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ORIGINAL PAPER

# Effect of delayed first feeding on larval performance of the spider crab *Maja brachydactyla* assessed by digestive enzyme activities and biometric parameters

**Guiomar Rotllant · Francisco Javier Moyano · Mireia Andrés · Alicia Estévez · Manuel Díaz · Enric Gisbert**

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**Abstract** The effect of food deprivation on larval performance of the spider crab *Maja brachydactyla* was studied in terms of survival, moulting capacity, size, weight and enzymatic activities. Five feeding treatments that differed in the initial age of first feeding larvae (fed from hatching, 2, 4 and 6 days post-hatching and unfed) were tested for 20 days. Newly hatched larvae kept without food supply lasted for 10 days and did not moult; with 50% survival observed at 6 days post-hatching. Larvae (zoea I stage) were only able to tolerate 2 days of food deprivation after the onset of exogenous feeding without their performance being compromised. Multivariate analyses suggest that digestive enzyme activities may be good indicators of the nutritional condition of larvae.

## Introduction

Many biological and physical factors affect the growth and survival of marine species with complex life histories that involve larval dispersal and settlement to the seabed, both before and after settlement. It has been suggested that the main causes of crustacean mortality during early life stages are starvation, predation, unfavourable environmental conditions and pathogens (Anger 2001; Wahle 2003). The survival of planktonic organisms is closely associated with their ability to locate food patches (Ciannelli et al. 2009). The commencement of first feeding once all yolk resources are exhausted is a key factor for crustacean larval performance (Giménez and Anger 2005). This capacity of larvae to grow and survive directly affects their recruitment and population size (Wahle 2003).

The spider crab *Maja brachydactyla* is an important commercial species along Western European Atlantic coasts (Freire et al. 2002). Like other species of the superfamily Majoidea, *M. brachydactyla* has a short planktotrophic larval development characterised by three stages: two zoeae and one megalopa (Guerao et al. 2008). As larvae in this species hatch without yolk reserves (Lang 1973), the onset of exogenous feeding is a pivotal issue for their development and growth, as these both depend on the proper nutrient input provided by the diet. Therefore, periods of food deprivation can result in abnormal behaviour and morphological development, degeneration of the alimentary tract and musculature and reduced food utilisation efficiency and feeding activity as reported for larvae of different aquatic organisms (i.e. Lemieux et al. 1999; Anger et al. 1981; Catalán and Olivar 2002; Gisbert et al. 2004; Figueiredo and Narciso 2006; Figueiredo et al. 2008; Shan et al. 2009).

The effect of food restrictions on aquatic organisms is routinely assessed by a number of indicators commonly called "condition indices". Nevertheless, while a great variety of morphometrical, histological and biochemical parameters have been routinely applied to evaluate condition and feeding history in wild and cultured populations of larval fish (Ferron and Legget 1994; McFadzen et al. 1997; Catalán and Olivar 2002; Gwak and Tanaka 2001; Gisbert et al. 2004, 2008), few studies have assessed the effect of

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G. Rotllant (✉) · M. Andrés · A. Estévez · E. Gisbert
Unitat Operativa de Cultius Experimentals, IRTA,
Carretera del Poblenou km 5.5, 43540 Sant Carles de la Ràpita,
Tarragona, Spain
e-mail: guiomar.rotllant@irta.cat

F. J. Moyano · M. Díaz
Department of Biología Aplicada, Escuela Politécnica Superior,
Edificio CITE II-B, Universidad de Almería, Campus
Universitario de la Cañada, 04120 Almería, Spain

food supply on the physiology and development of larval decapods (McConaugha 1985; Storch and Anger 1983; Anger et al. 1985; Ritar et al. 2003; Johnston et al. 2004a, b; Figueiredo and Narciso 2006). Among the different biochemical and nutritional condition indices frequently used, digestive enzymes are considered reliable indicators of the nutritional state of individuals due to their species and age specificity, sensitivity and short latency (Harms et al. 1991, 1994; Ueberschär 1995; Johnston et al. 2004a; Cara et al. 2007; Rotllant et al. 2008; Shan, et al. 2009). In addition, due to their key role in digestion, measuring the activity of certain digestive enzymes provides insight into the digestive physiology of the larva, which can potentially limit the growth of the whole organism (Lemieux et al. 1999). However, using them in either natural or cultured populations requires detailed experimental investigations under controlled situations in which parameters can be standardised and validated for a known nutritional history.

The present work was oriented towards determining the maximum period for which feeding could be delayed for newly hatched *M. brachydactyla* larvae and assessing the suitability of the different morphometric and biochemical parameters as indicators of changes in the nutritional status of the larvae in relation to food deprivation and feeding resumption conditions.

## Materials and methods

### Broodstock maintenance

Adult specimens (6 females and 2 males) of *Maja brachydactyla* of approximately 1 kg were captured by a commercial fleet using trammel nets in the Ria de A Coruña (Atlantic north-west coast of Spain) at 25–30 m depth and transported by road to IRTA (Sant Carles de la Ràpita, Tarragona, Spain) in high humidity containers at a temperature below 8°C. Once at IRTA, the broodstock was acclimatised and kept in a 2,000-L tank connected to a recirculation unit at a constant salinity and temperature of 35 ± 1 and 18 ± 1°C, respectively. The broodstock was fed on fresh or frozen mussels (*Mytilus* sp) and frozen crab (*Liocarcinus depurator*) to satiation. Spawning was observed after a few days and hatching occurred in a month and a half.

### Larval rearing

Newly hatched larvae (zoea I, ZI) were collected from the outflow of broodstock tanks immediately after hatching and transferred to rearing baskets. Baskets were PVC cylinders (31 × 45 cm) with a mesh bottom (150 μm) filled with 35 L of sea water. An air lift in each basket provided aeration and water renewal. The baskets were immersed in 1,500-L holding tanks connected to recirculation units. The temperature and salinity were kept constant at 18 ± 1°C and 35 ± 1, respectively, whereas light intensity and photoperiod were kept natural.

### Preliminary experiment: the effect of total food deprivation

A short preliminary experiment was run to assess the maximum survival time for food-deprived larvae. Twelve thousand newly hatched larvae from a single batch were individually counted and transferred to 24 baskets (500 each) immersed in 2 holding tanks and kept without food supply. Two baskets from each tank were sampled daily until day 11 when all the larvae died. Samples of larvae were counted every day to calculate their survival rate and observed to determine their developmental stage according to Guerao et al. (2008).

### Main experiment: the effects of delayed food supply

Newly hatched larvae from a single batch were individually counted and transferred to thirty baskets immersed in 3 holding tanks at an initial density of 60 larvae $L^{-1}$ (2,100 larvae per basket). Enriched *Artemia* metanauplii (EAM) were obtained by incubation for 24 h with EasySelco (INVE, Belgium). These live prey were provided once a day (each morning) at a rate of 60 EAM $larva^{-1}$. Different feeding treatments were established by changing the first day food was supplied to newly hatched zoea I: F0: fed from hatching (positive control); F2: fed from 2 dph; F4: fed from 4 dph; F6: fed from 6 dph; UF: unfed (negative control). Larvae were sampled on days 0, 4, 8, 12 and 20 dph to evaluate survival, growth, biometry and digestive enzyme activities. On the day of sampling, two baskets (replicates of each treatment) were removed from the culture, emptied and drained, and all the larvae counted and staged, while all the other baskets remained in the experimental conditions for later samplings.

