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Morphometric study of embryonic development of *Macrobrachium borellii* (Arthropoda: Crustacea)

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Summary

Embryo morphometry and developing time from just-laid eggs until hatching were described in the palaemonid prawn, *Macrobrachium borellii*, using a rapid non-invasive staging method. Embryos were kept in the laboratory under controlled conditions and development divided into seven stages according to major morphological characteristics. This lecithotrophic, freshwater shrimp has a highly abbreviated type of hatching development after 39 ±2 days as postlarvae at 24°C. Morphometry was recorded using a stereoscopic microscope with an image analyzer. Area, perimeter, maximum and minimum diameters and shape were measured in yolk sac, egg-coat and eye, respectively, and they were statistically selected as the best to define the stages. Eggs are ovoid with a maximum diameter that varies from the moment of oviposition to the time of hatching from 1.5 to 2.0 mm, respectively. Water content and egg size increase along with development, whereas egg shape only varies just before hatching when the egg becomes strongly ovoid. Egg coat and eye variables significantly increase as the embryo develops while all yolk variables decrease as the embryo consumes the vitellus. Yolk represents more than 95% of the egg at the time of oviposition, falling to 22% by the time of hatching. The major yolk area decrease is observed between stages 4 and 5, which is coincident with a marked increase in the catabolism. Using only egg coat, yolk and eye shape and maximum diameter, a researcher can straightforwardly identify a developing stage with an accuracy ranging from 70 to 100%. This tool may be employed in other species provided they have transparent chorion.

Key words: Prawn embryogenesis, development, crustacean egg, morphometry

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Introduction

One of the reasons for the reproductive success of Crustacea is related to the fact that many of them carry their eggs until hatching resulting in a high survival rate for the eggs (Giese and Pearse, 1974; Charniaux Cotton et al., 1992). In palaemonids, the eggs are large and laid in bulk (Nazzari et al., 2000), making it possible to follow the embryonic development through the transparent chorion (Sandeman and Sandeman, 1991; Odinetz-Collart and Rabelo, 1996).

Macrobrachium borellii is an endemic South American lecithotrophic freshwater shrimp living in turbid, template water streams in Argentina, Paraguay and Uruguay (approximately from 22°S to 37°S and 53°W to 60°W) (Boschi, 1981). This species' reproductive season is from November to February. Females breed large, yolky eggs cemented to the pleopods in a brooding chamber. Under natural conditions with temperatures ranging from 20 to 25°C, egg development takes around 50 days (Boschi, 1981). Sollaud (1923) described three types of larval development in the genus *Macrobrachium* based on the number and size of eggs and early life history: (1) typical type, with large number of small-sized eggs and numerous zoeal stages, some of which are free-swimming, e.g., *M. rosenbergii*; (2) semi-abbreviated type, with slightly fewer, larger sized eggs, and fewer zoeal stages, some of which are also free swimming; (3) highly abbreviated type, with complete suppression of free-swimming zoeal stages. According to this classification, *M. borellii* falls into the last type, hatching directly as postlarvae, though it keeps some primitive structures (Boschi, 1981). Clutches are small, 30 to 100 translucent eggs and hatchlings are 5–6 mm long postlarvae of benthonic habits which are omnivorous scavengers (pers. obs.). There is no available report dealing with the embryonic development in *M. borellii*.

The present paper describes for the first time the embryo morphometry of *M. borellii* from just-laid eggs until hatching using a non-invasive method that could be employed to distinguish the phases of this shrimp's embryonic development in relation to incubation time under controlled conditions. Thus, toxicological or physiological tests based on such phase differences could be performed.

Materials and Methods

Sample collection

Eggs from *M. borellii* were sampled from ovigerous females collected between October and February in a watercourse with turbid waters and temperatures ranging from 22 to 26°C. It is close to the Rio de La Plata River, Argentina (20 km SW of La Plata city). They were taken to the laboratory and the eggs were removed from pleopods for the experiments.

