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CYTOCHEMICAL STUDIES OF RNA AND BASIC NUCLEAR PROTEINS IN LYCOSID SPIDERS

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ABSTRACT. Tissues of lycosid spiders were studied for RNA distributions with the basic dye Azure B. Changes in the basic proteins associated with DNA during spermiogenesis were identified with alkaline fast green staining after DNA extraction with trichloroacetic acid and by cytochemical tests for arginine. Tissue glycoproteins of the gut diverticula and the ducts of silk glands were resistant to diastase digestion and required periodic acid hydrolysis to localize reaction products with the Schiff reagent for aldehydes. Spiders possess novel types of cells that are in need of further study and may be useful as models for developmental biology.

Keywords: Araneidae, RNA, histone proteins, protamines, spermiogenesis

In conjunction with studies of polyploidization in lycosid and araneid spiders (Rasch & Connelly 2004), a number of standard cytochemical procedures were used to identify RNA, histone and non-histone nuclear proteins in several organ systems of lycosid spiders. Aside from the recent cytological studies in several species of spiders from Taiwan (Chen 1999) and the quantitative determination of genome sizes for 115 species of spiders by image analysis densitometry (Gregory & Shorthouse 2003), there have been few general studies of arachnid cytochemistry since the early reports by Millot (1926, 1949) on the histophysiology of many different types of cells from several species of spiders and the reports on the cytochemistry of silk glands of mygalomorph spiders by Palmer et al. (1982) and Palmer (1985). The most recent reviews of the microscopic anatomy of spiders are those by Felgenhauer (1999) and the excellent descriptions of the fine structure of major organ systems by Foelix (1996). Both are comprehensive in their coverage and emphasize the sometimes unique adaptations of cells to the particular life style of these carnivorous animals. The present study is a brief description of the cytomorphology and nucleoprotein cytochemistry of several lycosid spiders. It points out that these understudied animals have many interesting and novel types of cells that may be useful for future studies of invertebrate tissue development and cell differentiation.

METHODS

Tissue imprints were obtained as touch preparations from the severed prosoma and abdomens of 1 male and 3 females of undetermined medium-sized members of the family Lycosidae collected from a small wooded lot in Johnson City TN (36.2923 latitude and −82.3773 longitude). Specimens were immobilized by chilling at 4 °C for 30 min before sacrifice. Bodies were separated at the pedicel and touched to a glass slide to obtain one or two droplets (1–3 μl) of hemolymph. Severed abdomens were routinely fixed for 3–4 hr in 3:1 methanol/acetic acid (v/v), dehydrated and embedded in PolyFin (Polysciences Inc., Warrington PA). Others were fixed for 3–4 hrs in 10% neutral formalin or in MFA (methanol/formalin/acetic acid; 85:10:5, v/v) and washed overnight in tap water before dehydrating, embedding and sectioning in the usual manner at 8 μm with an A&O Model 820 rotary microtome. Block faces varied between 3–7 mm in diameter. Serial sections were mounted on albumen-coated slides for routine staining with Harris hematoxylin and eosin, or for processing through the cytochemical procedures outlined below.

Tissue samples of testes of rainbow trout (*Onchorynchus mykiss*) and 6 month-old cat (*Felix domesticus*), for which cytochemical