### Survival, biometric measurements and growth indices

Survival rate, developmental stage (in terms of individual frequency per stage of development and sampling day), carapace length (CL) and width (CW), abdominal width (AW) or eye index (EI), wet weight (WW), dry weight (DW) and water content (WC) were measured for each treatment in both experiments on each sampling day. CL and CW and EI were measured ($n = 10$) to the nearest 0.01 μm using an image analysing system (AnalySIS, SIS, Münster, Germany) connected to a stereomicroscope (Nikon SMZ800). EI was calculated as indicated by Perkins (1972) for lobster eggs, i.e. by adding the length and

the width of the pigmented eye spot and dividing it by 2. Six larvae per replicate ($n = 5$) were gently rinsed in distilled water and dried on filter paper for WW estimation. After 24 h at 60°C, DW was determined using a Sartorius BP211D scale (Sartorius, Germany) to the nearest 0.01 mg. Water content (WC) was calculated as the percentage of DW versus WW. Specific growth rate (SGR) and percentage of dry weight gain (WG) were used as growth indices at each developmental stage and were calculated using the following formulas:

$$\mathrm{SGR}(\%\,\mathrm{day}^{-1}) = ((\ln \text{final DW} - \ln \text{initial DW}) / \text{days between stages}) \times 100$$

$$\mathrm{WG}(\%\,\mathrm{day}^{-1}) = ((\text{final DW} - \text{initial DW})/\text{initial DW}) \times 100$$

Digestive enzyme analysis

A fluorometric assay was used to quantify digestive enzyme activities due to the low number of individuals available in each sampling period and treatment. Total protease, trypsin, amylase and esterase were measured individually ($n = 16$) at different developmental stages in each treatment and on each sampling day. Extracts were prepared by homogenisation of a single individual in 100 µL distilled water by sonication in an ice bath with three short pulses of 2 s (Vibra-cell., Sonics, USA). The homogenates were then centrifuged for 5 min at 13,000×*g* at 4°C, and the extracts used for the analysis of the four enzyme activities studied. All the enzymatic activities were measured in a Fluoroskan reader (ThermoFisher Scientific; USA) using 96-well CLINIPLATE black flat bottom microplates (Thermo Scientific). The enzymatic activities were expressed in international units (IU = µmol of substrate hydrolysed in 1 min per mL of enzymatic extract) per individual.

*Total protease analysis*

A 10 µL volume of the diluted homogenate from spider crab larvae and juveniles (1:10) was incubated with 10 µL of 0.3% FTC-casein (Molecular Probes; C-2990) in 100 µL buffer (Tris–HCL 0.1 M, $CaCl_2$ 10 mM, pH 8.0) at 37°C for 1 h. The reaction was stopped with 30 µL TCA (20%). After 5 min at 4°C, the mixture was centrifuged for 5 min at 13,000×*g* at 4°C. Fifty microliters of the supernatant were added to a microplate well with 200 µL buffer (Tris–HCl 0.5 M pH 8.5). Fluorescence was measured at $\lambda_{ex}/\lambda_{em}$ of 485/538 nm.

*Trypsin analysis*

Homogenates were diluted to 1:50 in the case of ZI and first crab (C), to 1:100 in zoea II (ZII) and up to 1:200 in megalopa (M). Trypsin activity was assayed using 20 µM Boc-Glan-Ala-Arg-methylcoumarin hydrochloride (SIGMA; B4153) in dimethyl sulfoxide (DMSO). A 10 µL volume of the diluted homogenate was mixed with 5 µL of the substrate and 195 µL of 50 mM Tris–HCl, 10 mM $CaCl_2$ buffer (pH 8.0), in microplate wells for analysis. Fluorescence was measured at $\lambda_{ex}/\lambda_{em}$ of 380/440 for 5 min at 30°C.

*Amylase analysis*

Homogenates were diluted to 1:10 in Z I and C and to 1:50 in ZII and M. Amylase was assayed using the Ultra Amylase Assay Kit (Molecular Probes; E33651) following the manufacturer's instructions. Fluorescence was measured at $\lambda_{ex}/\lambda_{em}$ of 485/538 nm for 5 min at 30°C.

*Non-specific esterase analysis*

Non-specific esterase was assayed using 4-methylubelliferyl butyrate (MUB; Fluka, 19362) following a method modified from Vaneechoutte et al. (1988). In brief, a stock solution was made by dissolving 100 mg of MUB in 10 mL DMSO, to which 100 µL Triton X-100 was added; the mixture was stored at −80°C. This stock solution was diluted in phosphate buffer pH 7.0 reaching a final concentration of 0.4 mM MUB. A 10 µL volume of the diluted homogenate from larvae and juveniles (1:10) was added to the microplate well and mixed with 250 µL of MUB 0.4 mM for analysis. Fluorescence was measured at $\lambda_{ex}/\lambda_{em}$ of 355/460 nm for 2 min at 30°C.

Experimental design and statistical analysis

The effect of total food deprivation (preliminary experiment) on growth parameters was assessed by one-way ANOVA using age (days post-hatching, dph) as factor. To analyse the effects of delayed food supply on ontogenic development (main experiment), the age factor (dph) was associated with the developmental stage, since ontogenic changes (moulting) are a physiological consequence of age. A nested ANOVA was selected for analysing biometric measurements and digestive enzyme activities, as levels of one factor (age-stage) are not the same at each of the levels of the other factors (treatment or feeding regime) imposed by the experimental conditions (Montgomery 2001; Underwood 1997). Type IV sums of squares were used to test the significance of the ANOVA model because this is the recommended method for missing cell layouts (Quinn and Keough 2002). Partial–Eta-squared (partial-$\eta^2$) was used to interpret the ANOVA results and consider the effect of size. This is a correlation ratio that measures the proportion of total variability that can be attributed to a factor or interaction, taken as if it were the only variable

(Pierce et al. 2004). We used repeated ANOVA measures over time (4, 8, 12 and 20 dph) as the within-subject factor and feeding regime as the between-subject factor to assess the effect of the feeding regime on the shape of the trajectories of the survival rate in tanks. The hypothesis of sphericity for the repeated measures factor was assessed with Mauchly's test. One-way ANOVA was used to assess the effect of feeding regime on SGR and WG at each interval at each developmental stage.

ANOVA assumptions were determined for each dependent variable: homogeneity of variances across groups was tested with Levene's test, and normal distribution was tested with the Kolmogorov–Smirnov test of the studentised residuals. When these assumptions were not satisfied, the data were transformed and re-tested. Total protease, trypsin and amylase activities were transformed with square root and esterase activity with Ln. All weights and lengths were log-transformed (with Ln). When the variables contained values below 1.0, a constant was added to move the minimum value of the distribution. Arcsine transformation (arcsine of the square root of the number) was used for percentage data (survival rates, SGR and WG). A post hoc Tukey HSD test was used to find significant differences between means when the ANOVA indicated that means were not all equal. For all tests, a probability of 5% ($P < 0.05$) was considered significant. The statistical software packages SPSS (SPSS Inc., Chicago, IL) and STATISTICA (StatSoft Inc., Tulsa, OK) were used for all statistical analyses.

In the present study, a large number of morphometric and enzymatic variables were used to assess changes in the nutritional status of spider crab larvae and first juveniles affected by different delays in receiving their first food. Besides the information provided by each individual parameter, we considered that a wider scope could be obtained using two different multivariate analyses. The similarity among individuals at different stages and feeding patterns was then evaluated by cluster analysis. Single linkage was selected as the amalgamation rule, and Euclidean distances were used for computing distances between objects in the multidimensional space. In addition, a canonical discriminant analysis (CDA) was used to assess which variables could best predict the nutritional condition of the different developmental stages when feeding was initiated at different dph. Prior to running the CDA, data were checked for multivariate normality, homogeneity of variances and covariances and correlations between means and variances (StatSoft 2002). Differences between developmental stages were measured directly by means of Wilk's $\lambda$-criterion. The $\chi^2$-test for Wilk's $\lambda$ was used to test the significance of the overall difference between the centroids in the feeding regime. The relative importance of the original variables in separating the developmental stages of the groups was gauged by standardised values.

