



## Twin embryos in the larger benthic foraminifer *Nephrolepidina praemarginata*

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**ABSTRACT** - Twin embryos were recovered in *Nephrolepidina praemarginata* populations from the upper Rupelian of the Caltavuturo Formation (Sicily). Biometric data and photographic evidences suggest that Siamese-twins embryos result from joined cells produced from the same reproductive cyst during the multiple fissions. The fusion of juveniles may occur before the complete calcification of the first chamber walls and before the release by the parent cell.

The frequency of the twin embryos, commonly related to stressed conditions, such as high salinity, water motion, pollution, low pH etc., can be linked also to the reproductive strategies. In unpolluted environments, the occurrence of multiembryonic individuals is herein explained as related to the extreme depths of the total distribution range of a larger foraminiferal taxon, where asexual reproduction and r-strategy dominate far from the ecological optimum.

**Keywords:** larger foraminifera; *Nephrolepidina praemarginata*; Oligocene; twin embryos; reproductive strategies; paleoenvironment.

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### 1. INTRODUCTION

Twin and multiple tests are known since Rhumbler (1902) both in recent (Stouff et al., 1999; Meriç et al., 2004; 2008; Briguglio et al., 2014) and in fossil benthic (Cole, 1960; Nguyen, 1080; Meriç, 1971; Schiavinotto and Verrubbi, 2000; Ferrández Cañadell et al., 2012; Omaña et al., 2012; Benedetti and Pignatti, 2013) or planktic foraminifera (Boltovskoy, 1982; Ballent and Carignano, 2008).

Anomalies in foraminiferal tests, including Siamese twins, are commonly explained as linked to environmental stress such as high salinity (Almogi-Labin et al., 1992; Stouff et al., 1999; Ballent and Carignano, 2008), low pH (Geslin et al., 2002), dysoxic condition (Leiter and Altenbach, 2010) and pollution (Yanko et al., 1994; Samir, 2000; Geslin et al., 2002; Frontalini and Coccioni, 2008; 2012; Carboni et al., 2009).

Recently, twinning and deformities have commonly been related to increases in pollution, even if a certain cause of abnormalities in foraminiferal tests is often obscure (Rajeshwara Rao and Nandhakumar, 2012). Polyvalent individuals in larger foraminifera, resulting from the union of two or more young individuals (twins, triplet, quadruplet and Siamese twins), have been described as teratological (Rhumler, 1902; Nguyen, 1980) being, unfortunately, abnormal test morphology

and multiembryonal juveniles often considered as undifferentiated (e.g., Carboni et al., 2009); but while the first is commonly related to environmental factors and affects the cell during its ontogeny (e.g., Yanko et al., 1994), the latter is an instantaneous event that happens short time after cell birth (e.g., Krüger et al., 1997). In this work only multiembryonal tests are considered and their consequent abnormal shape is only due to the growth of one test possessing two or more juveniles.

Twins formed by coalescence of tests of different species are known from recent shallow-water environments (e.g., Meriç et al., 2012) and they should be simply linked to biological accident rather than to the influence of environmental factors (Meriç et al., 2008; 2012). Twin tests characterized by two or more embryonic apparatuses are considered in dependence with several factors such as salinity, water turbulence, type of substrate, seasonality and availability of nutrients, influencing the reproductive strategies of the parents and to the amount of stress of the environments in which they live (e.g., Krüger et al., 1997; Stouff et al., 1999).

Aim of this work is to investigate the unusual occurrence of twin embryos in some populations of the extinct larger foraminifer *Nephrolepidina praemarginata* recently documented from the Oligocene of north-western Sicily (Benedetti and Pignatti, 2013), and to give an interpretation of its environmental significance.

## 2. GENERAL FEATURES

### 2.1. Multiple embryos

Multiple embryonic chambers in larger foraminifera are well documented in the Cretaceous orbitoids (Meriç, 1971; Omaña et al., 2012), in discocyclinids (Meriç, 1992), in recent nummulitids (Hofker, 1927; Briguglio et al., 2012) and in lepidocyclinids (Cole, 1960; Schiavinotto and Verrubbi, 2000; Benedetti and Pignatti, 2013). An unambiguous interpretation of this phenomenon is lacking; possible explanations include the development of a thick-walled multiple fission cyst (Cole, 1960; Stouff et al., 1999), or the slower emission of pseudopods in hypersaline environments (Stouff et al., 1999) as explained below.

