UNIVERSIDADE ESTADUAL DE CAMPINAS

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Estrutura, ultra-estrutura e citoquímica da espermatogênese, dos ductos e ovidutos do lagarto *Tropidurus itambere* durante o ciclo reprodutivo

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural na área de Histologia.

Orientação: Profa. Dra. Mary Anne Heidi Dolder

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Dedico esta tese

Aos meus pais

Que um dia me disseram...

"Se os teus sonhos estão nas nuvens,

Não se preocupe, pois eles estão no lugar certo...

Mas comece agora mesmo a construir os degraus..."

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Deus... obrigada por colocar tantos anjos em minha vida.

Índice

Resumo

O estudo do conjunto de características estruturais, ultra-estruturais e citoquímicas das fases iniciais da espermatogênese, assim como das células presentes nos canais condutores de esperma durante um ciclo reprodutivo podem contribuir de forma significativa para o entendimento da morfologia e fisiologia da reprodução, do processo de maturação dos espermatozóides e de aspectos comportamentais e ecológicos de populações de lagartos. Com o objetivo de contribuir para essas diferentes áreas do conhecimento, o presente trabalho traz novos dados do lagarto *Tropidurus itambere,* sobre o ciclo reprodutivo testicular e epididimário, a espermatogênese, diferenciação das espermátides e dos espermatozóides analisados com o uso de técnicas de citoquímica estrutual e ultra-estrutural e com o uso de imunocitoquímica ultra-estrutural. Além disso, espermatozóides testiculares e espermatozóides presentes no receptáculo seminal de fêmeas foram observados com a utilização de lectinas, para localização de açúcares específicos. Também são caracterizados os aspectos morfológicos e histoquímicos (localização de glicoproteínas, polissacarídeos, glicosaminoglicanos ácidos) dos canais condutores de esperma em machos e fêmeas. Esses dados foram obtidos para animais durante o ciclo reprodutivo anual, capturados num fragmento de Mata Atlântica do estado de São Paulo. O ciclo reprodutivo é anual, com breve período de descontinuidade, dados muito semelhantes aos estabelecidos em outros trabalhos prévios de ecologia. A espermatogênese é mostrada pela primeira vez em lagartos desde suas fases iniciais da diferenciação celular. Algumas variações puderam ser notadas quanto à ultra-estrutura do espermatozóide deste lagarto Tropidurídeo e outros das famílias Iguanidae e Agamidae, reforçando a revisão sistemática proposta recentemente. O oviduto é constituído por três regiões distintas, das quais a mais distal é responsável pela estocagem dos espermatozóides por um curto período do ciclo anual. Foram detectados em eventos da espermatogênese, durante o ciclo anual proteínas básicas (principalmente no cone subacrosomal, centríolos da peça intermediária, corpos densos, proteínas periféricas do axonema e bainha fibrosa do flagelo), polissacarídeos (célula de

Sertoli e sua organização durante os picos do ciclo reprodutivo, e na formação do complexo acrosomal), glicogênio (formação do complexo acrosomal), fosfatase ácida (em regiões claras do complexo acrosomal e em volta de espermatozóides em degeneração no período quiescente) e lipídeos (na vesícula pró-acrosomal e em vesículas espalhadas no citoplasma da célula de Leydig), açucares específicos (durante a formação do complexo acrosomal em espermátides e em quase todas as estruturas do espermatozóide). Taís dados inexistiam para o grupo de Lacertílios e guardam algumas semelhanças, principalmente quanto à formação do acrosomo, com alguns anuros e aves. Foi detectada através de imunocitoquímica a presença de actina no cone subacrosomal e perforatório do complexo acrosomal, o que confirma a homologia destas organelas quanto à estrutura e função no espermatozóide de lagartos.

Abstract

The study of the structural, ultrastructural and cytochemical characteristics of the initial phases of spermatogenesis was undertaken, also including the cells present in the sperm throughout the reproductive cycle. This data brings significant contributions toward a better understanding of the morphology and physiology of reproduction, of the maturation process of the spermatozoa and the behavior and ecology of lizard populations. With this objective, this study brings new data on the lizard, *Tropidurus itambere*, its testicular and epididymal characteristics during the reproductive cycle, its spermatogenesis showing the differentiation of spermatid and spermatozoon structure and ultrastructure, as analyzed by cytochemical techniques and with ultrastructural immunocytochemistry. A comparison of testicular spermatozoa and those found in the female's seminal receptical was made using lectins to locate specific sugars. Morphological and histochemical methods located glycoproteins, polisaccharides, acid glycosaminoglycans in the sperm ducts of males and females. This data was studied throughout the reproductive cycle of animals obtained from a fragment of the Atlantic forest in the state of São Paulo. The cycle is annual with a brief descontinuous period, as previously very similar of the established by ecological studies. Spermatogenesis is shown for the first time in a lizard, including the first stages of cell differentiation. Some variations were observed for the spermatozoon of this Tropiduridae lizard in relation to the Iguanidae and Agamidae, which reinforces recent revision of their systematic positions. The oviduct is divided into three portions, the most distal one being responsible for spermatozoon storage during a short period in the annual cycle. Basic proteins were detected in specific structures during the annual cycle (especially the subacrosomic cone, centrioles in the midpiece, dense bodies, peripheral proteins of the axoneme and fibrous sheath of the flagellum). Polisaccharides were found in the Sertoli cell, showing peak organization of this cell during the reproductive period and its disorganization in quiescence, as well as occurring during acrosomal complex formation, while glycogen was identified only in the acrosomal complex.. Acid phosphatase was

verified in the clear regions of the acrosomal complex and surrounding the degenerating sperm cells of the quiescent period. Lipids are plentiful in the Leydig cells of this period and in the pro acrosomic vesicle. Specific sugars were verified during the acrosomic complex formation in spermatids and mark almost all structures in the spermatozoon. This data is new among lizards and can be compared with anurans and birds, especially for acrosome formation. Actin was detected with an immunocytochemical method in the subacrosomic cone and the perforatorium of the acrosomic complex, thus confirming their homology as to structure and function.

