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THE CONTENT OF ASCORBIC ACID AND TOCOPHEROL IN THE TISSUES AND EGGS OF WILD *MACROBRACHIUM ROSENBERGII* DURING MATURATION

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ABSTRACT Variations in the concentrations of ascorbic acid (AA) and tocopherols in association with the gonadal development of the freshwater prawn *Macrobrachium rosenbergii* were investigated in females captured in the Mae Klong River, Thailand. Mean ovarian AA levels ranged from 210 to 540 μg/g dry weight (dw) and were at least 11-fold higher than midgut gland (MG) levels. Variations in ovarian AA levels are believed to be related to the biosynthesis of steroid hormones, the formation of collagen, and the deposition of egg yolk compounds. α-Tocopherol (α-T) was the predominant form of vitamin E in prawn tissues and eggs. The level of α-T in the MG was constant, whereas in the ovaries, it ranged from 143 to 425 μg/g dw. The incorporation of α-T into the ovary was highly correlated ($r^2 = 0.87$) to ovarian lipid levels, which probably reflects the role of this vitamin as a major antioxidant agent. The present results provide further evidence of the essentiality of these vitamins in crustacean reproduction.

KEY WORDS: ascorbic acid, tocopherols, *Macrobrachium rosenbergii*, wild, reproduction, nutrition

INTRODUCTION

Although in the last two decades much progress has been achieved in the understanding of vitamin metabolism in crustaceans (Conklin 1997), knowledge concerning the role of vitamins in crustacean reproduction is still limited (Harrison 1990, Harrison 1997). As a result, most information on vitamin functions and requirements are adopted from literature on fish and other vertebrates, rather than being derived from studies with crustaceans (Harrison 1990).

Vitamin E (tocopherol) and vitamin C (ascorbic acid [AA]) are considered essential dietary components for crustaceans (Conklin 1997). The biological activity of vitamin E is widely accepted to be at least partially related to its antioxidant properties, as it reacts rapidly with organic free radicals that may damage membrane-bound polyunsaturated fatty acids (PUFA) (Burton & Trabor 1990). Vitamin E and C are known to act synergistically, with vitamin E reacting with lipid peroxy radicals donating a hydrogen atom and forming a vitamin E radical, which is then regenerated by AA (Packer et al. 1979). The importance of vitamin E for fish reproduction has long been recognised (Watanabe & Takashima 1977, Watanabe et al. 1985), but has only been recently demonstrated in crustaceans (Cahu et al. 1991, Alava et al. 1993b, Cahu et al. 1995).

Aside from its role in the recycling of vitamin E, AA also participates in the enzymatic processes involved in the formation of collagen (Barnes 1975; Hunter et al. 1979) and in the biosynthesis of steroid hormones (Hilton et al. 1979; Seymour 1981). Although the need for AA in diets for fish broodstock has been well established (Watanabe & Takashima 1977, Sandnes et al. 1984, Soliman et al. 1986, Waagbo et al. 1989, Dabrowski 1991, Blom & Dabrowski 1995), the essentiality of AA in crustacean reproduction was initially inferred from a study evidencing its variation in the ovary of *Palaemon serratus* Pennant (Guary et al. 1975). More recent studies on penaeid shrimps have confirmed its importance in crustacean reproduction (Alava et al. 1993a, Alava et al. 1993b, Cahu et al. 1995).

Under rearing conditions, feed regimes for the freshwater prawn *Macrobrachium rosenbergii* (de Man) range from fresh food to formulated feed, and thus vitamin rations may vary considerably. As no information is available on the status of vitamin E and C in adult prawn tissues during maturation, it is not possible yet to establish criteria for evaluating the ovarian status of these vitamins or to recommend modifications in the dietary vitamin content so as to optimize broodstock performance and offspring quality. With this in mind, this paper aims to present baseline data on the concentrations of AA and tocopherols in the midgut gland, ovary, and eggs of wild *M. rosenbergii* throughout sexual maturation, and it discusses the possible roles that these vitamins may have in the reproduction of this species.