### 2.2. Reproduction in larger foraminifera

Reproduction in larger foraminifera is controlled by ecological and especially environmental factors. Intensity of light, temperature, salinity, pH, turbidity, food availability, competition or the chemical composition of the water, could disturb the reproductive process (e.g., Boltovskoy and Wright, 1976).

Foraminifera can reproduce asexually by binary fission, producing many hundreds of identical offspring (Krüger et al., 1997), or sexually consisting on the production, release, and fusion of gametes to form zygotes (e.g., Krüger, 1997; Hohenegger, 2011).

Asexual reproduction is well documented in laboratory culture of symbiont-bearing larger foraminifera such as *Heterostegina depressa* (Röttger, 1974; Röttger et al., 1990), *Cycloclypeus carpenteri* (Krüger et al., 1997) and *Amphistegina gibbosa* (Dettmering et al., 1998), and the epiphytic porcelanaceous *Sorites orbiculus* (Kloos, 1984).

Reproduction by multiple fission generally takes place from an undifferentiated protoplasmic mass surrounding the parent cell. The broods remain on the parental test and grow until they develop the second chambers, after which they are released into the seawater (Krüger et al., 1997). Each offspring cell gets part of the parental cytoplasm containing symbionts and all organelles that are necessary for its life (e.g., Röttger, 1974).

Larger foraminifera are dimorphic, with two forms resulting from an alternation of generations between a haploid, uninucleate megalospheric gamont (the sexual generation which produces gametes – A form) and a diploid, multinucleate microspheric agamont (the asexual generation which produces offspring by multiple fission – B form) (Röttger et al., 1990; Dettmering et al., 1998). In addition to these two generations, a third generation is documented in recent larger foraminifera: a megalospheric schizont (Hofker, 1927). This may explain why most larger foraminiferal assemblages are dominated by megalospheric individuals (Röttger, 1974); the schizonts, produced by multiple fission, receive symbiotic algae from the parent, and begin their ontogenetic life as symbiont-bearing cells (Dettmering et al., 1998), whereas the gametes produced by meiosis are too small to carry

symbiont in the formation of zygotes.

In other worlds, the agamont (B form) is diploid and by multiple fission produces haploid gamonts (A2 form) or schizonts (A1 form) which in turn may produce a number of successive asexual generations. The recognition of the two megalospheric (A1 and A2) generations in the fossil record is often very difficult, since proloculi may present similar size (e.g., Röttger, 1974), and requires the occurrence of two morphologically distinct type of tests on the base of the embryonic diameter.

In benthic foraminifera which reproduce sexually, two gamonts may join together by their umbilical sides, face to face, documenting plastogamy, a particular kind of sexual reproduction, in which the haploid gametes are not freely released in the water column, but are kept within the joined tests until the formation and liberation of zygotes (Lipps and Erskian, 1969). The nuptial couple resulting by this joining is well documented in recent Glabratellidae (Lipps and Erskian, 1969; Hottinger et al., 1993; Hottinger, 2006) and Rosalinidae (Hottinger et al., 1993) and was reported by Mukhopadhyay (2003; 2007) to explain the morphology of abnormal-shaped fossil *Nummulites*.

### 2.3. Strategies in larger foraminifera and ecological implication

Larger foraminifera, among all foraminifera, are generally considered as K-strategists adapted to stable, oligotrophic environments (e.g., Hottinger, 1997), but within the same taxon A-forms are considered more opportunists (r-strategists) and B-forms more specialists (K-strategists) (e.g., Hottinger, 1999).

Environmental stress (low temperature, insufficient food or light, high salinity, water turbidity, oxygen depletion and low pH) tends to favor individuals which invest resources in reproduction and are r-strategists. High fecundity, large number of offspring, small-sized tests are general features of r-selection. In these conditions smaller embryos characterize the megalospheric communities, whereas individuals with larger embryos are produced in more suitable conditions (Pécheux, 1995; Benedetti et al., 2010; Benedetti and Pignatti, 2013).

According to Pécheux (1995), recent *Operculina ammonoides* from the Gulf of Aqaba has the large microspheric, sexual individuals, occurring only in the mid-depth zone of the distribution range of the species, near the ecological optimum. In other words K-strategists, with high carrying capacity, are encountered in mid-depth water, whereas in shallow water live the fecund, opportunistic r-strategists with high growth rate. The deeper water, characterized by limited light, are dominated by r-strategists capable to survive in adverse conditions.

