Sperm Storage and Degradation in the Spermathecae of the Salamander *Eurycea cirrigera*

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**ABSTRACT** Spermathecae are exocrine glands in the roof of the female cloaca that store sperm. Cytological and histochemical data indicate that the one type of secretion into the lumen is a glycoprotein. After a period of stasis in the summer, production of the secretion is initiated in the fall, coincident with an increase in ovarian follicular size. By the time of maximal follicular development and most intense mating activity in March and April, the spermathecal epithelium is filled with secretory granules. The secretory material is released into the lumen, enveloping the sperm. Many sperm remain in the spermathecae after oviposition, and most of these sperm are degraded in the spermathecal epithelium or pass through interruptions in the spermathecal walls caused by desquamation. Sperm in contact with the stromal environment are phagocytized by leukocytes. Some sperm, however, may survive in the lumen until at least the following fall. These sperm retain normal cytology, but whether or not they remain fertile and intact until a subsequent ovipository cycle is unknown.

The storage of viable sperm in the female urogenital tract for variable periods of time prior to fertilization of ova is known in every vertebrate class except Agnatha (Howarth, '74). Sperm were first noted in exocrine glands in the cloacal wall of female salamanders by Siebold (1858) who studied several species of *Salamandra* and *Triturus*. Siebold (1858) called the group of glands the *receptaculum seminis*, adopting a name already established for sperm-storage organs in arthropods. The name “spermatheca” was first applied to sperm-storage organs in salamanders by Kingsbury (1895), who found the term a “euphonious mononym” for *receptaculum seminis*. Also, Kingsbury (1895) proposed that each individual tubule that functions as a sperm reservoir is a spermatheca. As many such tubules occur in female salamanders, “spermathecas” (Kingsbury, 1895) or “spermathecae” (Sever, '87) are present. Numerous studies have dealt with the anatomy of spermathecae in salamanders (see reviews of Dent, '70; Boisseau and Joly, '73; Sever, '87, '91).

Some salamanders (such as those in the Plethodontidae and Salamandridae) apparently can store sperm in their spermathecae for months or years (Boisseau and Joly, '75; Marynick, '71; Massey, '90). Baylis ('39) reported fertilization of ova in *Salamandra salamandra* (Salamandridae) at least two years after the last possible mating. Ova of oviparous species are fertilized by sperm released from the spermathecae as the ova pass through the cloaca during oviposition (Boisseau and Joly, '75). In viviparous salamanders, fertilization occurs at the caudal end of the uterus in *S. atra* and in the middle and caudal parts of the oviduct in viviparous *S. salamandra* (Hafeli, '71; Boisseau and Joly, '75).

Benson ('68), Marynick ('71), and Boisseau and Joly ('75) believed that sperm receive nourishment during storage in the spermathecae from their being in contact with spermathecal epithelium. However, Dent ('70), Pool and Hoage ('73), and Brizzi et al. ('89) felt that only sperm found in the lumen are bathed with nourishing secretions, whereas those sperm in contact with the epithelium are in the process of being phagocytized. Dent ('70), Pool and Hoage ('73), Boisseau and Joly ('75), and Brizzi et al. ('89) all stated that sperm left in the spermathecae after oviposition degenerate or are resorbed or phagocytized, but none of these authors provided any cytological details about the process.

In the present study, transmission electron microscopy (TEM) was used to help resolve issues of sperm nourishment and deg-
radiation in spermathecae of a plethodontid salamander, *Eurycea cirrigera*. The histological features of the spermathecae of this species, or its sibling *E. bislineata*, were described by Kingsbury (1895), Koering ('25), and Sever ('87, '88). The reproductive cycle of the population utilized in the present study has been described by Sever ('88). Mating occurs in March and April, and oviposition is in late April and early May. Sperm are usually found in the spermathecae of animals collected between March and May, but they are not evident in spermathecae of animals collected in June, August, and October (Sever, '88).

**MATERIALS AND METHODS**

TEM was used to examine the spermathecae of 16 additional specimens of *Eurycea cirrigera* (Green). All specimens were collected from a 100 m stretch of stream used by Sever ('88) in Shades State Park, Montgomery County, Indiana. Snout-vent length (SVL) was measured to the nearest 0.1 mm from snout to posterior end of vent. Data on SVLs, collection dates, matings (if any) while maintained in the laboratory, dates of sacrifice, and presence of spermatozoa in the spermathecae are given in Table 1.