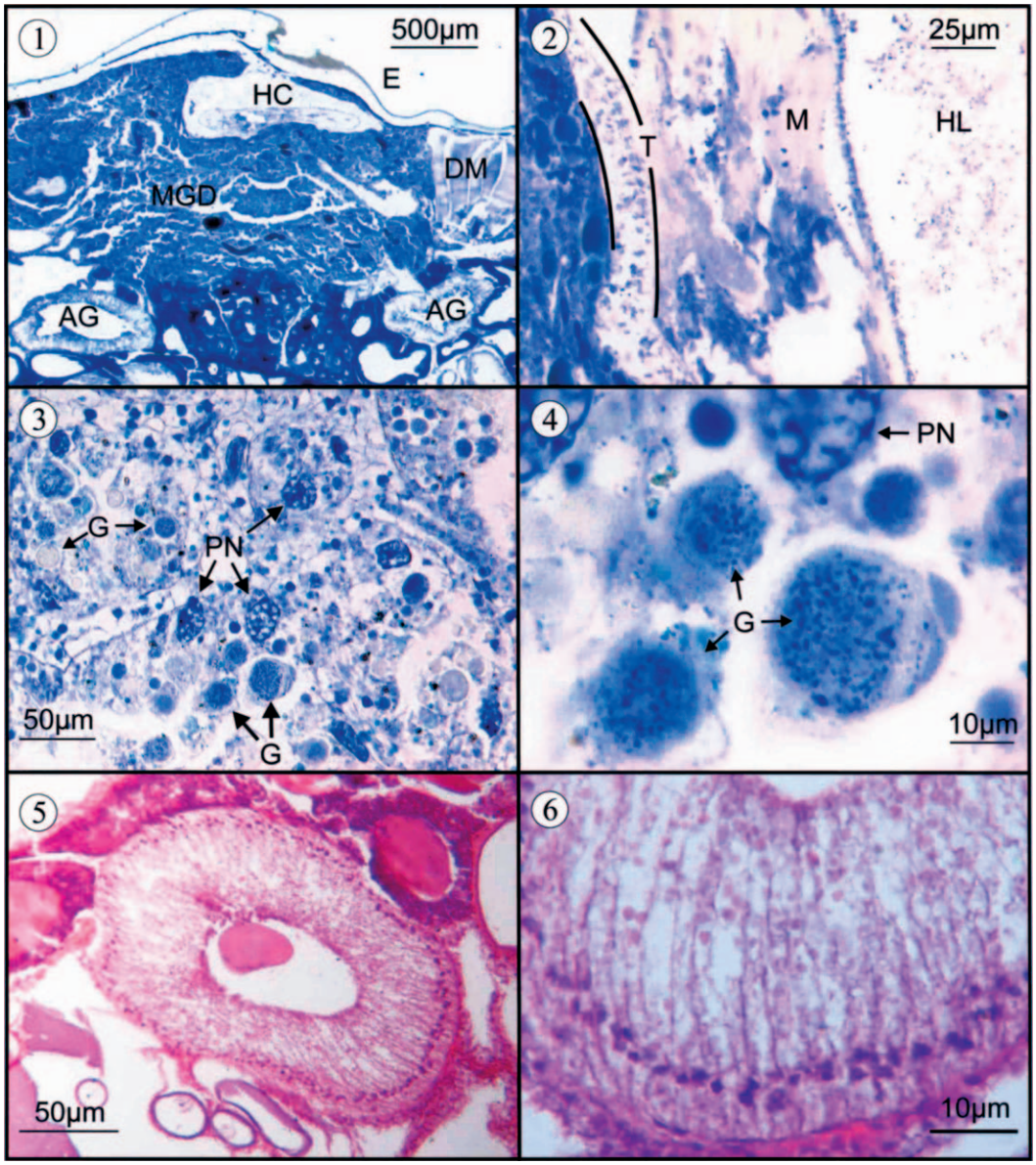
staining reactions for basic nucleoproteins are well recognized (Alfert & Gescshwind 1953; Rasch & Woodard 1959), were fixed in 10% neutral formalin and processed as above for tissue sections. Chicken blood smears (*Gallus domesticus*) were also used as a representative reference tissue for evaluating acid extraction procedures and thereby establishing an operational definition for histone proteins and protamines (Alfert 1956). All cytochemical reactions on spider tissues were applied in sequence to sections which had been part of the same paraffin ribbon in order to minimize differences due to variations in the fixation history of tissue blocks. Slides of the reference tissues were routinely processed simultaneously with each set of spider tissues.

RNA staining.—The RNA/DNA staining procedure of Flax & Himes (1952) was modified to stain sectioned spider tissues in a 0.025% solution of Azure B bromide (C.I. 52010) in 0.01 M acetate buffer at pH 4.0–4.1 for 2 hr at 37 °C. After a brief rinse in distilled water to remove excess dye, slides were dehydrated quickly through 3 rinses of tertiary butyl alcohol (TBA) for 2–3 min in each change and then allowed to stand overnight in a fresh change of absolute TBA before clearing in xylene and mounting in immersion oil or plastic resin for viewing and photography.