3. Introdução

Nos últimos anos, em conseqüência dos estudos de Tinkle *et al.* (1970), foi grande o estímulo para o estudo dos padrões reprodutivos dos répteis e como resultado tem se acumulado um grande corpo de dados. Contudo, grande parte do que é conhecido atualmente sobre a reprodução em lagartos refere-se a espécies de zonas temperadas, havendo muito pouca informação sobre as formas tropicais (Sherbrooke, 1975). A insuficiência de estudos, especialmente sobre as espécies de ambientes tropicais sazonais, torna ainda difícil estabelecer um padrão para as espécies destes ambientes (Magnusson, 1987). Além disso, a maior parte dos dados que Tinkle *et al.* (1970) consideravam fundamentais para se representar às estratégias reprodutivas dos lagartos, não estão disponíveis em grande parte dos estudos, inclusive naqueles realizados nas zonas temperadas (Vitt, 1977). A fauna de lagartos no Brasil é uma das mais ricas do planeta (Rocha, 1994) e diversos estudos foram feitos abordando aspectos dos mais variados, tais como: ecologia de populações, padrões de atividade, comportamentos defensivos e parasitismo (Rocha, 1994). Apesar disso os dados pertinentes à morfologia e bioquímica da reprodução são extremamente escassos e os existentes são muito específicos, e diretamente voltados ao entendimento da sistemática filogenética.

3.1. Caracterização da espécie em estudo

Foi escolhido como modelo para este estudo o lagarto *Tropidurus itambere* (figura 1). Esta espécie possui ampla distribuição geográfica no Brasil e é geralmente encontrada em locais onde há afloramentos rochosos, ocorrendo em campos rupestres, cerrados e demais formações abertas na região do domínio florestal Atlântico nos estados de São Paulo e Minas Gerais. Possuem coloração marrom escuro com várias manchas negras e brancas no dorso (Rodrigues, 1987). Há dimorfismo sexual no tamanho corporal, sendo os machos maiores que as fêmeas. Embora a coloração seja bastante semelhante entre os sexos, os machos apresentam manchas escuras no ventre, na face ventral das patas

posteriores e na aba anal (Van Sluys, 1993). É um lagarto diurno, ativo o ano todo, mas que possui uma variação sazonal no seu padrão de atividade (Van Sluys, 1992). São onívoros e se alimentam predominantemente de insetos (formigas) e aranhas (Van Sluys, 1993). A dieta varia sazonalmente, em função da variação na disponibilidade de alimento no ambiente (Van Sluys, 1995). Variações na densidade populacional ao longo do ano refletem o padrão sazonal da reprodução de *T. itambere*. São ovíparos e a época reprodutiva coincide com a estação chuvosa e a eclosão dos jovens ocorre entre janeiro e abril (Van Sluys, 1993).

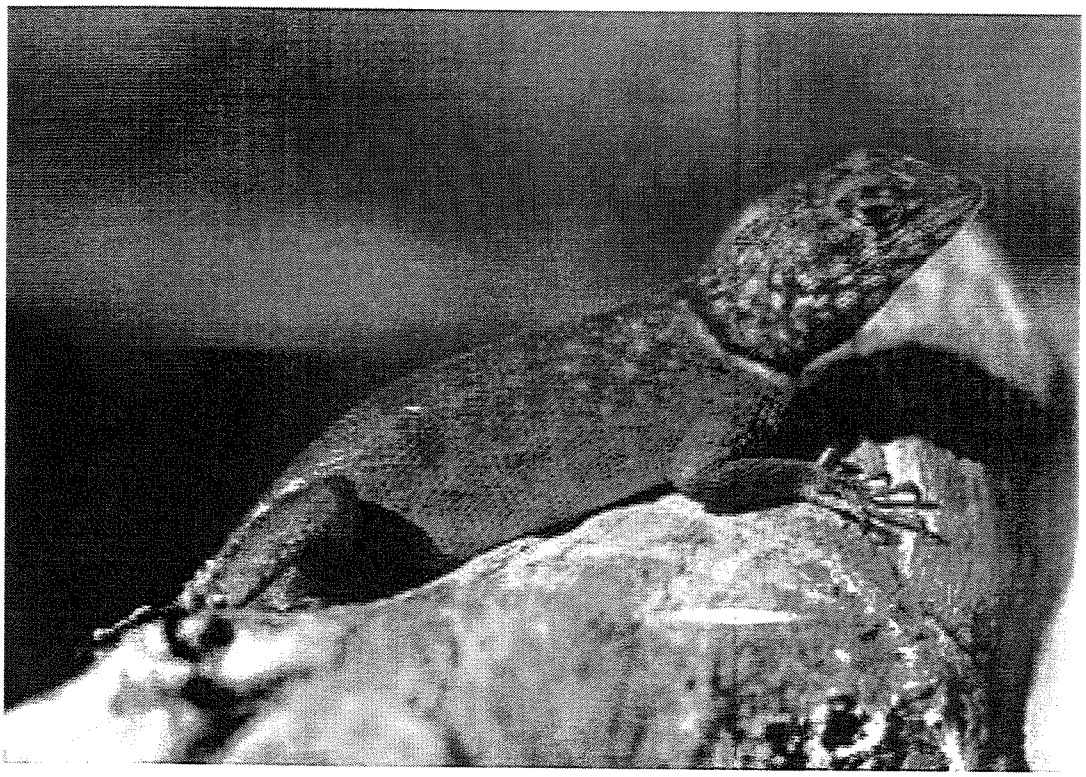


Figura 1: Macho adulto de *Tropidurus itambere*. Observar manchas claras na região do pescoço e cabeça, características do período reprodutivo.

