MECHANISMS OF NURSE EGG FORMATION IN THE SPIONID POLYCHAETE *BOCCARDIA PROBOSCIDEA*: AN ULTRASTRUCTURAL ANALYSIS

by

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Abstract

Nurse eggs are commonly produced by spionid polychaetes and gastropod molluscs and serve as sources of extra-embryonic nutrition for developing young. Although the influence of nurse eggs on offspring development has been well documented, the origin of nurse eggs is not understood. I examined nurse egg production in the spionid *Boccardia proboscidea* using brightfield and fluorescence microscopy. Nurse eggs in this species appear to arise as do viable oocytes. After spawning, nurse eggs produce fertilization envelopes indicating that development has been activated. Nurse eggs are also capable of producing polar bodies which indicates completion of meiosis in those eggs. In addition, I examined ultrastructural changes that accompany nurse egg formation using fluorescence and electron microscopy. Nurse eggs in *B.proboscidea* maintain the fertilization envelope with surface granules; however, microvilli are lost, and the plasma membrane invaginates and compartmentalizes the cytoplasm into vesicles that are ingested by developing siblings. Vesicle formation in nurse eggs involves the energy producing and synthetic organelles, mitochondria and endoplasmic reticulum. Additionally, nuclear DNA in nurse eggs is cleaved into fragments that stain with the apoptosis-specific fluorescent probe BODIPY-dUTP, which are packaged into the cytoplasmic vesicles. These morphological changes in *B.proboscidea* nurse eggs are indicative of apoptosis, which suggests that this process is involved in nurse egg formation in this species. Thus, nurse egg production in *B.proboscidea* is an active developmental process and not simply an artifact of sperm limitation.

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Chapter 1

General Introduction

Nurse Egg Origin

Nutrition is a major factor contributing to larval development in benthic marine invertebrates. Many species of marine invertebrate produce microlecithal eggs that develop into planktotrophic offspring and must rely on extrinsic food, such as phytoplankton, to support larval development. Other marine invertebrates provide developing young with enhanced nutritive resources (yolk) by either making larger eggs (lecithotrophy) or by supplementing young with non-developing nurse eggs as extra-embryonic food (adelphophagy) (Levin and Bridges 1995). Larval trophic mode is associated with major life-history consequences, such as survivorship, fecundity, dispersal, and length of the developmental period. Adelphophagy is a relatively common mechanism by which larval food is enhanced, and the consequences of adelphophagy in shaping larval development and survivorship have been described (Blake and Kudenov 1981, Rivest 1983, Gibson 1997). Despite their importance, the origin of nurse eggs is not understood.

Polychaete annelids and prosobranch molluscs commonly produce nurse eggs and, in most cases, nurse eggs constitute the majority of spawned eggs (Rivest 1983, Gallardo and Garrido 1987, Hadfield 1989, Levin and Bridges 1995, Gibson 1997). Nurse eggs have been described as abortive oocytes (Rivest 1983, Guérin 1991), nutritive or food eggs (West 1979, 1983, Gallardo and Garrido 1987), non-developing eggs (Rasmussen 1973, Rivest 1983, Gibson 1997), and unfertilized eggs (Blake 1969, Rice 1976, Clark 1977, Blake and Kudenov 1981). In most species, nurse eggs and viable

eggs are indistinguishable until activation of development (Rivest 1983, Smith and Gibson present study). Nurse eggs are often assumed to be normal ova, which are fated to become a nutritive resource. In many species, nurse eggs contain nuclear DNA; however, the fate of the chromatin and nucleoli is variable. In the slipper shell, *Crepidula dilatata*, nurse eggs contain a large nucleus that appears to remain at the germinal vesicle stage with no maturation divisions (Gallardo and Garrido 1987). Chromatin in *C. dilatata* nurse eggs degenerates as sibling embryos undergo normal development (Gallardo and Garrido 1987). In two polychaete species, *Pygospio elegans* (Rasmussen 1973) and *Boccardia proboscidea* (Smith and Gibson present study) presumptive nurse eggs and embryos are both nucleated before fertilization and spawning while in the female's coelom, but nurse eggs lose the nucleus immediately after release into egg capsules. In contrast, nurse eggs produced by the snail *Colus stimpsoni*, undergo karyokinesis to produce multiple nuclei but do not undergo cytokinesis as do embryos (West 1979, 1983). Additionally, in some nurse eggs produced by the snail *Fasciolaria tulipa*, chromatin condenses before degenerative processes occur which are accompanied by fragmentation of the nucleolus (Burger and Thornton 1935).

Nurse eggs appear to be produced as viable ova, in most species however; nurse eggs do not undergo cleavage. Instead, nurse eggs disintegrate or dissociate into small vesicles that are ingested by developing embryos in the polychaete, *Pygospio elegans* (Rasmussen 1973) and *Boccardia proboscidea* (Gibson 1997), and in the snails, *Fusinus closter* (Miloslavich and Penchaszadeh 1997), *Dendropoma corrodens* (Milsolavich and Penchaszadeh 1992), *Crepidula dilatata* (Gallardo and Garrido 1987), and *Colus stimpsoni* (West 1979, 1983). In other species, nurse eggs undergo abnormal cleavage or

a few divisions that are typical of zygotic cleavage, then abort development. Such cleavage divisions have been observed in *Searlesia dira* (Rivest 1983), *Vermetus* sp. (Miloslavich and Penchaszadeh 1992), *Nucella crassilabrum* (Gallardo and Garrido 1987), *Buccinum cyaneum* (Miloslavich and Dufresne 1994), and *Fasciolaria tulipa* (Burger and Thornton 1935). In these species nurse eggs are either swallowed whole (*S. dira*), are broken up by early veligers (*Vermetus* sp.), or contribute to a nurse egg mass that is consumed by embryos (*B. cyaneum*).

In some species, nurse eggs show evidence of fertilization. In *Colus stimpsoni* for example, West (1979, 1983) reports that nurse eggs are female gametes that incorporate sperm. These fertilized nurse eggs undergo karyokinesis but do not undergo cytokinesis. Another interesting example involves *Fusinus closter* nurse eggs. In this species, Miloslavich and Penchaszadeh (1997) describe migration, to the animal pole, of follicular cells that surround both viable and nurse eggs. Migration occurs after fertilization and before polar body release, thus, Miloslavich and Penchaszadeh (1997) suggest that nurse eggs are fertilized. The present study, on the polychaete *Boccardia proboscidea*, suggests that development is activated in nurse eggs. Fertilization was not confirmed since male pronuclei were not observed. Evidence for activation, however, included the elevation of a fertilization envelope, presence of a hyaline layer (indicative of the cortical reaction), and the occasional appearance of polar bodies in nurse eggs (often obscured by nurse egg vesicles upon dissociation of nurse egg cytoplasm).

Evidence for Apoptosis

Apoptosis, or programmed cell death, is an active process by which cells self-

destruct in an organized, predictable manner. New evidence suggests that all nucleated cells are capable of apoptosis, and the decision of whether to undergo apoptosis or mitosis may be influenced by either intrinsic or extrinsic factors (Raff 1992, 1998, Weil *et al.* 1996, Earnshaw 1999, Susin *et al.* 1999). Inactive forms of apoptotic proteins are maintained in healthy cells but are only activated when the cell is triggered to die (Raff 1998). Cells induced to undergo apoptosis follow a complex set of integrated pathways involving different families of proteins and genes. Many components of the apoptotic machinery have also been conserved; for example the CED-3 protein in *Caenorhabditis elegans* is similar to ICE (interleukin-1-converting enzyme) in humans. These proteins, and others in the same family, function as proteases during apoptosis (Raff 1998). Proteases cleave proteins contained within the nuclear membrane as well as components of the cytoskeleton in the process of disassembling the cell (Raff 1998).

Another event that is characteristic of apoptosis is DNA fragmentation by endonucleases at the internucleosomal linker regions (Li *et al.* 1995, Bär 1996, Bowen *et al.* 1998). Fragmented DNA in apoptotic cells is often packaged into small vesicles which become incorporated into the cytoplasmic apoptotic bodies that are eventually engulfed by neighboring cells (Bär 1996, Darzynkiewicz *et al.* 1997). A number of assays have been developed to detect apoptosis in cells or to visualize the fragmented DNA. A single-step procedure used to label DNA fragments involves the fluorophore BODIPY-FL 14-dUTP (Molecular Probes) (Li *et al.* 1995, Li *et al.* 1998, Li and Darzynkiewicz 1995). BODIPY-dUTP specifically stains apoptotically cleaved DNA, and produces yellow-green fluorescence easily visualized with fluorescence microscopy. The present study stained nurse eggs and embryos of *Boccardia proboscidea* with

BODIPY-dUTP. Ultra-violet fluorescence demonstrated BODIPY-dUTP-stained fragments in nurse eggs indicating that the DNA loss occurring in nurse eggs may be due to endonucleases cleaving DNA in a manner characteristic of apoptosis.

In addition to fragmentation of DNA, apoptotic cells also show characteristic ultrastructural changes involving the plasma membrane, microvilli, mitochondria, and endoplasmic reticulum (ER), as observed with electron microscopy (Honma and Hamasaki 1996, Bär 1996, Superti *et al.* 1996). During apoptosis, microvilli are lost and the plasma membrane blebs inward to compartmentalize the cytoplasm and nuclear vesicles into apoptotic bodies (Honma and Hamasaki 1996, Bär 1996, Superti *et al.* 1996, Darzynkiewicz *et al.* 1997). Mitochondria and ER are also intimately involved in energy producing and synthetic activities required during apoptosis (Bowen *et al.* 1998). In addition to ATP production, mitochondria also release molecules, normally found between the inner and outer mitochondrial membranes, into the cytoplasm where they play key roles in apoptosis (Raff 1998, Earnshaw 1999, Susin *et al.* 1999). Susin *et al.* (1999) demonstrated that cytochrome C, involved in cell respiration within mitochondria, is released from mitochondria in cells triggered to undergo apoptosis. In the cytoplasm, cytochrome C binds with another molecule to activate the apoptosis initiator protein caspase-9. Another factor released from mitochondria, AIF (apoptosis-inducing factor) accumulates in the nucleus and leads to chromatin condensation and DNA fragmentation as well as inducing the release of cytochrome C from mitochondria (Earnshaw 1999, Susin *et al.* 1999). Other evidence demonstrating the presence of apoptotic machinery in healthy cells is the expression of a protein involved in regulation of the cell cycle. Li *et al.* (1998) report that cells entering the Gap2/mitosis phase of the cell cycle express the

protein survivin. The interaction of survivin with the microtubules of the mitotic spindle is essential for cell division. Interference in the survivin-microtubule interaction leads to apoptosis (Li *et al.* 1998). These are only a few examples of the complex cascade of proteins, molecules and induction/suppression events that occur during cell-cycle regulation and apoptosis. Numerous factors are involved, many of which are similar between *C. elegans* and the human (Bär 1996, Raff 1998) and morphological changes characteristic of apoptosis in many cell types suggest that the apoptotic machinery, or at least the process, has been conserved.