### 2.4. Influence of environmental conditions on the frequency of twin embryos

The fusion of megalospheric embryos is referred to the result of a reduced dispersion of the juveniles following

schizogony (Stouff et al., 1999). Ecological causes such as hypersalinity (Almogi-Labin et al., 1992; Stouff et al., 1999), low pH (Geslin et al., 2002), dysoxia (Leiter and Altenbach, 2010), pollution (Yanko et al., 1994), very low water energy (Geslin et al., 2000), nutrients (Meriç et al., 2004), inhibit or slow the movements and dispersion of the young megalospheres. Under such conditions twins should be more abundant than under non-stressed conditions.

Foraminifera may also produce twins and multiple specimens because the embryos could share the same reproductive cyst (Cole, 1960; Meriç et al., 2008).

During multiple fission, reproductive cysts are formed around the microspheric test, and the thickness of the cysts could be higher under stressed conditions (Cole 1960; Stouff et al., 1999; Meriç et al., 2008). This should cause a delay in the release of each juvenile megalospheric individuals which may join together and start the construction of periebrionic chamberlets, and so the young megalospheric embryos are released as twins or polyvalent embryos (e.g., Meriç, 1992; Stouff et al., 1999; Meriç et al., 2008). Test fusion between post-juvenile individuals is a rare phenomenon as documented in extant or fossil populations (Ferrández-Cañadell et al., 2014).

### 3. MATERIALS AND METHODS

This study was based on five *Nephrolepidina* populations (PC16, PC17, PCs0, PC0 and PC060620) described in Benedetti and Pignatti (2013) and on three populations (MM9, MM10 and MM13) described in Benedetti (2010) from the upper Rupelian of the Caltavuturo Formation (Fig. 1). The sampled sections have been described in

detail (Benedetti, 2010; Benedetti and Pignatti, 2008; 2009; 2013) and a complete biometrical analysis of the six *Nephrolepidina praemarginata* populations has been given in Benedetti and Pignatti (2013). This study revealed the occurrence of the largest embryos among the known populations, suggesting that the investigated specimens come from water depth close to the ecologically optimal environment (e.g., Benedetti et al., 2010). The embryo size is strictly linked to the water depth in which the population grows (Benedetti et al., 2010; Benedetti and Pignatti, 2013), thus altering the efficiency of the biometrical investigation of the embryonal and periebrionic (nepionic) stages.

Except for sample PCs0, the study is based on thin sections of consolidated rocks and the measurements were taken on equatorial and axial sections of *Nephrolepidina* in which the embryos appear fully developed and visible. Individuals with unclear embryonic stage were not counted, so the number of twin embryos could be overestimated.

In addition some individuals from the sample PCs0 were dissolved in a HCl solution and the silicified embryos were investigated by a FEI Quanta 400 MK2 scanning electron microscope (SEM).

As stated by Benedetti and Pignatti (2013) no microspheric forms occur in the investigated samples; the absence of B forms is mainly due to a differential selection of the tests with transport (Aigner, 1985). In displaced assemblages B forms are usually absent or very rare (Benedetti and D'Amico, 2012) and their size is not that much larger than that of A forms.

An uncommon occurrence of twin embryos has been previously documented and some specimens have been illustrated in Benedetti and Pignatti (2013). The abnormal

