CHARACTERIZATION OF THE MEGALOPAL PREMOULT STAGES OF THE GREEN CRAB, *CARCINUS MAENAS* (DECAPODA, PORTUNIDAE), FROM LABORATORY CULTURE

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A B S T R A C T

The premoult stages of *Carcinus maenas* (L.) megalopae were described from laboratory-reared larvae. Characterization of the premoult stage sequence was based upon a time sequence of morphological modifications of the distal segment of the second maxilliped, the uropod, and the telson. An intermoult plus seven premoult stages were identified, following a sequence of morphological modifications similar to the one described in previous studies of brachyuran decapods. The later premoult stages D_3 and D_4 mentioned by Drach were not observed, possibly owing to both the low calcification of the megalopae cuticle and the short duration of these moult stages. The knowledge of the time series of moult events will assist in the evaluation of the development state and competence of megalopae throughout the several phases of dispersal and recruitment, based on the observation of their morphological characters.

Brachyuran decapod crustaceans usually have a planktonic larval phase that is responsible for dispersal (Forward *et al.*, 2001). The larval phase normally comprises several planktotrophic zoeal stages. The last zoea moults to a specialised planktotrophic settlement stage called megalopa (also known in the literature as postlarva). Megalopa is the stage responsible for the selection of the habitat in which to settle and metamorphose to the first juvenile crab instar (see Anger, 2001, for exceptions).

Crab megalopae are active swimmers that, during the dispersion and habitat-selection phases, perform diel (e.g., Epifanio, 1988; Hobbs and Botsford, 1992; Abelló and Guerao, 1999) and tidally-synchronized vertical migrations (e.g., Cronin and Forward, 1979; Tankersley and Forward, 1994; Zeng and Naylor, 1996a). These migrations assist in retention or in directional transport towards appropriate coastal habitats as, for example, in the tidal stream transport used to travel upstream by species that spend their adult life in estuaries (Olmi, 1994). Older megalopae may be more numerous than younger megalopae in the neuston layer of shelf waters during the night (Hobbs and Botsford, 1992). All available studies (Hatfield, 1983; Lipcius *et al.*, 1990;

Metcalf and Lipcius, 1992; Morgan *et al.*, 1996; Zeng *et al.*, 1997) show that as megalopae approach the settlement habitat they show a more advanced stage of development within the moult cycle. Collectively, these studies establish a relationship between dispersal processes and the physiological state of the last larvae.

Several factors, including the substrate of adult habitat, aquatic vegetation, biofilms, conspecific odours, humic acids present in estuarine waters, and odour of related crab species and prey, induce settlement and accelerate the development of crab megalopae, reducing the time to metamorphosis (reviewed by Forward *et al.*, 2001). Competence is defined as the ability of larval forms to respond to settlement stimuli (Crisp, 1974). However, there is a period before metamorphosis when the larvae are not competent. It is not known how early in the megalopal stage the larvae become competent and respond to settlement cues. Nonetheless, it seems clear that behavioural responses involved in transport of megalopae and habitat selection are generally related with the development stage within the intermoult cycle. Therefore, studies addressing mechanisms of transport, supply, and settlement of

crab megalopae should take into account the larval development stage, in order to obtain meaningful results concerning the timing of recruitment to benthic habitats (Hasek and Rabalais, 2001).

Drach (1939, 1944) was the first to describe integument changes in decapod crustaceans during the intermoult cycle. This scheme was originally applied to record the sequence of the morphologic changes in the integument and setagenesis in adults of brachyuran and caridean decapods. The sequence of events was grouped in four main stages: A and B (postmoult stages), C (intermoult stage), and D (premoult stage). The latter classification was later improved by Drach and Tchernigovtzeff (1967). Several other crustacean groups were enclosed by this work (e.g., amphipods, Charniaux-Legrand, 1952; cirripeds, Davis *et al*., 1973; stomatopods, Reaka, 1975), showing that this series of morphological changes could be generally applied to the moult cycle of crustaceans. Recent studies have also described the moult sequence in megalopae of brachyuran species in order to compare the physiological status (competency) of megalopae from different areas and in different periods of time during the larval season, or from different habitats (Hatfield, 1983; Metcalf and Lipcius, 1992; Hasek and Rabalais, 2001).