Chapter 2[1]

Nurse egg origin in the polychaete *Boccardia proboscidea* (Spionidae)

Introduction

A number of benthic marine invertebrates, including gastropod molluscs, echinoderms, and polychaetes (Levin and Bridges 1995) provide extra-embryonic nutritive resources for offspring, often in the form of nurse eggs. Nurse eggs have been described as non-developing eggs (Rasmussen 1973, Rivest 1983, Strathmann 1987, Gibson 1997), abortive oocytes (Rivest 1983, Guérin 1991), nutritive or food eggs (West 1981,1987, Gallardo and Garrido 1987), and unfertilized eggs (Rice 1976, Clark 1977, Blake and Kudenov 1981), but the mechanism underlying their origin is not well understood. Nurse egg ingestion (adelphophagy) enhances offspring survivorship by generating larger offspring at hatching with a reduced planktonic phase (Gibson 1997). Nurse egg production decreases fecundity as egg production is finite and an increase in nurse egg number decreases the number of developing young. The trade-off between nurse egg production and fecundity and associated consequences (dispersal, survivorship) are well described (Rivest 1983, Levin and Bridges 1995, Gibson 1997); however, we know very little about nurse egg origin. The main objective of this study is to investigate the origin of nurse eggs in the polychaete *Boccardia proboscidea.*

Nurse egg production and adelphophagy has been described in several species of spionid polychaetes and gastropod molluscs (Thorson 1946, Rivest 1983, Gallardo and Garrido 1987, Strathmann 1987, Radashevsky 1994, Gibson 1997). These species have internal fertilization and deposit eggs in egg capsules containing both developing young

[1]The material in Chapter 2 is reproduced with permission from Balaban Publishers International Science Services. Smith, H. and Gibson, G. Nurse egg origin in the polychaete *Boccardia proboscidea*, Spionidae, Invert. Repro. Dev. 35 (3), in press.

capsules within the same brood (e.g. *Pygospio elegans*, Thorson 1946; *Searlesia dira*, Rivest 1983; *Crepidula dilatata*, *Nucella crassilabrum*, Gallardo and Garrido 1987; *Polydora cornuta*, MacKay and Gibson submitted). This variation in resources results in a range of offspring sizes at hatching (Rivest 1983). Differences in the ability of larvae to feed on nurse eggs also result in different larval sizes. In *Boccardia proboscidea*, for example, females produce both planktotrophic and adelphophagic offspring (Blake and Kudenov 1981, Gibson 1997). Planktotrophic larvae do not feed on nurse eggs and hatch at an earlier developmental stage than the adelphophagic offspring, which hatch as juveniles.

We examined nurse egg origin in *Boccardia proboscidea* because nurse egg production varies among females (Gibson 1997). Some females do not produce nurse eggs (Type 1 morphs), few nurse eggs are produced by Type 2 morphs, and 90-95% of the eggs produced by Type 3 morphs are nurse eggs. In Type 1 reproduction, offspring rely on yolk contained in the original egg to support embryonic development and all eggs develop into planktotrophic larvae. In Type 2 reproduction, few nurse eggs are produced and are ingested by the developing larvae which results in a slightly larger hatching size and a reduced planktonic period. In Type 3 reproduction, both planktotrophic (no nurse eggs consumed) and adelphophagic (nurse egg consumers) offspring develop.

Nurse egg production was examined by comparing oogenesis and early embryonic development between Type 1 (no nurse eggs produced) and Type 3 (nurse eggs produced) females and broods. Oogenesis and post-spawning changes in nurse egg structure were examined using fluorescent probes and 1μm sections through a series of early developmental stages. Our results indicate that nurse eggs originate as viable eggs

and are activated when spawned. Nurse eggs do not cleave but undergo the active processes of nuclear DNA loss and compartmentalization of ooplasm into small vesicles.

Materials and Methods

Collection and Maintenance of Adults

Adults were collected from Victoria, British Columbia, Canada and from San Diego, California, USA. Cultures were maintained in 250 mL Pyrex crystallizing dishes and 1500 mL Pyrex loaf pans. The worms were provided with approximately 1 cm of sand for tube construction. Sea water was changed twice weekly and the cultures were sieved and given new sand approximately once per month. The laboratory temperature was maintained at 20°C with a photoperiod of 16 hours daylight. Adults were fed *Artemia* nauplia 3 times per week with supplements of Tetramin® fish food and dehydrated, ground *Enteromorpha* sp.

Brood Collection and Fixation

Cultures were checked daily for the presence of broods. Broods were removed from the females' tubes and cultured until the eggs reached the desired developmental stage. Egg capsules were fixed in 4% paraformaldehyde in Millonig's phosphate buffer. Eggs and embryos were examined in whole mount using brightfield microscopy. Capsules were serially sectioned at 15μm in a Reichert Histostat cryostat microtome, sections were stained with Hoechst 33342 (Molecular Probes 1996) and observed with UV excitation (wavelengths 330 - 380nm) to visualize nuclear DNA. Serial sections of nurse eggs were examined to determine the presence or absence of nuclear DNA.

Capsules were prepared for 1μm sectioning by fixation in 2.5% glutaraldehyde, and post-fixed in 1% osmium tetroxide, both buffered with 0.45μm filtered sea water and 0.1M sodium cacodylate (Buckland-Nicks 1993). Specimens were embedded in TAAB resin, sectioned at 1μm on a LKB Ultramicrotome III, and stained with either toluidine blue or Richardson's stain. Serial cross sections of specimens were observed.

Fertilized eggs were identified by the presence of fertilization envelopes, polar bodies and / or polar lobes (Gould and Stephano 1989, Gilbert 1997). The same criteria were used as evidence for egg activation in nurse eggs, but fertilization as such was not demonstrated because we did not test for fusion of the male and female pronuclei.

Adult Fixation

Adult females were fixed in Carnoy's fixative (Carson 1990) for paraffin embedding and serially sectioned at 10μm. The slides were stained with methyl green and pyronin Y (Carson 1990) to visualize DNA (green or blue) and RNA (red). This allowed for observation of oocyte nuclear development during oogenesis within the coelom of each female. Oocyte diameter was measured through the center of the oocytes (where a germinal vesicle was observed). Oocytes were measured with an ocular micrometer to the nearest 1.3 μm at 400X magnification.

Results

Oogenesis and Fertilization

No differences in oogenesis were observed between Type 1 (non-nurse egg producers) and Type 3 (nurse egg producers) *Boccardia proboscidea* females. In both reproductive morphs, oocytes proliferated in paired, discrete ovaries located ventro-

laterally within most setigers, beginning at setiger eight and continuing to approximately the 15th setiger from the pygidium. The ovaries were associated with internal septa, as well as with nephridia (Fig. 1a and b). In *B. proboscidea* oogenesis is extra-ovarian, as described by Eckelbarger (1984) in other spionids. Oocytes proliferated within ovaries and detached when they reached approximately 33 μm in diameter. Detachment was followed by growth and vitellogenesis (yolk accumulation) in oocytes free-floating within the coelomic fluid (Fig. 1a and b). All oocytes observed, from both Type 1 and Type 3 females, contained a large germinal vesicle and nucleolus (Fig. 1a and b). Oocytes within the ovary had smaller germinal vesicles (n = 744, 8.1μm ± 1.9; mean ± standard deviation) than those measured in the coelomic oocytes (n = 457, 13μm ± 2.8).

Oocytes were fertilized internally and spawned into egg capsules. Fertilization envelopes and polar bodies, two general characteristics of fertilization (Gould and Stephano 1989, Gilbert 1997), were used in this study as indicators of fertilization or egg activation in zygotes and nurse eggs. Polar lobes, which occur prior to cleavage (Gould and Stephano 1989), were also used as evidence for fertilization and activation of development. All Type 1 eggs were fertilized with the majority exhibiting fertilization envelopes (1cell stage 99.5%; 2-4 cell 100%), polar bodies (1cell 76.7%; 2-4cell 75.8%) and polar lobes (Fig. 2a, Table 1). In Type 3 broods, 97.2% of the eggs observed produced fertilization envelopes immediately after spawning, indicating activation of development in both zygotes and nurse eggs, nurse eggs forming 93% of the eggs per brood (Fig. 2b, Table 1; Gibson 1997). In the 2-4 cell stage of Type 3 broods, polar bodies were observed but in less than 20% of the embryos and nurse eggs (Fig. 2b, Table

1). In the nurse eggs that released polar bodies, generally two were observed although occasionally only one was produced.

Post-spawning Events

Zygotes at the 1 cell stage in type 1 broods exhibited brightly fluorescing nuclear material and polar bodies (Fig 3a). DNA was either in a condensed form, as mitotic chromosomes aligned at the metaphase plate (not shown), or more diffuse within the nucleus (in interphase) (Fig. 3a). As development proceeded in the Type 1 eggs, DNA was visible in all cells of all embryos (Fig. 3b).

Initially, Type 3 eggs appeared similar to Type 1 eggs and exhibited nuclear DNA (Fig. 3c, and 4a, b). Given that 93% of type 3 eggs become nurse eggs, this indicates the presence of DNA in nurse eggs as well as embryos. Nurse eggs and embryos were indistinguishable at the one cell stage. As development proceeded, however, embryos exhibited spiral cleavage whereas nurse eggs did not cleave. As in Type 1 embryos, nuclei of Type 3 embryos were brightly fluorescent (Fig. 3d, e) and clearly visible (Fig. 4c, d) at all stages of early development. In contrast, nurse eggs underwent loss of nuclear DNA and vesiculation of the cytoplasm. As cleavage began in the embryos, the cytoplasm of associated nurse eggs began to break apart into small vesicles, which often included DNA (Fig. 3d, and 4b). As development progressed within a particular brood (embryos at the trochophore stage) the DNA was either no longer visible in the nurse eggs or was reduced to small fragments, visible as flecks of fluorescent material (Fig. 3e). Thus, it appears that the DNA was either broken down or that the vesicles containing the DNA had been exocytosed from the nurse eggs.

Compartmentalization of nurse egg cytoplasm continued with development of the associated embryos. Vesicles began to form around the periphery of the nurse eggs (Fig. 4b) and progressed inward until all cytoplasmic components were enveloped (Fig. 4c and d). At about the 16 cell stage of the embryos, nurse egg dissociation was complete (Fig. 4d). The nurse egg vesicles averaged 14 μm in diameter in section and contained variable numbers and sizes of yolk granules, observed under brightfield microscopy. As nurse eggs deteriorated, embryos continued to develop. Large, yolky macromeres were observed in the embryonic gut and were surrounded by smaller, mainly yolk-free micromeres. A mitotic spindle apparatus is shown in Figure 4d and indicates that cleavage was occurring in that embryo. Additionally, nuclei were clearly visible in the micromeres of the embryos but not in the nurse eggs (Fig. 4d). Thus, nurse eggs are readily distinguished from embryos at early developmental stages.

Discussion

A central theme in life-history theory is the trade-off between per-offspring investment and fecundity. An increase in per-offspring investment is associated with a decrease in fecundity, assuming that reproductive resources are finite (Roff 1992). Nurse egg production greatly reduces fecundity but adelphophagic offspring may have greater survivorship due to a larger size at hatching (Rivest 1983, Strathmann 1987, Jaeckle 1995). Although the consequences related to nurse egg production have been well documented, the origin of nurse eggs is not understood. Hypotheses on the mechanism of nurse egg production in gastropod molluscs and spionid polychaetes suggest that nurse eggs are unfertilized eggs (Clark 1977, Blake and Kudenov 1981), fertilized eggs unable

to undergo cytokinesis (Miloslavich and Penchaszadeh 1997), or early embryos that abort development (Gallardo and Garrido 1987, Guérin 1991).

Our results indicate that nurse eggs in *Boccardia proboscidea* originate as do developing eggs, are activated at the same time as zygotes are fertilized, and undergo a predictable, organised pattern of cell death. Oogenesis in *B. proboscidea* is typical of solitary, extra-ovarian oogenesis in polychaetes as described by Eckelbarger (1984). Oocytes of *B. proboscidea* proliferate in the ovaries and increase in size to 33 μm in diameter before detaching from the ovaries and entering the coelom to undergo vitellogenesis as free-floating oocytes. All oocytes observed, both in the ovaries and in the coelom, contained a large germinal vesicle, each with a distinct nucleolus which, as described by Eckelbarger (1992), is typical of oocytes throughout oogenesis.