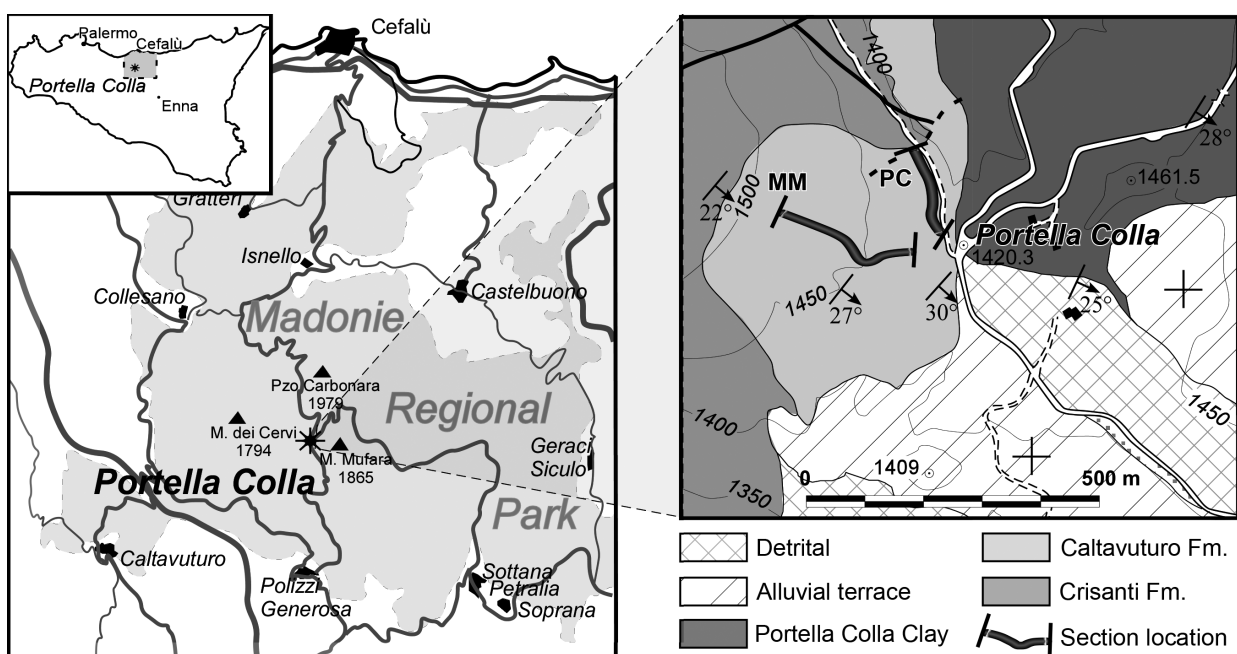


Fig. 1 - Sketch map of the investigated area. The location of the two stratigraphic sections is indicated.

individuals have been counted and photographed to investigate their growth.

The A-form of *Nephrolepidina* is normally characterized by a lenticular test and by the occurrence of a bilocular embryo constituted by a subspherical proloculus (protoconch) partially embraced by a subcircular to reniform deutoconch. The embryonic chamberlets are separated by a thin common wall and are followed by a nepionic stage constituted by two Primary Auxiliary Chambers (PAC), a variable number of adauxiliary chamberlets developed on the deutoconch and nepionic spirals starting from these chamberlets. Ogival to hexagonal chamberlets are arranged in concentric annuli in the neanic stage. Lateral chamberlets are fully developed and visible in axial section.

#### 4. RESULTS

The internal molds of embryonal apparatuses of *Nephrolepidina praemarginata*, isolated from the sample PCs0 (Fig. 2), permit the description of the distribution of stolons between the two embryonic chambers. The protoconch appears subspherical in outline, and it is only partially embraced by a bean-shaped deutoconch (Fig. 2 a,b). The stolon between the two chambers may be in central (Fig. 2f) or excentric position (Fig. 2g).

The adauxiliary chamberlets communicate with the deutoconch by a robust stolon measuring about 35-55  $\mu\text{m}$  in diameter (Fig. 2 c-e).

Twin embryos are rare in the isolated tests obtained from the sample PCs0 (1.2%, n=82), and absent in some of the investigated samples (PC16, n=74; MM9, n=27; MM10, n=47). Their occurrence is variable with an abundance of 3.5% in PC060620 (n=119), 3.7% in MM13 (n=28), and 4.7% in two samples (PC17, n=156; PC0, n=102).

Each embryo has its own PAC (Fig. 3 e,f) and possibly its nepionic spiral. Twin embryos might grow in the same plane (Fig. 3d) or in different planes resulting in irregular tests with Y, V or stellate shapes (Fig. 3a). The dimensions of the embryos, and their biometrical characteristics, such as the grade of enclosure of the protoconch by the deutoconch (van der Vlerk, 1959), and the number of auxiliary chamberlets on the deutoconch are similar (Fig. 3e). The diameter of the protoconchs ranges from 322 to 380  $\mu\text{m}$  in the specimen d of figure 3, the diameter of the deutoconchs is very similar (438-449  $\mu\text{m}$ ) considering that the two embryos are not perfectly centered. The grade of enclosure of the protoconch by the deutoconch (Ai) measures 34.3% in the embryo on the right of the picture, and it is 35.1% in the embryo on the left. These simple biometrical analysis could be