## Results

### Preliminary experiment: the effect of total food deprivation

Newly hatched larvae deprived of food remained at the ZI stage, and all individuals had died by day 11 post-hatching. Survival data were adjusted to a sigmoid curve on which 50% survival was observed by 6.24 dph, with a dramatic increase in mortality from then on (Fig. 1). After one-way ANOVA of growth parameters using age (dph) as factor, no significant differences were observed in the biometric parameters ($CL_1 = 627.12 \pm 14.61$ μm → $CL_{10} = 633.48 \pm 49.24$ μm; $CW_1 = 490.35 \pm 12.61$ μm → $CW_{10} = 493.27 \pm 39.45$ μm; $AW_1 = 172.43 \pm 1.53$ μm → $AW_{10} = 156.63 \pm 13.15$ μm), although the DW of larvae tended to decrease ($DW_0 = 100.00 \pm 13.93$ μg →$DW_{10} = 86.25 \pm 7.41$ μg) as their WC increased ($WC_0 = 81.52 \pm 2.61\%$ → $WC_{10} = 84.53 \pm 1.65\%$), but WW remained the same ($WW_0 = 0.54 \pm 0.01$ mg → $WW_{10} = 0.56 \pm 0.02$ mg).

### Main experiment: the effects of delayed food supply

As expected, the statistical analyses showed that the mean survival rates for each age were different and also that the feeding regime had a significant effect on this parameter ($F_{3,5} = 21.49$, $P = 0.002$, partial $\eta^2 = 0.945$) (Fig. 2). Larvae belonging to the UF group and F6 larvae showed high mortality by day 8. A higher survival rate was obtained for groups F2 and F4, with no significant differences between them. Larvae in group F0, which received food from the moment of hatching, showed a significantly higher survival rate than the rest of the experimental groups

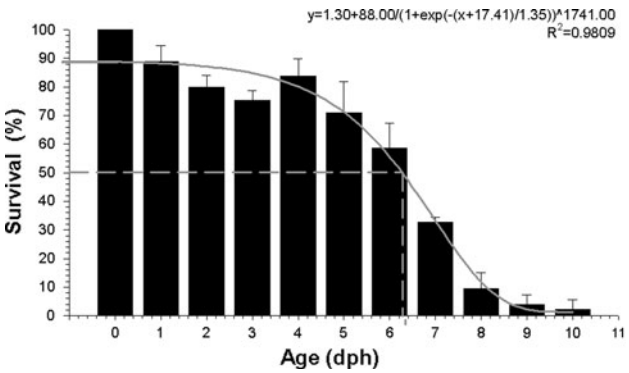


**Fig. 1** Survival rate (%) of newly hatched larvae of *Maja brachydactyla* reared during 11 days (*dph* days post-hatching) without food supply

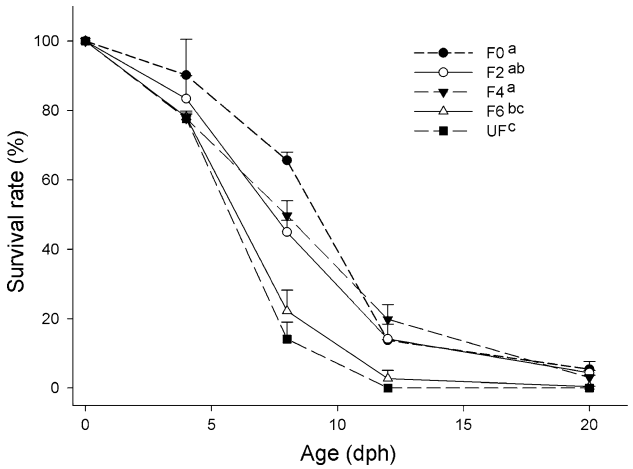


**Fig. 2** Survival rate (%) of the larvae and juveniles of *Maja brachydactyla* reared under different feeding conditions. Food deprivation treatments: *F0* fed EAM (enriched *Artemia* metanauplii) from hatching; *F2* fed EAM from day 2; *F4* fed EAM from day 4; *F6* fed EAM from day 6; *UF* unfed; *dph* days post-hatching. Letters indicate significant differences between feeding treatments (repeated measures ANOVA, $P < 0.05$; post hoc: Tukey HSD test)

until day 8; surprisingly, from this moment on, their mortality increased dramatically, which resulted in no significant differences by the end of the experiment (day 20) in relation to the values obtained for groups F2 and F4. In addition to the differences in survival rates, delayed feeding also had a significant effect on the development of larvae in each treatment, which was evidenced by differences in the number of individuals at a given developmental stage (ZI, ZII, M or C) in each group at the moment of sampling (Fig. 3). Thus, at 4 dph, the larvae in all groups were in ZI, although a small number of individuals in group F0 (12.4%) were already in ZII. At 8 dph, all the larvae in group F0 had moulted to ZII, while in group F2 26.7% of the individuals were still in ZI. In fact, a significant number of individuals were still at the ZI stage until 12 dph in all the groups affected by a delay in food supply, which was as much as 36.0% of the total larvae in group F6. By 12 dph, almost all larvae in group F4 were still in ZII (93.3%); in contrast, all the larvae in group F0 had moulted to M. By 20 dph, larvae in group F6 moulted to M, while crabs represented nearly the whole population in groups F0 (94.7%) and F2 (98.9%). A small amount of crabs were also sampled in F4 (29.4%).

The variability in the morphometrical (CL, CW and EI) and gravimetric (WW and DW) parameters during ontogenic development caused by the different feeding regimes was analysed by nested ANOVA. To determine the effect of age associated with moult stage, moult stage was nested in feeding regime, and to assess the effect of feeding regime, it was not nested (Table 1). With this model, more than 95% of the variance in dependent variables could be

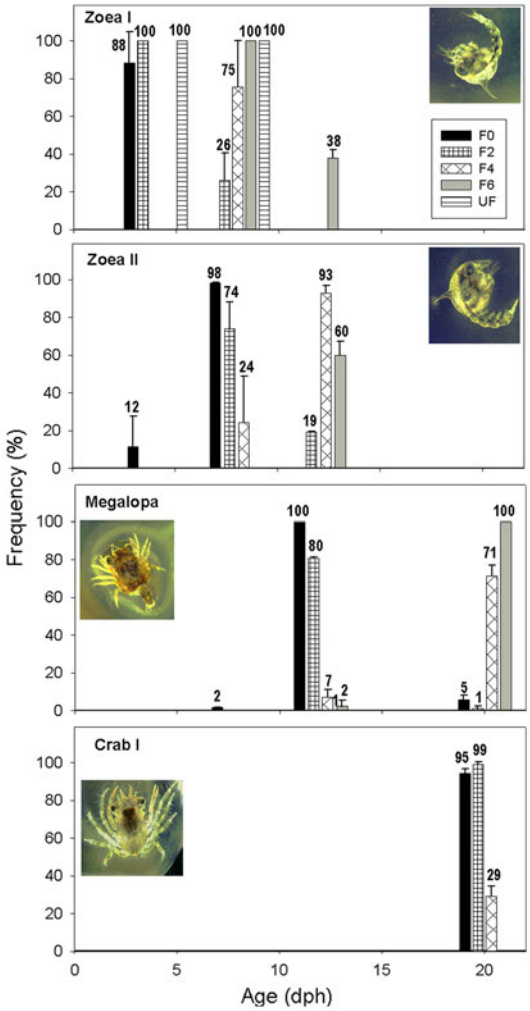


**Fig. 3** Frequency occurrence of different developmental stages of larvae and juveniles of *Maja brachydactyla* reared under different feeding conditions. Food deprivation treatments: *F0* fed EAM (enriched *Artemia* metanauplii) from hatching; *F2* fed EAM from day 2; *F4* fed EAM from day 4; *F6* fed EAM from day 6; *UF* unfed; *dph* days post-hatching

explained. However, age-stage had a highly significant effect on CL ($F_{21,420} = 897$, $P < 0.001$, partial $\eta^2 = 0.978$), CW ($F_{21,420} = 401$, $P < 0.001$, partial $\eta^2 = 0.952$), WW ($F_{21,202} = 383.7$, $P < 0.001$, partial $\eta^2 = 0.976$) and DW ($F_{21,202} = 259.6$, $P < 0.001$, partial $\eta^2 = 0.964$). The post hoc Tukey HSD test showed no significant effect of feeding regime on the different developmental stages, except for DW. There were significant differences between moult stages. However, feeding regimes had a large effect on the morphometric and gravimetric variables when age was not taken into account, except for EI ($F_{4,420} = 33$, $P < 0.001$, partial $\eta^2 = 0.241$). Although the ANOVA showed that the means were significantly different, the effect size was 24%. From a general point of view, two remarkable results were obtained (Table 1). First, a significantly larger carapace size