Pilot morphological study and developing time recording

Eggs from three clutches laid within the day were placed in 24-well, 2-ml plates (Corning, USA) in 0.2 µm filtered freshwater, one egg per well with a 10 h dark:14 h light photoperiod. The plates were placed in a 25°C water bath (New Brunswick Sci. Co., Edison, USA) essentially using the procedure described for *Palaemonetes pugio* by Rayburn et al. (1996) but without shaking and with a water exchange every second day. Every 2 days, the plates were removed from the bath and embryo overall morphology recorded until hatching with a stereoscopic microscope (Nikon SMZ-10, Tokyo, Japan).

Morphometrical analysis

Eggs were placed in 96-well plates in 0.2 µm filtered water (one egg per well). The plate was placed onto the flat stage of a stereoscopic microscope (Olympus SZ40, Tokyo, Japan) with epi-illumination. An analogical RGB camera (Sony DXC-151A CCD, Tokyo, Japan) attached to the microscope captured the images that were digitized and acquired into a computer. Images were then processed with Image-Pro Plus v4.1 (Media Cybernetics, Silver Spring, MA). At least 30 images were obtained from each developing stage (from 1 to 7). Yolk sac, egg-coat and eye were analyzed separately. The variables measured were: (1) area which accounts for the area of each object minus any holes; (2) maximum diameter accounts for the length of the longest line joining two outline points, passing through the centroid; (3) minimum diameter reports the length of the shortest line joining two outline points and passing through the centroid; (4) mean diameter reports the average length of the diameters measured at two degree intervals joining two outline points and passing through the centroid; (5) perimeter accounts for the length of the outline of each object; (6) roundness is determined by the formula: $\text{perimeter}^2/(4\times\pi \times \text{area})$; (7) maximum axis reports the length of the main axis of the ellipse equivalent to the object; (8) minimum axis reports the length of the minor axis of the ellipse equivalent to the object; (9) aspect ratio reports the ratio between the major axis and the minor axis of the ellipse equivalent to the object; (10) mean density reports the mean

average optical density of the object; (11) IOD reports the average intensity/density of each object; (12) max radius accounts for the maximum distance between each object's centroid pixel position and its perimeter; (13) min radius reports the minimum distance between each object's centroid pixel position and its perimeter; (14) radius ratio reports the ratio between max radius and min radius for each object.

Wet weight and moisture

Wet weight and moisture were obtained from egg masses at each developing stage and analyzed following the IAFMM method (1979). Egg masses were rinsed with filtered water, blotted on filter paper and then weighed to 0.01 g precision. Samples were analyzed in triplicate and each replica was composed of pools from three or four egg clutches.

Statistical analysis

Normality and homoscedascity of each variable were determined by a Kolmogorv–Smirnov and Bartlett analysis, respectively.

In order to study differences among previously defined stages, ANOVAs of variables measured on egg coat, eye and vitellus were performed (Tukey's HSD, $p<0.05$).

A Pearson correlation analysis was carried out among all variables to eliminate those that presented high correlation indexes. A principal component analysis (PCA) was performed to reduce the 14 variables analyzed, selecting those variables that best defined the groups. Finally, a discriminant analysis was made considering the stages defined to test if the selected variables allowed discrimination among naturally different occurring groups, leading to classification of samples into the above-mentioned groups. Data were transformed using (value−standard deviation/media).

Statistical analyses were performed using the Statistica 4.5 software (Statsoft Inc., USA).

