarian polypeptides occurred until the late vitellogenic stage (large oocytes>1.8 mm). Three predominant female-specific, SDS-PAGE separated, polypeptides were observed (103, 78 and 73 kDa) that may represent vitellin subunits. The most abundant carotenoid in the ovary was astaxanthin, while β-carotene was present at a lower concentration. The strong correlation between the increasing diameter of the oocyte and the concentration of astaxanthin in the ovary and in the hemolymph suggested an association of astaxanthin with vitellin and vitellogenin.

Key words: *Cherax quadricarinatus*, Decapoda, Crustacea, ovigerous setae, gonad maturation, oocyte growth, ovarian polypeptides, ovarian carotenoids

Introduction

The Australian red-claw crayfish *Cherax quadricarinatus* is relatively new to the scientific world, and the very few citations that are available do not provide a satisfactory description of its reproductive processes. With the recent growing interest in this species (Jones, 1990; Rouse et al., 1991; Ackefors, 1994), a need has emerged for a better understanding of its mode of reproduction. *C. quadricarinatus* reaches sexual maturity within 7–9 months, and more than one reproductive cycle per year may occur. After spawning, the eggs are firmly attached to "fine hairs" on the margins of the pleopods (Jones, 1990). These

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fine hairs, commonly termed ovigerous setae, represent one of the most remarkable sexual characteristics in the brood chamber of maturing female crustaceans (Charniaux-Cotton and Payen, 1985).

Preparation for mating involves not only external morphological changes but also a period of ovary conditioning, during which yolk accumulation (vitellogenesis) takes place within the oocytes (Quackenbush, 1989). Vitellogenesis is characterized by a rapid deposition of yolk and other proteins in the oocyte, which results in a fast increase in oocyte diameter (Eastman-Reks and Fingerman, 1985). Yolk — the main component of the laid eggs, which are approximately 2 mm in length and olive green in color — is later used as a nutritional source for embryonic development which in *C. quadricarinatus* lasts between 6–8 weeks after egg laying (Jones, 1990). Crustacean yolk contains proteins, lipids, and carbohydrates (Adiyodi and Subramoniam, 1983; Adiyodi, 1985). The major lipoprotein in the yolk is the vitellin. Vitellogenin is the precursor to egg yolk protein and is one of two lipoproteins known in crustacean hemolymph (Kerr, 1969; Lee, 1991; Quackenbush, 1991). Its concentration in the hemolymph is correlated to yolk accumulation in the oocyte (Quackenbush, 1989; Lee 1991; Okumura et al., 1992). Together with oocyte growth and the accumulation of protein in the *C. quadricarinatus* vitellogenic ovary, there is a dramatic accumulation of carotenoids, mostly non-esterified astaxanthin (Sagi et al., 1995a). Ovarian carotenoids are thought to be part of a carotenoid-protein complex — responsible for the green-brown appearance of the egg — termed "ovoverdin" by some researchers (Zagalsky, 1985; Ghidalia, 1985).

To contribute to a holistic description of some of the basic processes responsible for female maturation in *C. quadricarinatus,* we studied concurrent morphological, anatomical and biochemical changes during the first reproductive cycle and ripening of the ovary. These included changes in the morphology of the pleopods and the ovigerous setae, anatomy of the ovary, oocyte growth, and changes in the profiles of accumulated protein and carotenoids.

Materials and Methods

Animals

Seven- to nine-month-old *C. quadricarinatus* females (n=15) were harvested from earthen ponds at the Aquaculture Research Station, Dor, Israel. Four- to six-month-old females (n=14) were obtained from our laboratory at Ben-Gurion University. All animals were kept in 100 L freshwater tanks maintained at 28±2°C. The water was recirculated through a gravel biofilter. Food provided *ad libitum* consisted of minced fish flesh (Nile Perch), carrots and fish pellets containing 42% protein. Each female was weighed (± 0.1 g), and one of the third pair of pleopods was removed and placed on a glass slide. The widths of the endopod and of the exopod were measured with a pair of calipers (±0.01 mm), and the endopod width index was calculated [EWI=(Endo. width)/(Exo. width)]. The morphology of the pleopod setae was examined under a light microscope (×40). The gonads were dissected and weighed (±0.001 g), and the gonadosomatic index (GSI) was calculated as a percentage of gonad to body weight. Mean oocyte diameter (±SE) was calculated from a sample of 15 oocytes per ovary, and the difference between populations was tested by one-way ANOVA, $P \leq 0.05$.