**Table 1** Growth biometric parameters of the different feeding treatments at each developmental stage—age (in days post-hatch-dph) of the spider crab *Maja brachydactyla*

| Stage, dph | ZI-0 | ZI-4 | ZI-8 | ZI-12 | ZII-4 | ZII-8 | ZII-12 | M-8 | M-12 | M-20 | C-20 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Treat* | | | | | | | | | | | |
| CL: carapace length (μm) | | | | | | | | | | | |
| $^{A}$F0 | 854.6 ± 25.9$^{a}$ | 862.6 ± 31.1$^{a}$ | | | 1123.4 ± 62.1$^{c}$ | 1120.3 ± 51.8$^{c}$ | | 1571.6 ± 81.3$^{d}$ | 1669.1 ± 45.7$^{e}$ | 1667.4 ± 75.2$^{de}$ | 2119.0 ± 74.8$^{f}$ |
| $^{B}$F2 | | 855.1 ± 52.8$^{a}$ | 935.8 ± 50.3$^{b}$ | | | 1131.4 ± 45.6$^{c}$ | 1134.0 ± 57.0$^{c}$ | | 1638.7 ± 56.1$^{de}$ | | 2144.6 ± 55.5$^{f}$ |
| $^{A}$F4 | | | 939.6 ± 50.6$^{b}$ | | | 1081.2 ± 40.6$^{c}$ | 1104.1 ± 49.2$^{c}$ | | 1690.8 ± 76.9$^{e}$ | 1683.7 ± 86.9$^{e}$ | 2108.8 ± 70.4$^{f}$ |
| $^{C}$F6 | | | 938.8 ± 40.8$^{b}$ | 970.6 ± 72.9$^{b}$ | | | 1133.7 ± 44.6$^{c}$ | | | 1676.4 ± 39.4$^{e}$ | |
| $^{D}$UF | | 844.2 ± 32.6$^{a}$ | 936.8 ± 60.4$^{b}$ | | | | | | | | |
| CW: carapace width (μm) | | | | | | | | | | | |
| $^{A}$F0 | 736.5 ± 32.0$^{a}$ | 752.7 ± 31.0$^{a}$ | | | 928.0 ± 20.2$^{c}$ | 955.7 ± 40.2$^{c}$ | | 1203.8 ± 59.4$^{f}$ | 1087.9 ± 85.7$^{de}$ | 1130.5 ± 90.3$^{def}$ | 1537.5 ± 91.4$^{g}$ |
| $^{C}$F2 | | 755.1 ± 29.8$^{a}$ | 823.4 ± 45.0$^{b}$ | | | 956.6 ± 33.2$^{c}$ | 963.7 ± 40.0$^{c}$ | | 1063.9 ± 56.2$^{d}$ | | 1600.6 ± 47.9$^{g}$ |
| $^{B}$F4 | | | 824.2 ± 27.8$^{b}$ | | | 929.1 ± 30.2$^{c}$ | 950.9 ± 38.5$^{c}$ | | 1074.9 ± 56.7$^{de}$ | 1134.9 ± 69.3$^{e}$ | 1577.1 ± 81.7$^{g}$ |
| $^{D}$F6 | | | 829.9 ± 27.4$^{b}$ | 840.5 ± 50.8$^{b}$ | | | 925.3 ± 61.4$^{c}$ | | | 1060.6 ± 45.7$^{d}$ | |
| $^{E}$UF | | 726.8 ± 34.2$^{a}$ | 826.6 ± 31.8$^{b}$ | | | | | | | | |
| EI: eye index (μm) | | | | | | | | | | | |
| $^{A}$F0 | 257.4 ± 22.1$^{b}$ | 282.5 ± 12.9$^{c}$ | | | 332.0 ± 11.0$^{fg}$ | 320.2 ± 11.4$^{efg}$ | | 287.1 ± 11.9$^{c}$ | 289.9 ± 10.8$^{c}$ | 290.9 ± 16.2$^{cd}$ | 240.6 ± 12.8$^{a}$ |
| $^{B}$F2 | | 283.8 ± 13.6$^{c}$ | 308.9 ± 12.9$^{de}$ | | | 324.4 ± 9.8$^{efg}$ | 321.9 ± 12.7$^{efg}$ | | 289.5 ± 11.6$^{c}$ | | 237.5 ± 10.3$^{a}$ |
| $^{C}$F4 | | | 321.3 ± 12.3$^{efg}$ | | | 331.7 ± 10.8$^{f}$ | 321.7 ± 11.9$^{efg}$ | | 282.1 ± 12.7$^{c}$ | 292.6 ± 13.4$^{c}$ | 247.9 ± 15.8$^{ab}$ |
| $^{D}$F6 | | | 317.0 ± 9.1$^{efg}$ | 317.5 ± 8.8$^{efg}$ | | | 326.5 ± 11.5$^{efg}$ | | | 279.0 ± 13.1$^{c}$ | |
| $^{CD}$UF | | 291.6 ± 13.5$^{c}$ | 312.7 ± 11.8$^{eg}$ | | | | | | | | |
| WW: wet weight (mg) | | | | | | | | | | | |
| $^{A}$F0 | 0.50 ± 0.01$^{a}$ | 0.58 ± 0.08$^{ab}$ | | | 0.83 ± 0.02$^{c}$ | 0.87 ± 0.07$^{c}$ | | 1.57 ± 0.10$^{de}$ | 1.68 ± 0.10$^{de}$ | 1.51 ± 0.01$^{de}$ | 2.46 ± 0.32$^{f}$ |
| $^{B}$F2 | | 0.55 ± 0.05$^{a}$ | 0.57 ± 0.04$^{ab}$ | | | 0.82 ± 0.04$^{c}$ | 0.87 ± 0.07$^{c}$ | | 1.57 ± 0.13$^{de}$ | | 2.78 ± 0.31$^{f}$ |
| $^{B}$F4 | | | 0.60 ± 0.05$^{ab}$ | | | 0.80$^{ab}$ ± 0.05$^{ab}$ | 0.79 ± 0.08$^{c}$ | | 1.44 ± 0.13$^{d}$ | 1.82 ± 0.14$^{e}$ | 2.70 ± 0.36$^{f}$ |
| $^{C}$F6 | | | 0.59 ± 0.06$^{ab}$ | 0.57 ± 0.01$^{ab}$ | | | 0.72 ± 0.01$^{bc}$ | | | 1.59 ± 0.18$^{de}$ | |
| $^{D}$UF | | 0.54 ± 0.02$^{a}$ | 0.56 ± 0.01$^{a}$ | | | | | | | | |
| DW: dry weight (μg) | | | | | | | | | | | |
| $^{A}$F0 | 92.77 ± 6.13$^{ab}$ | 123.62 ± 16.54$^{bc}$ | | | 155.50 ± 22.89$^{ef}$ | 206.42 ± 15.79$^{gh}$ | | 260.47 ± 28.15$^{ij}$ | 443.28 ± 28.59$^{lm}$ | 327.56 ± 10.52$^{jk}$ | 541.54 ± 102.44$^{n}$ |
| $^{B}$F2 | | 108.87 ± 12.65$^{abc}$ | 116.27 ± 18.26$^{bcd}$ | | | 159.12 ± 23.02$^{ef}$ | 180.39 ± 9.27$^{fgh}$ | | 328.67 ± 29.37$^{k}$ | | 674.08 ± 72.02$^{o}$ |
| $^{B}$F4 | | | 116.78 ± 10.79$^{bc}$ | | | 140.80 ± 8.80$^{de}$ | 176.12 ± 25.02$^{fg}$ | | 229.58 ± 17.48$^{hi}$ | 370.33 ± 9.64$^{kl}$ | 490.88 ± 84.71$^{mn}$ |
| $^{C}$F6 | | | 104.96 ± 11.77$^{abc}$ | 91.30 ± 6.93$^{abc}$ | | | 121.67 ± 12.37$^{bcde}$ | | | 363.53 ± 16.14$^{kl}$ | |
| $^{D}$UF | | 93.77 ± 3.64$^{a}$ | 95.06 ± 2.64$^{a}$ | | | | | | | | |