Nurse eggs in *B. proboscidea* are activated during the process of spawning. Activation may occur through fertilization of nurse eggs, although male pronuclei were not observed in nurse eggs or embryos. Our evidence for egg activation, based on characteristics described by Gould and Stephano (1989) in *Urechis* sp. eggs, included the presence of fertilization envelopes and polar bodies. Egg activation in *Urechis* sp. eggs includes completion of the second meiosis, elevation of the surface coat, germinal vesicle breakdown, and polar body release (Gould and Stephano 1989). Kay and Shapiro (1985) stated, in relation to sea urchin eggs, that a fertilization envelope does not mean that an egg will develop but egg activation occurs only in eggs with a fertilization envelope. In the present study, almost all spawned eggs (zygotes and nurse eggs) studied exhibited a fertilization envelop in both Type 1 (zygotes only, 100%) and Type 3 (zygotes and nurse eggs, 97%) broods. Polar bodies were also observed on most embryos as well as on some

nurse eggs. In 2-4 cell Type 3 broods, 16% of the nurse eggs exhibited two polar bodies. At this stage polar bodies become rapidly obscured by the formation of cytoplasmic vesicles at the periphery of the nurse eggs. In addition, the germinal vesicle, observed in all eggs in the coelom, was lost after spawning. These observations suggest that nurse eggs are activated, begin to complete meiosis, and thus, may even be fertilized, although this remains to be tested.

The consistent and highly organized pattern of nuclear DNA loss and vesicle formation within the cytoplasm observed in *Boccardia proboscidea* nurse eggs suggests that nurse egg formation is an active form of cell death, similar in many regards to apoptosis (Bowen *et al.* 1998). Nuclear DNA loss in nurse eggs has also been noted in the gastropod *Buccinium cyaneum* (Miloslavich and Dufresne 1994). In *B. proboscidea*, all eggs exhibit a germinal vesicle before spawning. After spawning, DNA is packaged into vesicles in the nurse eggs at the same time as early cleavage occurs in associated embryos. At later stages of embryonic development, DNA is no longer visible in nurse eggs or only fragments remain. Two possible explanations for the disappearance of DNA in nurse eggs are that the vesicles of DNA may be exocytosed from the nurse eggs or that digestive enzymes (DNAases) are secreted into the vesicles which break down the DNA. Further investigation is required to determine the mechanism.

Cytoplasmic vesiculation is also characteristic of nurse egg formation in *Boccardia proboscidea*. The resulting vesicles are readily ingested by small adelphophagic larvae since entire nurse eggs are too large to be swallowed intact (Gibson pers. obs.). The break up of nurse eggs after spawning has also been described for the molluscs *Dendropoma corrodens* (Miloslavich and Penchaszadeh 1992) and *Fusinus*

closter (Miloslavich and Penchaszadeh 1997) as well as for the spionid polychaete *Pygospio elegans* (Rasmussen 1973). Rasmussen illustrated the appearance of nurse eggs after they had gone through the disintegration process (p. 98, Rasmussen 1973). The nurse eggs appear to be similar to those from *Boccardia proboscidea* broods after vesiculation has been completed. As in *B. proboscidea*, nurse eggs are not swallowed whole in *P. elegans* so disintegration may aid in nurse egg ingestion by developing embryos.

It is not clear why Type 3 females produce nurse eggs while Type 1 females do not. Possible explanations include genetic differences or maternal influences (ooplasmic determinants, chemical signals) that vary among eggs. Paternity (sperm) differences are not likely since crosses of Type 1 and Type 3 offspring always show the maternal development type (Gibson 1997). Environmental cues are also unlikely since both Type 1 and Type 3 females and broods were cultured under the same laboratory conditions. Offspring that ingest nurse eggs hatch at a larger size and have higher survivorship than those that do not ingest nurse eggs and hatch as small planktotrophic larvae (Gibson and McCauley, in prep). In general, increased offspring survivorship may compensate for the decrease in fecundity associated with nurse egg production in many species (Rivest 1983, Jaeckle 1995, Strathmann 1995, Gibson 1997).

Although the mechanism that activates this alternative mode of development is not yet known, nurse egg production in *Boccardia proboscidea* is an active process. Nurse eggs originate in the ovaries as do viable oocytes. However, they undergo nuclear DNA loss and vesiculation of the cytoplasm, which inhibits development and enhances their role as extra-embryonic nourishment for developing siblings. The processes of

vesicle formation and DNA degeneration suggest that a form of apoptosis may be involved in nurse egg formation in this species. Observation of similar processes in other species of polychaetes and molluscs suggests that an apoptotic-like mechanism may be widespread in nurse egg production in benthic marine invertebrates. Our understanding of nurse egg origin would benefit from further work on mechanisms that activate or determine the fate of nurse eggs and embryos.

Chapter 3

Mechanism of nurse egg formation in *Boccardia proboscidea*: DNA fragmentation and ultrastructural changes as evidence for apoptosis

Introduction

Nurse eggs are non-developing eggs that provide a source of extra-embryonic nutrition for developing young. They are commonly produced by polychaete annelids and prosobranch molluscs but are also produced by the kinorhyncs and nemertines (Levin and Bridges 1995). The consequences of adelphophagy (nurse egg ingestion) are well described and include enhanced offspring survivorship, larger hatching sizes with a shorter planktonic phase, as well as decreased fecundity (Rivest 1983, Levin and Bridges 1995, Gibson 1997), however, nurse egg origin is not well understood. Nurse eggs have variously been described as non-developing eggs (Rasmussen 1973, Rivest 1983, Strathmann 1987, Gibson 1997), abortive oocytes (Rivest 1983, Guérin 1991), nutritive or food eggs (West 1981, 1983, Gallardo and Garrido 1987), and unfertilized eggs (Rice 1976, Clark 1977, Blake and Kudenov 1981). Our previous work on nurse egg origin in the spionid *Boccardia probosidea* suggests that nurse eggs originate as do viable eggs but at spawning, nuclear DNA degenerates (Chapter 2). These results suggest the potential for apoptosis, or programmed cell death, as a mechanism underlying nurse egg production. The objectives of this study are to: 1) determine whether apoptosis occurs during nurse egg formation, and 2) describe the ultrastructural changes that accompany nurse egg formation in *B. proboscidea*.

In general, apoptotic cells are characterized by morphological changes in both nuclear DNA and cytoplasmic components. DNA in apoptotic cells is cleaved by endonucleases into fragments of a consistent length (Bär 1996, Raff 1998, Li *et al.* 1995).

Fragmented DNA separated onto agarose gels produces a characteristic ladder or a comet pattern (Bär 1996). Other assays use fluorescent probes to label DNA fragments or apoptotic cells with flow cytometry or fluorescence microscopy (Telford *et al.* 1992, Bär 1996, Darzynkiewicz *et al.* 1997, Li *et al.* 1995). The present study utilizes the fluorescent probe BODIPY-FL 14 dUTP (BODIPY-dUTP; Molecular Probes, Haugland 1996) to directly label any DNA fragments in nurse eggs produced by *Boccardia proboscidea* females. BODIPY-dUTP is a probe that binds to the 3'OH end of DNA fragments cleaved by endonucleases at the internucleosomal linker regions during apoptosis. The labeled fragments can then be visualized with UV excitation at wavelengths 505-515 nm (Li and Darzynkiewicz 1995, Li *et al.* 1995, Haugland 1996).

Other characteristic morphological features of apoptotic cells include preservation of organelle integrity, blebbing of the plasma membrane and packaging into apoptotic bodies, and loss of microvilli (Bär 1996, Honma and Hamasaki 1996, Darzynkiewicz *et al.* 1997, Superti *et al.* 1996). In addition to using BODIPY-dUTP to visualize DNA fragments in nurse eggs, the present study also utilizes transmission electron microscopy (TEM) to visualize changes in nurse egg morphology.

Our results indicate that DNA is in fact fragmented in nurse eggs and becomes dispersed throughout the egg. TEM observations have also revealed morphological changes characteristic of apoptosis, such as loss of microvilli, and formation of cytoplasmic vesicles (similar to apoptotic bodies) by incorporation of the plasma membrane. Nurse eggs, however, maintain a fertilization envelope which provides structural support until they are ingested by developing siblings, rather than blebbing of apoptotic bodies as occurs in most apoptotic cells. These results suggest that nurse egg

production is an active process, initiated at spawning. Although nurse eggs appear to be activated (Chapter 2) they do not undergo normal cleavage (mitosis) as do fertilized zygotes. Mitosis seems to be inhibited in nurse eggs since nurse eggs follow an alternate pathway of organized or programmed cell death (apoptosis).

Materials and Methods

Collection and Maintenance of Adults

Adults were collected from Victoria, British Columbia, Canada and from San Diego, California, USA. Cultures were maintained in 250 mL Pyrex crystallizing dishes and 1500 mL Pyrex loaf pans. The worms were provided with approximately 1 cm of sand across the dish for tube construction. Seawater was changed twice weekly and the cultures were sieved and sand was replaced approximately once per month. The laboratory temperature was maintained at 20°C with a photoperiod of 16 hours daylight. Adults were fed *Artemia* nauplia 3 times per week with supplements of Tetramin® fish food and dehydrated, ground *Enteromorpha* sp.

Brood Collection and Fixation

Cultures were checked daily for the presence of broods. Broods were removed from the females' tubes and cultured until the eggs reached the desired developmental stage. Egg capsules were fixed in 4% paraformaldehyde in Millonig's phosphate buffer. Eggs and embryos were examined in whole mount using brightfield microscopy. Serial sections of nurse eggs were examined for the presence of nuclear DNA. DNA was visualized with BODIPY-FL 14-dUTP, a probe that is specific for DNA fragments produced by endonucleases in apoptotic cells, and counter-stained with Hoechst 33342,

which labels double-stranded DNA and early apoptotic DNA fragments (Haugland 1996). Capsules were serially sectioned at 15μm in a Reichert Histostat cryostat microtome and incubated in 70% ethanol for 1 hour before staining with BODIPY-dUTP and Hoechst. BODIPY-dUTP-labeled DNA fragments and Hoechst-labeled DNA in nurse eggs were visualized with fluorescence microscopy (UV excitation at wavelengths 505-515nm and 330-380nm, respectively) (Haugland 1996). At least 2 broods were examined with fluorescence microscopy (BODIPY-dUTP and Hoechst labeling) at the 2 cell, 16-32 cell, mid-cleavage, and gastrula stages.

Capsules were prepared for transmission electron microscopy (TEM) by fixation in 2.5% glutaraldehyde, followed by 1% osmium tetroxide, both buffered with 0.45μm filtered sea water and 0.1M sodium cacodylate (Buckland-Nicks 1993). The sea water was previously filtered through a 0.45 μm millipore filter, to remove bacteria. Specimens were embedded in TAAB resin (Marivac) and sectioned on a LKB Ultramicrotome III. Ultra-thin sections were mounted on 200 mesh copper grids (Marivac) and were reverse stained with lead citrate and uranyl acetate (Buckland- Nicks pers. comm., 1993, Carson 1990). Ultra-thin sections were observed with a Philips transmission electron microscope (model EM 301) at 60kV. Broods at the 1-cell, 2-4 cell, and 16-32 cell stages were examined.