3.2. Ciclo reprodutivo em lagartos e sazonalidade

Entre os lagartos tropiciais, três tipos de ciclos reprodutivos são conhecidos: contínuo (Wilhoft, 1963; Inger & Greenberg, 1966), contínuo com variação na atividade reprodutiva (Licht & Gorman, 1970; Sexton *et al.*, 1971; Ruibal *et al.*, 1972), e descontínuo (Licht & Gorman, 1970; Marion & Sexton, 1971). A sazonalidade na reprodução é comum

entre lagartos da zona temperada (Fitch, 1980). A temperatura e o fotoperíodo são fatores determinantes para a atividade reprodutiva dos lagartos em zonas temperadas, onde a amplitude térmica anual e a grande variação no fotoperíodo podem determinar um período de hibernação para muitas espécies (Bartholomeu, 1953; Mayhew, 1964; Licht, 1973). Já nos trópicos, em que a temperatura e o fotoperíodo sofrem menor variação e onde a seca é comparável ao frio prolongado das zonas temperadas (Tinkle, 1969; Pianka, 1970), a precipitação tem sido demonstrada como principal fator influenciando o ciclo reprodutivo de várias espécies de lagartos (Licht & Gorman, 1970; Sexton *et al.*, 1971; Ruibal *et al.*, 1972). A importância das chuvas como fator regulador do ciclo reprodutivo, se expressa tanto pela maior umidade, fundamental para impedir a dessecação dos ovos, como pela maior produtividade animal e vegetal, garantindo a disponibilidade de recursos alimentares (Vallejo & Vallejo, 1981). A maior produtividade vegetal durante as chuvas deve ainda ser de grande importância na produção das ninhadas (Ferreira *et al.*, 2002).

3.3. Anatomia dos aparelhos reprodutores de T. itambere

Os aparelhos reprodutores de machos e fêmas de *T. itambere* estão localizados dorso-longitudinalmente na cavidade abdominal. Nos machos (figura 2) é constituído por um par de testículos ovalados, conectados interna e lateralmente ao epidídimo, seguido pelo canal deferente em íntimo contato com os rins. O canal deferente é inserido em uma região que leva aos órgãos copulatórios, os hemipênis, que são pares e inseridos na base da cauda (Ferreira *et al.*, 2002). Nas fêmeas (figura 3) é constituído por um ovário, e ovidutos, os quais, nas fêmeas jovens são pares idênticos, e nas adultas apresentam uma regreção do oviduto esquerdo que se torna vestigial e inativo, enquanto o oviduto direito é dividido em três regiões, o infundíbulo apical, o útero medial e o receptáculo seminal (ou vagina) na região próxima à abertura da cloaca. A morfologia dos ductos e ovidutos, em especial as regiões em que ocorre a estocagem de espermatozóides, é bastante preservada nas diferentes espécies de lagartos vivíparos e ovíparos (Cuellar, 1966; Conner & Crews, 1980; Adams & Cooper, 1988; Kumari *et al.*, 1990; Srinivas *et al.*, 1995; Girling *et al.*, 1997; Blackburn, 1998; Sever & Hamllet, 2002). No entanto, são encontradas diferenças na

constituição das glândulas, da quantidade de cílios, na altura de epitélio e na distribuição dos túbulos de estocagem de espermatozóides em regiões específicas.

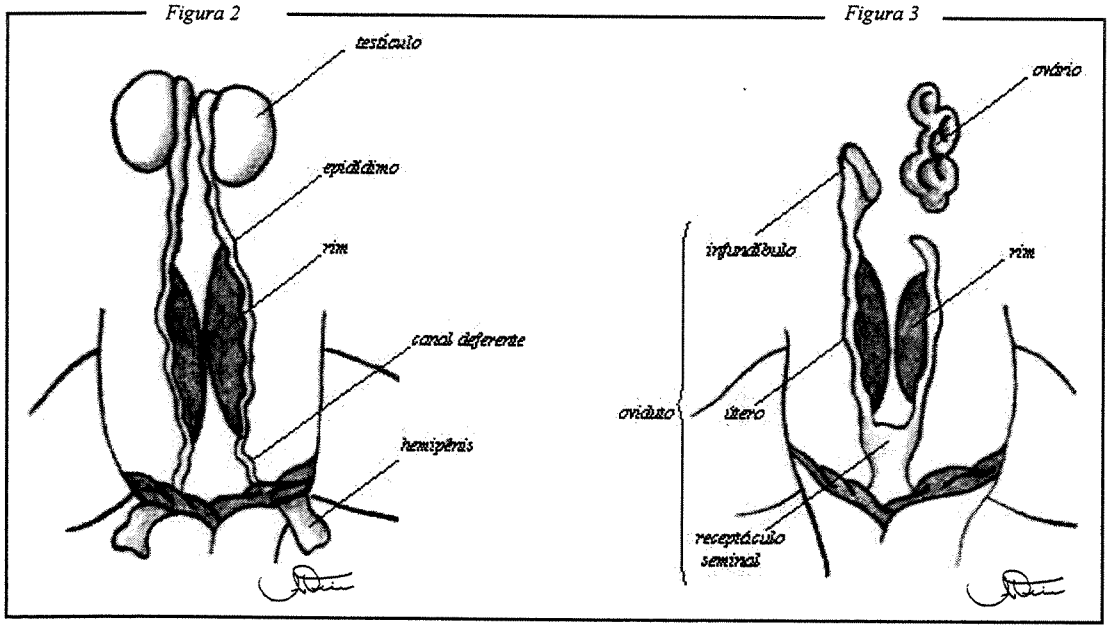


Figura 2: Desenho esquemático do aparelho reprodutor masculino de *T. itambere.*

Figura 3: Desenho esquemático do aparelho reprodutor feminino de *T. itambere.*

3.4. Espermatogênese em lagartos

Apesar de estarem sendo desenvolvidos vários trabalhos sobre o processo reprodutivo em lagartos (Van Sluys, 1993; Amat *et al.*, 2000; Ramirez-Batista *et al.*, 2002; Wiederhecker *et al.*, 2002), poucos exploraram as características histológicas ou ultra-estruturais das gônadas (Amey & Whittier, 2000; Vieira *et al.*, 2001; Ferreira *et al.*, 2002). Os estudos em espermiogênese de lagartos do gênero *Tropidurus* limitam-se a uma descrição feita para *Tropidurus torquatus* (Cruz-Landim & Cruz-Höfling, 1977; Cruz-Höfling & Cruz-Landim, 1978; Vieira *et al.*, 2001). O espermatozóide foi descrito apenas para *Tropidurus semitaeniatus* e *Tropidurus torquatus* (Teixeira *et al.*, 1999). A espermiogênese envolve os processos de alongamento nuclear, condensação da cromatina, formação do complexo acrosomal, eliminação de citoplasma residual, desenvolvimento flagelar, alongamento e formação do axonema e estruturas acessórias como corpos densos e

bainha fibrosa (Vieira *et al.*, 2001; Ferreira & Dolder, 2002). Nos poucos trabalhos existentes o que mais chama a atenção é a associação intercelular por evidentes pontes citoplasmáticas e o retículo endoplasmático bastante desenvolvido cujas cisternas formam pequenas estruturas reunidas numa forma semelhante a rosetas (Cruz-Landim & Cruz Höfling, 1977; Vieira *et al.*, 2001). Espermátides e espermatozóides são ancorados a extensos prolongamentos da célula de Sertoli. Estas características são compartilhadas entre os lagartos do grupo Iguania (Saita *et al.*, 1988; Ferreira & Dolder, 2002).