*CL* carapace length (μm), *CW* carapace width (μm), *EI* eye index (μm), *WW* wet weight (mg), *DW* dry weight (μg)

Results are expressed in terms of mean ± SD. Values with different superscript are significantly different from each other (capital letters for the effect of treatment and small letters for the effect of age-stage; nested ANOVA, $P < 0.05$, Tukey HSD test post hoc). Feeding treatments: *F0*, *F2*, *F4* & *F6* newly hatched larvae fed from 0, 2, 4 and 6 dph, respectively; *UF* unfed larvae during the whole experiment

of ZI was measured in all the delayed feeding treatments for ages ZI-8 and ZI-12 (CL around 930 μm; CW around 830 μm) compared to the same stage in groups F0 and F2 for ages ZI-0 and ZI-4 (CL around 855 μm; CW around 750 μm). Surprisingly, this larger size of ZI found in all the food delayed treatments was not correlated with an increase in their biomass, measured either as WW or as DW, which indicates that in delayed feeding treatments the carapace may be separated from the epidermis. Second, significantly higher values of DW were measured in ZII and M of group F0 compared with those of groups F2 and F4 sampled on the same day. In contrast, a significantly higher DW of the C stage was measured in F2 (674.1 ± 72.0 μg) compared to F0 (541.5 ± 102.4 μg). The aforementioned delay in reaching each developmental stage as a result of the differences in the initial moment of food supply after hatching was also evidenced by the SGR and percentages of weight gain calculated between stages in each feeding group (Table 2). The faster development and weight gain of larvae in group F0 was evident only up to 12 dph, since during the transition from M to C, a significant reduction in both SGR and dry weight gain was observed when compared to values calculated for larvae in groups F2 or F4. The highest values of these two parameters were observed during the transition from ZII to M in all the experimental groups.

The enzymatic activities measured in each experimental group at different moments during larval development are shown in Fig. 4. The nested ANOVA of the four enzymatic activities showed significant differences related to the two factors. The effect of feeding regime was similar on all enzymes: total protease activity ($F_{4,227} = 37.24$, $P < 0.001$, partial $\eta^2 = 0.396$), trypsin ($F_{4,228} = 30.60$, $P < 0.001$, partial $\eta^2 = 0.349$), amylase ($F_{4,228} = 56.37$, $P < 0.001$, partial $\eta^2 = 0.497$) and esterase ($F_{4,226} = 224.80$, $P < 0.001$, partial $\eta^2 = 0.799$). The intensity of fasting induced a significant effect on enzymatic activities independently of the moult stage (decrease in activity after fasting and increase after a time of feeding); however, age determined the recovery of enzyme levels. The effect of age-stage nested in feeding regime was more evident for specific enzymes, trypsin ($F_{11,228} = 13.72$, $P < 0.001$, partial $\eta^2 = 0.398$), amylase ($F_{11,228} = 24.38$, $P < 0.001$, partial $\eta^2 = 0.540$) and esterase ($F_{11,226} = 85.90$, $P < 0.001$, partial $\eta^2 = 0.809$), than for total protease activity ($F_{11,227} = 5.53$, $P < 0.001$, partial $\eta^2 = 0.211$).

**Table 2** Specific growth weight (SGR) and percentage of dry weight gain (WG) between stages of the spider crab *Maja brachydactyla*

| | dph | $ZI_0$-$ZI_n$ | dph | $ZI_n$-$ZII_n$ | dph | $ZII_n$-$M_n$ | dph | $M_n$-$C_{20}$ |
|---|---|---|---|---|---|---|---|---|
| SGR (% day$^{-1}$) | | | | | | | | |
| F0 | 0–4 | 6.98 ± 3.25$^{a}$ | 4–8 | 12.75 ± 1.92$^{a}$ | 8–12 | 19.06 ± 1.67$^{a}$ | 12–20 | 6.87 ± 0.28 |
| | | | | | 8–20 | 3.85 ± 0.27$^{b}$ | | |
| F2 | 0–4 | 3.86 ± 2.78$^{b}$ | 4–8 | 9.20 ± 4.14$^{b}$ | 8–12 | 18.04 ± 2.26$^{a}$ | 12–20 | 7.83 ± 4.27 |
| | 0–8 | 2.68 ± 2.02$^{bc}$ | 4–12 | 6.30 ± 0.63$^{bc}$ | | | | |
| F4 | 0–8 | 2.83 ± 1.24$^{bd}$ | 4–8 | 3.21 ± 1.55$^{cd}$ | 8–12 | 12.17 ± 1.88$^{c}$ | 12–20 | 9.35 ± 2.00 |
| | | | 4–12 | 5.16 ± 3.46$^{c}$ | 8–20 | 8.06 ± 0.22$^{d}$ | | |
| F6 | 0–8 | 1.47 ± 1.46$^{bc}$ | 4–12 | −0.24 ± 1.25$^{d}$ | 8–20 | 7.90 ± 0.37$^{d}$ | | |
| | 0–12 | −0.14 ± 0.63$^{bc}$ | | | | | | |
| UF | 0–4 | 0.25 ± 0.97$^{c}$ | | | | | | |
| | 0–8 | 0.30 ± 0.34$^{cd}$ | | | | | | |
| WG (% day$^{-1}$) | | | | | | | | |
| F0 | 0–4 | 33.26 ± 17.783$^{a}$ | 4–8 | 66.98 ± 12.77$^{a}$ | 8–12 | 114.75 ± 13.85$^{a}$ | 12–20 | 73.32 ± 3.03$^{a}$ |
| | | | | | 8–20 | 58.69 ± 5.10$^{b}$ | | |
| F2 | 0–4 | 17.35 ± 13.63$^{abc}$ | 4–8 | 46.16 ± 21.14$^{abc}$ | 8–12 | 106.56 ± 18.46$^{a}$ | 12–20 | 106.65 ± 22.28$^{b}$ |
| | 0–8 | 25.33 ± 19.69$^{ab}$ | 4–12 | 65.70 ± 8.51$^{ab}$ | | | | |
| F4 | 0–8 | 25.89 ± 11.63$^{ab}$ | 4–8 | 13.90 ± 7.12$^{bc}$ | 8–12 | 63.06 ± 12.42$^{b}$ | 12–20 | 113.81 ± 36.90$^{b}$ |
| | | | 4–12 | 57.42 ± 54.91$^{ac}$ | 8–20 | 163.02 ± 6.84$^{c}$ | | |
| F6 | 0–8 | 13.15 ± 12.68$^{bc}$ | 4–12 | −1.58 ± 10.01$^{c}$ | 8–20 | 158.19 ± 11.47$^{c}$ | | |
| | 0–12 | −1.58 ± 7.47$^{bc}$ | | | | | | |
| UF | 0–4 | 1.08 ± 3.92$^{c}$ | | | | | | |
| | 0–8 | 2.47 ± 2.84$^{c}$ | | | | | | |

Results are expressed in terms of mean ± SD. Letters indicate significant differences between feeding regimes for changes in the stage of development (nested ANOVA, $P < 0.05$, Tukey HSD test post hoc). *C* first crab, *dph* days post-hatching; *F0*, *F2*, *F4* & *F6* newly hatched larvae fed from 0, 2, 4 and 6 dph, respectively, *n* number of dph in each treatment and sampling day, *M* megalopa, *UF* unfed larvae during the whole experiment, *ZI* zoea I, *ZII* zoea II