Results

Morphological changes

Egg capsules containing embryos at various developmental stages (1 cell, 2-4 cell, 32 cell) and nurse eggs were observed by electron microscopy. Immediately after

spawning, one-celled zygotes and nurse eggs are indistinguishable. All eggs exhibit fertilization envelopes with surface granules, microvilli, and a regular distribution of organelles throughout the cytoplasm (Fig. 5). Nurse eggs could be distinguished from zygotes after the first cleavage occurred in embryos because nurse eggs did not cleave.

In nurse eggs, the fertilization envelope was maintained throughout their formation, to the 32 cell stage in sibling embryos, and showed prominent surface granules (Figs. 6a, b and 7b). Other structures including the plasma membrane, microvilli, mitochondria, and endoplasmic reticulum (ER) were involved in the organized, dissociation processes that were followed by nurse eggs. Morphological changes in nurse eggs began after the first or second embryonic cleavage, and nurse egg dissociation into cytoplasmic vesicles was completed at the 32 cell stage of sibling embryos.

The first changes in nurse egg ultrastructure included invagination of the plasma membrane to compartmentalize the cytoplasmic components (Fig. 6a-c) and loss of microvilli (Fig. 7b). Invagination of the plasma membrane resulted in vesicle formation, or cytoplasmic blebbing (Figs. 6, and 7a-c). Vesicles began to form around the periphery of nurse eggs and progressed inward until the entire cytoplasm was compartmentalized (Figs. 6 and 7a). Clusters of ER and mitochondria were observed in areas where the plasma membrane was invaginating (Fig. 6b-d). These energy producing and synthetic organelles maintained structural integrity during vesicle formation. As vesicle formation was completed in nurse eggs cross sections of the mitochondria and ER profiles were less frequently observed. Within the vesicles the mitochondria and ER profiles were concentrated along the vesicle membrane (Figs. 6d and 7c). Yolk granules and lipid were

also compartmentalized into the vesicles during nurse egg formation (Figs. 6a, b, d, and 7a, c).

Embryonic development in *Boccardia proboscidea* is quite different from nurse egg formation. Embryos undergo holoblastic, spiral cleavage which results in two major cell types, micromeres (relatively yolk free) and macromeres (contain large numbers of yolk granules) (Fig. 7a, d). Nuclei were often observed in section in embryonic micromeres and often contained multiple nucleoli (Fig. 7d). Although Hoechst 33342 demonstrated nuclear DNA in early nurse eggs (1 cell stage) (Chapter 2) followed by loss of nuclear DNA in nurse eggs as they dissociated, nuclei and DNA were not observed by electron microscopy.

Throughout embryonic development, up to the 32-cell stage, the fertilization envelope with surface granules was maintained as in nurse eggs (Figs. 5 and 7). Microvilli were also maintained in embryos, also up to the 32 cell stage, as folds in the plasma membrane (Fig. 7b). In cross sections of embryos the micromeres appeared to maintain an even distribution of organelles throughout the cytoplasm (not shown) while the macromeres appeared similar to nurse eggs with few organelle profiles, however, cytoplasmic blebbing did not occur. In macromeres, mitochondria and ER became concentrated along the plasma membrane (Fig. 7b,d). Yolk granules became confined to embryonic macromeres, similar to nurse egg vesicles, and micromeres remained relatively yolk-free (Fig. 7a, d).

DNA fragmentation

Nurse eggs and embryos were stained with the probes Hoechst 33342 (Hoechst) and BODIPY-FL 14-dUTP (BODIPY-dUTP) to visualize intact nuclear DNA and

apoptotic DNA fragments, respectively. Broods were examined between the 1 cell and the trochophore stages.

In 2 cell stage broods, nurse eggs showed fluorescence in localized areas, adjacent to the nuclear region, and in one or two areas along the cortex of the egg (Fig. 8a, b). Perikaryal fluorescence indicates degradation of DNA not associated with the nurse egg nucleus, such as polar bodies and possibly sperm. As cleavage continued in embryos, nurse eggs showed localized Hoechst-labeled DNA and a broad distribution of BODIPY-dUTP-labeled DNA in areas surrounding the Hoechst-labeled area (Fig. 8c, d). At mid-cleavage stages, nurse eggs maintained a broad distribution of BODIPY-dUTP-labeled DNA but the fragments were confined to the cytoplasmic vesicles (Fig. 5e, f). Fluorescence was more localized in earlier stages and gradually became more dispersed throughout the nurse eggs suggesting that DNA was progressively degraded into smaller fragments as nurse eggs underwent vesiculation.

Hoechst was used as a counter-stain for double-stranded DNA. In nurse eggs, Hoechst frequently stained DNA remnants (Fig 8c, and 9a) and BODIPY-dUTP stained fragmented DNA in areas where Hoechst-labeled DNA was not visible (Fig. 8c,d, and 9a, b). In embryos, Hoechst-labeled DNA was clearly visible in micromeres but fluorescent nuclei were not visible in embryonic macromeres (Fig 9a). However, BODIPY-dUTP-labeled DNA was visible in the macromeres and not in the micromeres (Fig 9b). These preliminary observations suggest that apoptosis may occur in macromeres as in nurse eggs. However, further investigation is required to determine the exact processes involved in larval gut formation.

Discussion

Adelphophagy is a relatively common larval trophic mode in benthic marine invertebrate species that produce encapsulated young (Levin and Bridges 1995). Embryos are provided with extra-embryonic yolk, often in the form of nurse eggs. Production of nurse eggs reduces female fecundity, but nurse egg consumption increases hatchling size, which may result in higher survivorship to metamorphic competence (Rivest 1983, Levin and Bridges 1995, Gibson 1997). Despite the common occurrence of nurse eggs, there have been few descriptions of nurse egg formation and the mechanism underlying nurse egg production is not understood.

Nurse eggs produced by prosobranch molluscs and polychaete annelids appear to be normal ova and are indistinguishable from viable eggs until zygotes undergo normal cleavage (Rivest 1983, Chapter 2). In most species, nurse eggs do not cleave but dissociate into small vesicles that are consumed by developing embryos (e.g. *Pygospio elegans*, Rasmussen 1973; *Colus stimpsoni*, West 1979, 1983; *Dendropoma corrodens*, Miloslavich and Penchaszedeh 1992; *Boccardia proboscidea*, Gibson 1997; *Fusinus closter*, Miloslavich and Penchaszedeh 1997). In some species, however, nurse eggs undergo a few abnormal cleavages and then abort development (e.g. the gastropods *Fasciolaria tulipa*, Burger and Thornton 1935; *Searlesia dira*, Rivest 1983; *Nucella crassilabrum*, Gallardo and Garrido 1987; *Vermetus* sp., Miloslavich and Penchaszedeh 1992; *Buccinum cyaneum*, Miloslavich and Dufresne 1994). Our results show that nurse eggs of the polychaete *Boccardia proboscidea* dissociate into small vesicles. The process of vesicle formation involves an invagination of the plasma membrane and loss of microvilli. Vesicles begin to form around the cortex of nurse eggs and progress inward

into the egg until the entire cytoplasm has been compartmentalized.

Loss of microvilli and blebbing of the cytoplasm, involving the plasma membrane, are characteristic features of cells undergoing apoptosis (Bär 1996, Honma and Hamasaki 1996, Superti *et al.* 1996, Darzynkiewicz *et al.* 1997). Numerous mitochondria and endoplasmic reticulum (ER) are also observed in areas of vesicle formation in *Boccardia proboscidea* nurse eggs. This observation is consistent with the idea that nurse eggs may be undergoing apoptosis since Bowen *et al.* (1998) report that these energy producing and synthetic organelles appear to play a vital role in apoptotic cells. Recent evidence additionally links molecules located within the intermembrane space of mitochondria to apoptosis (Earnshaw 1999, Susin *et al.* 1999). For example, cytochrome C, which is normally involved in cellular respiration in mitochondria, is released into the cytoplasm in cells triggered to undergo apoptosis. Cytochrome C binds with another cytoplasmic molecule and activates caspase-9, an initiator of apoptosis (Earnshaw 1999, Susin *et al.* 1999). Another molecule, survivin, has been shown to inhibit apoptosis and allow mitosis to proceed. Survivin expression is regulated during the cell cycle and survivin interacts with microtubules of the mitotic spindle during early stages of mitosis (during the Gap 2/mitosis phase). If the survivin-microtubule interaction is disrupted, there is an increase in activity of the mitotic spindle protease, caspase-3, which leads to apoptosis. Thus survivin may inhibit apoptosis through suppression of caspase-3 activity and maintenance of microtubule structure (Li *et al.* 1998). The large numbers of mitochondria and the presence of ER in *B. proboscidea* nurse eggs suggest that nurse eggs are undergoing processes typical of apoptosis. Since nurse eggs do not undergo cleavage (mitosis) perhaps factors such as survivin are

involved in inhibiting normal development by interrupting the cell-cycle and initiating apoptosis.

In many species, nurse eggs contain nuclear DNA; however, the fate of the chromatin varies between species. Gallardo and Garrido (1987) describe a large nucleus that appears to remain in the germinal vesicle stage in *Crepidula dilatata* nurse eggs. Chromatin in these nurse eggs degenerates as development is initiated in zygotes. Nurse eggs are also nucleated before spawning, in the coelom, of *Pygospio elegans* females (Rasmussen 1973) but the nucleus is lost immediately after spawning. Alternatively, nurse eggs produced by *Colus stimpsoni* undergo karyokinesis and become multinucleated, without undergoing cytokinesis (West 1979, 1983). Nurse eggs in *Boccardia proboscidea* (Chapter 2) are also nucleated before spawning but the DNA degenerates after spawning.

DNA in apoptotic cells is fragmented by endonucleases into characteristic lengths and packaged into apoptotic bodies (Li *et al.* 1995, Bär 1996, Bowen *et al.* 1998). DNA fragmented in apoptotic cells can be labeled with specific fluorochromes to visualize the distribution of fragments throughout the cells (Li *et al.* 1995, Li and Darzynkiewicz 1995, Li *et al.* 1996). One fluorochrome, BODIPY-FL 14-dUTP (Haugland 1996) has been used to visualize DNA fragmentation during apoptosis in vertebrates. For example, HL-60 human promyelocytic leukemia cells and human breast cancer cell line MCF-7, induced to undergo apoptosis by Li *et al.* (1996) in an investigation of techniques for labeling DNA strand breaks associated with apoptosis. BODIPY-dUTP was shown to be specific for apoptotic DNA fragments in comparison with two other deoxynucleotides, fluorescinated-dUTP, and dCT conjugated to cyanine dye CY-3 (Li *et al.* 1996). The

protocol used by Li *et al.* (1996) was modified in the present investigation of nurse egg origin and produced compatible results, although the present study investigates an invertebrate, rather than a vertebrate system. The results show localized DNA fluorescence near or within the nuclear area in early nurse eggs followed by a gradual spreading of DNA fragments throughout nurse egg vesicles. Staining with Hoechst 33342 also demonstrates packaging of DNA in nurse egg vesicles. These observations indicate that nurse eggs are undergoing apoptotic processes involving degeneration of nuclear DNA. Future investigations should consider further validation of the protocol used including induction of apoptosis in early embryos from broods lacking nurse eggs to determine if similar results are obtained.

BODIPY-dUTP fluorescence was also observed in the macromere region of mid-cleavage stage embryos. Although the macromeres do not undergo vesicle formation (cytoplasmic blebbing), they have a similar appearance to nurse egg vesicles. Yolk granules, mitochondria and scattered ER were visible in macromeres adjacent to the plasma membrane, similar to the nurse egg vesicles. Thus, it is possible that macromeres, in addition to giving rise to the endoderm, undergo apoptotic processes and serve as yolk reserves for developing cells. Early formation of the embryonic gut has not been described in polychaetes. Further work is required to determine the exact role of macromeres in embryonic development and to determine whether apoptosis is involved.