MATERIAL AND METHODS

Live mature *M. rosenbergii* females were obtained from fishermen in the Mae Klong River, Amphur Muang, Province of Samut Songkhram, Thailand. Captures were made in single collections on July and September 1998. After capture, female prawns were grouped in five stages of gonadal development, according to the size, colour, and aspect of the ovary (Chang & Shih 1995), i.e., (I) no ovarian tissue is visible, which is characteristic of both nondeveloped and spent females; (II) the ovary has a small yellow-colored spot near the posterior part of the carapace; (III) the ovarian tissue turns orange and is visible from the posterior part of the carapace to the area just in front of the epigastric tooth; (IV) the ovarian tissues have grown and extended to the area of the epigastric tooth; and (V) the ovarian tissues have extended to the anterior part of the carapace. Females in all five stages of gonadal development were sampled on July and September.

Females were then blotted dry and were individually measured (total length from the tip of the rostrum to the end of the telson) and weighed (to the nearest 0.1 g). The ovary and midgut gland were quickly dissected, weighed, and immediately frozen at –20°C. Grayish, eyed-eggs were also sampled and conserved in a

TABLE 1.

Weight, total length, ovary weight, and midgut gland weight of wild *M. rosenbergii* females at different stages of gonadal development. Each value is the mean of four separate prawn samples analyzed individually, except for stages I and II where tissues of three prawns were pooled. Within rows, values with different superscript letters indicate significant differences ($P < 0.05$).

	Stage of Gonadal Development				
	I	II	III	IV	V
Prawn weight (g)	33.4 ± 12.4	34.3 ± 10.1	41.4 ± 12.5	36.7 ± 11.6	38.8 ± 10.2
Prawn length (cm)	14.4 ± 1.1	14.7 ± 1.4	15.2 ± 1.9	15.4 ± 1.7	15.5 ± 1.4
Midgut gland weight (g)	1.37 ± 0.50	1.38 ± 0.39	1.79 ± 0.54	1.53 ± 0.48	1.73 ± 0.45
Ovary weight (g)	0.17 ± 0.06^{c}	0.53 ± 0.15^{c}	1.16 ± 0.35^{bc}	1.62 ± 0.51^{ab}	2.33 ± 0.60^{a}

similar manner. The gonado-somatic index (GSI) and midgut somatic index (MSI) were calculated as the percentage of gonad and midgut gland to total body weight, respectively. All samples were conditioned in a Styrofoam box with dry ice and were transported by air to Belgium. Samples for AA and tocopherol analysis were then maintained at –80°C and –20°C until analysis, respectively. The content of AA of the samples was determined according to Nelis et al. (1997), while α-tocopherol (α-T), γ-tocopherol (γ-T), and δ-tocopherol (δ-T) levels were estimated following Huo et al. (1999). Tissue samples from the same wild *M. rosenbergii* females were utilized in a parallel study describing the variation of total lipids, lipid classes, and fatty acids (Cavalli et al. 2001).

Statistical analysis of the data was undertaken with one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test. An alpha level of 0.05 was used to identify significant differences. Correlations were determined using linear regression analysis. A minimum of three replicates for each tissue and eggs were analyzed. When tissues of one individual were insufficient for analysis, tissues were pooled from 2 to 4 individuals. This was especially true for the early stages of gonadal development. Results are presented as means ± SD.

RESULTS

Prawn weight, total length, and midgut gland weight presented no significant differences between the various stages of maturation, but ovarian weight tended to increase, especially after stage III (Table 1). Changes in MSI and GSI during maturation are shown in Figure 1. MSI was constant irrespective of maturation stage, whereas GSI presented a significant increase from stages I to V.

Figure 2 summarizes the data on the concentrations of AA in the midgut gland (MG) and ovary throughout maturation. The content of AA in the MG was constant in the early stages of maturation, but decreased significantly from stage III (mean of 24.9 μg/g dw) to stage V (12.8 μg/g dw). In the ovary, the AA concentrations were stable at around 540 μg/g dw at stages I and II, but declined significantly between stages II to IV. At stage V, ovarian AA levels increased significantly to 370 μg/g dw. Within the same stage of gonadal development, mean AA levels were 11- to 29-fold higher in the ovary than in the MG. Eggs contained 128.2 ± 28.3 μg AA/g dw (Table 3).

The main form of tocopherol present in the MG, ovary, and eggs was α-T (Tables 2 and 3). In the MG, there were no significant differences in tocopherol content, as the variations were large. The content of α-T in the ovary increased significantly during the initial stages of gonadal development (from stages I to III), and decreased afterwards. The levels of γ-T were not significantly different at any given stage of maturation, and δ-T levels were below the detection limit during the initial stages of maturation. The mean content of α- and γ-T in the eggs was 324.7 and 22.8 μg/g dw, respectively (Table 3). No δ-T was detected in the eggs.