Individuals collected on 13 August and 25 April were held in a refrigerator at 5°C prior to sacrificing them on 31 August and 25 April, respectively. Individuals collected on 30 September were maintained in the laboratory prior to their sacrifice. These salamanders were kept in an environmental chamber at 15°C, housed singly in 17 × 31 × 9 cm plastic containers supplied with wet paper towels, and fed ad libitum from vials of *Drosophila*. As noted in Table 1, two individuals were sacrificed on 6 October and one on 13 December. Matings with males from the population were staged for the other females by placing a male and a female together in a container overnight. Protrusion of a spermatophore cap from the cloacal orifice of the female on the following morning was evidence of successful mating. One female underwent mating on 19 January and was sacrificed the following morning. Another female (43.7 mm SVL) underwent mating on 23 January by using an injection of pregnant mare serum gonadotropin (Sigma) as described by Davitt and Larsen ('88). This animal received 100 IU on both 30 and 31 March, but she did not lay eggs and was sacrificed on 15 April.

All salamanders were sacrificed by immersion in 5% MS-222. For the animals sacrificed on 31 August, the spermathecae were removed and transferred to a solution of 2.5% glutaraldehyde in Millonig's phosphate buffer at pH 7.4. For the other animals, cir-

<table>
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<tr>
<th>Breeding condition</th>
<th>SVL</th>
<th>Date collected</th>
<th>Follicle size</th>
<th>Mated in lab</th>
<th>Date sacrificed</th>
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<td>0.58</td>
<td>N</td>
<td>31 Aug</td>
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<td></td>
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1Measurements are in mm; follicle size is a mean based on 10 follicles; N, no; S, spent; X, missing data; Y, yes.
cstances dictated that preservation and storage of the entire animal was necessary before further processing of tissue. These animals were transferred to a 3.7% formaldehyde solution buffered to pH 7.2 with monobasic and dibasic sodium phosphate, and spermathecae were removed later. Formaldehyde is not the best fixative for TEM, but it penetrates larger tissue blocks well and allows for temporary storage of tissue (Dawes, '88; Hayat, '89). Following a water rinse, the spermathecae were postfixed in 2% aqueous osmium tetroxide, dehydrated in a graded series of acetone, and embedded in Spurr epoxy resin. Thin sections of 65–80 nm were cut with diamond or glass knives using a RMC 1000XL ultramicrotome, stained with 2% uranyl acetate, and examined with a Hitachi H-300 electron microscope. The diameters of 10 ovarian follicles from each female were measured to 0.01 mm at 20x with an ocular micrometer in a dissecting microscope.

RESULTS

Specimens killed between 19 January and 25 April were in a sexually active reproductive condition (Table 1). These animals had mean ovarian egg sizes of 2.27–2.64 mm and sperm in the spermathecae. Bishop ('41) found egg diameters of 2.5–3 mm in oviposited clutches in a related species, Eurycea bislineata. Specimens caring for oviposited eggs were spent, but still had sperm in their spermathecae. Specimens collected in August and September were found to be sexually inactive. These specimens had ovarian follicles smaller than those in sexually active animals, and with one exception, lacked sperm in their spermathecae (Table 1). The one exception, described below, contained a few sperm that presumably remained from mating in the previous spring.

The production of secretory product in the spermathecae coincides with an increase in ovarian follicular size and sexual activity. Individuals collected and sacrificed in August have inactive spermathecae (Fig. 1). Their lumina are empty, and epithelial cells have an apical border comprised of short microvilli. The columnar nuclei are large relative to the amount of cytoplasm present and nearly fill the cell. Heterochromatin occurs around the nuclear borders and multiple dense nucleoli are prominent (Fig. 1A). Intercellular canaliculi are wide and interdigitating. However, spot desmosomes are frequent, and the intercellular canaliculi narrow to tight junctions at the luminal border (Fig. 1B).