The green crab, *Carcinus maenas* (L., 1758), is one of the most characteristic shore crabs in both soft and rocky bottoms of European waters. It has large populations in estuaries, especially when large areas of intertidal mud and sand flats occur. *Carcinus maenas* has four zoeal stages and one megalopal stage (Rice and Ingle, 1975). The export of hatched larvae from estuaries and rocky shores to shelf waters is enhanced by selective tidal-stream transport during nocturnal ebb tides (Zeng and Naylor, 1996b; Queiroga *et al*., 1997). To enter and migrate upwards in the estuaries, megalopae also use a selective tidal transport strategy (Zeng and Naylor, 1996b; Queiroga, 1998). However, in order to avoid seaward transport, megalopae move near the bottom during ebb. In estuarine waters of the Portuguese coast, several episodes of recruitment take place during each reproductive season (Almeida and Queiroga, 2003), which are partially regulated by the strength of along-shore winds. It is probable that not all the larvae arriving to the shore are sufficiently physiologically developed to respond to settlement; in other words, not all of them are competent, and thus not all of these settle.

The aim of this work is to establish a simple and easily recognisable chronological series of integumentary changes occurring before the ecdysis of megalopa to first juvenile instar in the green crab, *Carcinus maenas*. The knowledge of this sequence of premoult stages will be a useful tool for the assessment of development stage in relation to the different phases of the dispersal process.

MATERIALS AND METHODS

In order to examine the sequential changes of the integument previous to ecdysis of the megalopa, larvae of *Carcinus maenas* were reared in the laboratory. The larvae were obtained from four females bearing eggs in similar embryonic development stage. The adult females were caught in a shallow inlet of the saltmarsh area of the Bay of Cádiz (SW Spain: 36°23′–37′N, 6°8′–15′W) in February 2002, and identified according to Zariquiey (1968) and Clark *et al*. (2001). They were kept together in a 15-L tank with well-aerated, filtered natural sea water (35 salinity) with no food supply. All females released larvae between 48 h and 68 h after its collection, in a total amount of approximately 270,000 larvae. The actively swimming larvae from the four broods were transferred to a unique 150-L conical trunk fiberglass tank, slightly aerated, so that a density of 100 larvae L^{-1} was obtained. The temperature and salinity of the larval culture were kept at $19 \pm 1°C$ and 35, respectively, and the photoperiod was set to 8L:16D. The tank was filled with new sea water and dead larvae were removed every two days, while food was supplied daily in excess. Larvae were fed during the first two days after hatching with a mixture of the rotifer *Brachyonus plicatilis* and the alga *Nannochloropsis gaditana*, and with *Artemia* nauplii for the remaining days of the rearing process.

First megalopae were recorded 22 d after hatching. To avoid the collection of megalopae with a fast developmental time anomaly, all megalopae released during the first 24 h were discarded. In order to facilitate the collection of megalopae during the experiment, 600 randomly selected newly moulted megalopae were carefully pipetted from the culture tank and distributed into three 2-L glass bottles (200 megalopae/bottle) maintained in similar initial laboratory conditions of temperature, salinity, and light. Ten live megalopae were sampled every day from each replicate, fixed in 4% neutral Formalin, and labelled according to the number of days that megalopae were reared in each replicate. Thus, three samples of ten individuals each were collected representing megalopae with the same number of days since moulting to megalopae. First juveniles were recorded nine days after the beginning of the megalopal culture, and the experiment finished on the eleventh day, when the daily percentage of megalopae moulting to juveniles was high, with the aim of recording the final morphological changes that took place immediately before the metamorphosis.