Results

Under laboratory controlled conditions, postlarvae of *M. borellii* hatched after approx. 39 days. In this pilot experiment we tentatively separated embryonic development into seven stages as shown in Fig. 1. Stage 1 (0–7 days) comprises embryos from 4-cell morula to gastrula with a highly viscous vitellus that takes up most of the egg; Stage 2 (8–15 days): vitellus

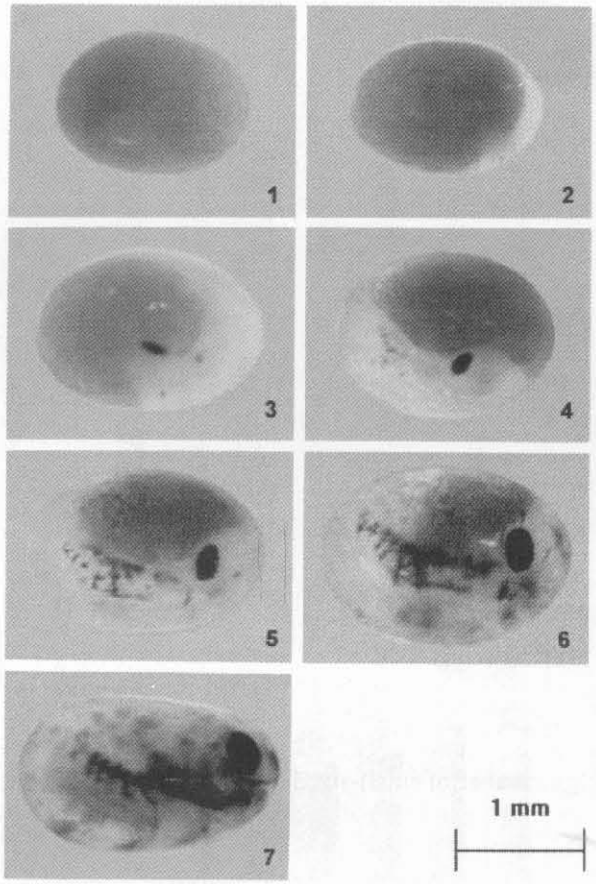

Fig. 1. Typical morphology of *M. borellii* eggs at different developing stages.

starts diminishing and embryo is at the tissue-cap stage (clear region of the egg in Fig. 1); Stage 3 (15–21 days): embryos show incipient eyes, and the vitellus fills the remaining egg volume. At stage 4 (21–29 days) the vitellus is being engulfed by the embryo and vitelline mass is divided into four lobules. Eyes are elongated; Stage 5 (29–32 days): the reddish pigmentation becomes evident and embryo eye is bigger and more circular in shape; at stage 6 (32–37 days) blue from hemolymph hemocyanin becomes visible and the vitellus is almost embodied into the embryo and the amount of yolk is markedly diminished; Stage 7 (37–40 days) comprises embryos thoroughly formed and ready to hatch. In general, vitellus decreases its viscosity along development.

M. borellii embryo morphometry was described by measuring 14 variables on which we performed statistical analyses to select the ones that best define the stages and at the same time are easy to measure. Thus, major morphological events during development of embryos could be followed.