Linear regression analysis of the total lipid content in the ovary (data from Cavalli et al. 2001) against the concentration of α-T produced a correlation coefficient (r^2) equal to 0.87 (Fig. 3). Correlations between ovarian total lipids and γ- and δ-T levels were not significant.

DISCUSSION

Although only a limited number of studies report AA levels in crustaceans, it is well documented that tissue levels vary with season, ontogenetic development, dietary intake, moulting, and reproductive cycles (Guary et al. 1975, Magarelli & Colvin 1978, Coglianese & Neff 1981, Merchie et al. 1995). In fish, several

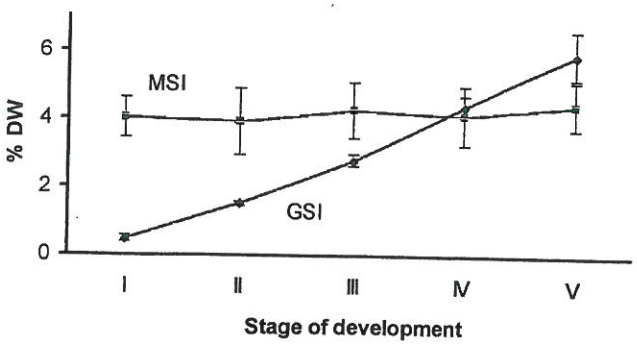

Figure 1. Changes in GSI and MSI of wild *M. rosenbergii* females at different stages of gonadal development.

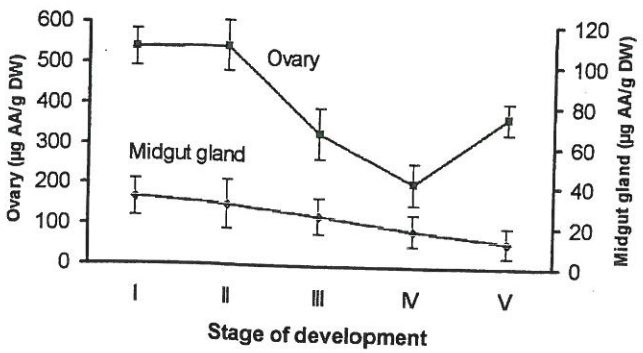

Figure 2. Concentration of ascorbic acid (micrograms per gram of dw) in the midgut gland and ovary of wild *M. rosenbergii* females according to the stage of gonadal development.

TABLE 2.

Concentration of tocopherols (micrograms per gram of dry weight) in the midgut gland and ovary according to the stage of gonadal development of wild *M. rosenbergii* females. Each value is the mean of four separate prawn samples analyzed individually, except for stages I and II where tissues of three prawns were pooled. Within rows, values with different superscript letters indicate significant differences ($P < 0.05$).

	Stage of Gonadal Development				
	I	II	III	IV	V
Midgut gland					
α-Tocopherol	153.7 ± 72.2	174.9 ± 70.3	136.3 ± 92.8	31.6 ± 12.3	102.3 ± 26.5
γ-Tocopherol	21.1 ± 18.1	13.0 ± 11.3	9.8 ± 1.6	2.7 ± 0.9	9.7 ± 1.6
δ-Tocopherol	0.5 ± 0.7	0.3 ± 0.3	n.d.	n.d.	1.0 ± 0.5
Ovary					
α-Tocopherol	142.6 ± 13.0^{c}	334.0 ± 79.9^{ab}	425.0 ± 67.7^{a}	260.3 ± 33.5^{bc}	279.1 ± 37.1^{bc}
γ-Tocopherol	11.0 ± 1.9	22.1 ± 2.1	13.1 ± 0.8	16.3 ± 1.5	17.9 ± 7.1
δ-Tocopherol	n.d.	n.d.	n.d.	n.d.	0.6 ± 0.4

n.d. = not detected

authors (Seymour 1981, Sandnes & Braekman 1981, Dabrowski 1991) have demonstrated that the levels of AA in the ovaries change during the reproductive cycle. Sandnes and Braekman (1981) showed a rise in ovarian AA concentration during ovarian growth followed by a decrease in the final stages prior to spawning, and they discussed whether this variation could be related with sex steroid synthesis. Guary et al. (1975) also postulated that the decrease in AA levels in the maturing ovary of *P. serratus* could be connected to steroidogenesis. In the present study, the decline of AA levels in the ovary between stages II to IV coincides with an active phase of ecdysteroid hormone accumulation in the maturing ovaries of *M. rosenbergii* (Wilder et al. 1991). Furthermore, significant levels of cholesterol, the chief precursor of steroid hormones (Kanazawa & Teshima 1971), were present in the ovary of *M. rosenbergii* throughout maturation (Cavalli et al. 2001). These findings reflect a possible demand for AA by the hydroxylating reactions needed for steroidogenesis in the ovarian follicle cells, and they agree with results revealing the possibility of endogenous production of steroid hormones in crustaceans (Kanazawa & Teshima 1971, Shih & Liao 1998).