Polypeptide analysis

Ovaries from females in different reproductive stages were dissected on ice. Ovarian samples were homogenized with 0.05 M Tris-HCl buffer (tissue: buffer, approximately V:V), and the protease inhibitors benzamidine 1.0 mM, 1.0 mM ε-amino caproic acid, 4.0 mM EDTA, 10.3 mM leupeptin, 0.2 mM PMSF and 18.2 mM pepstatin (pH 7.4). The samples were centrifuged (10,000 g, 15 min at 0°C), and aliquots of the supernatant were removed and assayed for total protein content (Bradford, 1976) with bovine serum albumin as the standard. Aliquots were stored at -70°C prior to SDS-PAGE separation, according to Laemmli (1970) on 7% polyacrylamide gel. High molecular mass (205, 116, 97, 66, 45 and 29 kDa) markers (Sigma, St. Louis, USA) were used. Proteins were stained with Coomassie brilliant blue R-250 (Hames, 1990). The same procedure was performed for newly hatched eggs and a sample of male hemolymph.

Carotenoid analysis

A representative sample of nine females from various reproductive stages, with oocyte diameters of 0.31±0.02 to 1.55±0.74 mm, were selected to study changes in ovarian and hemolymph carotenoids. Ovarian fragments (up to 0.6 g per ovary), dissected on ice, and hemolymph samples (1 ml) were placed in glass tubes (5 ml) containing acetone, on ice, in the dark, and stored at 0°C until analyzed. The samples were homogenized in acetone and subjected to phase separation in acetone (80%)/hexane. The hexane phase was dried under nitrogen, and the residue was redissolved in acetone and analyzed using an RP-HPLC system according to Sagi et al. (1995a).

Pigments were identified at 470 nm by their retention times (RT) and by absorbance spectra in comparison with the following known markers: β-carotene (SIGMA, USA) and astaxanthin (Hoffmann LaRoche, Switzerland). The absorption spectra were carried out concurrently during each HPLC run. Quantitative determination was performed by peak area integration; calibration being performed against the absorbance of known concentrations of the above pure standards.

Results

Oocyte growth during maturation

Oocytes in ovaries reaching a GSI of up to 0.5 had a milky white shaded color and a diameter of 0.24± 0.05 mm (Fig. 1). In the process of gonad ripening prior to the first egg laying (GSI values of 1–5), the development of two distinctly-sized oocyte populations, significantly different in diameter ($p \leq 0.05$), was observed in each ovary. The large oocytes grew in correlation with the increase in GSI (r=0.838) from 0.73 to 2.55 mm and usually had a darker color (deep orange, olive green or creamy brown). The small oocytes hardly increased in diameter (0.42–0.60 mm) during maturation, and their growth was poorly correlated with the increase in GSI (r=0.391). The small oocytes were usually yellowish (with much less color variation).

Pleopod growth and development of reproductive setae

In immature *C. quadricarinatus* females, both the endopod and exopod were approximately equal in width and had identical plumose setation (Fig. 2B). Each plumose seta consisted of two distinct rows of densely packed delicate setules whose insertions were directly opposite in the setal shaft (Fig. 2C). In mature females, the endopod became longer and wider than the exopod (Fig. 2A). A mixture of plumose setae and long thin simple setae was present on the endopod (Fig. 2D), while the exopod had only plumose setae. Simple setae were also observed on the protopodite of each pleopod in mature females (Fig. 2A). The laid eggs were attached only to the simple setae on the endopod and on the protopod; the simple setae are thus also termed "ovigerous setae". No eggs were attached to the exopod or to any of the plumose setae.