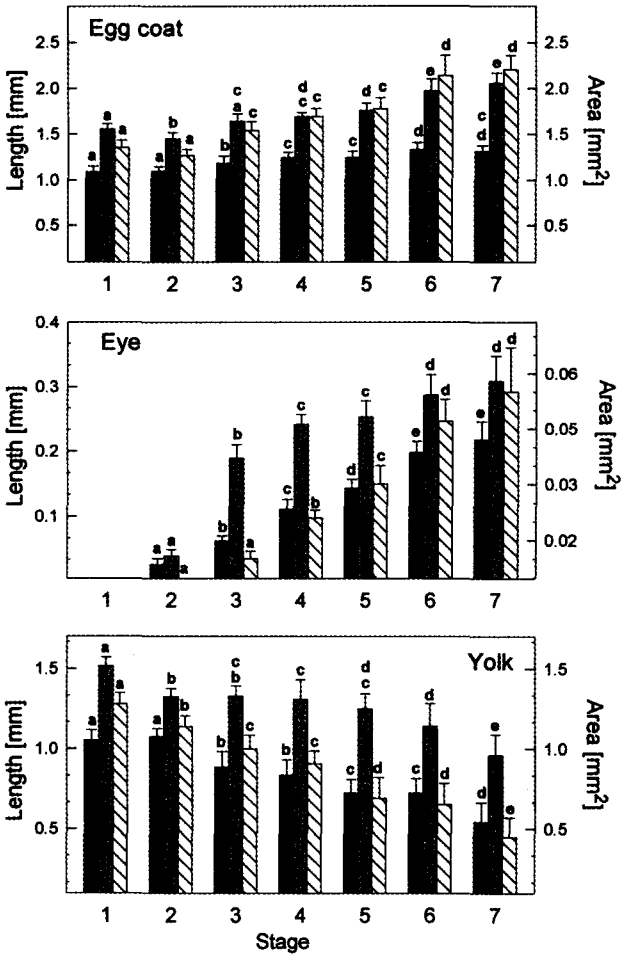

Fig. 2. Changes in egg coat, eye and yolk sac during development. Black: diameter (min); striped: area.; grey: diameter (max). Values are the mean ± 1 SD, N = 160. Observed differences among stages ($p<0.05$) are indicated by different letters.

Newly-laid eggs are ovoid with a maximum diameter that varies from oviposition to hatching from 1.5 to 2.0 mm, respectively (Fig. 2). This increase in size is coincident with an increase in the water content that is statistically significant between stage 1 and the last three stages (Table 2). In spite of this significant increase in egg size, egg roundness is the same along development, except at stage 7 when the egg becomes more ovoid (Table 1, Fig. 1). Fig. 2 shows the ANOVA analysis of the most relevant morphological variables selected using the Pearson correlation analysis. Variables with great dispersion were discarded. When two variables with high correlation between them were found, we selected the easiest one to measure. Thus, area (a), maximum diameter (x), minimum diameter (m), roundness (r) and in some cases perimeter (p) were selected. When we analyzed

Table 1. Variation of roundness of egg coat, eye and yolk during development

Stage	Egg coat	Eye	Yolk
1	$1.07 \pm 0.05^{a,b}$	nd	$1.12 \pm 0.05^{a,b}$
2	1.04 ± 0.02^{a}	$1.02 \pm 0.04^{a,d,f}$	1.07 ± 0.05^{a}
3	$1.05 \pm 0.02^{a,b}$	1.49 ± 0.10^{b}	$1.20 \pm 0.14^{b,c}$
4	$1.04 \pm 0.01^{a,b}$	1.28 ± 0.10^{c}	$1.24 \pm 0.16^{c,d}$
5	$1.08 \pm 0.02^{b,c}$	$1.20 \pm 0.09^{c,d,e}$	$1.22 \pm 0.10^{c,d}$
6	$1.09 \pm 0.02^{c,d}$	1.07 ± 0.04^{f}	$1.20 \pm 0.08^{b,d}$
7	1.09 ± 0.02^{d}	$1.13 \pm 0.15^{e,f}$	1.37 ± 0.16^{e}

Observed differences among stages are indicated by different letters ($p<0.05$).
nd: not detected.

Table 2. Dry weight of egg at different developing stages

Stage	Dry weight , % (w/w)
1	48.8 ±2.5
2	44.9 ±1.6
3	44.2 ±3.5
4	41.5 ±3.5
5	39.9 ±2.0
6	37.5 ±4.0
7	38.5 ±4.0

Values are the mean of triplicate analysis ±1 SD.

those variables in egg coat and eye we found a significant increase along development whereas all yolk variables decreased (Fig. 2). Yolk represents more than 95% of the egg at the time of oviposition and falls to 35% (negatively correlated with the increase in egg size). The proportion of yolk consumed as energy was also reflected by the decrease in the dry weight observed (Table 2). Roundness of yolk decreases slightly along development (Table 1), while the only anatomical characteristic in the embryo that strikingly varied were the eyes which were therefore analyzed individually.