3.5. Aspectos citoquímicos ultra-estruturais da espermatogênese

O processo de espermatogênese envolve a remodelação celular e uma série de mudanças químicas para a formação de uma célula altamente especializada, o espermatozóide (Fawcett *et al.*, 1971). A detecção *in situ* dos diferentes componentes químicos da célula, pode contribuir para o entendimento de seu papel biológico *in vivo*, assim como da relação entre a função e a localização em compartimentos subcelulares (Hayat, 1993). Vários componentes químicos foram localizados em espermatozóides de vertebrados, como polissacarídeos, em especial glicoproteínas e glicogênio (Guraya, 1971; Depeiges *et al.*, 1985; Martinage *et al.*, 1996), proteínas básicas (Gordon & Bensch, 1968; Kasinsky *et al.*, 1987; Báo *et al.*, 1991), fosfatase ácida (Fernandes & Báo, 1998), resíduos de diferentes açúcares específicos (Báo *et al.*,2001; Calvo *et al.*, 2000; Sáez *et al.*, 2000; Labate & Desantis, 1995) e lipídio nas células de Leydig (Upadhyay & Guraya, 1972; Rune *et al.*, 1991), além de actina na composição de estruturas do acrosomo (Courtens *et al.*, 1991; Fouquet *et al.*, 1991; Fouquet *et al.*, 1992; Paranko *et al.*, 1994; Guerra *et al.*, 1994). Todos estes componentes químicos fazem parte de características envolvidas na capacitação espermática, tendo como resultado final o sucesso ou não da fecundação. Também nos conduzem ao entendimento de alguns mecanismos de regulação da diferenciação celular da espermatogênese, dados dessa natureza inexistem para lagartos.

3.6. Problemática e justificativa

Um grupo de dados quanto a aspectos de biologia reprodutiva pode ser reunido para a família Tropiduridae (i.e., *T. albermalensis* (Stebbins *et al.*, 1967), *T. hispidus* (Prieto *et*

al., 1976), *T. delanonis* (Werner, 1978), *T. quadrivittatus* and *T. theresioides* (Goldberg & Rodrigues, 1986), *T. itambere* (Van Sluys, 1993) and *T. torquatus* (Vieira *et al.*, 2001; Wiederhecker *et al.*, 2002). Estes dados estão direcionados geralmente para estudos de ecologia e estratégias reprodutivas. Com base neste conhecimento, tentamos evidenciar através da utilização de metodologia histológica e ultra-estrutural a organização dos componetes celulares durante um ciclo reprodutivo anual. Dados sobre a ultra-estrutura da espermiogênese de lagartos do gênero foram relatadas na literatura (Furieri, 1974; Cruz-Landim and Cruz-Höfling, 1977; Cruz-Höfling and Cruz-Landim, 1978; Vieira *et al.*, 2001) assim como da ultra-estrutura do espermatozóide. No entanto detalhes das fases iniciais da espermatogênese são pouco descritos. Nenhum dado foi encontrado na literatura quanto às características citoquímicas das células germinativas e das células de Sertoli e Leydig em lagartos. Estes dados podem contribuir para um maior conhecimento de aspectos relacionados à atividade reprodutiva dos lagartos em diferentes condições climático-sazonais.

4. Objetivos

Este trabalho teve como objetivo geral o estudo da biologia reprodutiva de *Tropidurus itambere* (Reptilia, Squamata, Tropiduridae), habitantes da região do domínio florestal Atlântico do estado de São Paulo, visando promover uma associação da morfologia com aspectos fisiológicos, comportamentais e adaptativos da espécie.

Teve como objetivos específicos:

1. Relacionar a variação estrutrual e ultra-estrutural do testículo e epidídimo durante o ciclo reprodutivo com a influência da sazonalidade;
2. Caracterizar histológica e ultra-estruturalmente a espermatogênese e o espermatozóide coletado no epidídimo;
3. Caracterizar histológica, histoquímica e ultra-estruturalmente os ovidutos das fêmeas, com ênfase no receptáculo seminal e estocagem de espermatozóides.
4. Caracterizar por histoquímica e citoquímica ultra-estrutural a espermiogênese e o espermatozóide coletado no epidídimo de machos e no receptáculo seminal de fêmeas, durante o ciclo reprodutivo.

5. Referências

Adams CS, Cooper WE (1988) Oviductal morphology and sperm storage in the keeled earless lizard, *Holbrookia pronpinqua*. **Herpetologica 44**: 190-197.

Amat F, Llorente GA, Carretero MA (2000) Reproductive cycle of the sand lizard (Lacerta agilis) in its southwestern range. **Amphibia-Reptilia 21**: 463-476.

Amey AP, Whittier JM (2000) The annual reproductive cycle and sperm storage in the bearded dragon, Pogona barbata. **Australian J. Zool. 48**: 411-419.

Báo SN, Dalton GC, Oliveira SF (1991) Spermiogenesis in *Odontophrynus cultripes* (Amphibia, Anura, Leptodactylidae): ultrastructural and cytochemical studies of proteins using E-pta. **J. Morphol. 207**: 303-314.

Báo SN, Vieira GHC, Fernandes A (2001) Spermiogenesis in *Melanophryniscus cambaraensis* (Amphibia, Anura, Bufonidae): ultrastructural and cytochemical studies of carbohydrates using lectins. **Cytobios 10**: 203-216.

Bartholomeu JrGC (1953) The modification by temperature of the photoperiodic control of gonadal development in the lizard *Xantusia vigilis*. **Copeia 1953**: 44-50.