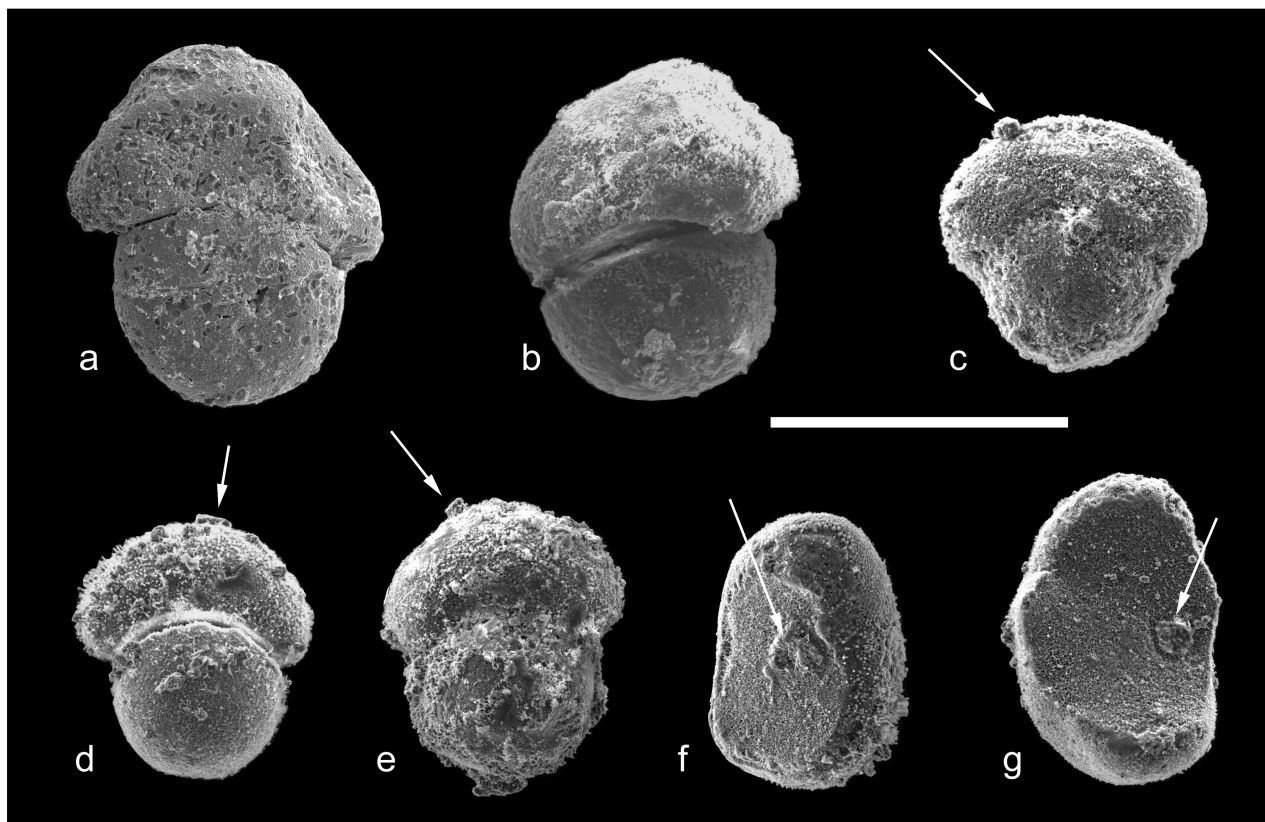


Fig. 2 - Scanning electron micrographs of silicified internal molds of embryonic chambers of *Nephrolepidina praemarginata* from sample PCs0. a-e. Bilocular embryos, the white arrows indicate the stolon between deutoconch and the adauxiliary chamberlet; f. Megalospheric protoconch, the arrow indicates the stolon between the two embryonic chamberlets; g. Deutoconch, the arrow indicates the stolon in excentric position. Scale bar = 0.5 mm.