Basic protein staining.—All slides in each experiment were treated simultaneously through the staining procedures described by Alfert & Geschwind (1953). Briefly, deparaffinized slides were rehydrated through graded ethanols, immersed for 1 min in distilled water at 90 °C and transferred immediately into 5% trichloroacetic acid (TCA) at 90 °C for 15 min. Following 3 rinses in chilled 70% ethanol, slides were given 2 changes in distilled water and placed for 1–2 min in distilled water at pH 8.1 before being placed in a freshly prepared 0.1% solution of fast green FCF (C.I. 42053) adjusted to pH 8.1 with a minimum amount of 1 N NaOH. After staining for 60 min, slides were quickly dipped in 2 changes of distilled water at pH 8.1 and then dehydrated rapidly through 3 changes of TBA and cleared in xylene. Specificity of the staining for basic proteins was verified by carrying matching slides through a distilled water bath at 90 °C while test slides were hydrolyzed in 5% TCA and then staining all slides simultaneously in the same fast green solution at pH 8.1. Controls included slides of chicken RBC nuclei, trout RBC nuclei and slides carrying sections of testis from rainbow trout and domestic cat to assess conversion from histone proteins to protamine-like proteins during sperm maturation (Alfert 1956). Replicate slides with or without the 5% hot TCA extraction were stained to confirm DNA extraction, using the Feulgen reaction for DNA as described elsewhere (Rasch 2003).

A modified protocol of the Sakaguchi histochemical reaction for arginine (Barka & Anderson 1965) was used to assess alteration of histone proteins to arginine-rich protamines during spermiogenesis in lycosid spiders. The procedure described by Rasch & Woodard (1959), required the incubation of deparaffinized and rehydrated sections for 3 min at 23 °C in 0.5% 8-hydroxyquinoline (Eastman Kodak) in absolute ethanol, followed by development of a bright orange reaction product within 30–45 sec in a 1:1:1 mixture of 10% NaOH, commercial Chlorox® and distilled water. The latter step was carried out at 0–1 °C in an ice bath. Preparations were immediately rinsed for 3–5 sec with 2 changes of ice-cold distilled water to remove the NaOH and dehydrated rapidly through 3 changes of TBA, cleared in 3 changes of xylene and air-dried or mounted in immersion oil for photography. Slides of chicken and trout RBC nuclei and sections of trout and cat testis were stained by the same protocol to serve as reference controls.

Periodic acid Schiff reaction (PAS).—Carbohydrate and glycoprotein deposits in sections of spider abdomens were localized by hydrolysis for 10 min in 0.5% periodic acid in distilled water, followed by a 10 min wash in running tap water and 15 min of staining in freshly prepared 1% Schiff reagent. After three 5 min rinses in sulfite water, another 10 min wash in running tap water and dehydration through absolute ethanol, preparations were air-dried and mounted in oil or resin. Matching preparations were pretreated with diastase or held in distilled water during the acid hydrolysis to serve as negative controls. All slides were stained simultaneously with the Schiff reagent and counterstained with 0.5% fast green in 95% ethanol.



Figures 1–6.—Histological sections of lycosid spiders. 1. Low power view of x-section through the abdomen of a female wolf spider to show portions of the dorsal chitinous exoskeleton (E), heart chamber (HC), midgut diverticula (MGD), ampullate glands (AG) and dorsal muscles (DM). Azure B, pH 4.1, for RNA. 2. Periphery of heart chamber at higher magnification to show the spiral pattern of supports (at T and outlined in black) that prevent collapse of ligaments and the pericardial space during pressure changes. Note that this structure runs immediately adjacent to the heart chamber. Several tangential profiles of muscles (M) are associated with the heart. The acidophilic hemolymph (HL) is essentially unstained. Azure B, pH 4.1, for RNA. 3. Low power view of cells from the posterior of the midgut diverticula to show the densely stained networks of chromatin strands of the large polyploid nuclei (PN) characteristic of this tissue in lycosid and araneid spiders. Note the many small droplets and large, amorphous globules