Mean Endopod Width Index (EWI) of young females was 1.25±0.23. The increase in EWI correlated (r=0.732) with an increase in GSI throughout maturation (Fig. 3). At the end of the maturation process, the EWI of the mature females (1.8± 0.2) was significantly different from the EWI of the immature females (p<0.001). No correlation between EWI and GSI in mature females (r=0.083) was found.

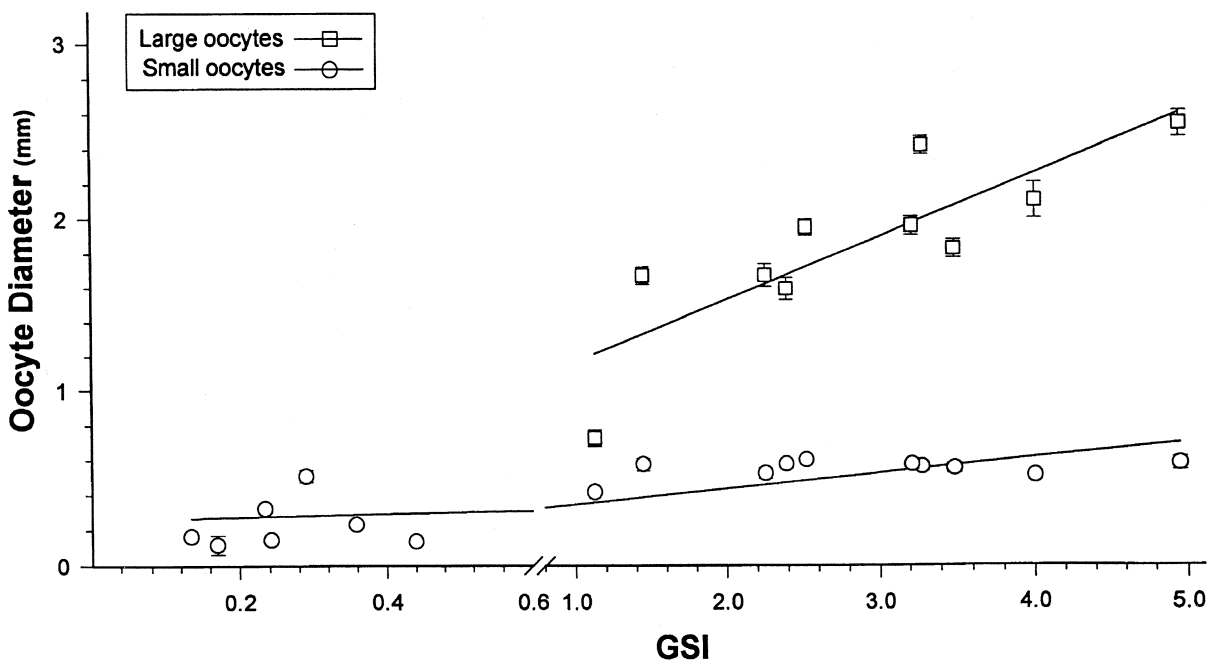

Fig. 1. Diameter of the oocyte in *Cherax quadricarinatus* females with different gonadosomatic indices. GSI=(gonad wt.)/(body wt)×100; mean±SE.