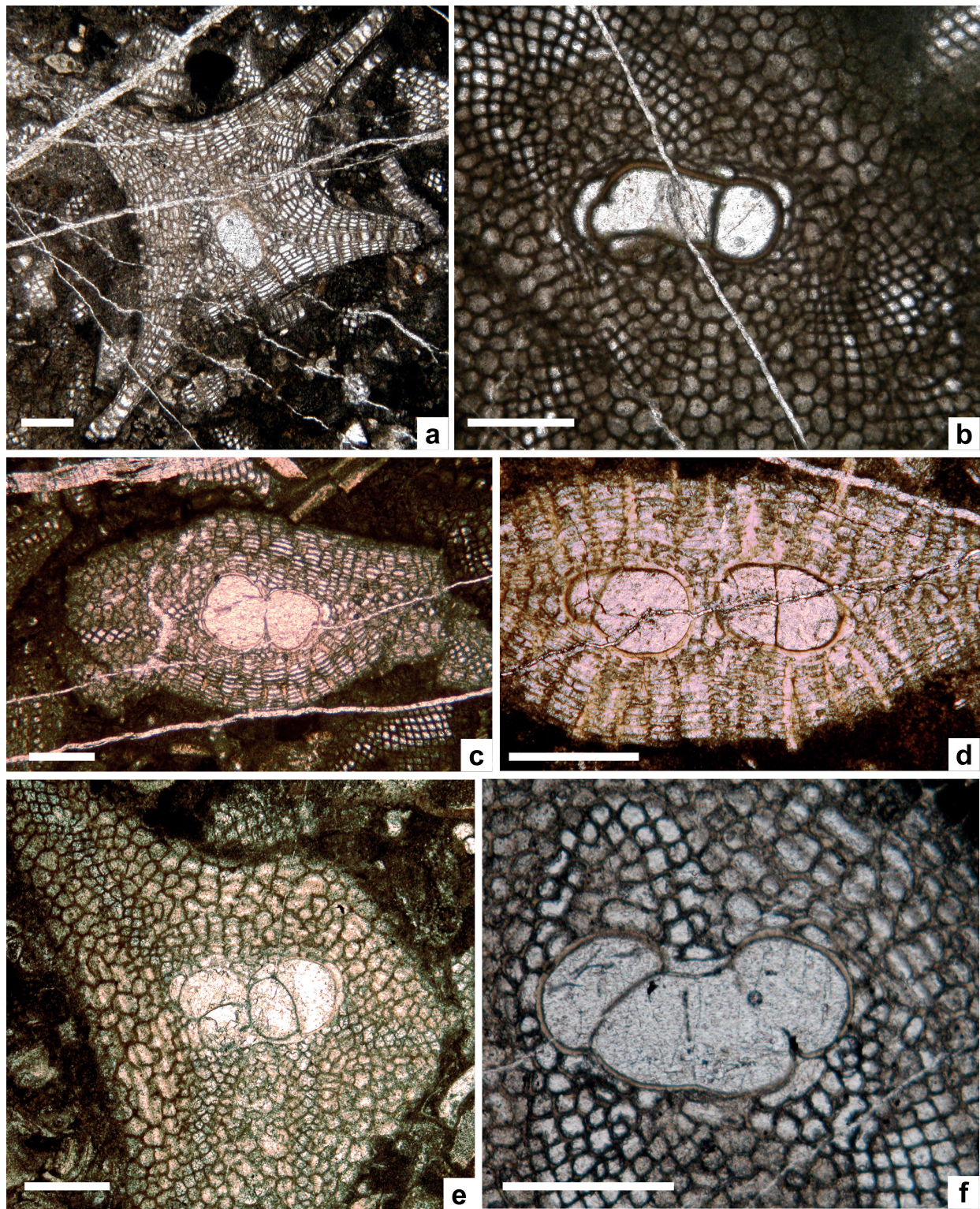


Fig. 3 - Twinned specimens of *Nephrolepidina praemarginata*. a. Irregular X-shaped test resulting by the simultaneous growth of two individuals, the second embryo lies in a different plane than the first one. Sample PB17, thin section C; b. Siamese twin embryos sharing the protoconch. Sample PB17 thin section 05; c. Twin embryos in axial section. Sample PB17 thin section 04; d. Axial section of one specimen showing two embryos lying in the same plane. Sample PC0, thin section B; e. Conjoined embryos showing a similar grade of enclosure of the deutoconch on the protoconch ( $Ai$  (left) = 35.1%;  $Ai$  (right) = 34.3%). Sample PC060620; f. Siamese twin embryos joined by the protoconchs. Sample PB17 thin section B. Scale bar = 0.5 mm.

sufficient to suggest a provenience of juveniles from the same brood.

Singular events are given by the Siamese twins (Fig. 3 b,f) in which the two proloculi appear joined together. Particularly in specimen f of figure 3, the protoconchs (dI=353-369  $\mu\text{m}$ ; diameter of deutoconch dII=385-391  $\mu\text{m}$ ) are clearly compenetrated and share one PAC. This suggests that the two embryos were merged into each other in an earlier stage in which the calcification of the single cells was not complete. The two cytoplasm are not fused into one single individual, and the two joined daughter cells develop independently, even if they seem to have a common PAC. Adauxiliary chamberlets and nepionic spirals are fully developed on both the deutoconchs suggesting that the two cells are grown at the same time, although the calcified proloculi are joined and connected, the cytoplasm of the two cells was independent and carried on its own life cycle.