To study the covariation of several variables and the grouping of samples, principal component analysis was performed with the standardized data set. Fig. 3 shows the original variables (vectors) and data set grouped by clutches (numbered dots). Vectors close to each other are positively correlated whereas opposite vectors indicate a negative correlation.

Only the first two components, principal component 1 (PC1) and 2 (PC2) were employed in this study as they account for 92% and 9% for PC1 and PC2, respectively). PC1 is mainly determined by the contribution of yolk Ya, Yx and Ym

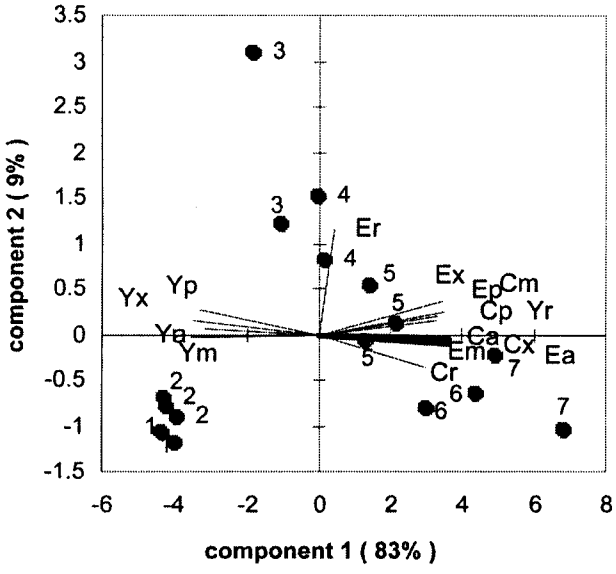

Fig. 3. Plot of the first two components of a principal component analysis (PCA). Data set was grouped by clutches (circles) and the relative contribution of the 14 variables was plotted. Vectors represent each variable. Ya, yolk area; Yx, yolk maximum diameter; Ym, yolk minimum diameter; Yp, yolk perimeter; Ea, eye area; Ex, eye maximum diameter; Em, eye minimum diameter; Ep, eye perimeter; Ca, egg coat area; Cx, egg coat maximum diameter; Cm, egg coat minimum diameter; Cp, egg coat perimeter; 1 to 7: developing stages of the clutches.

(−PC1) and of egg coat Cm, Cx, Ca, Cp, as well as eye Ea, Em, Ex, Ep and yolk Yp (+PC1). PC2 presents a strong contribution of Er and of less magnitude Ex (+PC2), and Cr (−PC2). Both components discriminate the clutches according to developing stage, the early developing stages being better defined by yolk content of the egg, stages 3 and 4 by the shape of the eye, and the late stages by the above mentioned increase in egg size and shape as well as eye area (0 to 0.05 mm^2) and size (0.04 to 0.30 mm max diameter). Although stages 6 and 7 fall apart in this analysis, Fig. 1 shows another embryo feature that separates these stages from each other which is the marked blue color of the hemolymph, perceived even with naked eye at stage 7. This blue color is due to the respiratory pigment hemocyanin that is first observed at stage 5 embryos as small dots. This variable was not included in the study because the optical density was strongly influenced by light, and therefore the analysis of this variable showed a great variance.

A discriminant analysis was then performed selecting those variables that were at the same time easy to measure and with low correlation between them. Variables were tested with the whole data set.

Table 3. Discriminant analysis of data set using selected variables (% correction of predicted classification)

Stage	Without eye variables	With eye variables
1	84.2	
2	100	100.0
3	57.1	100.0
4	35.3	73.3
5	85.2	82.6
6	69.2	70.0
7	78.6	84.6
Total	76.7	82.9

Table 3 shows that using only four variables (Cr, Cx, Yx, Yr) we were able to group the previously assigned sample stage (1 to 7) with great predicting classification power, except for stages 3 and 4 where these variables were not enough. For these two stages, as shown in PCA analysis, eye variables (Er and Ex) were included. In this analysis, only stages 2–7 were analyzed, as stage 1 has no eyes. Including eye variables, the predicting power of the analysis let us assign a sample with a high degree of confidence to stage 3 or 4 with an accuracy of 100 and 73%, respectively.