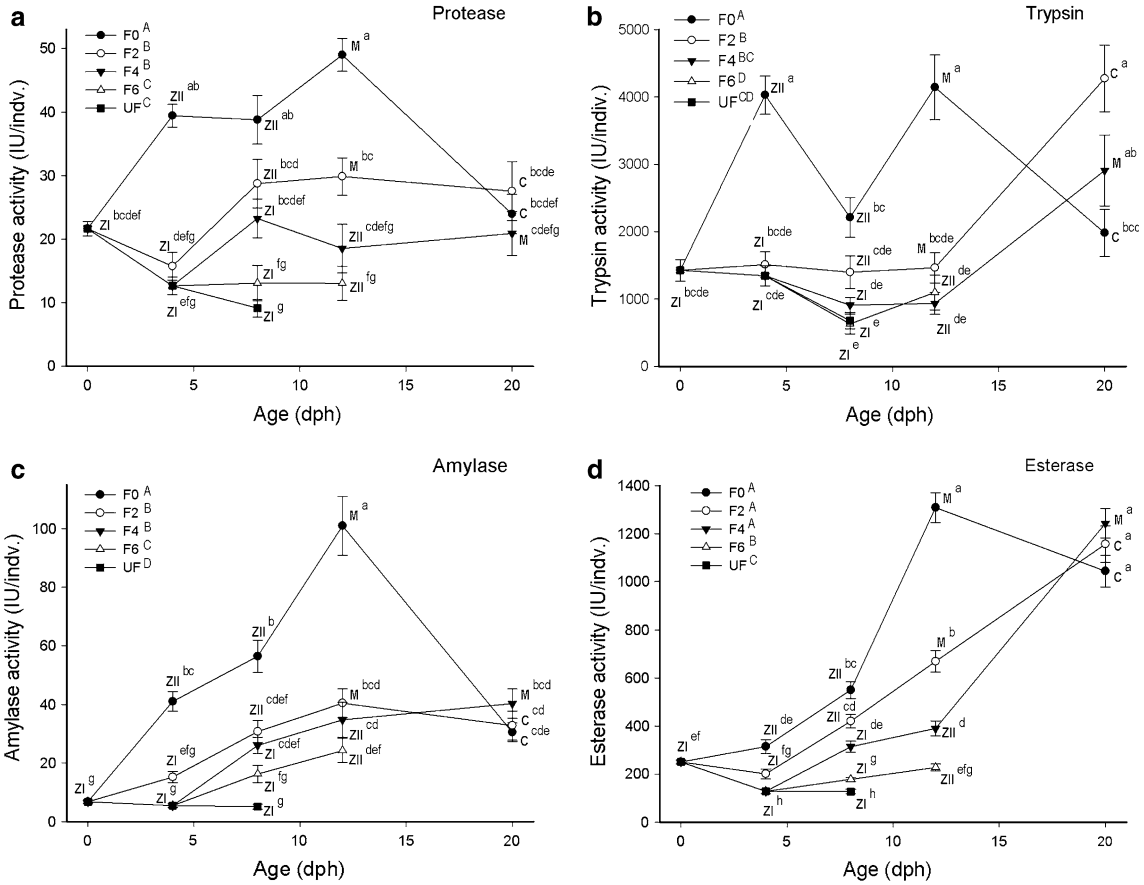


**Fig. 4** Total protease (**a**), trypsin (**b**), amylase (**c**) and esterase (**d**) activities (units per min and individual) in the most representative spider crab *Maja brachydactyla* larval stages reared under different feeding conditions. Results are expressed in terms of mean ± SE. Values with *different superscript* are significantly different from each other (*capital letters* for the effect of treatment and *small-letters* for the effect of age-stage) (nested ANOVA $P < 0.05$; post hoc: Tukey HSD test). Feeding treatments: *F0* fed EAM (enriched *Artemia* metanauplii) from hatching; *F2* fed EAM from day 2; *F4* fed EAM from day 4; *F6* fed EAM from day 6; *UF* unfed. Stages of development: *ZI* zoea I; *ZII* zoea II; *M* megalopa; *C* first crab; *dph* days post-hatching

Although nested ANOVA showed that the means of protease activity were significantly different, the partial $\eta^2$ was low, so the effect on this variable was small.

The post hoc Tukey HSD test of age-stage nested in feeding regime showed quite similar patterns for all the assayed enzymes. In all cases, group F0 showed higher activity values irrespectively of the developmental stage until 12 dph, but a sharp decrease was observed in all the activities for this group when M moulted to C. In contrast, larvae in groups F2 and F4 showed no changes until 12 dph, but from this moment on there were sharp increases in the trypsin and esterase activities, which reached or exceeded the values measured in F0 larvae. Larvae in groups F6 and UF showed significantly lower activities for all the assayed enzymes.

The cluster analysis used to assess whether the biometrical variables or the digestive enzyme activities were able to classify the condition of the different developmental stages of *M. brachydactyla* with different nutritional histories is detailed in Fig. 5. When biometrical variables were considered, the individuals were classified by their developmental stage better than by their feeding treatment (Fig. 5a). In contrast, enzymatic activities provided a better classification of the individuals in relation to their feeding treatment (Fig. 5b). Nevertheless, at 20 dph, crabs in groups F0 and F2 were placed closer to M present in groups F4 and F6, which suggests that larval stages were better indicators of nutritional condition after metamorphosis than enzymatic activities.

The ability of enzyme activities to evidence changes in the nutritional condition of the larvae was also supported by the results obtained when data were analysed with CDA, in which all the variables were used to calculate discriminant functions that could predict the nutritional state of the larvae at each developmental stage (Table 3). In the case of ZI, 4 functions representing 99.5% of the total variance observed among six different experimental groups significantly discriminated the moment of first feeding (Tables 3, 4; Fig. 6a). The first 3 discriminant functions accounted for 63.4, 23.0 and 11.4% of the total

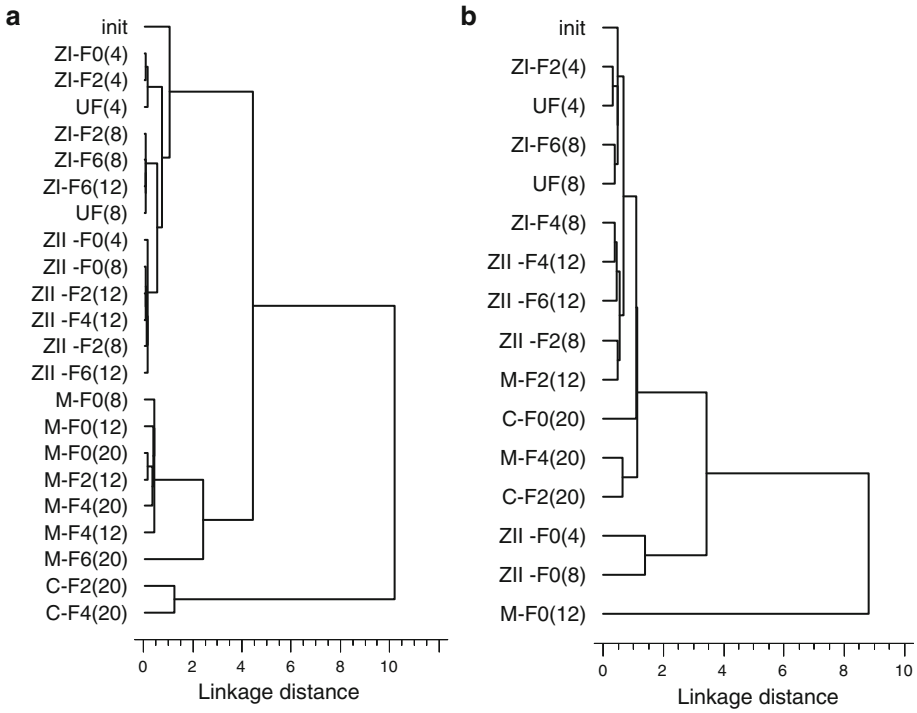


**Fig. 5** Result of the cluster multivariate analysis generated by biometric (**a**) and enzymatic activity (**b**) data at the different development stages reached after following several food deprivation treatments: *F0* fed EAM (enriched *Artemia* metanauplii) from hatching; *F2* fed EAM from day 2; *F4* fed EAM from day 4; *F6* fed EAM from day 6; *UF* unfed. Stages of development: *ZI* zoea I; *ZII* zoea II; *M* megalopa; *C* first crab. Days post-hatching indicated between *brackets*. *Init* newly hatched zoea