The analysis was focused to the transparent edge of the distal parts of the second maxilliped, following the suggestions of Hatfield (1983), and also on the posterior margins of the uropod's exopod and the telson. These appendages allow the clear observation of apolysis, which is the first observable event in ecdysis. It corresponds to the

early separation of epidermis from the old exoskeleton and generally occurs first in the distal structures (Drach, 1939). The appendages were mounted in permanent slides using Faure's liquid (Reyne, 1949) and studied under 40× magnification using a microscope (Zeis Axioscop) equipped with a digital photographic camera (5 megapixel resolution).

The stages of intermoult and premoult cycle were established according to a combination between the degree of the development of the appendages' integument and the progression of the setagenesis, resulting in modified criteria as close as possible to the ones from Drach and Tchernigovtzeff (1967). The characteristics of each moult stage and its correspondence with the Drach stages are described later in the text.

RESULTS

According to the development of the integument and the progression of the setagenesis of the second maxilliped, uropod, and telson, we have recognized eight successive events that cover the intermoult and premoult periods of the *Carcinus maenas* megalopa, herein referred to as moult stages (Table 1). To each moult stage is assigned a nominal variable which is defined as a particular combination of development stages of each of the appendages considered. Because the different appendages developed independently of one another at different rates and had different maximum numbers of stages (6, 4, and 4 for the maxilliped, uropod, and telson, respectively), we coded each appendage's stage separately with Roman numerals (Table 1).

Moult Stage 1.—There is no discontinuity between the epidermal layer of all the appendages and the cuticle (Figs. 1I, 2I, 3I). Maxilliped setae are completely filled with matrix. This characteristic was considered typical for the C intermoult stage by Drach (1939).

Moult Stage 2.—The epidermis of the maxilliped begins to separate from the cuticle, and the new epicuticle starts to form. This is evidenced by the appearance of a double-bordered integument, with a light band between membranes (Fig. 1II). Drach and Tchernigovtzeff (1967) established these features to be representative of the D_o premoult stage. Separation of the epidermis in the uropod and telson is not observed.

Moult Stage 3.—The matrix of the maxilliped setae is fully retracted within the setae, giving rise to a thin fibrous axis (Fig. 1III). This characteristic was considered by Drach and Tchernigovtzeff (1967) to belong to D_o stage. The separation of the epidermis begins in the uropod and telson (Figs. 2II, 3II).

Moult Stage 4.—No changes are observed in the

Table 1. Moult stages identified for the megalopa of the crab *Carcinus maenas*. See Results for description of development stages. Universal stage refers to general scheme proposed by Drach and Tchernigovtzeff (1967). Roman numerals in brackets refer to exceptional cases found.

Moult stage	Universal stage	Maxilliped stage	Uropod stage	Telson stage	Megalopal rearing time (d)
1	C	I	I	I	0–1
2	D_0	II	I (II)	I (II)	0–3
3	D_0	III	II	II (III)	2–5
4	D_0	III	III (II)	III	5–7
5	$D_{1'}$	IV	III, IV	III	5–9
6	$D_{1''}$	V (IV)	IV	III	7–11
7	D_2	VI	IV	III	9–11
8	D_2	VI	IV	IV	9–11

second maxilliped. The separation between cuticle and epidermis is progressing in the uropod and the telson (Figs. 2III, 3III). In the distal end of the uropod, this separation is as wide as the width of the distal setae. These changes were not recorded by Drach (1939).

Moult Stage 5.—New setae begin to form in the second maxilliped. Below each old seta, a slightly deep outline of the circular invaginations is visible in the matrix (Fig. 1IV), while the shaft of the seta is not yet clearly visible. These features are similar to the ones assigned by Drach and Tchernigovtzeff (1967) for the $D_{1'}$ premoult stage. The uropod and the telson do not show any changes.