Discussion

Under controlled laboratory conditions, *M. borellii* eggs took 39 days to hatch, which is an incubation time about 10 days faster than the one reported for wild-caught specimens (Boschi, 1981). This is most probably due to water fluctuation that in the environment ranges between 20–28°C. In a study on the effect of environmental temperature on *M. rosenbergii* embryonic development, Gomez Diaz (1987) found that eggs raised at 25°C and 31°C took 26 and 16 days to hatch, respectively. Moreover, it is clear that only a slight change in temperature is sufficient to significantly alter the time taken for egg development (Wear, 1974). The incubation period in *M. borellii* is significantly longer than that for other Palaemonids, and Wear (1974) suggests that among decapods which are closely related, increasing egg size (and consequently yolkiness, as it is *M. borellii* case) slows down the rate of development.

Major morphological events during development of embryos were followed, measuring the most conspicuous embryo features (i.e., egg coat, yolk and eye). Maximum diameter increases along development, as well as the water content and egg coat area. Along with embryonic development there was an increase in water

content. It is known that the egg coat alters its permeability during development probably allowing water uptake (Glas et al., 1997; Narashimha et al., 1981).Therefore, the observed 33% egg coat area increase in relation to development could be attributed to both embryonic expansion and osmotic swelling via water uptake as it was found in other decapods (Pandian, 1970; Marangos and Vincent, 1988; Narashimha et al., 1981). Increases of this magnitude have been reported for other Palaemonids (Gore, 1985). As the egg enlarges, it becomes more ovoid as it been reported in other Palaemonids (Nazari et al., 2000).

The eyes appear at around 15 days of development, but they become more visible and conspicuous later, becoming more spherical before hatching. All yolk variables decreased during development, due to the utilization of yolk in *M. borellii* as energy source (Heras et al., 2000). The proportion of yolk consumed was also reflected by the decrease in the dry weight observed (Table 2). The major yolk area decrease observed between stages 4 and 5 is simultaneous with a marked increase in the activity of many enzymes involved in the lipid catabolism (major energy source) (Gonzalez Baró et al., 2000). Both of them coincide with an active embryo organogenesis (Fig. 1). At hatching, egg still possesses 35% yolk, which supports the first postlarval stage while postlarva starts feeding. In the 17-day development embryos of *M. rosenbergii*, Clarke et al. (1990) reported that at hatching the zoea larvae retains about 90% of the original yolk that is consumed in the following 24 h. *M. rosenbergii* has small (0.55× 0.57 mm) lecithotrophic eggs, but this species does not have abbreviated development (Rabalais and Gore, 1985).

We can thus conclude that using only egg coat, yolk and eye roundness and maximum diameters a researcher can determine the seven stages of *M. borellii* embryos with an accuracy ranging from 70 to 100%. This non-invasive method measuring basic shrimp embryo morphometry allows to quickly select embryos of the desired developing stage without killing them, which is particularly important for biochemical, toxicological or physiological studies that require living organisms.

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References

Boschi, E.E., Decapoda: Natantia. In: Fauna Argentina de Agua Dulce, Vol. 26, E.E. Boschi (ed.), FECIC, Buenos Aires, 1981, pp. 19–61.

Clarke, A., Brown, J.H. and Holmes, L.J., The biochemical composition of eggs from *Macrobrachium rosenbergii* in relation to embryonic development, Comp. Biochem. Physiol., 96B (1990) 505–511.

Charniaux Cotton, H., Payen, G.G. and Gisburger-Vogel, T., Arthropoda–Crustacea: sexual differentiation. In: Reproductive Biology of Invertebrates, Vol. 5, K.G. Adiyodi and R.G. Adiyodi (eds.), Wiley, New York, 1992, pp. 281–324.