**Table 3** Standardised function coefficients for each of the significant discriminant functions used to identify and classify the nutritional condition of different developmental stages of *Maja brachydactyla*

| Variable | ZI | | | | ZII | | | M | | C |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 1 | 2 | 1 |
| **DW** | 0.614 | 0.164 | −0.319 | 0.391 | −0.413 | 0.189 | **−0.761** | −0.279 | 0.689 | **1.731** |
| **WC** | 0.435 | −0.209 | −0.011 | 0.628 | 0.467 | −0.069 | −0.145 | 0.805 | 0.073 | 0.808 |
| **CL** | 0.027 | 0.026 | 0.186 | 0.073 | **0.688** | −0.048 | **0.748** | −0.073 | 0.219 | **1.573** |
| **CW** | 0.302 | −0.262 | 0.355 | **−0.747** | −0.836 | 0.023 | −0.281 | 0.011 | −0.008 | −1.243 |
| **EI** | 0.289 | **−0.598** | 0.196 | 0.441 | 0.311 | 0.221 | −0.432 | −0.139 | −0.002 | −0.171 |
| **Protease** | **−1.014** | −0.327 | **1.636** | **0.403** | 0.390 | **1.990** | 0.409 | −0.436 | **−1.774** | **2.621** |
| **Trypsin** | −0.763 | −0.319 | −0.719 | −0.413 | −0.168 | 0.212 | **−0.642** | **0.920** | **1.114** | **−3.130** |
| **Amylase** | **1.235** | 0.143 | **−1.519** | −0.355 | 0.032 | **−1.539** | 0.014 | **−0.600** | −0.837 | 0.813 |
| **Esterase** | 0.468 | **1.094** | 0.554 | 0.172 | **−0.730** | −0.534 | **0.669** | 0.036 | **1.382** | −0.739 |

Coefficients with more weight inside the discriminant function are in bold. *C* first crab, *CL* carapace length, *CW* carapace width, *DW* dry weight, *EI* Eye index, *M. megalopa*, *Z* zoea, *WC* water content (%)

variance, respectively. Specific activities of amylase and protease were the variables with the highest relevance in the first and third constructed functions, while esterase had a higher weight in the second function (Table 3). Discriminant functions were also successful in reassigning cases to their level of food deprivation, correctly classifying as much as 93.9% of the total observations for ZI.

Four statistically significant functions were able to predict the nutritional condition of ZII fed on different days after hatching (Tables 3, 4), and most of the variability (95.6%) was explained by 3 of these functions, which accounted for 69.5, 20.9 and 5.2% of the total variance, respectively. CL and esterase activity were the variables that best identified the nutritional condition of the larvae and were included in the first and third functions, while protease and amylase activities had a higher relevance in the second function. Considering the first discriminant function (axis *x*, Fig. 6b), ZII from group F6 at 12 dph showed extreme and centroid values that were the opposite of those of ZII fed from hatching (F0-8 dph). The other groups with delayed food supply (i.e. F2-8 dph and F4–12 dph) showed intermediate centroid values along the axis. In contrast, the second discriminant function (axis y)

**Table 4** Summary of the results of the canonical discrimination analysis (only significant functions are shown) used to establish discriminant functions in order to identify and classify the nutritional condition of *Maja brachydactyla* larvae at different developmental stages

| Stage | Function | Eigen value | Percentage of variance | Canonical correlation | Wilks Lambda | Chi-Square | d.f. | *P* |
|---|---|---|---|---|---|---|---|---|
| Zoea I | 1 | 9.15 | 63.37 | 0.95 | 0.006 | 537.87 | 45 | <0.0001 |
| | 2 | 3.32 | 22.98 | 0.88 | 0.065 | 290.99 | 32 | <0.0001 |
| | 3 | 1.65 | 11.41 | 0.79 | 0.281 | 135.15 | 21 | <0.0001 |
| | 4 | 0.25 | 1.75 | 0.44 | 0.744 | 31.44 | 12 | 0.0017 |
| Zoea II | 1 | 3.42 | 69.51 | 0.88 | 0.073 | 240.61 | 36 | <0.0001 |
| | 2 | 1.03 | 20.90 | 0.71 | 0.323 | 103.93 | 24 | <0.0001 |
| | 3 | 0.26 | 5.20 | 0.45 | 0.655 | 38.91 | 14 | 0.0004 |
| Megalopa | 1 | 12.38 | 55.50 | 0.96 | 0.007 | 259.18 | 18 | <0.0001 |
| | 2 | 9.92 | 44.50 | 0.95 | 0.092 | 124.33 | 8 | <0.0001 |
| Crab | 1 | 13.48 | 100.00 | 0.97 | 0.069 | 89.52 | 9 | <0.0001 |

*df* discriminant function, *P* probability

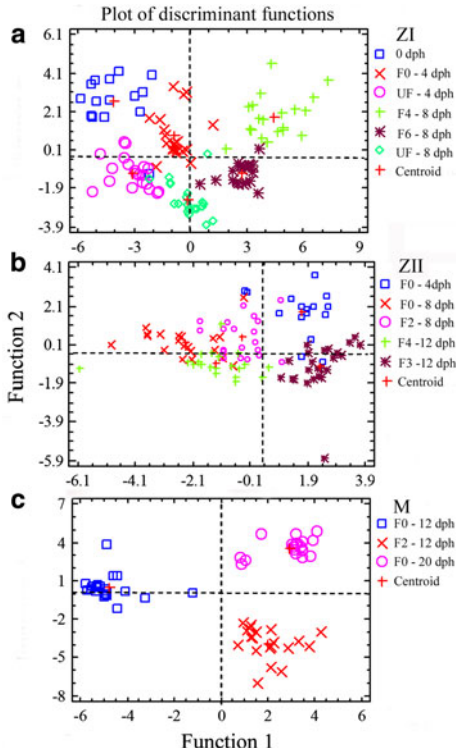


**Fig. 6** Plots of the first two axes for the canonical discriminant analysis represented by different feeding treatments and stage of development in spider crab *Maja brachydactyla*. Data were created using five biometrical measurements and four hepatopancreatic enzyme activities. Food deprivation treatments: *F0* fed EAM (enriched *Artemia* metanauplii) from hatching; *F2* fed EAM from day 2; *F4* fed EAM from day 4; *F6* fed EAM from day 6; *UF* unfed. Stages of development: *ZI* zoea I; *ZII* zoea II; *M* megalopa. Days post-hatching (dph) indicated after the feeding treatment

discriminated among experimental groups of different ages rather than the nutritional condition of the larvae or the length of the delay in food supply. Using the results derived from discriminant functions, 84.0% of the cases were correctly classified into different experimental groups.

In the case of M, the CDA showed two functions, which discriminated between individuals fed at different times (Table 4). The first function accounted for about 55.5% and the second function for about 44.5% of the variability among the different experimental conditions. The variables with the greatest weight in each function were trypsin and esterase activities, respectively (Table 3). M from the group fed from hatching (F0–12 dph) showed extreme and centroid values in function 1, which were the opposite of those in groups F2 (12 dph) and F4 (20 dph), which allowed us to discriminate between the control group and the rest of the groups; whereas M from the group fed from hatching (F0–12 dph) had intermediate centroid values in function 2, so that this function could discriminate this group from the other two (Fig. 6c). Discriminant functions were also successful in reassigning cases to their level of food deprivation, with all the cases correctly classified (100%). In the first crab stage, only one function was able to discriminate between the F0 and F2 crabs (Table 4), and total protease and trypsin had the highest relevance. All the cases were correctly classified (100%) into the experimental groups (F0 and F2 at 20 dph) with the results derived from the discriminant function.