Moult Stage 6.—The invaginations of the matrix of the maxilliped reach its maximum depth, establishing the length of the new seta. The outline of the shaft of the new seta is evident owing to the secretion of new cuticle; however, the proximal end of the new seta is still not well defined (Fig. 1V). According to Drach and Tchernigovtzeff (1967), these features are typical of the $D_{1''}$ premoult stage. The matrix of the uropod is strongly retracted from the distal end, filling up less than half of the uropod volume (Fig. 2IV). No changes are observed in the telson.

Moult Stage 7.—The new setae are completely formed, and their barbules and proximal end are clearly visible. The preecdysial procuticle is well developed (Fig. 1VI). Drach (1939) assigned these features to the D_2 premoult stage. No changes are observed in the uropod and the telson.

Moult Stage 8.—The telson appears very retracted. In the telson and the uropod, the space between the old and the new cuticles is
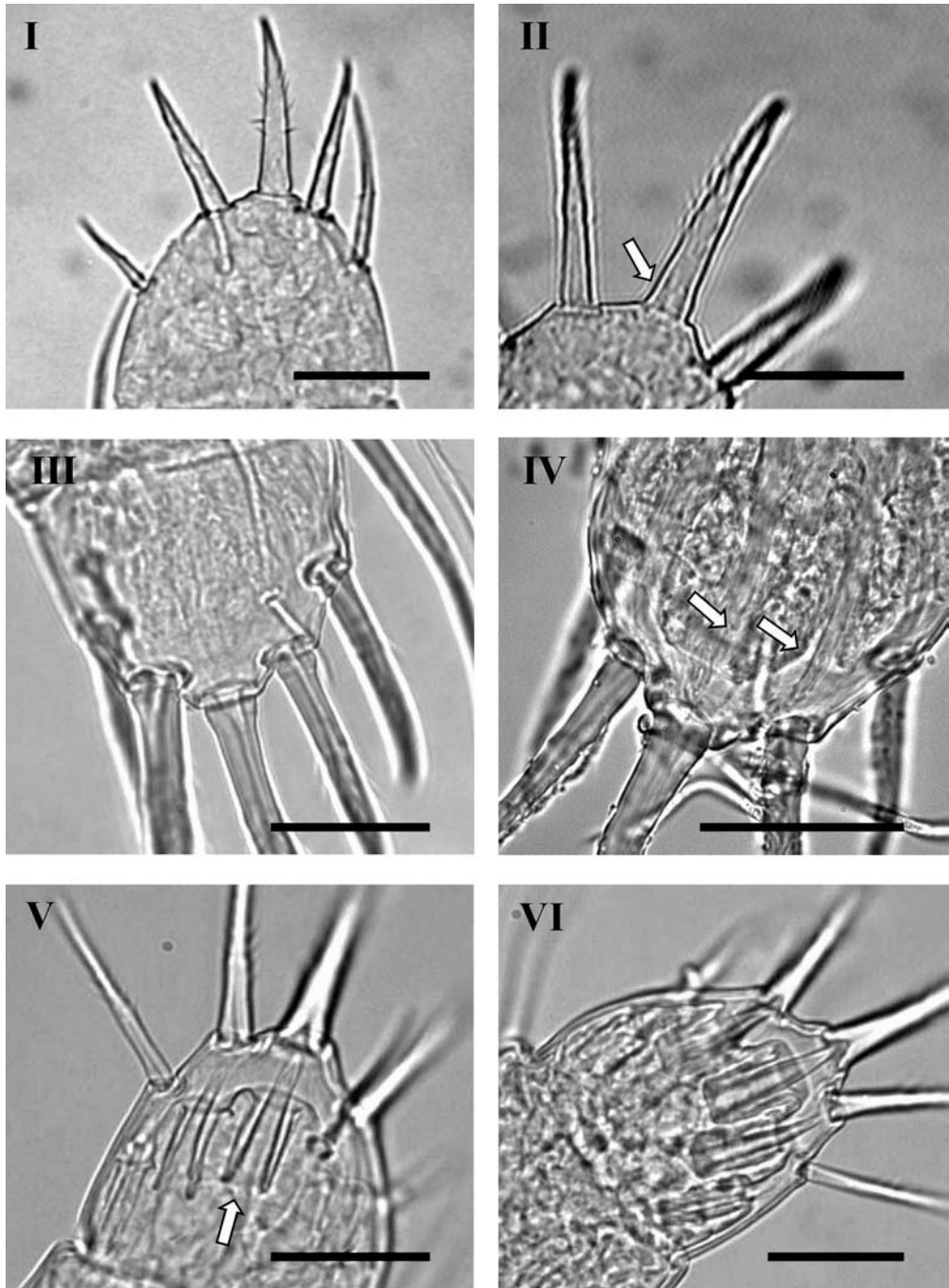
Fig. 1. Sequence of changes in the distal segment of the second maxilliped of *Carcinus maenas* megalopa. Roman numerals I to VI refer to each development stage considered. Arrow shows: II) Double-bordered integument, with a light band between membranes; IV) Circular invaginations of new seta in the matrix; V) Shaft of the new seta. Scale bars = 50 μm.
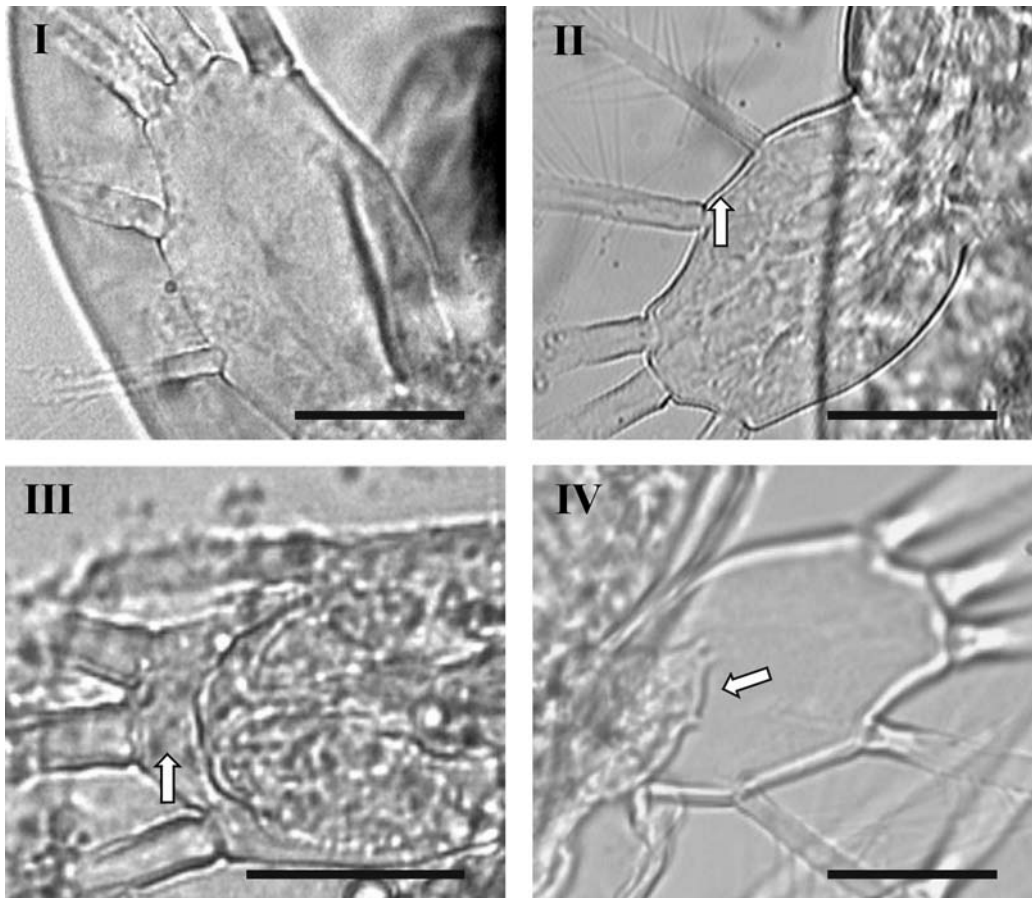
Fig. 2. Sequence of changes in the uropod of *Carcinus maenas* megalopa. Roman numerals I to IV refer to each development stage considered. Arrows show: II) Separation of the epidermis; III) Separation between cuticle and epidermis; IV) Matrix of the uropod. Scale bars = 50 μm.

very transparent owing to the complete digestion and reabsorption of the postecdysial procuticle (Fig. 3IV). These changes were not considered by Drach and Tchernigovtzeff (1967).