A fertilization envelope and hyaline layer were observed on all *Boccardia proboscidea* eggs and nurse eggs, through to the 32-cell stage. Although not examined at the ultrastructural level, it is assumed that the fertilization envelope is lost as embryos become swimming trochophore larvae. It is also assumed that nurse eggs maintain the

fertilization envelope until ingestion by early larvae within the egg capsule. As larvae begin to feed on nurse eggs within the egg capsules, the nurse eggs break apart and individual particles (i.e. vesicles) are ingested (Gibson 1997). In studies on vertebrates, apoptotic cells bleb into apoptotic bodies, which are engulfed by neighboring cells such as macrophages (Bowen *et al.* 1998). We suggest that the fertilization envelope functions to maintain structural integrity of nurse eggs, until larval ingestion of the vesicles, as a modification to the apoptotic process. Other species that produce nurse eggs demonstrate variations in nurse egg structural integrity. In *Searlesia dira* (Rivest 1983), nurse eggs undergo abnormal cleavage and are then swallowed whole by offspring. In *Vermetus* sp. (Miloslavich and Penchaszadeh 1992) early veligers break up the nurse eggs and swallow the particles. Finally, nurse eggs in some species dissociate and contribute to a mass of nurse egg vesicles (nurse egg mass) that is consumed by embryos (e.g. *Buccinum cyaneum*, Miloslavich and Dufresne 1994).

The present study demonstrates that nurse egg formation in *Boccardia proboscidea* is a complex process involving morphological changes of nurse egg structure and degeneration of DNA. These processes (blebbing of the plasma membrane, loss of microvilli, maintenance of organelle integrity, involvement of mitochondria and ER, DNA fragmentation) are characteristic features of apoptotic cells. Loss of nuclear DNA and break-up of the cytoplasm have been noted in nurse eggs of a few other species of marine invertebrates, suggesting that this mechanism of nurse egg production may be widespread (Rasmussen 1973, Miloslavich and Penchaszadeh 1992, 1997). Since the apoptotic machinery appears to have been conserved, and under the control of cell death genes that are homologous between the nematode *Caenorhabditis elegans* and the human

(Raff 1998), it seems most likely that cells may be capable of apoptosis in marine invertebrates as well. Future work should investigate the possibility of an apoptotic mechanism underlying nurse egg production among marine invertebrates.

Chapter 4

Conclusions

A number of hypotheses have been proposed to explain the origin of nurse eggs although the underlying mechanisms have rarely been investigated. Nurse eggs appear to be quite variable in different species, and development aborts at a range of early developmental stages from oogenesis to early cleavage.

This study demonstrates that nurse eggs in *Boccardia proboscidea* arise through active processes characteristic of apoptosis. Nurse eggs are produced as viable oocytes and are activated at spawning but fail to cleave. Nurse eggs lose nuclear DNA through fragmentation as visualized by the apoptosis-specific stain BODIPY-FL 14-dUTP. Nurse egg cytoplasm and DNA fragments are compartmentalized into small vesicles resembling apoptotic bodies through blebbing of the plasma membrane and loss of microvilli. Mitochondria and ER are also involved in the death program of nurse eggs and are present in areas where cytoplasmic vesicles are forming. Mitochondria and ER are also visible in formed vesicles and are localized along vesicle membrane.

Although apoptosis has not been identified as the mechanism underlying nurse egg origin in other species, nurse eggs have been described that show morphological features suggesting that apoptosis may be involved. For example, Rasmussen (1973) described loss of DNA in nurse eggs of *Pygospio elegans* as well as disintegration of nurse eggs into vesicles that resemble those observed in *B.proboscidea*.

Other studies suggest a variety of potential mechanisms that may trigger nurse egg formation, including fertilization, induction by siblings, or the production of defective eggs. Hyman (1935 cited in Miloslavich and Dufresne 1994) suggests that

nurse eggs arise from fertilization by abnormal spermatozoa in *Fasciolaria tulipa*. While spionids are known to produce polymorphic sperm (Rice 1992), paternal effects are unlikely to be involved in nurse egg formation in *Boccardia proboscidea* since crosses of type 1 (do not produce nurse eggs) and 3 (produce nurse eggs) males and females always result in offspring that exhibit the female reproductive mode (Gibson 1997). Hadfield (1989) suggests that in *Petaloconchus monteryensis* the fate of nurse eggs occurs in the egg capsule since only one embryo ever develops per capsule, despite the variable number of eggs spawned per female with the rest of the eggs always becoming nurse eggs. This suggests that factors or signals in the egg capsule trigger nurse egg formation, possibly after an embryo has been triggered to develop, otherwise egg capsules containing only nurse eggs and no embryos would occur. In *Boccardia proboscidea* egg capsules containing only nurse eggs (lacking embryos) are not uncommon, while most egg capsules normally contain multiple embryos. Thus it is unlikely that sibling competition, or sibling-associated inducers, are involved in nurse egg production. Environmental factors are also not likely to be involved in nurse egg formation in *B.proboscidea* since both development types are maintained under the same laboratory conditions.

West (1979, 1983) suggests that in *Colus stimpsoni*, nurse eggs are either defective or deficient in factors required for cytokinesis. This is quite plausible given the evidence that nurse eggs in *Boccardia proboscidea* may undergo apoptosis as developing siblings are undergoing the first mitotic division. Although it remains to be tested in nurse eggs, factors such as survivin, known to suppress apoptosis in other cell lines (Li *et al.* 1996), may be present and involved in mitosis during embryonic cleavage. These

factors may also be present in nurse eggs and similarly lead to apoptosis if interactions with the mitotic spindle microtubules are disrupted. Future work should investigate the presence and functioning of such factors through comparisons of nurse eggs and zygotes.

Since nurse eggs of many marine invertebrate species undergo processes characteristic of apoptosis, it would be interesting to investigate the role of organelles such as mitochondria and endoplasmic reticulum (ER), and the plasma membrane in nurse egg formation. Mitochondria and ER appear to play key roles in nurse egg formation in *Boccardia proboscidea*. It would be interesting to determine whether apoptosis-inducing factors such as cytochrome C and AIF are released from the mitochondria in nurse eggs. Other useful information on the role of ER in membrane synthesis during cytoplasmic blebbing may be obtained through utilizing membrane specific probes on nurse eggs dissociating into vesicles. Another possibility for nurse egg determination, also suggested by Staiger (1951, in Miloslavich and Penchaszadeh 1997) is the presence of genetic factors or ooplasmic determinants in the eggs. Ooplasmic determinants are often involved in shaping early development before zygotic genes are activated (Gilbert 1997); thus it is possible that females influence nurse egg formation or embryonic development through ooplasmic determinants placed in the oocyte cytoplasm. These factors may initiate the developmental program and inhibit apoptosis in zygotes or may inhibit development and induce apoptosis in nurse eggs, assuming that females are capable of partitioning these factors during oogenesis.

This study has provided insight into mechanisms involved in nurse egg origin and formation. Nurse egg production involves a complex set of events in *Boccardia proboscidea*, similar to apoptosis, as described in other systems. Additional studies are

needed to investigate nurse egg origin in other species before generalizations can be made. Survivin, or similar factors, may act as cell-cycle regulators in nurse eggs to inhibit mitosis and promote apoptosis. Since cleavage is inhibited in nurse eggs at various stages of early development, from oocytes to early cleavage, perhaps there are factors involved in cell-cycle regulation that induce apoptosis. These factors may be expressed or inhibited at different times during the cell-cycle, depending on the species, and result in nurse egg determination at different stages.

Table 1: Egg activation in *Boccardia proboscidea*, as the percentage of eggs (Type 1 and Type 3) with fertilization envelopes and polar bodies at the 2-4 cell stages.

Brood Type	Developmental stage		Egg number	Presence of fertilization envelope (%)	Presence of polar body (%)
Type 1	1 cell		210	99.5	76.7
	2-4 cell		198	100	75.8
Type 3	1 cell		142	97.2	14.3
	2-4 cell	embryos	26	92.3	19.2
		nurse eggs	93	51.1	16.7

Figure 1: Oogenesis in Types 1 and 3 *Boccardia proboscidea*. **A.** Photomicrograph of a longitudinal section through a Type 1 female showing the orientation of ovaries within a few setigers. Ovaries (ov) are each associated with a septum (s) which divides the coelom into setigers. Oocytes (oc) detach from the ovaries and fill the coelom of each setiger. Scale bar = 100μm **B.** Photomicrograph of a cross section through a Type 3 female showing a close up of the ovary and detached oocytes in the coelom each with a prominent germinal vesicle (gv). The ovary is associated with a darkly stained nephridium (d). Scale bar = 25μm

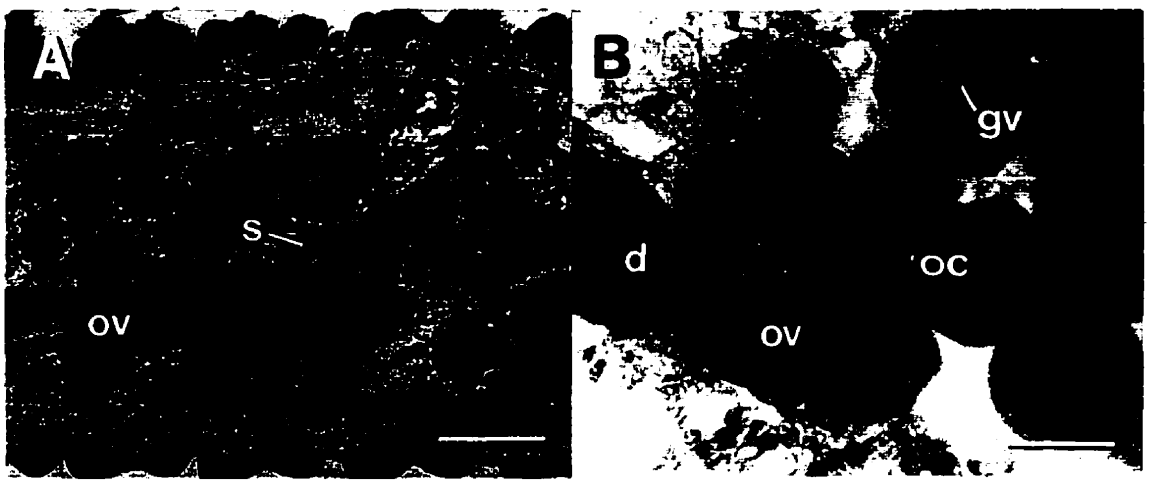
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Figure 2: Evidence for egg activation in *Boccardia proboscidea* after spawning. **A.** Photomicrograph of a Type 1, one-cell stage embryo exhibiting polar bodies (pb), a polar lobe (pl), and a fertilization envelope (f). Scale bar = 25μm **B.** Photomicrograph of a Type 3 brood, including a 4-cell stage embryo and two nurse eggs exhibiting fertilization envelopes (f). A polar body is visible on one nurse egg (pb) Scale bar = 50μm.