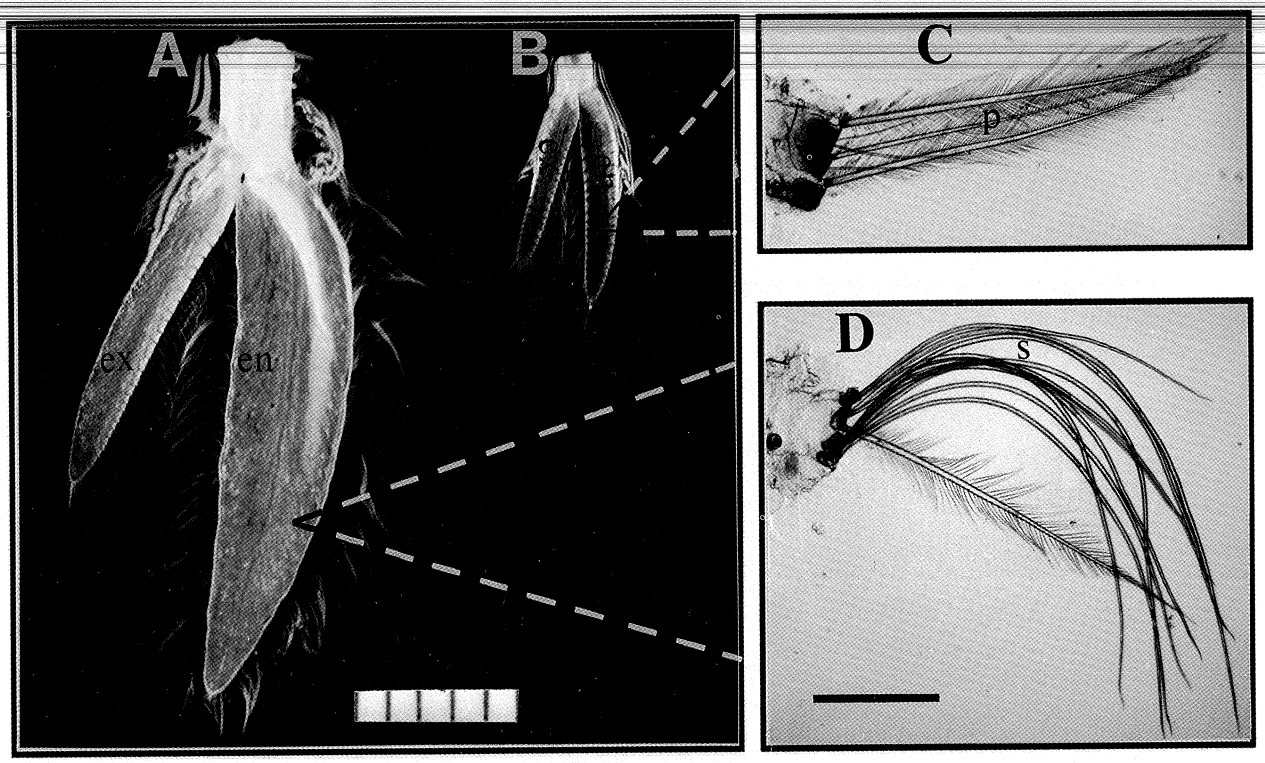

Fig. 2. Pleopods from mature (A) and young (B) *C. quadricarinatus* females. C: Section of a young endopod. D: Section of a mature endopod; ex, exopod; en, endopod; p, plumose setae; s, simple (ovigerous) setae. Bar = 0.5 cm for A and B and 0.5 mm for C and D.