RESULTS

Sections through the abdomens of lycosid females after staining with Azure B reveal large amounts of RNA basophilia present in cells of the midgut diverticula (MGD) that fill the space around and under the heart chamber, which is positioned just below the dorsal surface of the body (HC in Fig. 1). Tangential sections through the dorsal musculature (DM in Fig. 1) and the acidophilic hemolymph in the heart chamber (HL in Fig. 2) are only faintly stained. The thickened, spirally arranged reinforcements (T) that adjoin the heart chamber and its surrounding musculature also are slightly stained, possibly due to strongly acidic groups of their constituent glucosamine polymers, as is also evident in the thin walls that delimit air pockets in the book lungs (Fig. 9). At higher magnification, the polysomatic nuclei (PN) of the gastric diverticula are strongly basophilic, as are many of the small droplets and varying sizes of spherules shown in Fig. 3. These bodies are bluish pink in adjacent sections stained with hematoxylin and eosin and presumably are the zymogen granules released from secretory cells during food processing in this tissue. The vacuolate appearance of the cytoplasm of other cells is taken to reflect resorption by endocytosis and intracellular transport of digested materials. Identification of the interstitial cells that are so clearly delineated in electron micrographs (Foelix 1996) is uncertain here, probably because they are obscured by surrounding secretory and absorptive cells and lack appreciable basophilia. Some of the smaller droplets appear to be faintly azurophilic and have a homogeneous texture. Many of the larger spherules also appear to be basophilic, but the coloration is due in part to the small granules that coat the surfaces of these globules (Fig. 4). In adjacent unstained sections the large spheres are yellow to dark tan in color and are highly refractile when viewed by phase microscopy. The image in Fig. 4, when viewed at a higher magnification, shows part of a polysomatic nucleus (PN) and several large globules slightly under-focused to show the superficial caps of densely azurophilic granules in the optical plane above the spheres. The globules are resistant to extraction by ethanols, butanols, chloroform, ether, xylene, 5% trichloroacetic acid and 5 N HCl, which suggests that they consist of highly condensed and sequestered proteins that are related to enzymic processing and subsequent excretion of ingested food materials.

The ampullate silk glands of lycosid spiders are readily recognized by their size and characteristic morphology (Fig. 5). The tail portions of these glands show highly basophilic cytoplasm at the cell apex with nuclei restricted to the basal regions, as shown for higher power view of the sac of the ampullate gland (Fig. 6). Protein secretions in the lumen of both parts of the gland are strongly eosinophilic (Fig. 5).

The structure of the book lung (BL) in the abdomen of a lycosid spider is shown in Fig. 7, which was seen in the male, a portion of whose tubular testis lies adjacent to the connective tissue of the lung on the ventral surface of this animal. Several bundles of muscle (M) just above the exocuticle are evident from