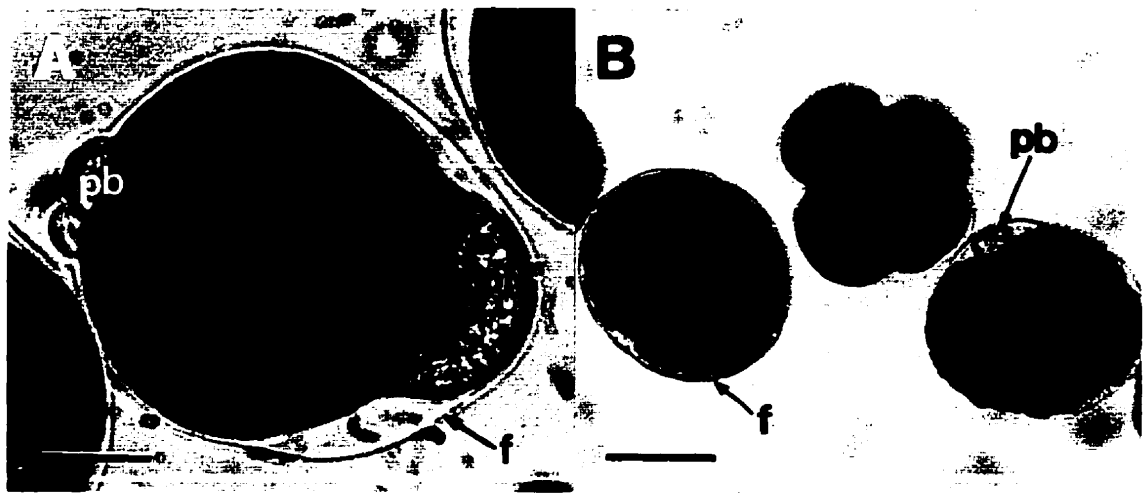
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Figure 3: DNA loss in *Boccardia proboscidea* nurse eggs. Fluorescence = DNA labeled with Hoechst 33342. **A.** Photomicrograph of whole mounts of Type 1, 1-2 cell embryos exhibiting fluorescent nuclear DNA (n) and polar bodies (pb). **B.** Photomicrograph of whole mounts of Type 1, mid-cleavage embryos exhibiting fluorescent DNA (n) in all cells. **C.** Photomicrograph of a section through a Type 3, 1 cell stage egg capsule showing fluorescent DNA (n). **D.** Photomicrograph of a section through a Type 3, 2-cell stage egg capsule showing embryos and nurse eggs (ne). Fluorescence indicates nuclear DNA in embryos (n). Nurse eggs also show fluorescent DNA within vesicles and in areas where vesicles have not yet formed (arrow heads). **E.** Photomicrograph of a section through a Type 3 trochophore stage egg capsule showing a trochophore larva and nurse eggs. The larva has one pair of eyes (e) anteriorly and a yolky gut (g) posteriorly. Brightly fluorescent nuclei are visible in the cells of the larva. The nurse eggs are essentially enucleate except for two flecks of fluorescent material (fl). Scale bars A-D = 100μm, Scale bar E = 50μm.

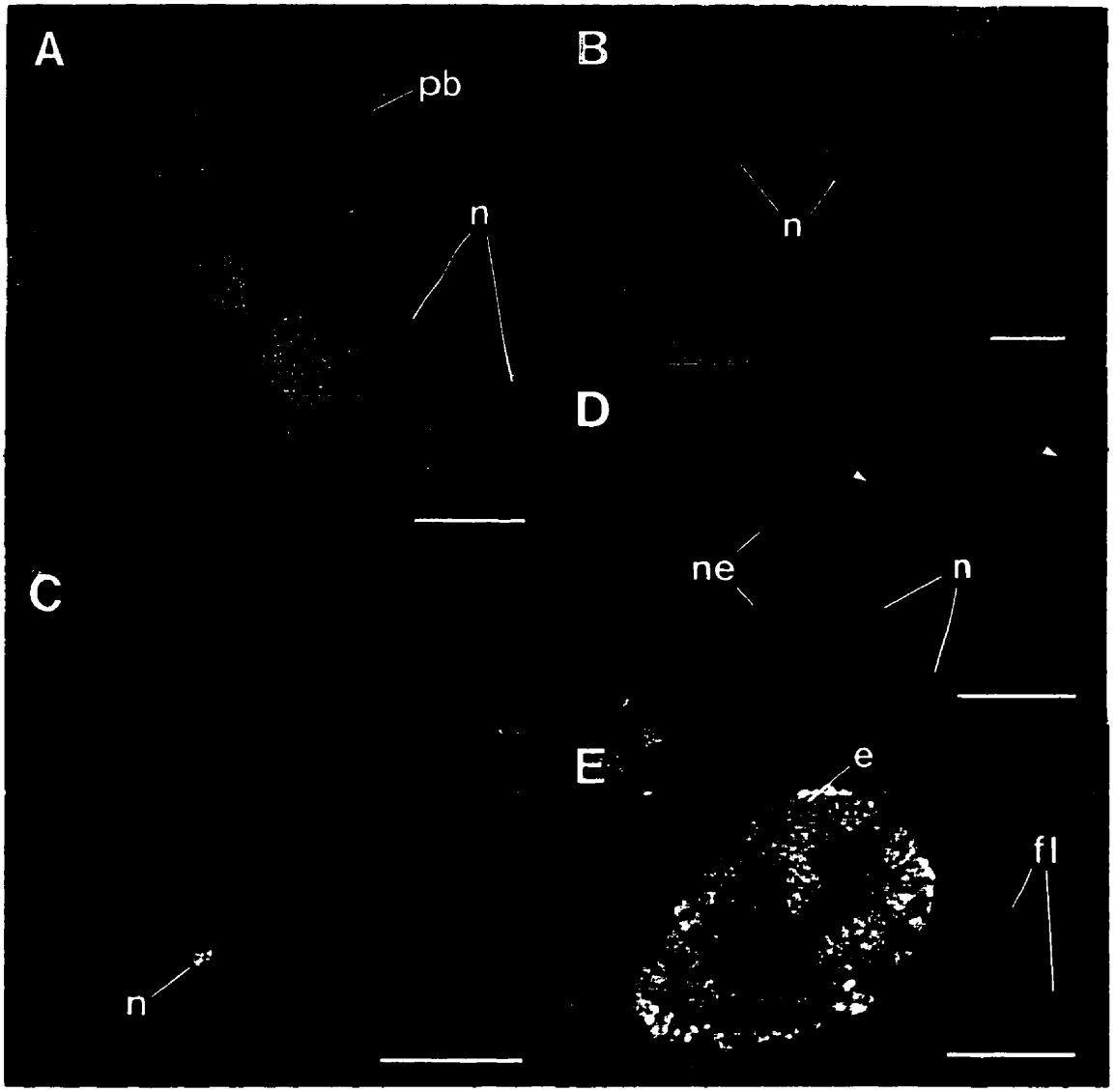
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Figure 4: Embryonic cleavage and nurse egg dissociation into vesicles in Type 3 broods. **A.** Photomicrograph of a 1μm section through a 1-cell stage egg capsule. The nucleus is clearly visible in many eggs (n). **B.** Photomicrograph of a 1μm section through a nurse egg from a 2-cell stage brood showing fluorescing DNA within a vesicle as well as within a region where vesicles have not yet formed (n). Note that vesicles are only apparent around the periphery of the nurse eggs at this stage (large arrow). **C.** Photomicrograph of a section through a 4-cell stage egg capsule showing part of an embryo and nurse eggs. A nucleus is visible in one cell of the embryo (n). Vesicles containing yolk granules and ooplasm are visible within the nurse eggs (large arrows point to vesicles). **D.** Photomicrograph of a 1μm section through a 16-cell stage egg capsule showing an embryo and nurse eggs. The embryo is undergoing cleavage (s = mitotic spindle apparatus). Yolky macromeres are visible in the gut region (ma) and are surrounded by micromeres (mi). Micromeres clearly show a nucleus (n). The nurse egg has completed vesicle formation and the vesicles are visible throughout (large arrows point to vesicles). Scale bars = 50μm.

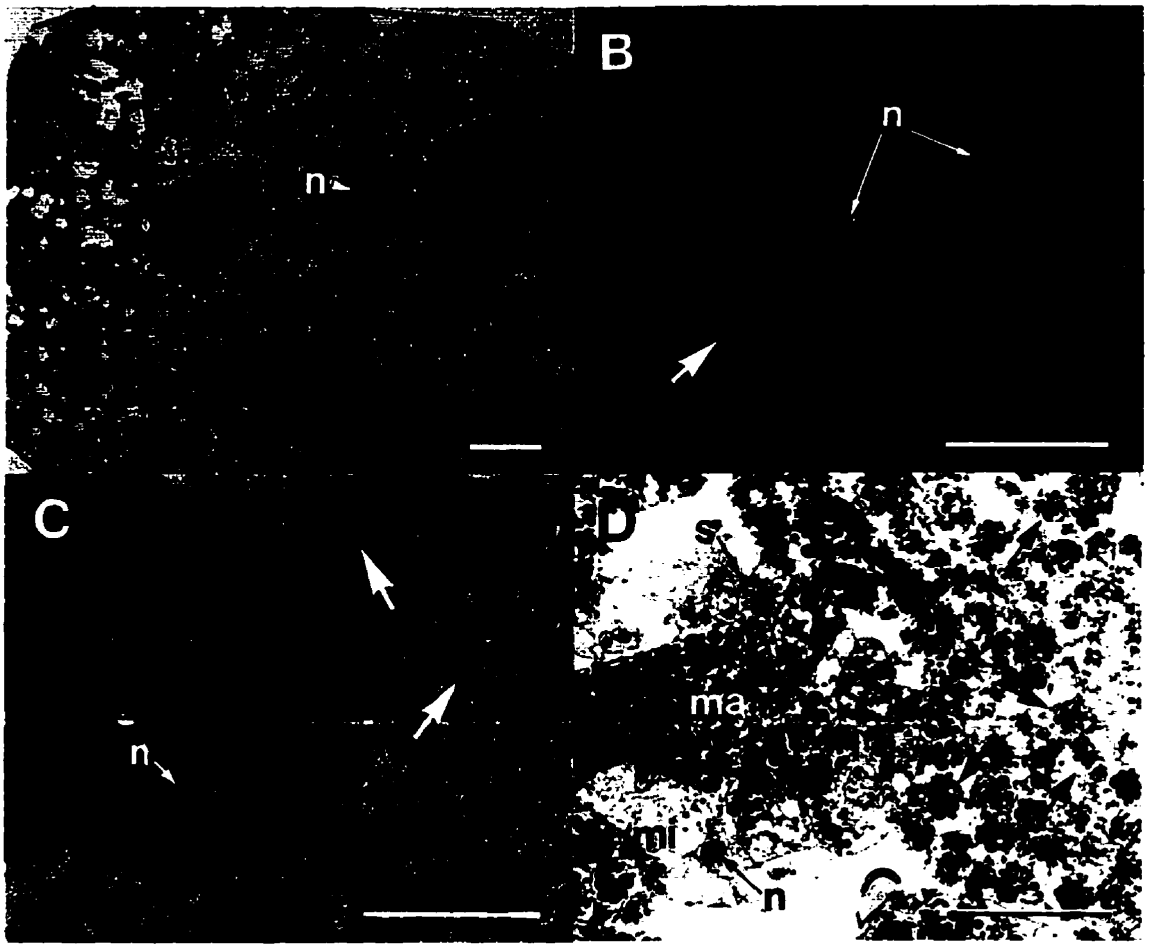
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Figure 5: Ultrastructure of *Boccardia proboscidea* eggs at the 1-cell stage. **A.** Electronmicrograph of a section through two, 1-cell stage eggs showing the fertilization envelope (fe), endoplasmic reticulum (er), numerous mitochondria (m) and yolk granules (Y) throughout the cytoplasm. 7500X magnification **B.** Electronmicrograph of a section through two, 1-cell stage eggs at a higher magnification (9800X) again showing the fertilization envelope with surface granules (large arrowheads). Microvilli (v) and a plasma membrane (p) are visible on both eggs. Mitochondria, endoplasmic reticulum, and a Golgi complex (g) are also visible in the cytoplasm.