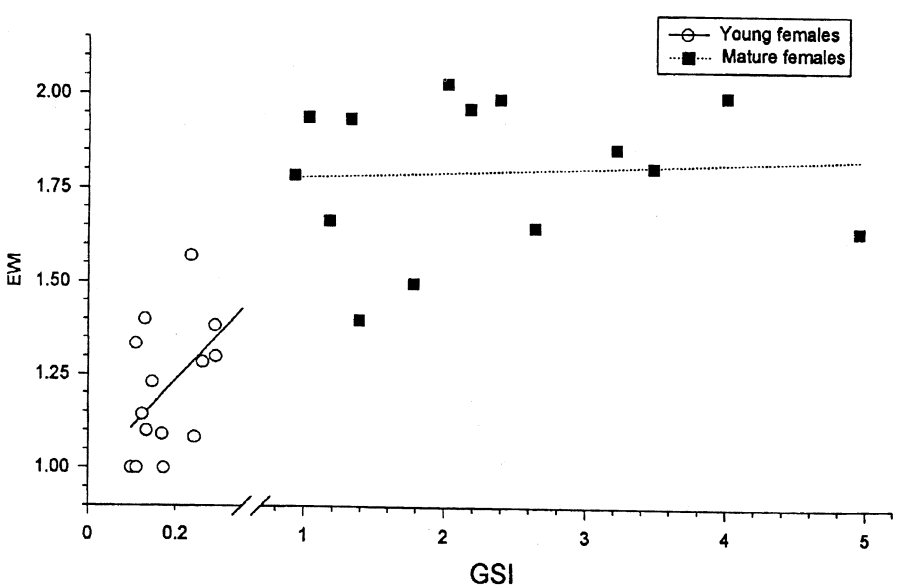

Fig. 3. Relative growth of the pleopod in young and mature *Cherax quadricarinatus* females. GSI = (gonad wt./body wt.) × 100; EWI=(endopod width)/(exopod width).

Ovarian polypeptide profile

In the course of the vitellogenic process, the ovarian and egg SDS-PAGE polypeptide profiles of the cytosolic fraction changed with the increase in oocyte diameter (Fig. 4A–D). Immature ovaries (GSI= 0.19) and ovaries at early stage of the reproductive cycle (GSI=1.13) showed similar polypeptide profiles (Fig. 4, lanes A and B, respectively). In the pre-spawn ovary (large oocytes>1. 6 mm) and recently laid eggs, there were three predominant specific polypeptides

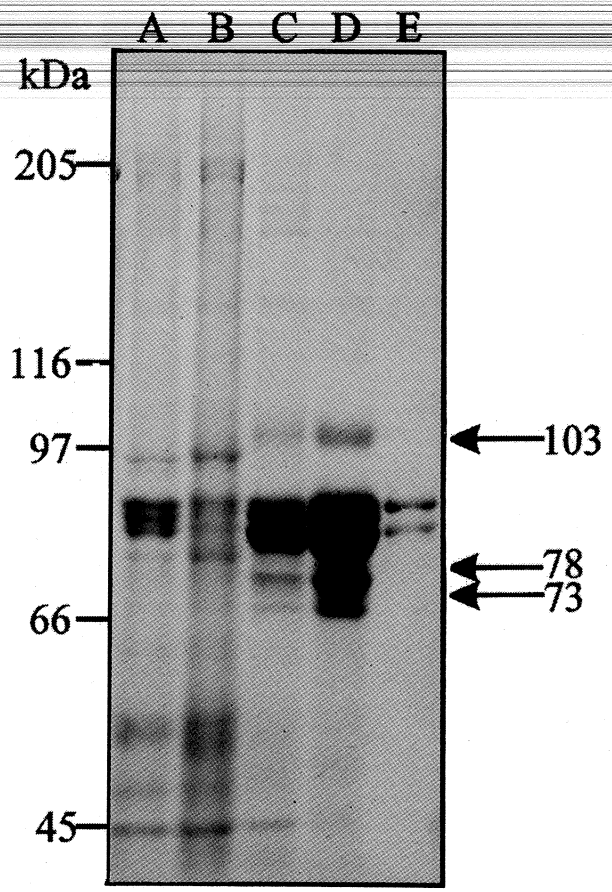

Fig. 4. Polypeptide profile of the cytosolic fraction from ovaries with oocytes of different diameters and newly laid eggs. A: Immature (oocyte diameter 0.21±0.01 mm, GSI= 0.19). B: Early stage of the reproductive cycle (large oocytes diameter 0.73±0.05 mm, small oocyte diameter 0.42± 0.02 mm, GSI-1.13). C: Pre-spawn (large oocyte diameter 1.83±0.05 mm, small oocyte diameter 0.55±0.03 mm, GSI= 3.49). D: Eggs immediately after laying. E: Male hemolymph (hemocyanin subunits). Seven percent SDS-PAGE; arrows indicate female-specific polypeptides at molecular masses of approximately 103, 78 and 73 kDa.

with molecular masses of approximately 103, 78 and 73 kDa (Fig. 4C,D). Male hemolymph showed only two polypeptide bands at molecular masses of approximately 90 and 87 kDa (Fig. 4E), which were also present in all other lanes (Fig. 4A–D).