## Discussion

*Maja brachydactyla* larvae, like other Majoidea species (Anger et al. 1981; Dawirs 1983; McConaugha 1985; Figueiredo et al. 2008), are planktotrophic; and consequently, a decrease in the food supply is associated with an increase in the duration of larval stages and a decrease in growth and survival rates. Under normal conditions, larvae fed with live prey from hatching complete their development until C stage in 18 days (Andrés et al. 2007, 2008). In the present study, unfed larvae kept under the same

environmental conditions (temperature of 18 ± 1°C, salinity 36 ± 1 and natural photoperiod) did not moult into ZII and died at 11 dph. This is in agreement with what has been observed in other crab species such as *Carcinus maenas* (Dawirs 1984, 1986) and *Chasmagnathus granulata* (Giménez 2002). In contrast, Figueiredo and Narciso (2006) found that unfed ZI of *Lysmata seticulata* moulted to ZII after 3 days and survived for 11 days, which suggests the existence of facultative lecithotrophy in this shrimp species.

Larvae of *M. brachydactyla* affected by the delay in the initial food supply after hatching showed a lower survival rate, a delay in moulting and an altered nutritional condition compared to larvae that had received food immediately. This last point was evidenced by differences in certain biometrical parameters measured at each developmental stage, such as carapace size and DW, but also largely by changes in the activities of the main digestive enzymes.

The survival rate measured in the control group (F0) until the larvae reached the ZII stage at 8 dph was significantly higher than in the rest of the experimental treatments. Nevertheless, a dramatic decrease was then observed at the M and C stages, with values resembling those measured in groups F2 and F4. Taking into account the good growth and digestive enzyme activity values measured in these larvae up until then, it is possible that the amount of food provided (60 EAM larva$^{-1}$) was not enough to properly support the rapidly growing population in the tanks. The delayed onset of feeding clearly affected the survival rate of larvae and also determined a noticeable delay in larval moulting (Fig 3), as well as the presence of ZI stages with a larger sized carapace than those fed normally (Table 1). Under normal feeding conditions, a longer duration of larval stages should imply that there is more time available for accumulating the body reserves needed for initiating moulting and successful development (Anger 1987). This occasionally results in further compensatory growth manifested by a higher biomass; however, in the present study, larvae under temporary food deprivation reached different developmental stages with a conspicuous delay, but showed no differences in WW or DW. In the case of the above-mentioned "large sized" ZI, this could be due to a detachment of the carapace and not with real growth.

However, although larvae in group F6 ingested food and filled their stomachs, their development was impaired since they were not able to metamorphose during the 20 days of the experiment and their mortality increased dramatically to over 50%. Taking this into account, a 6 day delay in providing first food to larvae in this species might be considered a physiological point of no return (PNR), resulting not in the inability to eat, but in irreversible damages which affect growth (as evidenced by the negative SGR and WG) and development (very few larvae reached M stage and none metamorphosed into crabs). According to Storch and Anger (1983) and Anger et al. (1985), surpassing this PNR always leads to an irreversible loss in the ability to store and metabolize lipids.

Although in the present study, the biochemical composition of larvae was not analysed, the activities of the main digestive enzymes provided insight into the use of the energy sources at each developmental stage as well as feeding conditions. Larvae in group F0 showed significantly higher activities of all the enzymes during almost the entire experiment, although the food restriction was also reflected in the lower values measured at C stage. In contrast, larvae in groups F2 and F4, which initially showed low values of digestive enzyme activities at stages ZI and ZII, showed a clear increase with age, which was particularly evident in the case of trypsin and esterase (Fig. 4b). This result could be explained as a "compensatory response", which was also illustrated by the significantly higher dry weight measured, for example, in the C stage of F2 group (Table 1). A similar response was also observed in the spiny lobster (*Jasus edwardsii*) by Johnston et al. (2004a). When these authors kept phyllosoma larvae without food supply for 8 days, at the beginning of stages I and IV they found higher activity of total protease, lower esterase activity and similar trypsin and amylase activities to those measured in fed larvae. In contrast, a reduction in digestive enzyme activities as an effect of food deprivation has been described in *Carcinus maenas* larvae (Dawirs 1983; Harms et al. 1994) and *Litopenaeus vannamei* juveniles (Sánchez-Paz et al. 2007). It seems that different physiological responses to food deprivation can be found during early life stages of crustaceans depending on their nutritional life history and their type and amount of nutrient reserves.

We considered that the nutritional condition of *M. brachydactyla* larvae might be better evidenced by a combination of the different parameters evaluated. In this sense, the cluster analysis clearly discriminated among different feeding treatments when enzyme activity values were used, while the classification obtained using morphological parameters only separated the individuals according to their developmental stage, irrespectively of their feeding treatment. Similarly, when a CDA was carried out for each developmental stage, the digestive enzyme activities were key variables in the construction of the functions, which explained the differences resulting from the changes in the feeding pattern. Total protease and esterase activities were the variables with the highest weight in the discrimination of newly hatched zoeae at different ages and in different dietary treatments, which was closely linked to the presence of lipids and proteins in larval stores. Unfed ZI at 4 and 8 dph showed lower esterase activity in comparison with larvae of group F2 at

4 dph, which indicates that maternal reserves had been completely exhausted. In contrast, amylase activity was an important discriminant variable in older larvae after first feeding but not so important in newly hatched ZI and unfed larvae. This could be related to both the progressive increase in the activities of all digestive enzymes during ontogeny in this superfamily of crustaceans (Hirche and Anger 1987a, b; Harms et al. 1991; Andrés et al. 2010) and the increase in the fraction of carbohydrates present in the larval food. In the case of ZII, esterase activity discriminated best among feeding treatments, which was probably linked to their increasing role in the digestion of the lipids of their live prey. At M stage, the CDA suggests that there are different digestion strategies depending on the nutritional status of the larvae. Well-fed larvae, which received food from the moment of hatching (F0), digested carbohydrates to use them as a source of energy to a greater extent than any of the deficiently fed larvae, which might have tended to use proteins as energy rather than carbohydrates or lipids. This is supported by the studies by Anger (1986) and Harms et al. (1991) who indicated that during starvation periods larval decapods use their own protein reserves once lipid reserves are completely depleted. Finally, results from the CDA of the two experimental groups that reached C stage (F0 and F2) distinguished the two treatments according to their biometrical parameters (DW, CL and CW) and the digestive enzyme activities related to protein digestion (total protease and trypsin). Considering the different ecological niches and physiology of planktonic larvae (ZI, ZII, M) and benthic juveniles (C1), the present results indicate that there is no universal index that can discriminate between them. Consequently, nutritional indices have to be defined for each developmental stage.

## Conclusions

As a summary, the results obtained in the present study suggest that a delay of 2 days in the onset of exogenous feeding might be considered the maximum allowable time that does not have negative effects on growth, survival and larval quality. These results are in agreement with Figueiredo and Narciso (2006), who found that *L. seticulata* larvae that were unfed for 2 days were able to metamorphose without any detrimental effect on their performance. Hence, detrimental effects may have occurred in larvae when feeding started after 2 dph as observed in the present study for larvae supplied with food at 4 dph. Six days without food supply could be considered a physiological point of no return because of the irreversible damage observed. Limitations in food availability would considerably increase the length of the vulnerable pelagic stages of development (Z and M), which might result in higher mortality. The faster the metamorphosis and transfer to benthic conditions the better in terms of final survival.

However, it has been demonstrated that in the spider crab, digestive enzyme activities are good indicators of the nutritional condition of larval decapods due to their species and age specificity, sensitivity and short latency, similarly to what has been reported for other crabs (Harms et al. 1994), lobsters (Johnston et al. 2004a), shrimps (Comoglio and Gaxiola 2004) and fish larvae and juveniles (Ferron and Leggett 1994). Metamorphosis implies large physiological changes, which means that larvae and juveniles occupy different ecological niches; therefore, in order to compare them, stage-specific nutritional indices are necessary.

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