Elongate mitochondria are scattered throughout the cytoplasm (Fig. 1A). Electron-dense bodies that appear to be membrane-bound are common in the apical cytoplasm (Fig. 1B). Endoplasmic reticulum, Golgi bodies, and secretory vacuoles are not apparent. Basally, epithelial cells are in contact by spot desmosomes to a layer of elongate myoepithelial cells (Fig. 1A,C). A thick layer of collagen fibers occurs superficial to the myoepithelial layer (Fig. 1A,C), and melanocytes are abundant in the stromal tissue surrounding the spermathecae (Fig. 1A).

In animals collected on 30 September and sacrificed on 6 October, the spermathecae appear to be approaching a secretory condition, although no condensing vacuoles occur (Fig. 2). Compared with inactive glands, the cytoplasm is denser, microvilli are more elongate, and nuclei are round, basal, and relatively smaller in relation to the amount of cytoplasm (Fig. 2A). Nucleoli seem less dense, although peripheral heterochromatin is still prominent in the nuclei. Tight junctions again exist at the luminal borders (Fig. 2A,B), but intercellular canaliculi are uniformly narrow and labyrinthine (Fig. 2C). Golgi complexes, especially around the apical border of nuclei, rough endoplasmic reticulum (RER), and polyribosomes are now present (Fig. 2C). Mitochondria lack crystalline material and their cristae are variable in shape.

Sperm are present in only one individual of this group. Some sperm are free in the lumen, and others are surrounded by microvilli or appear enclosed by endocytic vacuoles in the apical cytoplasm (Fig. 2B). Except for the presence of sperm, the spermathecae closely resemble those found in other animals of this group. As this individual was not in reproductive condition, the sperm probably represent remnants from mating in a previous breeding season (at least the previous spring).

In an animal collected on 30 September, but not sacrificed until 13 December, the spermathecae possess numerous secretory granules (Fig. 3). Some fine granular material is present in the lumen, but most of the product is contained in electron-dense secretory granules in the periluminal cytoplasm (Fig. 3A). Nuclei are basal and possess more irregular borders and relatively less heterochromatin than in earlier stages. RER is abundant in the supranuclear area adjacent to the secretory granules (Fig. 3B).
In animals sacrificed after mating, but before oviposition, secretory material bathes sperm in the lumen (Fig. 4A,B). Because of the very irregular and indented apical surfaces of the cells, the boundary between the apical cytoplasm and the lumen is difficult to discern in ultrathin sections (Fig. 4A). The nuclei of the epithelial cells are located basally; their central regions contain a predominance of euchromatin; and nucleoli are not prominent (Fig. 4A). Intercellular borders are so narrow that the epithelium appears syncytial at lower magnifications (Fig. 4A).

Sperm are still numerous in animals sacrificed shortly after oviposition (while tending eggs). Some sperm apparently are able to survive in the lumen for extended periods, as noted in one individual sacrificed in October. Most of the sperm, however, are removed from the lumen by endocytosis followed by desquamation of the spermathecal epithelium (Figs. 5, 6).

Endocytic vacuoles occur along scattered areas of the luminal border following the merocrine release of secretory material, and groups of sperm surrounded by secretory material are contained in the vacuoles (Fig. 5A). Golgi stacks and RER are prominent in the cytoplasm adjacent to these vacuoles (Fig. 5B), but these do not produce secretory granules resembling those described previously.

Indeed, the cells containing sperm-filled vacuoles seem to be degenerating and undergoing desquamation from surrounding epithelial cells (Fig. 6). Nuclei in desquamated cells appear pyknotic, as they possess irregular borders and light globular areas in their dense heterochromatin (Fig. 6B). Dent ('70) described sperm degradation in the spermathecae of Notophthalmus viridescens and using his criteria, sperm destruction is evidenced by decreased opacity of the sperm nucleus and axoneme and the loss of mitochondria associated with the middle piece of the tail (Fig. 6).

Desquamation occurs only in certain areas, and other portions of the epithelium retain a normal appearance and maintain secretory activity. Wherever desquamation occurs, however, the epithelial wall shows gaps and sperm move into the stromal environment of loose connective tissue (Figs. 6A, 7). Components of the immune system are important in the degradation of sperm and desquamated epithelial cells in areas in which channels appear in the spermathecal walls. Polymorphonuclear leukocytes (presumed neutrophils) and macrophages with degenerating sperm in their cytoplasm occur adjacent to masses of sperm that have moved into the stroma (Fig. 7).