The decrease in the ovarian AA content between stages II and IV could also be linked to the biosynthesis of collagen, as the hydroxylation reaction necessary for the synthesis of this fibrous protein requires the presence of AA at adequate levels (Hunter et al. 1979). In this respect, the concentration of total ovarian protein has been shown to increase linearly along with GSI in *M. rosenbergii* (Lee & Chang 1997).

Guary et al. (1975) suggested that the formation of egg yolk compounds, such as polysaccharides and glycogen, also require considerable amounts of AA and therefore could be an additional cause for the decrease in ovarian AA levels, particularly at the final stages of gonadal development. Although the possibility that these metabolic processes consume some AA cannot be ruled out, the observation that the levels of AA in the ovary of *M. rosenbergii* increased from stage IV to V suggests that the deposition of AA into the ovary at the final stages of maturation occurs at a much higher rate than its catabolism.

The raise in ovarian AA content in the final stages of maturation may be related to an increased requirement in the egg at a later stage of life (Hilton et al. 1979). Indeed, it was found in various fishes (Hilton et al. 1979, Dabrowski 1991, Blom & Dabrowski 1995) and crustaceans (Guary et al. 1975, Coglianese & Neff 1981, Alava et al. 1993a) that AA levels in the ovaries are usually higher than in other tissues. Similarly, in the present study, the ovary of *M. rosenbergii* contained significantly more AA than the MG. Sandnes et al. (1984) and Soliman et al. (1986) confirmed that an important share of the broodstock dietary AA intake is transferred to the oocytes where it is stored for use during embryogenesis and larval development. This clearly indicates a preferential transfer of reserve AA to the embryos, which is particularly important in *M. rosenbergii* since the embryos and early larvae are totally dependent on the yolk reserves for normal organogenesis and physiological functioning (Harrison 1990). Several authors have shown that the viability of fish eggs (Sandnes et al. 1984, Soliman et al. 1986, Waagbo et al. 1989) and shrimp eggs (Cahu et al. 1995) was directly related to their AA content. The improvement in egg viability with increased AA levels was attributed to the protection of membrane-bound lipids against oxidation and by the action of this vitamin in the synthesis of stable forms of collagen (Cahu et al. 1995), as suggested for fish (Waagbo et al. 1989).

In an earlier study, De Caluwé et al. (1995) collected *M. rosenbergii* eggs 2 days after fertilization and found that mean AA levels in the eggs varied between 210 and 382 μg/g dw. The upper limit of this range agrees well with the ovarian AA content at the final stages of maturation in the present study (around 370 μg/g dw), but is relatively higher than the 128 μg AA/g dw found in the eggs. However, as the eggs sampled here were "eyed" and gray colored, and were thus at the final stages of embryonic development (New & Singholka 1982), this suggests that the developing

TABLE 3.

Concentration of ascorbic acid and tocopherols in the eggs of wild *M. rosenbergii* females.

	Mean ± SD (μg/g dry weight)
Ascorbic acid	128.2 ± 28.3
α-Tocopherol	324.7 ± 77.3
γ-Tocopherol	22.8 ± 10.4
δ-Tocopherol	n.d.