Blackburn DG (1998) Structure, function, and evolution of the oviducts of squamate reptiles, with special reference to viviparity and placentation. **J. Exp. Zool. 282**: 560-617.

Calvo A, Pastor LM, Bonet S, Pinart E, Ventura M (2000) Characterization of the glycoconjugates of boar testis and epididymis. **J. Reprod. Fert. 120**: 325-335.

Conner J, Crews D (1980) Sperm transfer and storage in the lizard, *Anolis carolinensis*. **J. Morphol. 163**: 331-348.

Courtens, J. L., Biggiogera, M., Gabbiani, G., Fakan, S. (1991). The striated body: a new structure in the sperm head of the rabbit spermatozoon. **J. Exp. Zool. 259:** 128-137.

Cruz Höfling MA, Cruz Landim C (1978) The fine structure of nuclei during spermiogenesis in the lizard *Tropidurus torquatus* (Lacertilia). **Cytologia 43**: 61-68.

Cruz Landim C, Cruz Höfling MA (1977) Electron microscope study of lizard spermiogenesis in *Tropidurus torquatus* (Lacertilia). **Caryologia 30**: 151-162.

Cuellar O (1966) Oviducal anatomy and sperm storage structures in lizards. **J. Morphol. 119**: 7-20.

Depeiges A, Betail G, Coulet M, DufaureJP (1985) Histochemical study of epididymal secretions in the lizard, *Lacerta vivipara*: localization of lectin-binding sites. **Cell Tissue Res. 239**: 463-466.

Dufaure JP, Courty Y, Depeiges A, Mesure M, Chevalier M (1986) Evolution and testosterone content of the epididymis during the annual cycle of the lizard *Lacerta vivipara*. **Biol. Reprod. 35**: 667-675.

Fawcett DW, Anderson WA, Phillips DM (1971) Morphogenetic factors influencing the shape of the sperm head. **Develop. Biol. 26**: 220-251.

Fernandes AP, Báo SN (1998) Cytochemical localization of phosphatases in the germ and Sertoli cells of *Odontophrynus cultripes* (Amphibia, Anura, Leptodactylidae). **Biocell 22**: 93-101.

Ferreira A, Dolder H (2002) Ultrastructural spermiogenesis of *Iguana iguana* (Reptilia: Sauria: Iguanidae). **Eur. J. Mrophol.**: *(in press)*

Ferreira A, Laura IA, Dolder H (2002) Reproductive cycle of male green iguanas, *Iguana iguana* (Reptilia, Sauria, Iguanidae), in the Pantanal region of Brazil. **Braz. J. Morphol. Sci. 19**: 23-28.

Fitch HS (1980) Reproductive strategies of reptiles. *In*: Muphy JB, Collins JJ (eds) Reproductive biology and diseases of captive reptiles. **SSAR Contributions of Herpetology 1**. Society for the study of amphibians and reptiles.

Fouquet, J. P., Fraile, B., Kann, M. L. (1991). Sperm actin and calmodulin during fertilization in the hamster: an immune electron microscopic study. **Anat. Rec. 231:** 316-323.

Fouquet, J. P., Valentin, A., Kann, M. L. (1992). Perinuclear cytoskeleton of acrosome less spermatids in the blind sterile mutant mouse. **Tissue & Cell 24:** 655-665.

Girling JE, Cree A, Guillette LJ (1997) Oviductal structure in a viviparous New Zealand gecko, Hoplodactylus maculatus. **J. Morphol. 234**: 51-68

Gist DH, Congdon JD (1998) Oviductal sperm storage as a reproductive tactic of turtles. **J. Exp. Zool. 282**: 526-534.

Gist DH, Dawes SM, Turner TW, Sheldon S, Congdon JD (2001) Sperm storage in turtles: a male perspective. **J. Exp. Zool. 292**: 180-186.

Gordon M, Bensch KG (1968) Cytochemical differentitation of the guinea pig sperm flagellum with phosphotungstic acid. **J. Ultrast. Res. 24**: 33-50.

Guerra, R., Campos, B., Esponda, P. (1994). Analysis of the spermatozoa of four bivalves with particular reference to the acrosome and plasma membrane glycoproteins. **J. Submicrosc. Cytol. Pathol. 26:** 489-495.

Guraya SS (1971) Histochemical observations on the lizard spermiogenesis. **Acta Anat. 79**: 270-279.

Halpert AP, Garstka WR, Crews D (1982) Sperm transport and storage and its relation to the annual sexual cycle of the female red-sided garter snake, *Thamnophis sirtalis parietallis*. **J. Morphol. 174**: 149-159.

Hattan LR, Gist DH (1975) Seminal receptacles in the eastern box turtle, *Terrapene carolina*. **Copeia 3**: 505-510.

Hayat MA. 1993. **Stains and cytochemical methods**. 1rd ed. Plenum Edit. New York.

Hoffman LH, Wimsatt WA (1972) Histochemical and electron microscopic observations on the sperm receptacles in the garter snake oviduct. **Am. J. Anat. 134**: 71-96.

Inger RF, Greenber B (1966) Annual Reproductive Patterns of lizards from a bornean rain forest. **Ecology 47**: 1007-1010.

Kasinsky HE, Mann M, Huang SY, Fabre L, Coyle B, Byrd EW (1987) On the diversity os sperm basic proteins in the vertebrates: V. Cytochemical and Amino acid analysis in Squamata, Testudines, and Crocodylia. **J. Exp. Zool. 243**: 137-151.

Kohane AC, González-Echeverría FMC, Piñeiro L, and Blaquier JA (1980) Interaction f proteins of epididymal origin with spermatozoa. **Biol. Reprod. 23**: 737-742.

Kumari TRS, Sarkar HBD, Shivanandappa T (1990) Histology and histochemistry of the oviductal sperm storage pockets of the agamid lizard *Calotes versicolor*. **J. Morphol. 203**: 97-106.

Labate M, Desantis S (1995) Histochemical analysis of lizard testicular glycoconjugates during the annual spermatogenetic cycle. **Europ. J. Histochem. 39**: 201-212.

Licht P, Gorman GC (1970) Reproductive and fat cycles in Caribbean *Anolis* lizards. **Univ. California Publ. Zool. 95**: 1-52.