**DISCUSSION**

Results of the present study indicate that sperm embedded in spermathecal epithelium in Eurycea cirrigera are undergoing degradation rather than receiving nourishment. Benson ('68) found that the acrosomes of sperm are in contact with the spermathecal epithelium of Notophthalmus viridescens and suggested that the epithelial cells might have a nutritive function similar to that of the Sertoli cells. A similar suggestion was made by Marynick ('71) for Desmognathus auricula-tus, in which sperm heads are surrounded by epithelial cells. Both Benson's and Marynick's studies involved the use of light microscopy, with which cellular junctions cannot be discerned due to insufficient resolution. Thus, sperm surrounded by secretory material might appear to be embedded in the apical portions of the cells.

However, Boisseau and Joly ('75) provided ultrastructural evidence for nourishment of sperm embedded in the spermathecae of Salmandra salamandra. Sperm were deeply embedded in the epithelium, penetrating even to the myoepithelial layer. The "plasma membranes" of the epithelial cells invaginate and form "mantles" around the sperm, nearly all of which seem undamaged. Boisseau and Joly ('75) suggested that the epithelium provides nutrition for the embedded sperm, but did not provide cytological evidence for such a process.

The findings of the present study on embedded sperm in the epithelium agree more with those obtained in other ultrastructural studies. Dent ('70) examined the spermathecae of Notophthalmus viridescens and found that sperm are sometimes in close apposition to the epithelium. Nearly all the sperm deeply embedded in cytoplasm are degenerating,
Figure 2
however, and the epithelial cells involved appear desquamated and within the proximal tubular lumina. Dent ('70) concluded that other contacts between sperm and epithelium usually are casual, and “there is no direct interchange of materials between the sperm and the epithelium.” Rather, Dent ('70) believed that molecular exchanges between spermathecal secretions and sperm occur “by diffusion through the luminal fluid.”

Pool and Hoage ('73) described a complex process for production of secretory material in the spermathecae of Eurycea quadridigitata. Initially, a fusion of small, “single membrane vessels” occurs with mitochondria that have an inner crystalline matrix (presumably a protein), and these complexes become associated with extensive stacks of Golgi complexes in the perinuclear area. Then, on the immature face of the Golgi apparatus, a precursor derived from the mitochondria and transported by the small vessels becomes associated with other vesicles derived from smooth endoplasmic reticulum. From the mature face, a granular secretory product arises that fuses into large deposits that accumulate in the apical cytoplasm. This product is released into the lumen by a merocrine mechanism. No specializations of the luminal border occur, so the secretory products are simply released to diffuse and bathe the stored sperm, which are not in orderly arrays. In one cell, Pool and Hoage ('73) found the remnants of a sperm that was assumed to be undergoing dissolution due to the “complete encompassing of the cytoplasmic membrane.” Mitochondria with an inner crystalline matrix were not noted in the present study.

Davitt and Larsen ('88) conducted an SEM study on the luminal surface of spermathecal tubules in female Plethodon larselli collected just prior to oviposition. Some stored sperm are in contact with the epithelium, often adjacent to secretory granules and apocrine blebs, and apical extensions of cells occur around some individual sperm, although it remained unresolved whether or not contact represents a casual or a fundamental relationship. Four days after injection with pregnant mare serum gonadotropin, apocrine blebs and secretory vesicles (produced from the blebs) occur in the lumina of distal ends of tubules. The walls of vesicles apparently degenerate, allowing release of the contents. In the proximal portions of the tubules, in which sperm are most abundant, the cells have fewer blebs, and lumina contain numerous spherical vesicles and an amorphous material.

Brizzi et al. ('89) studied the TEM ultrastructure of spermathecae from Salamandra terdigitata, and their findings most resemble those of the present study. Interdigitated canaliculi lie between the “prismatic-shaped” epithelial cells, and an intense apical secretion of small, electron-dense granules forms on the mature side of periluminal Golgi stacks. In the lumen, secretory material appears as structureless dense masses containing bundles of sperm. Brizzi et al. ('89) found that secretory activity is reduced after oviposition, and the tubular lumina they contain degenerating sperm mixed with deteriorated cells and traces of secretory material.