Ovarian carotenoid profile

A significant accumulation of carotenoids was observed in ripening ovaries (up to 260 µg/ovary). The most abundant ovarian carotenoid was astaxanthin while the content of β-carotene was significantly smaller (ratio of 15:1). In the process of ovarian ripening, as the oocyte diameter increased, a significant (up to 50-fold) increase in the concentration of ovarian astaxanthin and β-carotene was recorded (Fig. 5). There was a relatively strong correlation (r= 0.826) between oocyte diameter and the concentration of astaxanthin in the ovary (Fig. 5A). Oocyte diameter and β-carotene concentration in the ovary were less strongly correlated (r=0.705, Fig. 5B).

The concentration of astaxanthin in the circulation reached up to 0.8 µg/ml of hemolymph and was very strongly correlated with the diameter of the oocyte (r= 0.951, Fig. 6A). The concentration of β-carotene in the hemolymph was much lower and only reached 0.15µg/ml. There was no correlation between the concentration of β-carotene in the hemolymph and the diameter of the oocyte (r=0.071, Fig. 6B).

Discussion

A *C. quadricarinatus* female retains the fertile eggs after laying on the ovigerous setae for approximately one month until the young hatch (Jones, 1990). In a number of crustaceans, the pleopods of the female are adapted to provide attachment sites for egg clusters (McLaughlin, 1982). The pleopod of the immature *C. quadricarinatus* female, which had pre-vitellogenic ovary, was similar in shape to the male pleopod, i.e., the endopod was equal in length and width to the exopod, and all the setae were plumose. During maturation, the pleopod changes in size and setation, and these changes correlated with the ongoing development of the ovary. At the end of the maturation process, the ovary was vitellogenic, and the endopod was approximately twice as wide and long as the exopod and had ovigerous setae.

The relationship between ovarian maturation and the endocrine regulation of female secondary sexual characteristics was demonstrated by ovariectomy and implantation experiments in several isopods and amphipods (Charniaux-Cotton, 1957, 1965, 1972; Legrand and Juchault, 1972). It has been suggested that during secondary vitellogenesis the ovary secretes a hormone that promotes the formation of the external characteristics (Charniaux-Cotton and Payen, 1985). In decapods, implantation of secondary vitellogenic ovarian tissue into an andrectomized *Macrobrachium rosenbergii* male resulted in the induction of female breeding characteristics, such as brood chambers and ovigerous and ovipositing setae (Nagamine and Knight, 1987). The findings of the present study agree with previous observations of *M. rosenbergii.* The ovigerous setae that were not present in pre-vitellogenic, immature *C. quadricarinatus* females appeared during the first reproductive cycle as gonad maturation and vitellogenesis progressed. However,