Licht P (1973) Environmental influences on the testis cycles of the lizards *Dipsosaurus dorsales* and *Xantusia vigilis*. **Comp. Biochem. Physiol. 15**: 7-20.

Magnusson WE (1987) Reproductive cycle of teiid lizards in Amazonian Savanna. **J. Herpetol. 21**: 307-316.

Marion KR, Sexton OJ (1971) Reproductive cycle of lizard *Sceloporus malachiticus* in Costa Rica. **Copeia 3**: 517-520.

Martinage A, Depeiges A, Wouters D, Morel L, Sautiere P (1996) Spermatogenesis of the lizard *Lacerta vivipara*: Histological studies and amino acid sequence of protamine lacertine 1. **Comp. Ren. Acad. Scien. III – Scien. Vie-Life Scien. 319**: 511-516.

Mayhew WW (1964) Photoperiodic responses in three species of the lizard genus *Uma*. **Herpetologica 20**: 95-113.

Paranko, J., Yagi, A., Kuusisto, M. (1994). Immunocytochemical detection of actin and 53 kDa polypeptide in the epididymal spermatozoa of rat and mouse. **Ana. Rec. 240:** 516-527.

Pianka ER (1970) Comparative autoecology of the lizard *Cnemidophorus tigris* in different parts of its geographic range. **Ecology 51**: 703-720.

Ramirez-Batista A, Ramos-Flores O, Sites JW (2002) Reproductive cycle of the spiny lizard Sceloporus jarrovii (Sauria: Phrynosomatidae) from north-central Mexico. **J. Herpetol. 36**: 225-233.

Ravet V, Courty Y, Depeiges A, Dufaure JP (1987) Changes in epididymal protein synthesis during the sexual cycle of the lizard, *Lacerta vivipara*. **Biol. Reprod. 37**: 901-907.

Rocha CFD (1994) A ecologia de lagartos no Brasil: status e perspectivas. *In*: Nascimento LB, Bernardes AT, Cotta GA (eds). **Herpetologia no Brasil I.** Fund. Biodivers. e Fund. Ezequiel.

Rodrigues MT (1987) Sistemática, ecologia e zoogeografia dos *Tropidurus* do grupo Torquatus ao sul do rio Amazonas (Sauria: Iguanidae). **Arq. Zool. S.P. 31**: 105-230.

Ruibal R, Philibosian R, Adkins JL (1972) Reproductive cycle and growth in the lizard *Anolis acutus*. **Copeia 1972**: 509-518.

Rune GM, Souza PH, Merker HJ (1991) Ultrastructural and histochemical characterization of marmoset (*Callitrhrix jacchus*) Leydig cells during postnatal development. **Anat. Embryol. 183**: 179-191.

Sáez FJ, Madrid JF, Aparicio R, Alonso E, Hernández F (2000) Glycan residues of N- and O- linked oligosaccharides in the premeiotic spermatogenetic cells of the urodele amphibian *Pleurodeles waltl* characterized by means of lectin histochemistry. **Tissue & Cell 32**: 302-311.

Saita A, Comazzi M, Perrota E (1988) New data at the E. M. on the spermiogenesis of *Iguana delicatissima* (Laurent) involving comparative significance. **Acta Embryol. Morphol. Exper. 9**: 105-114.

Sever DM, Hamlett WC (2002) Female sperm storage in reptiles. **J. Exp. Zool. 292**: 187-199.

Sever DM, Ryan TJ (1999) Ultrastructure of the reproductive system of the black swamp snake (*Seminatrix pygaea*): Part I. Evidence for oviducal sperm storage. **J. Morphol. 241**: 1-18.

Sever DM, Ryan TJ, Morris T, Patton D, Swafford S (2000) Ultrastructure of the reproductive system of the black swamp snake (*Seminatrix pygaea*). II. Anual oviductal cycle. **J. Morphol. 245**: 146-160.

Sexton OJ, Ortleb EP, Hathaway LM, Ballinger RE, Licht P (1971) Reproductive cycles of three species of anoline lizards from the Isthmus of Panama. **Ecology 52**: 201-215.

Sherbrooke WC (1975) Reproductive cycle of a tropical teiid lizard, *Neusticurus ecpleopus* Cope in Peru. **Biotropica 7**: 194-207.

Srinivas SR, Shivanandappa T, Hedge SN, Sarkar HBD (1995) Sperm storage in the oviduct of the tropical rock lizard, *Psammophilus dorsalis*. **J. Morphol. 224**: 293-301.

Teixeira RD, Vieira GHC, Colli GR, Báo SN (1999) Ultrastructural study of spermatozoa of the neotropical lizards, *Tropidurus semiaeniatus* and *Tropidurus torquatus* (Squamata: Tropiduridae). **Tissue & Cell 31**: 308-317.

Tinkle DW (1969) The concept of reproductive effort and its relation to the evolution of life histories of lizards. **Amer. Natur. 103**: 501-516.

Tinkle PW, Wilbur HM, Tilley SG (1970) Evolutionary strategies in lizard reproduction. **Evolution 24**: 55-74.

Upadhyay SN, Guraya SS (1972) Histochemical observations on the interstitial gland (or Leydig) cells of the lizard testis. **Gen. Comp. Endoc. 19**: 88-95.

Vallejo LR, Vallejo MS (1981) Contribuição ao estudo ecológico dos microartrópodos do "litter" na restinga de Maricá. RJ. **Rev. Bras. Biol. 41**: 539-543.

Van Sluys M (1992) Aspectos da ecologia do lagarto *Tropidurus itambere* (Tropiduridae), em uma área do sudeste do Brasil. **Rev. Bras. Biol. 52**: 181-185.

Van Sluys M (1993) The reproductive cycle of *Tropidurus itambere* (Sauria: Tropiduridae) in Southeastern Brazil. **J. Herpetol. 27**: 28-32.

Van Sluys M (1995) Dinâmica populacional, crescimento e área de vida do lagarto *Tropidurus itambere* Rodrigues, 1987 (Tropiduridae) em uma área de afloramentos rochosos no sudeste do Brasil. Unpubl. Ph.D. diss. Univ. Estadual de Campinas, São Paulo, Brasil.