The frequency of successive development stages was directly related with rearing time (Fig. 4). Therefore, as the rearing time increases the megalopae show a more advanced moult stage and become closer to metamorphosis, as it would be expected.

DISCUSSION

The moult cycle in the megalopa of *Carcinus maenas* can be staged in eight successive events prior to juvenile metamorphosis. This sequence, based in the general development of setagenesis and epidermal retraction from the integument, is consistent with the presently accepted sequence established by Drach (1939) for adult crabs and later completed by Drach and Tchernigovtzeff (1967). The premoult cycle proposed by Drach and Tchernigovtzeff (1967) established five stages for *Palaemon serratus* and seven stages for *Cancer pagurus*. Therefore, although the general succession of changes is similar for all crustaceans, the number of stages reported during the entire premoult period can be different. For instance, Aiken (1973) reported ten premoult stages when studying the pleopod of *Homarus americanus*, Hatfield (1983) reported nine premoult stages based on the analysis of the second maxilliped of the megalopa of *Cancer magister*, while Brumbaugh and McConaugha (1995) recorded four stages when studying the maxillipeds and the uropods of the megalopa of *Callinectes sapidus*. This lack of homogeneity in the number of stages reflects different methodological ap-
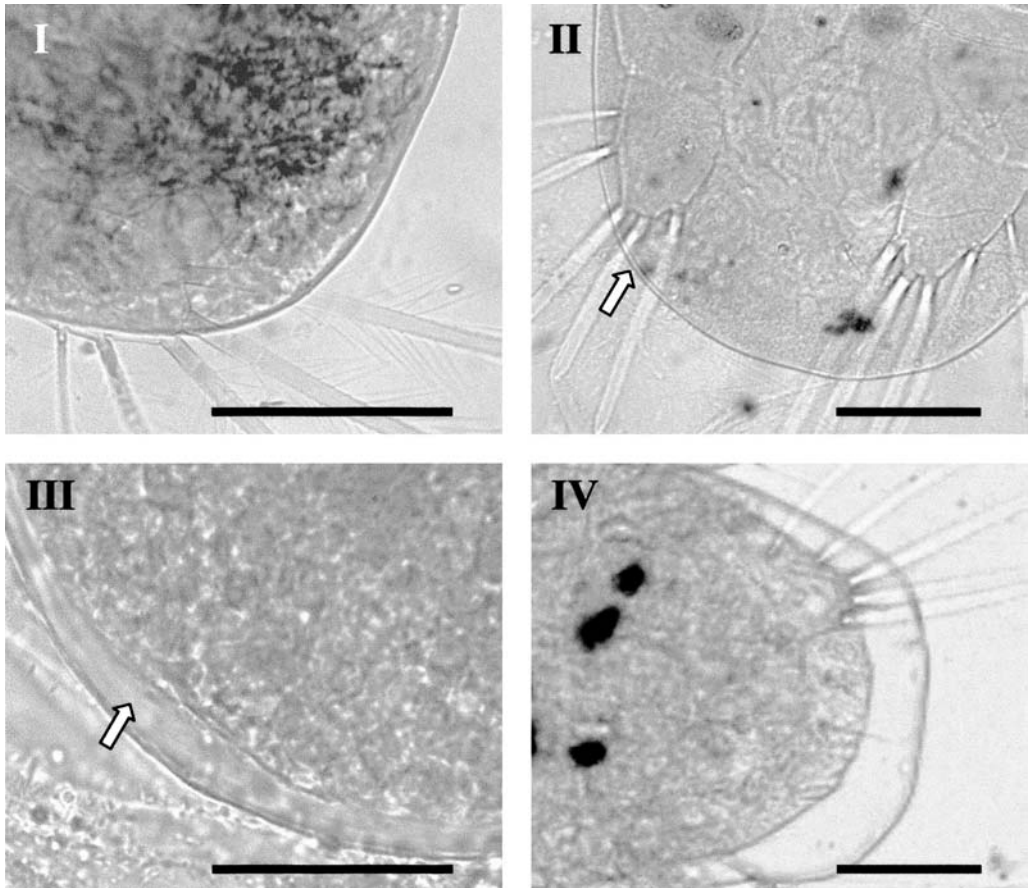
Fig. 3.   Sequence of changes in the telson of *Carcinus maenas* megalopa. Roman numerals I to IV refer to each development stage considered. Arrows show: II and III) Separation of the epidermis; Scale bars = 50 μm.