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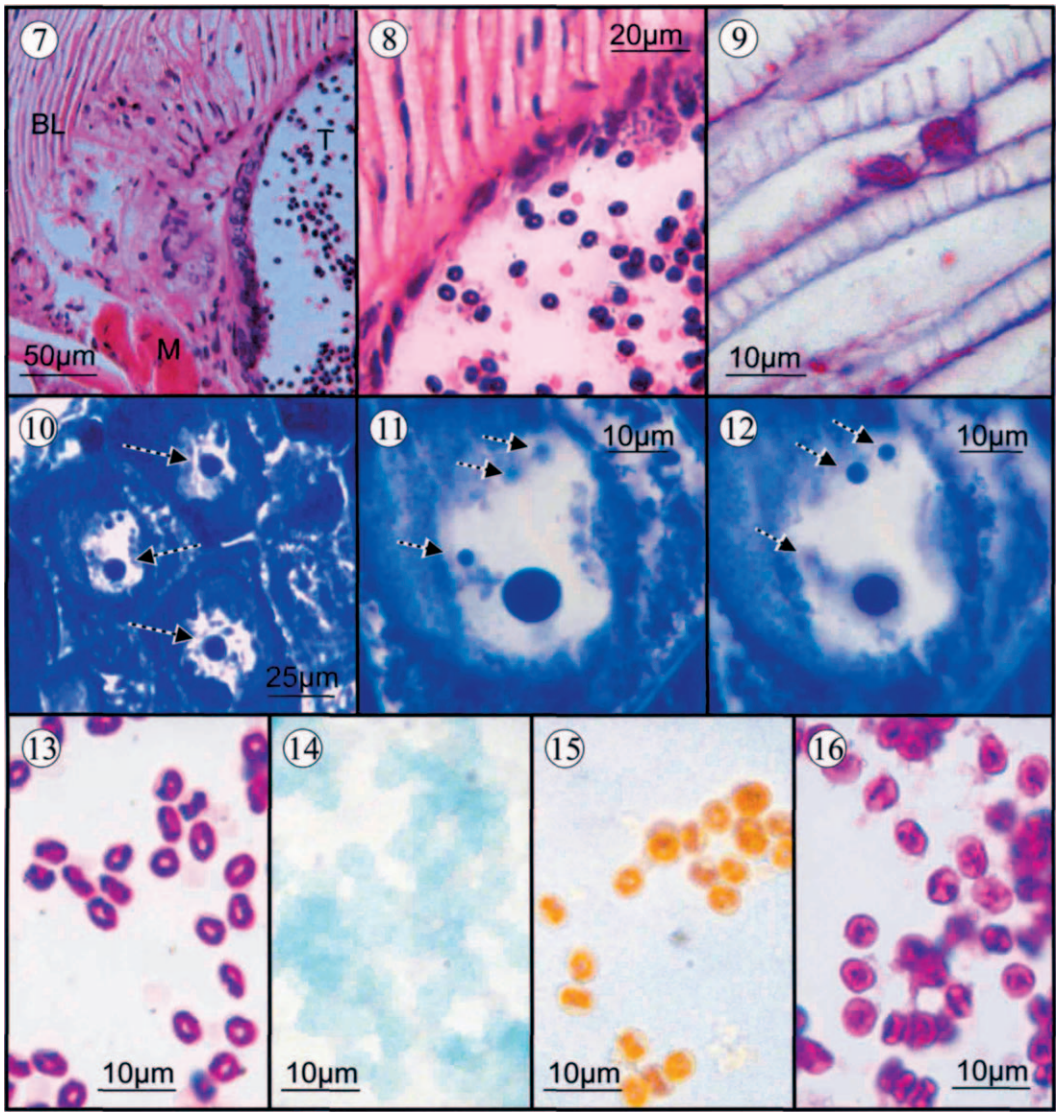
Figures 1–6. (continued)—(G) that accumulate in this tissue during food processing and absorption. Azure B, pH 4.1, for RNA. 4. Same as in Fig. 3, at higher magnification to show part of a polyploid nucleus (PN) and several large globules (G). The image is slightly under-focused to show the superficial caps of densely basophilic granules (arrows) in the plane above the globules. The granules are resistant to extraction by ethanol, butanol, chloroform, ether, xylene and 5N HCl. Azure B, pH 4.1, for RNA. 5. Cross sectional view of ampullate gland with a portion of the tail of the gland at upper right and chitin-lined ducts at lower left. Hematoxylin and eosin. 6. High power view of epithelial cells of ampullate gland to show nuclei at the base of highly elongated cells whose vacuolate cytoplasm contains only a few small eosinophilic droplets. Hematoxylin and eosin.



Figures 7–16.—Histological sections of lycosid spiders. 7. Low power view of a section through the abdomen of a mature male of lycosid spider to show the book lung (BL), an adjacent lobe of testis (T) and several bundles of muscle (M). Hematoxylin and eosin. 8. Same field as Fig. 7, at higher magnification to show differences in sizes, shapes and staining intensities of the nuclei of epithelial cells that separate organs, the elongated nuclei of hypodermal cells that line the air pockets of the book lungs and the small nuclei of hemocytes in the space between adjacent air spaces. Note the highly compacted, hollow centered sperm in the lumen of the testis at lower right. Hematoxylin and eosin. 9. High power view of book lung to show the oval nuclei of hemocytes in the hemolymph space between the air pockets of this tissue. Note the thin, chitinous pedestals or struts that maintain the patency of the air pockets. Periodic acid Schiff and Feulgen reactions with fast green counterstain. 10. Thick section of ovary from a young lycosid female. Note the prominent, highly basophilic nucleoli (arrows) and cytoplasm in these immature oocytes. Azure

Table 1.—Basic protein staining of spider tissue and controls with fast green at pH 8.1. Staining intensity is scored as 0 = no stain; + = trace; ++ = moderate; +++ = strong.