A
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er
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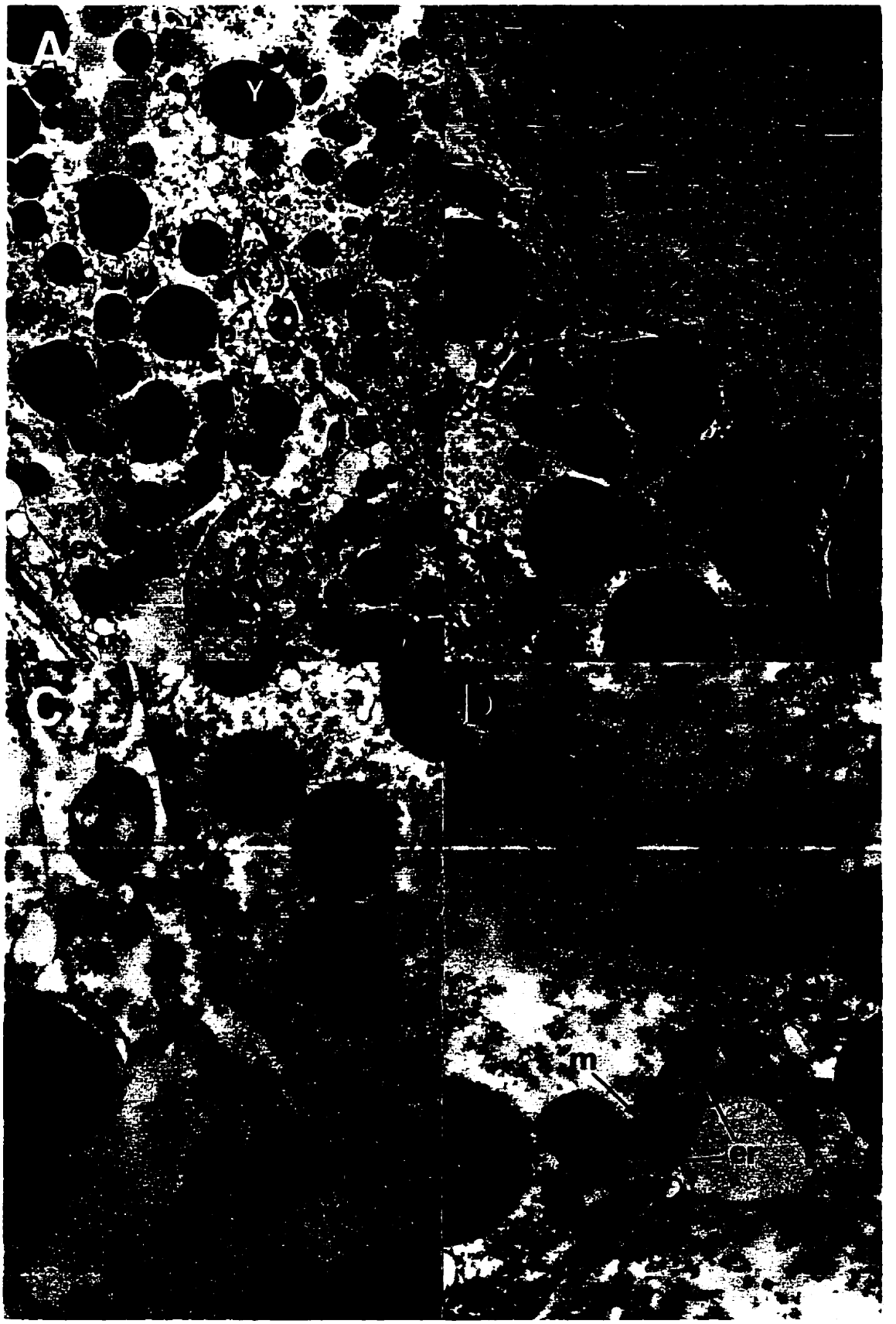
Figure 6: Vesicle formation in *Boccardia proboscidea* nurse eggs from 2-4 cell broods. **A and B.** Electronmicrographs of sections through part of two nurse eggs showing invagination of the plasma membrane (p) into the cytoplasm. Yolk granules (Y), lipid (L), and fertilization envelopes (fe) are also visible. Electronmicrograph **B** shows typical clustering of mitochondria (m) and endoplasmic reticulum (er) along the plasma membrane during vesicle formation. **A** = 3600X magnification, **B** = 5900X magnification. **C.** Electronmicrograph at a higher magnification (5900X) of part of the region in **A** showing mitochondria lined up along the invaginating plasma membrane. **D.** Electronmicrograph of a section through a nurse egg showing part of four completed nurse egg vesicles. Mitochondria (m) and endoplasmic reticulum (er) are still visible along the vesicle membrane. Much smaller vesicles are visible in the space between the vesicles. 9800X magnification.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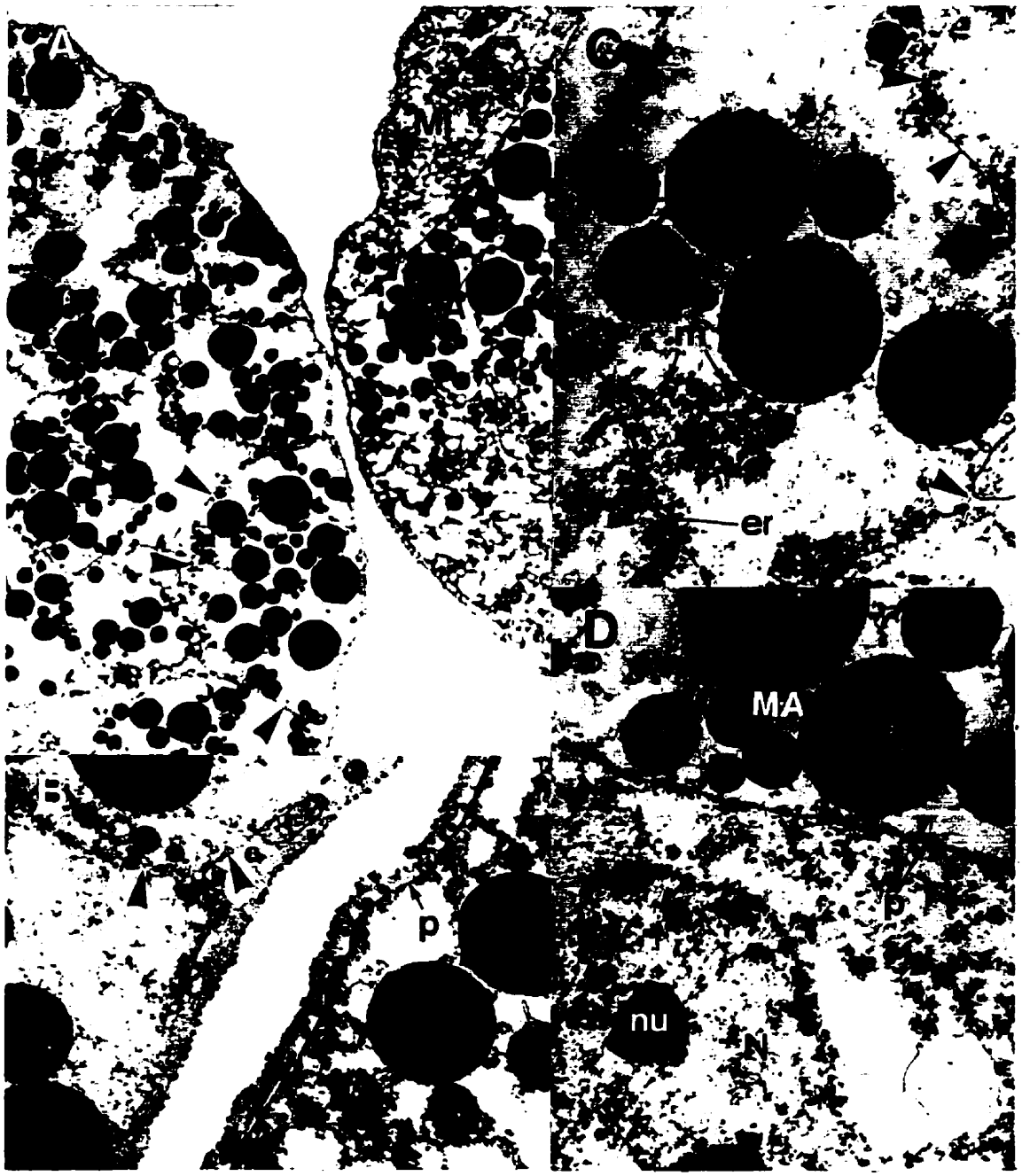
er

Figure 7: Ultrastructure of *Boccardia proboscidea* nurse eggs and 32-cell embryos. **A.** Low magnification electronmicrograph (2200X) of a nurse egg (left) and an embryo (right). Vesicle formation of the nurse egg cytoplasm is complete (large arrowheads point to the membrane of a peripheral vesicle). An outer layer of yolk-free micromeres (MI) surrounding an inner region of yolky macromeres (MA) is visible in the embryo **B.** Higher magnification electronmicrograph (4300X) of the nurse egg (left) and embryo (right) from **A** showing the presence of microvilli on the embryo (small arrowheads) and no microvilli on the nurse egg. Large arrowheads point to the membrane of a peripheral vesicle in the nurse egg. The plasma membrane (p) is visible in the embryo. **C.** Electronmicrograph of one nurse egg vesicle showing mitochondria (m) and endoplasmic reticulum (er) adjacent to the vesicle membrane (large arrowheads point to vesicle membrane). 7500X magnification. **D.** Electronmicrograph of a section through an embryo showing part of a macromere (MA) showing mainly yolk granules and two small mitochondria near the plasma membrane (p), and a micromere containing a nucleus (N) with nucleoli (nu; only one shown). 9800X magnification.