Vieira GHC, Wiederhecker HC, Colli GR, Báo SN (2001) Spermiogenesis and testicular cycle of the lizard *Tropidurus torquatus* (Squamata, Tropiduridae) in the Cerrado of central Brazil. **Amphibia-Reptilia 22**: 217-233.

Vitt LJ (1977) Observations on clutch and egg size and evidence for multiple clutches in some lizards of Southwestern United States. **Herpetologica 33**: 333-338.

Wiederhecker HC, Pinto ACS, Colli GR (2002) Reproductive ecology of *Tropidurus torquatus* (Squamata: Tropiduridae) in the highly seasonal Cerrado biome of central Brazil. **J. Herpetol. 36**: 82-91.

Wilhoft DC (1963) Gonadal histology and seasonal changes in tropical Australian lizard, *Leiolopisma rhomboidalis*. **J. Morphol. 113**: 185-190.

6. Artigos

6.1. "Seasonal changes in testicular and epididymal histology of the tropical lizard, Tropidurus itambere, during its annual reproductive cycle"

Ferreira A & DolderH - aceito para publicação: Zoocriaderos

6.2. "Sperm ultrastructure and spermatogenesis in the lizard, Tropidurus itambere"

Ferreira A & Dolder H - aceito para publicação: Biocell

6.3. "Histology, histochemistry and ultrastructure of the oviducts and seminal receptacle of Tropidurus itambere (Reptilia, Tropiduridae)"

Ferreira A & Dolder H - submetido para publicacão: Annals of Anatomy

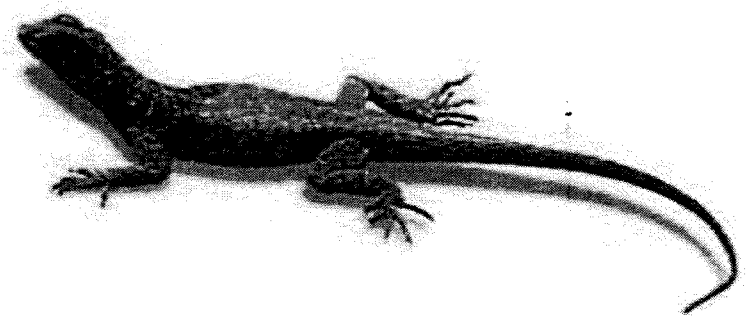
6.4. "Cytochemical study of the spermiogenesis and mature spermatozoon of the lizard, Tropidurus itambere (Reptilia, Squamata)"

Ferreira A & Dolder H - aceito para publicação: Acta Histochemica

6.5. "Ultrastructural imunocytochemistry to evidence actin in the acrosomal complex during the spermiogenesis of the lizard Tropidurus itambere (Reptilia, Squamata)"

Ferreira A, Joazeiro P & Dolder H - submetido para publicação: Caryologia

6.1. Seasonal changes in testicular and epididymal histology of the tropical lizard, *Tropidurus itambere*, during its annual reproductive cycle.



ABSTRACT. The reproductive cycle of some lizards, including some species of the genus *Tropidurus*, have been widely studied. However, few studies have shown the morphological aspects of the testis and epididymis during the annual reproductive cycle. In addition, there are no descriptions of the ultrastructural, epithelial variations in the epididymis. Using histology and transmission electron microscopy, the present study was designed to show seasonal changes in the testis and epididymis during the annual reproductive cycle. The cycle of *Tropidurus itambere*, from the Atlantic Forest domain of the Brazil, was followed from June 2001 to June 2002. While the males vary their annual reproductive activity, they were considered potentially reproductive in most months with the exception of February through April. During this nonreproductive period, there is a reduction in the mean seminiferous tubule volume and fewer sperm were found in both the testis and the epididymis.

Key Words: Male reproductive cycle, Seasonal variation, *Tropidurus itambere*, Tropical lizards, Histology, Transmission electron microscopy, Ultrastructure, Brazil.

INTRODUCTION

In general, reproductive studies of tropical lizards have dealt with three distinctive aspects of reproduction. First, the reproductive period has been investigated to determine whether a species reproduces continuously throughout the year, and if so, whether the level of reproductive activity is constant. Second, which environmental factors can be correlated with reproduction and may dictate the timing of these activities. Finally, which evolutionarily important selective pressures can be linked with successful reproduction at various times of the year. While some authors have focused on a single aspect of lizard reproduction, most studies have examined all three (Sherbrooke, 1975).

Tropidurus itambere (Squamata, Tropiduridae) is a medium-sized lizard (adult mean snout-vent length + 71.8 mm) which belongs to the *Torquatus* group. Their bodies are brown with many white and black spots. *T. itambere* commonly occurs in open, sometimes

rocky, areas in the Atlantic Forest domain, in central and southeastern Brazil (Rodrigues, 1987).

From June 1998 to June 1989, Van Sluys (1993) studied an ecological aspect of the reproductive cycle of this lizard in the Valinhos region (Brazil). In this study, we focused on the morphological details of the male reproductive cycle from June 2001 to June 2002, describing the structure and ultrastructure of the testis and epididymis.

MATERIAL AND METHODS

ANIMALS AND STUDY SITE

The adult male*T.itambere* (N=13) used in this study were collected from the Valinhos region (23°00'S, 47°00'W), São Paulo state, Brazil. The elevation of this area is approximately 670m above sea level. This approximately 1 km area is divided by a stream with a small riparian forest along it's banks. Mean monthlv rainfall in the region is strongly seasonal (1955-1988: winter (June to August) = 40.7 mm; summer (November to February) = 198.7 mm) (Van Sluys, 1983).

Lizards were deposited at the Museu de História Natural, Universidade Estadual de Campinas (ZUEC, n° 02690-02702).

LIGHT MICROSCOPY

From June 2001 to June 2002, lizards were collected at monthly intervals. First, testis and epididymis were fixed overnight at 4° C in a 0.1M sodium caccodylate buffer solution (pH = 7.2) containing 2.5% glutaraldehyde. Next, they were dehydrated overnight in acetone and embedded in hydroxyethyl methacrylate resin. Finally, the sections were stained with hematoxilin and eosin and observed with a light photomicroscope (Olympus, BX60).