proaches concerning the appendages analysed or the scale of observations. In the present work, we observed that the stage D_0 proposed by Drach and Tchernigovtzeff (1967) can be subdivided into three different stages, if the analysis include together the setagenesis and epidermis retraction of the second maxilliped, uropod, and telson (see Table 1).

Some of the premoult stages established by Drach (1939), namely D_3 and D_4, are difficult to recognise, mainly because of the short time gap between their occurrence (only a few hours). To avoid this constraint, in the peak of moulting percentage (eleventh day of culture), all the megalopae were collected for analysis. However, in this work it was not possible to observe the stage D_3. Stage D_3 corresponds to the last event of reabsorption of the procuticle, and in stage D_4 the epimeric line of the posterior end of the cephalotorax breaks into a fissure, allowing the animal to deprive the old cuticle. In poorly calcified species, these premoult stages are rarely observed (Drach and Tchernigovtzeff, 1967; Stevenson, 1985), suggesting that the duration of the last premoult stages depends on the amount of calcified material available. Thus, it is likely that these stages happen so quickly in the poorly calcified megalopae that it is difficult to record them.

The duration of the moult stages can also be determinant for staging. Davis *et al.* (1973), analyzing *Balanus amphitrite*, noted that the D stage (including D_0, D_1, D_2, and D_3 stages) can last from one to five days. Similarly, the duration of the intermoult stages of *Carcinus maenas* has a large variability. In the present study, stage 1 lasted from several hours to one day, which may lead to the collection of stage 2 megalopae in the same day that megalopa in stage 1 were recorded. Stages 2 and 3 lasted
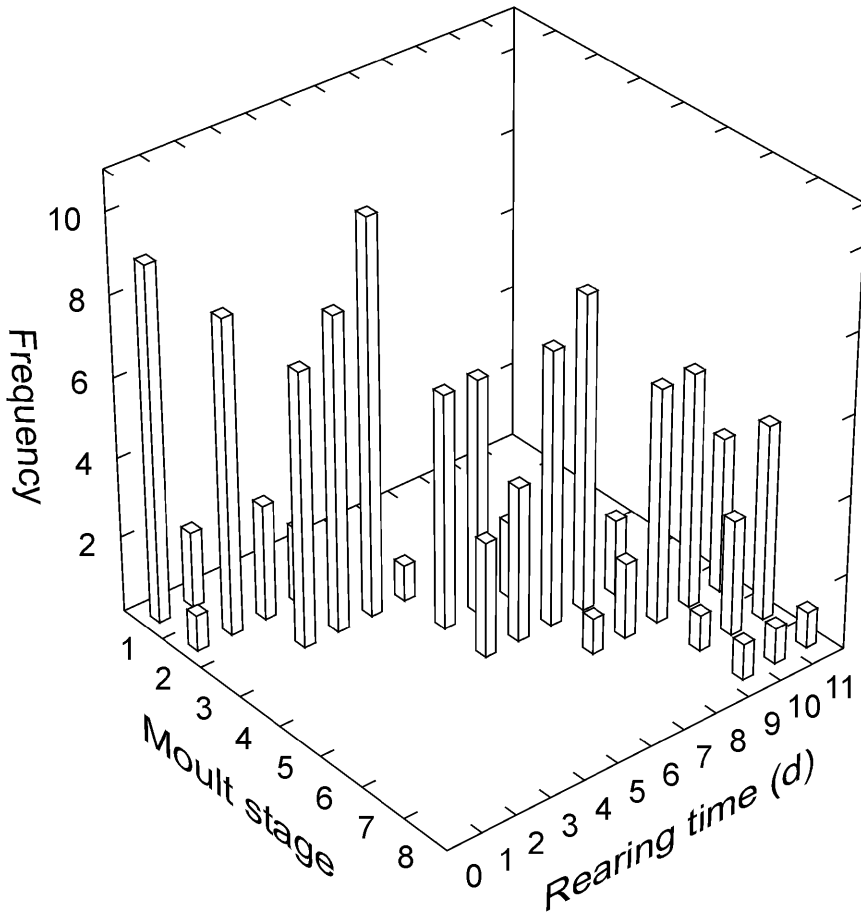
Fig. 4.   Frequency of moult stages of *Carcinus maenas* megalopa according to the rearing time.