Since no complete tridimensional tests have been investigated, it is not possible determine whether one of the two cells prevails during the ontogeny, but they both seem to reach alive the adult stage (no information are available about the gerontic stage).

## 5. DISCUSSION

The early joining of embryos has been long debated, but the occurrence of Siamese embryos with shared proloculi seems to be a prove of the origin of the conjunction before the release of the juveniles by the parent. The recovered twinned specimens of *Nephrolepidina praemarginata*, according to the results offered by the simple biometric data reported, seem to confirm that juvenile fusions occur during the first stage of growth, when the offspring embryos are attached to the parent cell and their walls are not completely calcified. The joined proloculi, however, calcify before the breackage of reproductive cysts containing two or more offspring cells. The occurrence of several twin embryos could be linked to an higher thickness of the cyst under stressed condition (Stouff et al., 1999) or more simply to the increase of the asexual reproduction and of the possibility to have repeated schizogonic cycles (Ferràndez-Cañadell et al., 2014).

Anyway, the open question concerns the causes that leads to the occurrence and the frequency of these twins.

In hypersaline culture, the analysis of abnormalities in a small foraminifer such as *Ammonia*, reveals the occurrence of about 25% of double or multiple tests (Stouff et al., 1999). In recent marine environments, twin tests are usually very rare in normal conditions and commonly not more than one percent of a sample (Meriç et al., 2008).

In fossil communities the percentage of twin or multiple tests is poorly known, for example Omaña et al. (2012) found 10-13% of abnormal tests in *Lepidorbitoides minima* populations also including other types of morphologies than multiple embryos.

The percentage of twin embryos measured in this work

does not reach values indicating abnormal conditions in the bottom water nor contaminated environments. The fusion of juveniles may accidentally occur during multiple fissions, but it appears to be favorite by local contamination of bottom waters and fluctuation in pH values (not constrained to the water depth); a slow motion of the water and low temperature, i.e., elevated water depth; increase in salinity, i.e., very shallow water (Fig. 4), thus suggesting also a control operating by the different reproductive strategies (e.g., Pechéux, 1995). In populations within the limit of their environmental tolerance the percentage of twin embryos are usually low; percentages close to 5% could indicate stressed environments, such as for samples PC17 and PC0, but these suffer an overestimation due to the impossibility to count all individuals, the observations are in fact limited to thin sections of rock. The most suitable measurement is that of sample PCs0 (1.2% of twin embryos), taken on free-matrix tests, resulting in an amount of abnormal tests compatible with that of actual unstressed areas.

According to the biometrical study in Benedetti and Pignatti (2013), the investigated populations come from depth close to the ecological optimum, far from unfavorable environmental condition. Under stable environment conditions sexual reproduction dominates, A and B forms are presents and megalospheric embryos are large.

The frequency of twin embryos is higher in unsuitable conditions; under stressed (even if unpolluted) environments, r-strategists and asexual reproduction dominate the assemblages, embryos are smaller but the possibility to have twinned tests increases. In the case of larger foraminifera, stressed environments are the deepest and shallowest depths of the total bathymetrical range of a species (Pécheux, 1995).

Although the definition of an unambiguous cause of the occurrence of multiple test is not the main aim of this paper, especially because too many factors contribute to create a stressed environments, the above discussed description of Siamese twins, the relative abundances of conjoined juvenile apparatuses are sufficient to link their higher frequency to an increase of adverse environmental conditions.

As concern larger foraminifera, such as *Nephrolepidina* or nummulitids, the stressed (paleo)environments correspond to the extreme-depth distribution of the taxa, below and above the ecological optimum. This poses a further question, i.e., what is, with reasonable certainty, the optimum depth distribution of extinct taxa such as lepidocyclinidids?

## 6. CONCLUSIONS

The twin embryos formation is likely the result of several biological (reproductive), ecological and environmental factors (Stouff et al., 1999; Meriç et al., 2008).