A
M
C
e
m
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e
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p
nu
N

Figure 8: DNA fragmentation in *Boccardia proboscidea* nurse eggs. Fluorescence in **A, B, D-F** reveals BODIPY-labeled DNA fragments. Fluorescence in **C** reveals Hoechst-labeled dsDNA. **A and B.** Photomicrographs of two nurse eggs from 2-cell stage broods showing localized BODIPY fluorescence in the nuclear area (central dark area) in **A** and fluorescence outside of the nuclear area as well as along the egg cortex in **B**. **C and D.** Photomicrographs of a nurse egg from a 16-cell stage brood stained with both Hoechst and BODIPY. Fluorescence in **C** reveals Hoechst and is located within the nuclear area. Fluorescence in **D** reveals BODIPY and is distributed throughout the nurse egg cytoplasm. **E and F.** Photomicrographs of nurse eggs from mid-cleavage broods. Vesicle formation is complete in the nurse eggs (large arrowhead points to a vesicle). BODIPY-labeled fragments are confined to vesicles at this stage. Scale bars = 20μm

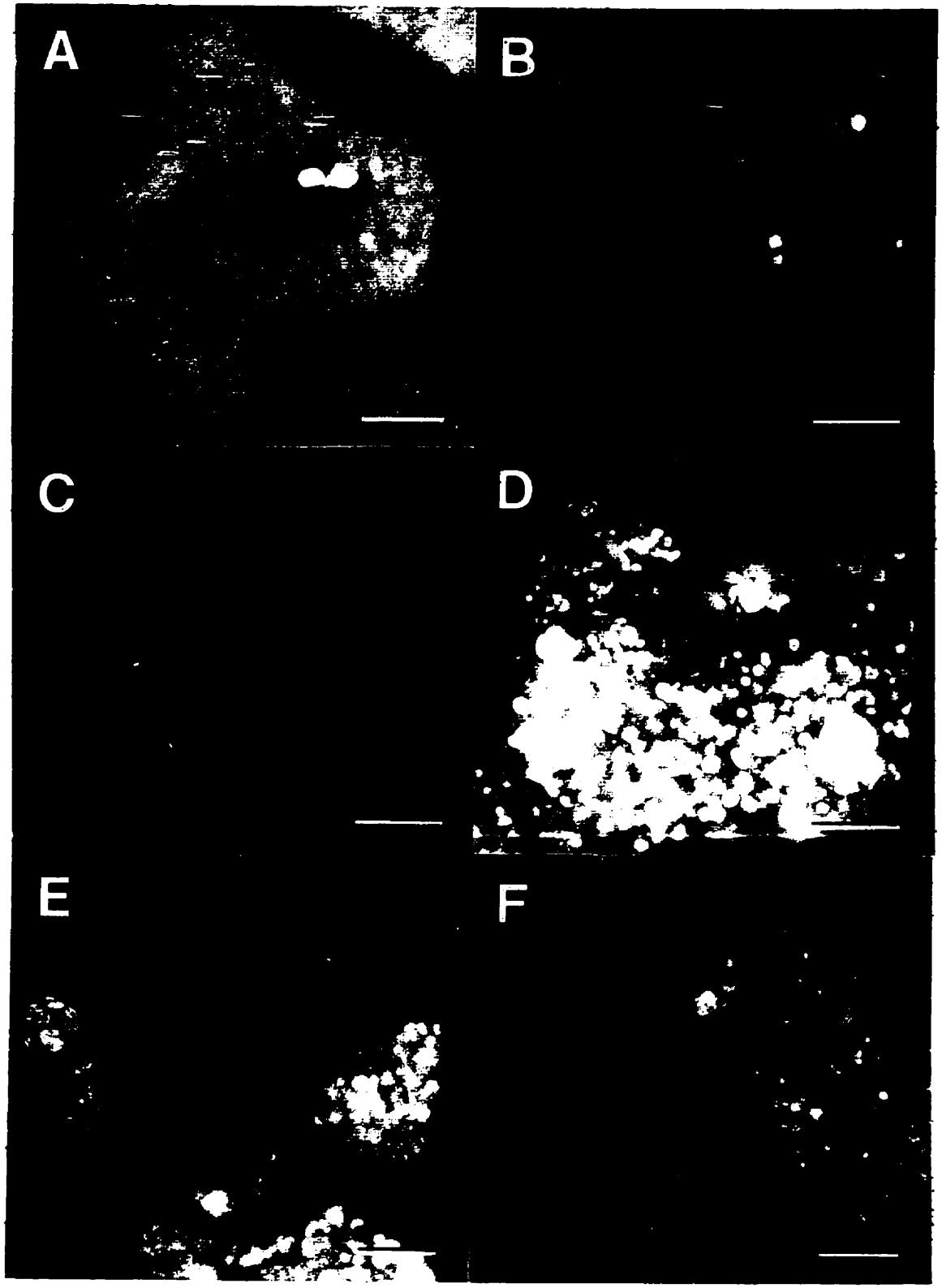
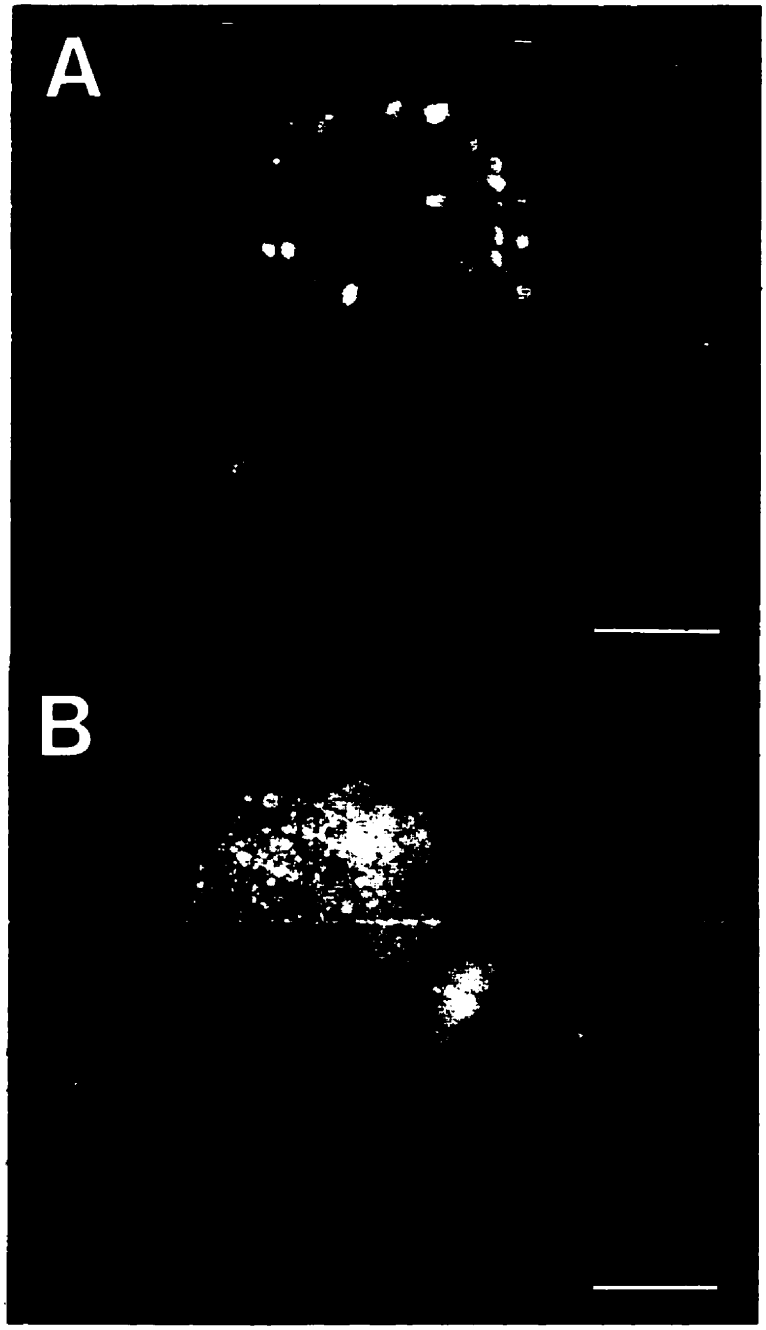

A
B
C
D
E
F

Figure 9: Hoechst- and BODIPY-labeled DNA in *Boccardia proboscidea* nurse eggs and an embryo at the gastrula stage. **A.** Hoechst-labeled DNA in nuclei of embryonic micromeres surrounding macromeres which do not show Hoechst fluorescence. DNA remnants are also visible in the nurse egg to the left of the embryo. **B.** BODIPY-labeled DNA is visible in the embryonic macromeres but not in the micromeres demonstrating the presence of fragmented DNA within the macromeres. BODIPY-labeled DNA is also visible in the nurse egg in areas not labeled with Hoechst. Differential staining with BODIPY and Hoechst demonstrates that they are not labeling the same fragments of DNA. Scale bars = 40μm


A
B

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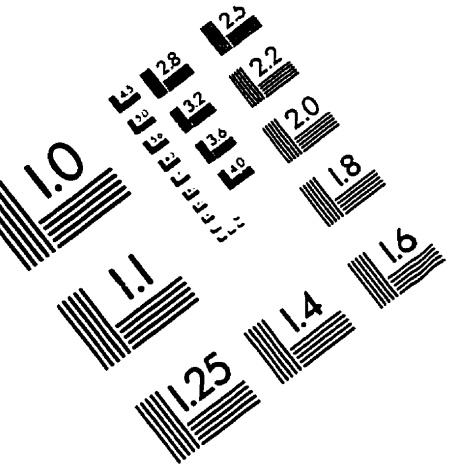

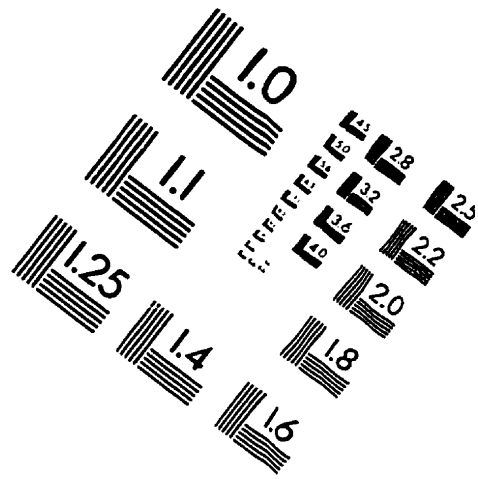

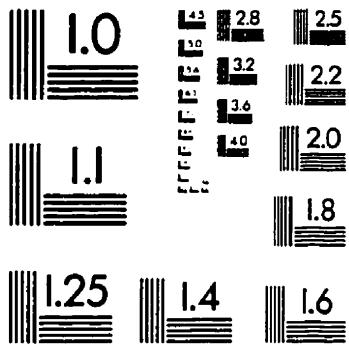

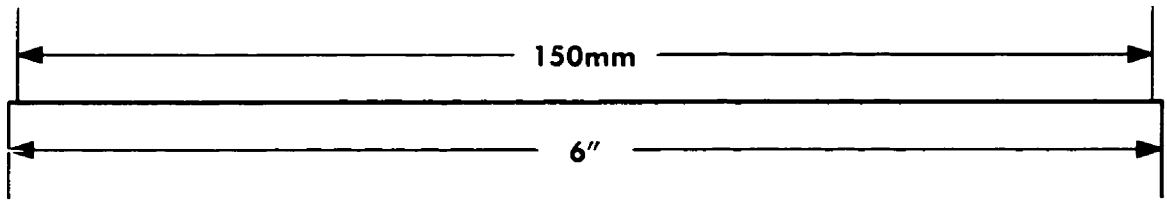

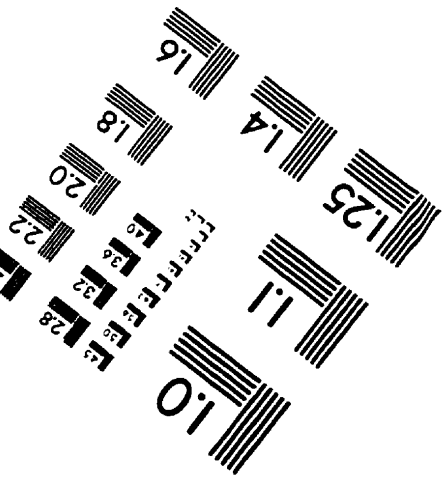

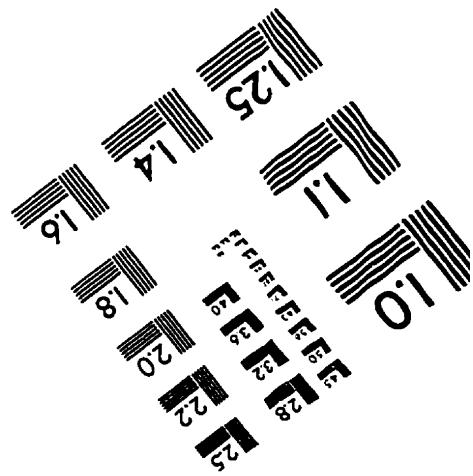

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