from one to three and from two to three days, respectively. However, stage 4 can occur very rapidly because at the same time that the first fourth stage megalopae were recorded, some fifth stage megalopae were already present. Similarly, stages 7 and 8 also seem to occur rapidly, as both were found in the same culture day, together with the first juveniles.

Several studies on coastal crab species with a larval phase that disperses offshore have collectively demonstrated a connection between development state of the megalopa and the step of the mechanism involved in habitat location and settlement. As expected, there is an inverse relationship between intermoult stage and time to metamorphosis in *Cancer magister* and *Callinectes sapidus* megalopae when the larvae are reared without moult-inducing cues (Hatfield, 1983; Zeng *et al.*, 1997; Hasek and Rabalais, 2001). Additionally, megalopae in intermoult

stage of *C. magister* (see Hatfield, 1983), *C. sapidus* (see Morgan *et al.*, 1996; Lipcius *et al.*, 1990; Metcalf and Lipcius, 1992), and *C. maenas* (see Zeng *et al.*, 1997) advance shoreward on the shelf and upstream in estuaries. However, development stage may not be a good predictor of time to metamorphosis because of the influence of moult cues. The presence of adult conspecifics, estuarine eelgrasses, and sediment have all been involved in the acceleration of the moulting process in *Uca* spp. (see Christy, 1989; O'Connor, 1993) and *C. sapidus* (see De Vries *et al.*, 1994; Forward *et al.*, 1994) megalopae. The factors that accelerate metamorphosis, at least in the case of estuarine vegetation, appear to be of a chemical nature and change in potency across estuaries (Forward *et al.*, 1996). Metamorphosis is also affected by the phases of day and tide. In the case of *C. maenas* (see Zeng *et al.*, 1997), there is an endogenous rhythm that synchronises

metamorphosis with high tide, whereas *C. sapidus* megalopae moult in higher proportion during the day under a simulated light : dark cycle (Forward *et al.*, 1996).

From the above evidence, it is clear that the prediction of time to metamorphosis of crab megalopae collected from the field is difficult. Although attempts can be made by rearing field-collected megalopae in the laboratory until moulting to first juvenile, this would hardly give rise to accurate information because the environmental conditions of the larval culture differ from the natural ones. The knowledge of the time sequence of the morphological modifications shown by laboratory-reared megalopae allows determining the development stages of natural megalopal assemblages, without resorting to time-dependent data. It also allows the comparison of the development state among different systems, steps of the dispersal process, phases of physical forcing factors, or recruitment pulses, in order to better define the nature of the mechanisms involved in dispersal and recruitment.

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