The nature of the secretion into the lumen and its function need further research. On samples examined by light microscopy, Sever ('87) found that the spermathecal epithelium of Eurycea cirrigera is stained by the periodic acid-Schiff (PAS) reaction for neutral carbohydrates, and does not react with osmic acid for lipids or the ninhydrin-Schiff reaction for proteins. The affinity of the spermathecal epithelium for carbohydrate stains has been noted in other salamanders. Sever et al. ('90) found that female bolitoglossines vary in their reaction with PAS, which reveals neutral carbohydrates, or alcian blue at pH 2.5, which reveals acidic mucosubstances. Lemaitre-Lutz ('68) found in Pleurodeles waltl that a granular secretion stained by alcian blue predominates; after mating a homogeneous secretion stained by the PAS reaction becomes abundant. Boisseau and Joly ('75), also noting variation with PAS and with alcian blue at low pH, concluded that although some heterogeneity exists, the secretion is composed of neutral or acidic polysaccharides. In Eurycea quadridigitata storing sperm, however, Pool and Hoage ('73) obtained no staining with the PAS reaction and inconclusive results with alcian blue and ruthenium red.

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Fig. 2. Eurycea cirrigera Spermatheca of a 49.6 mm specimen collected 30 September and sacrificed 6 October. Note initiation of secretory activity. Spermatозoa present in this individual probably were introduced in the previous spring. A: Overview of cytological features. B: Relation of spermatозoa to spermathecal epithelium. C: Organelles involved in secretory activity. Scale bar in lower right corner = approximately 2.5 μm for A, 1.5 μm for B, and 380 nm for C. Labels same as for Figure 1, plus: Go, Golgi apparatus; Po, polyribosomes; Sm, middle piece of the tail of sperm; Sv, portions of sperm in endocytic vacuoles.
Fig. 3. *Eurycea cirrigera*. Spermatheca of a 44.6 mm specimen collected 30 September and sacrificed 13 December. Note accumulation of secretory granules. **A**: Overview of cytological features. **B**: Detail of apical cytoplasm. Scale bar in lower right corner = approximately 3.7 μm for A and 570 nm for B. Labels same as for Figure 1, plus: Rer, rough endoplasmic reticulum; Sg, secretory granules.
Fig. 4. *Eurycea cirrigera*. Spermatheca of 38.3 mm specimen collected 22 April and preserved 25 April, after mating but before oviposition. Note sperm storage. **A**: Overview of cytological features. **B**: Detail of apical region. Unlabelled arrows indicate intercellular junctions. Scale bar in lower right corner = approximately 2.2 μm for A and 1.0 μm for B. Labels same as for Figure 1, plus: Nu, nucleus of an epithelial cell; Si, spermatozoon in the lumen.
Fig. 5. *Eurycea cirrigera*. Spermatheca of a 37.9 mm specimen collected 22 April after oviposition (while tending eggs) and preserved 25 April. Note endocytosis of luminal sperm. **A**: Overview of sperm in endocytic vacuoles. **B**: Detail of the border of a vacuole, showing adjacent intercellular junctions and secretory apparatus. Scale bar in lower right corner = approximately 1.3 μm for **A** and 390 nm for **B**. Labels same as for Figures 1, 2, and 3. Unlabelled arrows indicate intercellular borders.
Fig. 6. *Eurycea cirrigera*. Spermatheca of individual in Figure 5, showing desquamated epithelium. **A**: Overview of cytological features in an area where desquamation has resulted in a gap in the spermathecal wall. **B**: Detail of a desquamated epithelial cell. Scale bar in lower right corner = approximately 2.2 μm for **A** and 1.0 μm for **B**. Labels same as for Figures 1, 2, 3, and 4, plus: Ds, desquamated epithelium; Gp, gap in spermathecal epithelium due to desquamation of labelled epithelial cells. Unlabelled arrows indicate degenerating sperm outside of the lumen either in desquamated epithelium or free in the connective tissue stroma.
Fig. 7. *Eurycea cirrigera*. Spermatheca of individual in Figure 5, showing components of the loose connective tissue in an area where desquamation of spermathecal epithelium has resulted in contact between luminal sperm and the stromal environment. **A**: Overview of cytological features. **B**: Detail of a polymorphonuclear leukocyte and a macrophage shown in A. Scale bar in lower right corner = approximately 6.3 μm for A and 1.8 μm for B. Ma, macrophage; Pm, polymorphonuclear leukocyte; Rbc, red blood cell. Unlabelled arrows indicate degenerating spermatzoa.
They still concluded that a highly carboxylated polysaccharide was present because of the occurrence of a strong staining with toluidine blue (Pool and Hoage, ’73). Dent (’70) found glycogen in the spermathecal epithelium of Notophthalmus viridescens, but its role in the secretory process was not clarified. Finally, Brizzi et al. (’89) found that secretions in the spermathecae of Salamandrina terdigitata are stained strongly with the PAS reaction, alcian blue at pH 2.5 and toluidine blue, but they are stained weakly with ninhydrin-Schiff reaction for proteins. These data suggest that the secretory product contains an acid mucopolysaccharide conjugated with protein (a “proteoglycan”).