Treatment/Reaction	Chicken RBC	Trout RBC	Trout sperm	Cat sperm	Spider somatic	Spider sperm
Feulgen-DNA	+++	+++	+++	+++	+++	+++
Distilled water 90 °C, FG pH 8.1	0	0	0	0	0	0
Trichloroacetic acid 90 °C, FG pH 8.1	+++	+++	0	0	+++	0
Arginine	+++	+++	++	++	+++	++

their strong acidophilia after staining with hematoxylin and eosin. Fig. 8 is from the same section at higher magnification to show differences in sizes, shapes and staining intensities of the nuclei of the cells that delineate the separate organs and the slender elongated nuclei of hypodermal cells that line the air pockets of the book lungs. Both types of cells differ in size and basophilia from the small, oval nuclei of hemocytes that circulate through the hemolymph space between adjacent air pockets of the book lung (Fig. 9). All are easily distinguished from the densely stained, highly compacted, coiled sperm in the lumen of the adjacent testis (T, Fig. 7). The size of the small nuclei within the book lung is roughly equivalent to the small dark nuclei in the tangential section of the ventral musculature (M, Fig. 7). The delicate, supporting struts or pedestals of cuticle that maintain patent air passages in the book lung can be seen to good advantage after staining with the PAS reaction (Fig. 9).

Developing oocytes in the ovaries of all three lycosid females show highly basophilic staining of the cytoplasm and large, very prominent nucleoli with Azure B (Fig. 10). Many of the oocytes, however, also have up to three much smaller nucleolar bodies (Figs. 11 & 12), suggesting that there may be ribosomal gene transcription, possibly selective amplification, at more than one chromosomal site in these cells.

As shown in Fig. 8, the sperm of lycosid spiders often appear as small, densely stained rings or bars in paraffin sections, because their crescent-shaped nuclei are helically coiled in a head to tail fashion to form a nuclear bracelet or amulet that is thicker in its mid-region

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Figures 7–16. (continued)—B, pH 4.1, for RNA. 11–12. High power views at different focal planes of the oocyte that is marked by the middle arrow in Fig. 10 to show a single, large primary nucleolus and three smaller nucleoli in the same nucleoplasm. Azure B, pH 4.1 for RNA. 13. Mature sperm from an adult male of lycosid spider to show the distribution of DNA in the helically coiled, densely stained, hollow rings or bar profiles seen in paraffin sections of spider testis. The crescent-shaped sperm nuclei are coiled in a head to tail fashion to form a nuclear band or bracelet that is thicker in its mid region, thinner at its tips and show a hollow center when viewed face on. Feulgen reaction for DNA. 14. Loss of staining for basic nucleoproteins by mature sperm in an adjacent section of testis from the same male of lycosid spider following extraction of DNA by 5% trichloroacetic acid at 90 °C and staining for histone proteins with fast green at pH 8.1. See text for additional details. 15. Retention of arginine-rich proteins by mature sperm in an adjacent section of testis from the same male of lycosid spider following extraction of DNA by 5% trichloroacetic acid at 90 °C and staining with the Sakaguchi reaction for arginine. 16. Glycoprotein sheaths enclosing individual mature sperm from a male of lycosid spider after staining with the periodic acid Schiff reaction for polysaccharides.

and thinner at its tips. This unique configuration was described in detail by Millot (1949, fig. 456), which shows a helically coiled nucleus that accounts for the appearance of a compacted ring of DNA encircling a hollow center in face-on views of flattened sperm. It also explains the darker, but much thinner profiles seen in views from the topside (or underside) of the coiled bracelets formed by chromatin condensation during sperm maturation. The sperm shown in Fig. 13 were stained with the Feulgen reaction for DNA to illustrate aspects of the flattened coil vs. the vertical coil which is at 90° to the plane of the former. This spiral coiling of sperm nuclei in sectioned material is somewhat obscured after PAS staining, which reveals a thin glycoprotein sheath that envelopes individual sperm (Fig. 15). Reger (1970) has also described the development of a prominent acrosome and rounding up of the sperm nucleus during spermiogenesis in the spider *Pisaurina* sp.