This work confirms that the fusions of juveniles occur during the multiple fission when the first two embryonic chambers are not completely calcified according to the

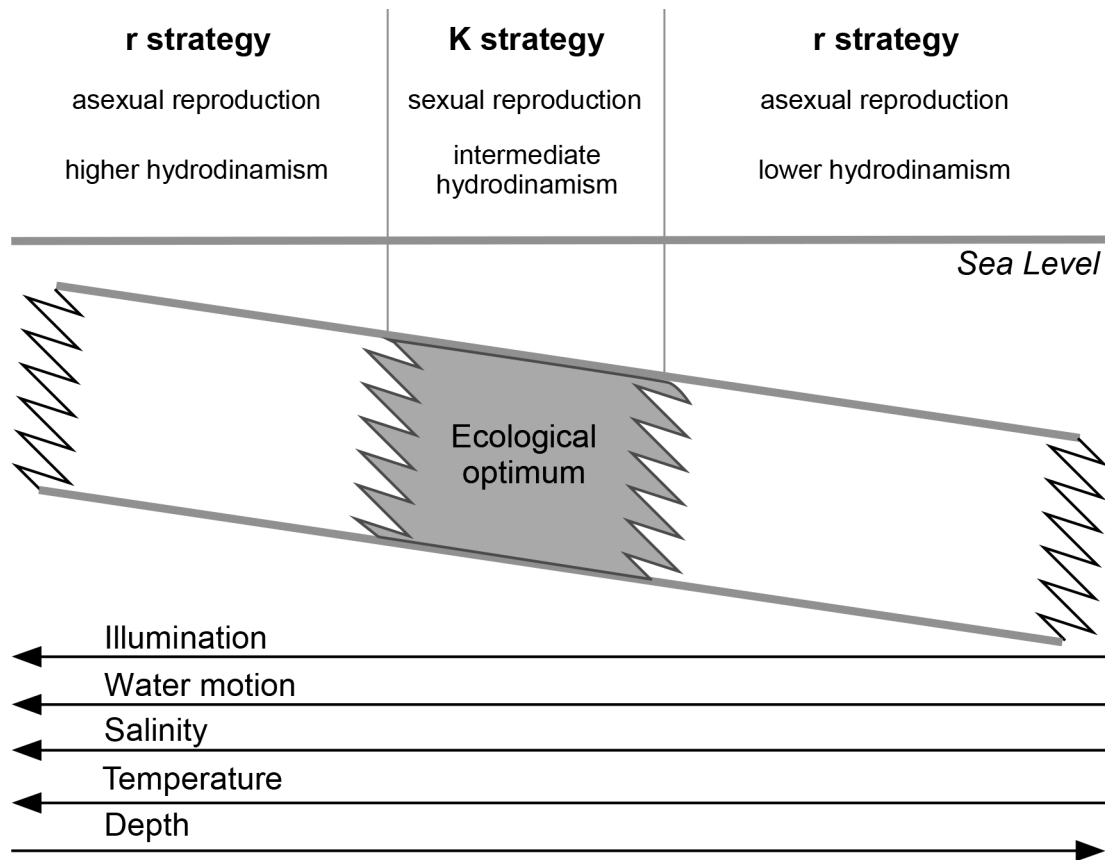


Fig. 4 - Summary of key environmental controls on the distribution of larger foraminifera and reproductive strategies along a depth gradient.

investigation of of twin embryos with similar biometrical parameters, and especially of compenetrated Siamese-twins. This occurs before the young are released from the reproductive cyst, where they are densely packed.

The frequency of twinned individuals is assumed higher under stressed environments, that in unpolluted conditions correspond to the shallower (higher water illumination, salinity and temperature) and deeper (lower water motion, illumination and temperature) distribution of a taxon. In this adverse condition asexual reproduction and r-strategy prevail contributing to the statistical increase in and accidental fusion of juveniles.

This study suggests that the usual biometric approach to the embryonic apparatus of orbitoidiforms, such as *Nephrolepidina*, and the recognition of an embryonic acceleration are hampered by environmental factors. The environmental conditions required for a successful sexual reproduction are restricted to the optimum bathymetrical range, specific for each taxon. To discriminate among depth- or evolutionary-dependent biometrical parameters the solution will be adopt the study of arrangement of the equatorial chamberlets (Benedetti, 2014) as suggested by the data on neanic acceleration (Schiavinotto, 1992; 2010).

Future works will be required to determine 1) the difference in percentage range of twin embryos between stable (ecological optimum) and stressed (i.e., extreme distributions of the taxon) environments, 2) the precise

ecological factors causing the occurrence of the twinned specimens; but this paper add a new perspective in the application of the occurrence of twin embryos in larger foraminifera for paleobathymetrical reconstructions.

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