The ultrastructural features of the epithelial cells of the spermathecae of Eurycea cirrigera suggest that the cells produce proteins or carbohydrates and proteins for export. The occurrence of extensive RER indicates peptide synthesis, but the secretory vacuoles are not as electron-dense as zymogen granules.

Whether or not this secretion actively “nourishes” stored sperm or merely holds them in a quiescent state prior to oviposition is unresolved. Hardy and Dent (’86a) found that sperm become quiescent in the spermathecae of Notophthalmus viridescens, perhaps due to high osmolality in the lumen of the tubules. Sperm found in one female Eurycea cirrigera from October were likely to be remnants from a mating some 5–6 months previously and apparently had survived through stages of summer stasis in spermathecal secretory activity. The viability of those sperm is unknown, and future experiments are planned to explore the survival of sperm through periods of sexual inactivity (summer and fall months) using long-term captives with known matings.

The role performed by the spermathecal secretory product is not completely clear. Jordan (1893), Noble and Weber (’29), and Lemaitre-Lutz (’68) believed that the secretory product attracts sperm after mating. Hardy and Dent (’86b), on the other hand, proposed that thigmotaxis is primarily responsible for the entry of sperm into the spermathecae and that if a chemical attractant exists, its range is limited. The results of the present study leave this matter unresolved. The release of the one type of secretion seems to coincide with the appearance of sperm in the lumen and thus to provide equivocal evidence for an attractant function, but this secretion persists throughout the entire period of sperm storage.

Davitt and Larsen (’88) proposed that capacitation (sensu Hamner and McLaughlin, ’74) of sperm occurs in the spermathecae as a result of secretions released by the spermathecal epithelium during oviposition, whereas Hardy and Dent (’86b) found that activation of sperm occurs after their release from the spermathecae into the cloaca. Again, the present study cannot resolve this issue, although it may be relevant that only one type of secretory product is released into the lumen, and that this substance is still present after oviposition while many sperm still persist.

This article provides the first evidence of the degradation of sperm remaining in the spermathecae after oviposition; more research is needed on this process. Many sperm remain in the spermathecae of Eurycea cirrigera during the period during which the females are tending recently oviposited eggs. Retention of sperm is at most negligible through the period of spermathecal inactivity in summer, but, as evidenced in one individual examined in the present study, some sperm may survive. Additional work is needed to obtain a better understanding of the cytological features of the spermathecae in such cases. The process of epithelial desquamation is selective, and research is necessary to explain the desquamation of some cells and their vacuolated spermatozoa and the persistence of others. Phagocytosis of sperm that pass through gaps in desquamated epithelial appears to be an immunological reaction to foreign bodies (sperm) in the stromal environment, but more work on the cues involved in this process is merited. Finally, no one has addressed cytologically the issue of sperm competition (Halliday and Verrell, ’84) or the possibility that features of sperm storage may not be the same in species of salamanders with different reproductive strategies regarding the length of time between mating and oviposition. If other salamanders are like Eurycea cirrigera, however, the “embedding” of a spermatozoon in the spermathecal epithelium assures that its fate is degradation.

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LITERATURE CITED