To determine if changes in the histone proteins associated with DNA occur during sperm maturation in spiders, the alkaline fast green technique introduced by Alfert & Geschwind (1953) was applied to slides carrying sections of the same male described above. Chicken and trout blood films and sections of trout and cat testis were processed simultaneously with sections of the spider tissues. In all cases, treatment for 15 min with just with distilled water at 90 °C prior to staining in alkaline fast green showed no staining of either somatic or sperm nuclei. Pretreatment with 5% TCA at 90 °C to extract DNA, followed by staining in fast green at pH 8.1 resulted in coloration only in nuclei of the book lung and other somatic tissues adjacent to the testis, but the sperm remained unstained (Table 1, Fig. 14). These sperm ''ghosts,'' recognized by their unique morphology and hollow centers, were readily visualized in the sections by phase microscopy and were well stained in a replicate preparation of sections from the same testis using the cytochemical reaction for arginine (Table 1, Fig. 15). As expected, blood cell nuclei of chicken and trout were well stained by alkaline fast green after acid extraction of DNA, but the sperm of trout and cat also remained unstained by alkaline fast green after the hot 5% TCA treatment. Both showed substantial concentrations of arginine reaction product in companion preparations (Table 1). Each of the tissues tested for histone staining was matched by a slide stained with the Feulgen reaction for DNA to demonstrate the stability of the nuclear basic proteins of sperm to hydrolysis in 5 N HCl (Table 1). The absence of fast green staining in sperm after DNA extraction by TCA and their retention of a high concentration of arginine reaction products suggests that a conversion from a primarily histone type of basic nucleoprotein to a more arginine-rich, protamine-like protein accompanies spermiogenesis in these spiders.

Glycoprotein staining.—Periodic acid hydrolysis followed by localization of reactive aldehyde groups with the Schiff reagent results in extensive staining in sections of spider tissues fixed in MFA. In addition to staining of the coating of individual sperm (Fig. 16) accumulation of reaction product is prominent in the zymogen granules of gut diverticula, the ducts of silk glands and the walls of the air pockets in the book lung (Fig. 9). Densely stained PAS-positive droplets and globules are characteristic of the secretory tissues of gut diverticula and appear similar in size and location to the azurophilic droplets and granules described earlier. Presumably, these deposits reflect the presence of acid hydrolases secreted by digestive tissues Control slides with sections cut from the same tissue block and treated only with distilled water before staining with the Schiff reagent remain unstained. Additional studies with histochemical reactions for lysosomal enzymes and more selective digestion of substrates with other aldehyde producing reagents are needed to characterize the large concentrations of glycoproteins seen in spider tissues. Pretreatment of sections with diastase did not produce detectable differences in the intensity of Schiff staining for glycol groups when compared with control slides.

DISCUSSION

The cytochemical observations chronicled here are just the beginning of more detailed studies to explore the large variety of cell types with very specific functions and developmental histories available among arachnids and other arthropods, such as centipedes, millipedes, mites or ticks. These studies complement our cytophotometric analysis of endonuclear DNA replication in lycosid spiders (Rasch & Connelly 2004).

The wide diversity of types of cells and staining properties of spider tissues is particularly well demonstrated by the intense basophilia with Azure B due to high concentrations of RNA in the large secretory cells of the mid gut epithelium (Figs. 3–4) and in the nucleoli and cytoplasm of immature oocytes (Figs. 10–12). The latter finding confirms an early study by Edström (1960) based on his analysis of ultraviolet absorption curves to assess levels of RNA in the nucleoli and cytoplasm of oocytes of the common house spider *Tegenaria domestica* (Clerck 1757). The synthesis and accumulation of ribosomal RNA in both organelles is to be expected for a tissue about to embark upon a course of extensive yolk deposition prior to egg maturation. Glycoprotein staining after using the PAS reaction clearly detailed many delicate deposits of chitin lining chambers of the book lung (Figs. 7–9). Our study of the histone proteins of mature sperm of lycosid spiders has demonstrated that their sperm undergo conversion to an arginine-rich, protamine-like protein during spermiogensis (Figs. 13–15), similar to the pattern of changes found for the sperm of trout and several other animal species (Alfert 1956). We also found that there is a thin glycoprotein sheath enclosing individual sperm (Fig. 16). The special fixation protocols and tissue maceration techniques used by Chen (1999) in his elegant analysis of chromosomes for 6 species of spiders from Taiwan were not employed in the present study and therefore preclude useful comparisons with his findings. No mitotic figures were observed in any of the tissues examined here.

As shown by previous cytochemical studies of silk glands in *Antrodiaetus* which is very distantly related to lycosids (Palmer et al. 1982) and *Euagrus* (Palmer 1985), cells of these organs are morphologically and functionally diverse. These and many other species of spiders with special cellular adaptations in poison glands, silk producing glands and other tissues that produce a variety of proteins (Palmer et al. 1982; Palmer 1985; Foelix 1996; Felgenhauer 1999; Vollrath & Knight 2001) provide a broad landscape for future research of potential cell models from a group of understudied arthropods with a long history on earth.

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