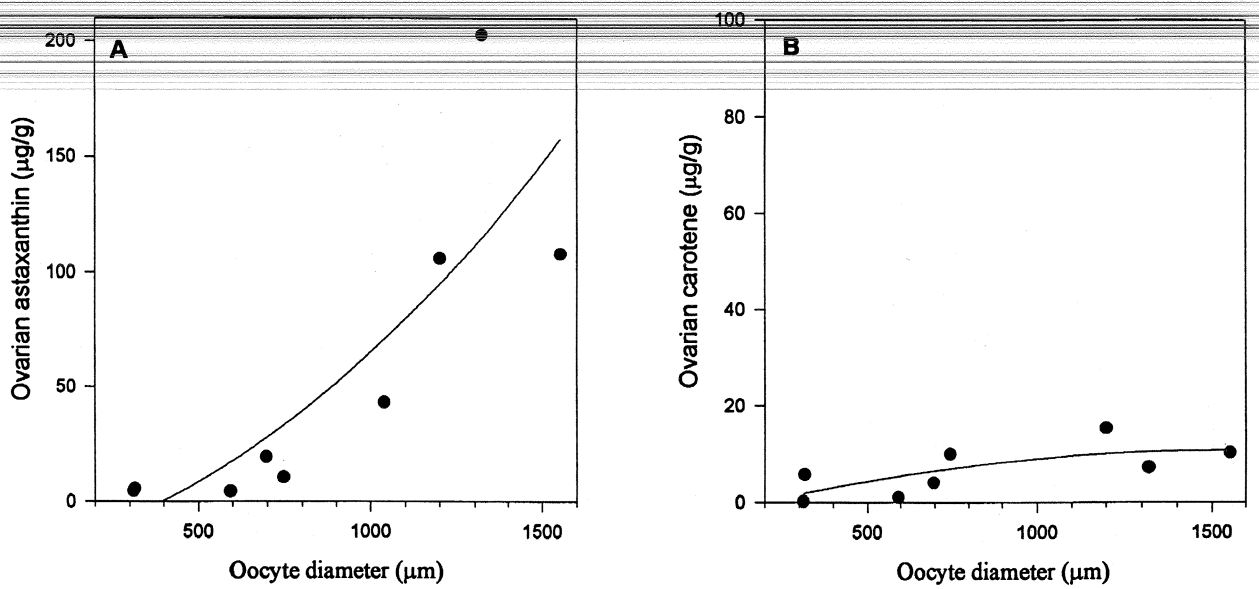

Fig. 5. Concentration of astaxanthin (A) and β-carotene (B) in ovaries with oocytes of different diameters from *Cherax quadricarinatus* females. In case two distinctly sized populations were found, "oocyte diameter" refers only to the average of the large oocytes.

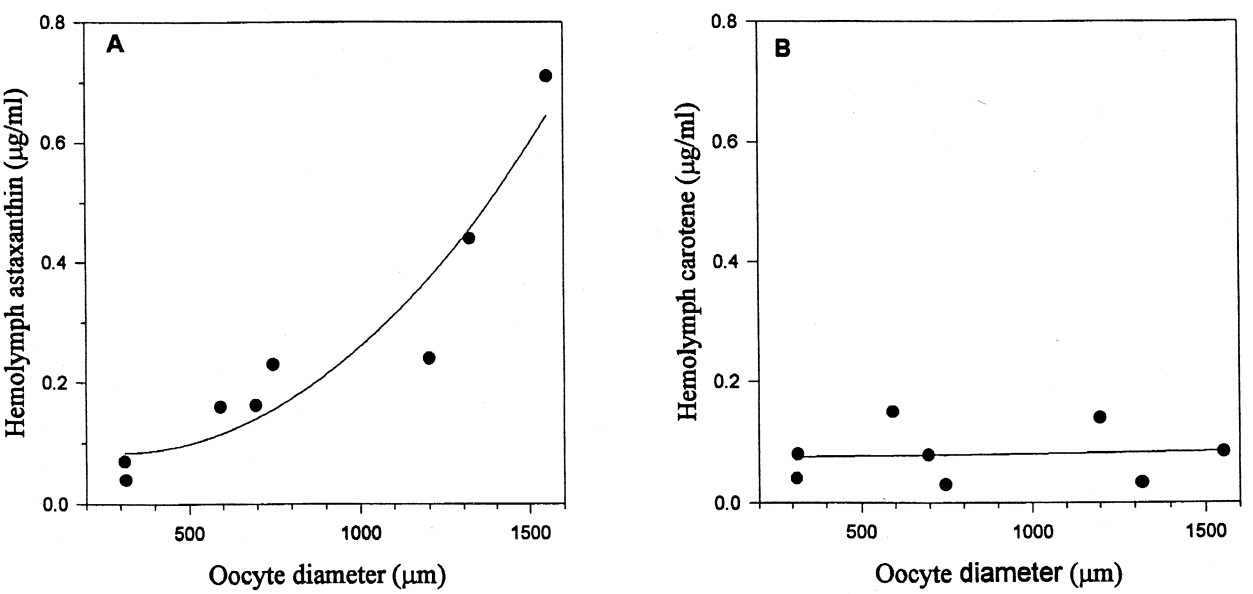

Fig. 6. Concentration of astaxanthin (A) and β-carotene (B) in hemolymph from *Cherax quadricarinatus* females with oocytes of different diameters. In case two distinctly sized populations were found, "oocyte diameter" refers only to the average of the large oocytes.

further research is needed for the identification of the responsible morphogenic hormone.