Giese, A.C. and Pearse, J.S., Reproduction of Marine Invertebrates, Vol. 1, Academic Press, New York, 1974, pp. 1–546.

Glas, P., Courtney, L., Rayburn, J. and Fisher, W., Embryonic coat of the grass shrimp *Palaemonetes pugio*. Biol. Bull., 192 (1997) 231–242.

Gomez Diaz, G., Effect of environmental embryonic temperature on larval development of *Macrobrachium rosenbergii* (De Man), J. Exp. Mar. Biol. Ecol., 114 (1987) 39–47.

Gonzalez Baró, M.R., Heras, H. and Pollero, R., Enzyme activities involved in lipid metabolism during embryonic development of *Macrobrachium borellii*. J. Ex. Zool., 286 (2000) 231–237.

Gore, R.H., Molting and growth in decapod larvae, In: Larval Growth, A.M. Wenner (ed.), A.A. Balkema, Rotterdam, Boston, 1985, pp. 1–65.

Heras, H., Gonzalez-Baró, M.R. and Pollero, R., Lipid and fatty acid composition and energy partitioning during embryo development in the shrimp *Macrobrachium borellii*. Lipids, 36 (2000) 645–651.

IAFMM, Recommended method of analysis for determination of moisture in fish meal, Vol. 9, In: International Association of Fish Meal Manufacturers, Technical Bulletin, 1979.

Ishikawa, K. and Imabayashi, H., Early larval development of the penaeus shrimp, *Trachypenaeus curvirostris* (Simpson) reared in the laboratory, J. Fac. Appl. Biol. Sci., 30 (1991) 1–11.

Marangos, Ch. and Vincent, M., Etude descriptive et biométrique des oeufs de la crevette grise Crangon crangon (L.) (Crustacea: Decapoda) au cours de l'embryogénese, La mer, 26 (1988) 53–60.

Narasimha Rao, C., Ponnuchamy R., Katre Shakuntala and Ravinchandra Reddy, S., Fecundity and energetics of embryonic metabolism of *Caridina weberi* (De Man) (Decapoda: Atyidae), Int. J. Invert. Reprod., 3 (1981) 75–85.

Nazari, E.M., Rauh Müler, Y.M. and Ammar, D., Embryonic development of *Palaemonetes argentinus* Nobili, 1901

(Decapoda, Palaemonide), reared in the laboratory. Crustaceana, 73 (2000) 143–152.

Odinetz-Collart, O. and Rabelo, H., Variation in egg size of the fresh-water prawn *Macrobrachium amazonicum* (Decapoda, Palaemonide), J. Crust. Biol., 16 (1996) 684–688.

Pandian, T.J., Ecophysiological studies on the developing eggs and embryos of the European lobster *Homarus gammarus*. Mar. Biol., 5 (1970) 154–167.

Rabalais, N.N. and Gore, R.H., Abbreviated development in decapods, In: Larval Growth, A.M. Wenner (ed.), A.A. Balkema, Rotterdam, Boston, 1985, pp. 67–126.

Rayburn, J.R., Glas, P.S., Foss, S.S. and Fisher, W.S., Characterization of grass shrimp (*Palaemonetes pugio*) embryo toxicity tests using the water soluble fraction of number 2 fuel oil, Mar. Poll. Bull., 32 (1996) 860–866.

Sandeman, R. and Sandeman, D., Development and identified neural systems in the crayfish brain. In: Frontiers in Crustacean Neurobiology, Vol. 1, W.D. Krezt, J. Tautz, H. Heichert and B. Mulloney (eds.), Birkhäuser Verlag, Basel, 1990, pp. 498–508.

Sollaud, E., Le développement larvaire des "Palaemoninae". I. Partie descriptive. La condensation progresive de l'ontogenèse, Biology Bulletin France Belg., 57 (1923) 509–603.

Wear, R.G., Incubation in British decapod crustacea, and the effects of temperature on the rate and success of embryonic development, J. Mar. Biol. Ass. UK, 54 (1974) 745–762.