n.d. = not detected

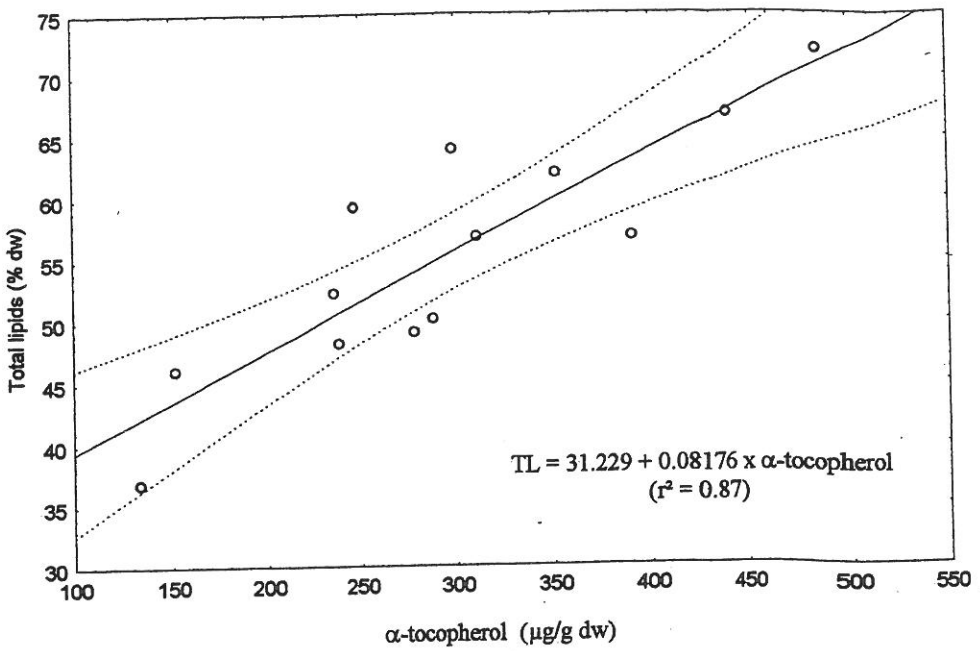

Figure 3. Linear regression analysis between the total lipids (TL) and the contents of α-T in the ovary of wild *M. rosenbergii* females.

embryos possibly consumed AA. Moreover, the fact that newly hatched, nonfeeding larvae of *M. rosenbergii* contained from 149 to 265 µg AA/g dw (Cavalli et al., 2000) further indicates that AA was indeed consumed by the developing embryo. This possibility is also supported by the results of Sato et al. (1987) who demonstrated a continuous decrease in AA content of rainbow trout eggs during embryonic development. Conversely, Guary and Guary (1975) reported that the eggs of *P. serratus* and *Cancer pagurus* (L.) seemed able to synthesize AA during the early stages of embryonic development, and hence, AA contents after spawning were found to be similar to those just before hatching. However, from a metabolic standpoint, it seems unlikely that a female shrimp would accumulate considerable amounts of AA into its gonad (Guary et al., 1975) if the eggs were able to biosynthesize it during the early stages of embryonic development. Therefore, it remains to be confirmed whether the biosynthesis of AA occurs during the embryonic development of crustaceans.

Watanabe et al. (1985) reported that vitamin E, together with lipids, was easily incorporated into red sea bream eggs. According to the present results, the incorporation of α-T into the ovary of *M. rosenbergii* was highly correlated to ovarian lipid levels. This finding is in agreement with the antioxidative role of this fat-soluble vitamin, which requires its close association with lipids, particularly membrane-bound PUFA. Therefore, it is possible that to fulfill its vitamin E requirements, reproductive *M. rosenbergii* females would depend more on the dietary intake than on body reserves, as was hypothesized for lipids (Cavalli et al. 2001). Nevertheless, the contribution of MG and muscle reserves may also be of some importance. Data from the studies of Castillo et al. (1989) and Alava et al. (1993b) indicate that α-T might have been transferred from these tissues to the eggs of *P. indicus* and *P. japonicus*, respectively. It is still unclear whether this is also true for *M. rosenbergii*.

De Caluwé et al. (1995) found that *M. rosenbergii* eggs had from 711 to 1,287 µg α-T/g dw. These concentrations are much higher than those found in the present study, and they suggest a comparatively lower dietary intake of vitamin E under natural conditions. In fact, the rate of incorporation of vitamin E into the eggs of *P. indicus* and *M. rosenbergii* was shown to increase with dietary levels of α-TA (Cahu et al. 1991, De Caluwé et al. 1995). *M. rosenbergii* females fed a diet containing 223 µg α-TA/g dw produced eggs with an average of 711µg α-T/g dw, while the content of α-T in the eggs almost doubled to 1,287 µg/g dw when dietary α-TA levels were increased to 2,025 µg/g dw.

In summary, the present study provides further evidence of the importance of AA and tocopherols in the reproduction of crustaceans, and consequently suggests that feeding a diet deficient in either vitamin C or E could virtually impair broodstock performance and offspring viability. More research is necessary to determine optimal dietary levels for crustacean broodstock.

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