During the reproductive cycle, which climaxes in egg deposition, two distinctly sized, and often pigmented, oocyte populations developed. It is possible that during ovarian development and maturation, certain oocytes are retarded or promoted via hormonal regulation. This enables one oocyte population to rapidly accumulate yolk proteins and increase in diameter (0.728–2.552 mm), while the other remains relatively dormant (0.122–0.603 mm) until the larger population is laid (Fig. 1). In several

insect species, the nonvitellogenic oocyte will not enter vitellogenesis until the vitellogenic oocyte has been laid (Huebner, 1981a, 1981b; Thomas, 1979). It has been suggested that this process is controlled by cellular inhibitory and stimulatory interactions — both direct and indirect — between the different oocytes (for review see Huebner, 1983). In contrast, in some decapods such as *M. rosenbergii,* the ovary is composed of homogeneously sized oocytes, which synchronously develop and increase in diameter until egg laying (O'Donovan et al., 1984).

It has been suggested that crustacean lipovitellin constitutes 60–90% of the proteins in the ripe ovary (Quackenbush, 1991). The molecular mass of vitellin may differ greatly between species, from 300 through 500 kDa, and may be composed of 2–11 subunits (Eastman-Reks and Fingerman, 1985). In *C. quadricarinatus* females, polypeptide profiles in different stages of ovarian maturation show a trend of increasing relative abundance of three predominant female-specific polypeptides at molecular masses of approximately 103, 78 and 73 kDa. A similar molecular mass range (84 and 92.2 kDa) of vitellin subunits was observed in *M. rosenbergii* (Derelle et al., 1986), and a similar pattern (74, 83 and 104 kDa) was observed in an ovary extract of the tiger prawn *Penaeus monodon* (Chen and Chen, 1993). The polypeptides observed in *C. quadricarinatus* may well represent vitellin subunits that increase in relative abundance with the gradual process of yolk accumulation by the developing oocytes. Further purification and characterization of *C. quadricarinatus* vitellin is currently under way in our laboratory.

Carotenoids are associated with ovarian glycoproteins as part of the glycocaroteno-protein, known as lipovitellin or ovoverdin (Wallace et al., 1967; Zagalsky et al., 1990). The correlation found in *C. quadricarinatus* between the concentration of astaxanthin in the ovary and the development of the growing oocytes (Fig. 5A) demonstrated that astaxanthin was the main carotenoid associated with vitellin. This finding is in keeping with the stoichiometry of the astaxanthin-lipovitellin association found in lobster ovoverdin (Zagalsky et al., 1985).

A controversy exists regarding the significance of extraovarian (Fainzilber et al., 1989; Sagi et al., 1995b) versus ovarian (Fainzilber et al., 1992) sites of yolk protein synthesis in crustaceans. In *C. quadricarinatus* it is not yet known whether vitellin is formed in the ovary or in extraovarian sites. Hence, evidently the site in which the carotenoid component is attached to the yolk protein is not known. The correlation between hemolymph astaxanthin and oocyte diameter (Fig. 6A) may suggest that astaxanthin is the carotenoidal prosthetic group of vitellogenin and that in *C. quadricarinatus* this complex may be formed in an extraovarian site and transported into the oocyte through receptor-mediated endocytosis, as has been suggested for lobster and crayfish (Laverdure and Soyez, 1988; Jugan and Van Herp, 1989). The presence of a specific astaxanthin-vitellogenin association in *C. quadricarinatus* is also supported by the fact that no such correlation was found with other carotenoids, e.g., β-carotene (Fig. 6B). However, we cannot rule out the possibility that astaxanthin may be carried by proteins through the circulation to the ovary, dissociate from the carotenoprotein complex and penetrate the hydrophobic membrane to form the association with vitellin within the oocyte.

More detailed studies of proteins and carotenoids in the hemolymph and the different sized oocyte populations should be performed to further elucidate the physiology of reproduction and its endocrine regulation in the *C. quadricarinatus* female.

Acknowledgments

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