TRANSMISSION ELECTRON MICROSCOPY

After the testes and epididymis were fixed following the above protocol, they were post fixed for 2 h in 0.1M sodium caccodylate buffer solution (pH = 7.2) containing 1% osmium

tetroxide. Next, they were dehydrated in acetone and embedded in LR White resin. Finally, the ultrathin sections were stained with uranyl acetate and lead citrate and observed with the transmission electron microscope (Zeiss, Leo 906).

RESULTS

During the year, the testes presented seminiferous tubules with germ cells in various degrees of differentiation including spermatogonia, spermatocytes, and various spermatid stages. From February to April, a period of degeneration for the seminiferous tubules, spermatogenesis was interrupted. The seminiferous tubules were small from February to April (fig. 1A) but increased progressively from June to October (fig. 1B, 1C); maximum seminiferous tubule diameter was reached in December (fig. 1D). While the seminiferous tubules were completely occluded from February to April (fig. 1A), transmission electron microscopy was required to clearly ascertained the intense degeneration of germ cells (fig. 1E). From June to December, the majority of testes contained active sperm (fig. 1F). At the peak of the breeding season there was tremendous testicular hypertrophy and very abundant sperm. This gradual increase in the amount of germ cells and sperm in the seminiferous tubules is associated with a reduction in the interstitial tissue (figs. 1A-1D).

The epithelium of the epididymis is composed of two main cell types: secretory cells, which are the more numerous, and basal cells, which are probably important for cell replacement. This differentiation is particularly noticeable as the secretory cells have a columnar appearance compared to the basal cells which remain much smaller and wedge shaped (figs. 2A-2F and 3A-3F). From April to June, in synchrony with the testis cycle, there is a cycle in the secretory cells of the epididymis. During this period there is an absence of spermatozoa in the lumen and a gradual epithelial stratification which results in an increase in the cell cytoplasm height and the production of secretion vesicles (figs. 2A, 2B, 3A, 3B). Throughout the year, the epididymis contains varying amounts of spermatozoa; for example, from June to December (figs. 2C, 2D, 2E, 3C, 3D, 3E) the

epithelial stratification is reduced to the point of total suppression of secretory activity (figs. 2F, 3F).

DISCUSSION

In order to understand variation in reproductive activity of tropical lizards, Sherbrooke (1975) grouped the reproductive strategies into three possible types: (1) continuous, without variation in reproductive activity, (2) continuous, with variation in reproductive activity, noted by seasonal variation either in testis size and/or spermatogenetic activity or the percentage of fertile or ovigerous females, and (3) non-continuous, with periods when all individuals are reproductively inactive, males lack spermatozoa in their testes and epididymis and females lack large yoked ova and/or oviductal eggs.

A variety of reproductive patterns have been documented for the genus *Tropidurus* which is widespread in South America and the Galapagos Islands (Rodrigues, 1987). Continuous reproduction has been found in *T. hispidus* in both the uniformly hot Caatinga region of northeastern Brazil (Vitt, 1993) and under highly unpredictable environmental conditions. In contrast, a well defined seven month reproductive season has been found in *T. itambere*, a species which experiences regular seasons in the Atlantic Forest domain in southeastern Brazil (Van Sluys, 1993). This seasonal variation in reproduction is also found in *T. etheridgei* which has a six month reproductive cycle and lives in the Argentinian Chaco (Cruz, 1997), a region with predictable seasonality.

Additional studies of other *Tropidurus* species (i.e., *T. albermalensis* (Stebbins *et al.*, 1967), *T. hispidus* (Prieto *et al.*, 1976), *T. delanonis* (Werner, 1978), *T. quadrivittatus* and *T. theresioides* (Goldberg & Rodrigues, 1986), and *T. torquatus* (Vieira *et al.*, 2001; Wiederhecker *et al.*, 2002)) living in seasonal localities have found that females reproduce only during the wet season while males are potentially reproductive throughout the year. *T. torquatus* and *Platynotus semitaeniatus* are an exception to this seasonal rule reproducing mainly during the dry season, in northeastern Brazil (Vitt & Goldberg, 1983). The small

number of females reproducing during the wet season suggests that this season is unfavorable for reproduction (Vitt & Goldberg, 1983). This finding demonstrates that, while reproduction has been closely related to wet-dry seasons, there is nosingle reproductive strategy.

There is an apparent tendency to concentrate reproductive activities during the regular rainy seasons (Wiederhecker *et al.*, 2002). However, the lack of information for a large number of localities hampers a better understanding of the association between reproductive activity and environmental factors for *Tropidurus* lizards. In *T. itambere*, the reduction of mean testis volume was associated with a drop in the frequency of males containing sperm in either the testes or epididymis (Van Sluys, 1993). A similar reduction in testis size has been associated with diminished sperm production in various tropical lizards which have continuous, year-round spermatogenesis (Daniel, 1960; Licht & Gorman, 1970; Sexton *et al.*, 1971). In *T. itambere*, the reproductive period coincides with the rainy season which occurs from January to April (Van Sluys, 1993). Van Sluys (1993) findings were confirmed in the present study by documenting testis and epididymis morphology. In addition, the epithelial epididymis cells were found to show variation in the secretion production, a factor which is considered important for sperm maturation.

The pattern of reproductive activities in Squamata has often been associated with limiting environmental factors. In temperate regions, reproduction is seasonal and dictated by temperature and daylight hours (Fitch, 1970; Duvall *et al.*, 1982). In tropical areas, however, Squamata exhibit a broad variety of reproductive patterns, ranging from continuous to strongly seasonal reproduction, making it difficult to identify the limiting environmental factors (Vitt, 1992; Clerke & Alford, 1993). Two main hypotheses have been advanced for these areas: (1) the lack of microhabitats with adequate moisture for egg development (Sexton et al., 1971; Andrews, 1988), and (2) the lack of food resources for reproduction and/or development of the young (Rocha, 1992; Van Sluys, 1993; Vrcibradic & Rocha, 1998). An association of all these factors could happen (Ferreira *et al.*, 2002) during an unfavourable season. In those species with broad or wide-ranging geographical

distributions, phenotypic plasticity in reproduction has been demonstrated (Seigel & Ford, 1991).

Of the few studies demonstrating epididymal variations during the annual reproductive cycle in lizards, most are experimental and investigate the effect of castration (Haider, 1985), various drugs (Haider & Rai, 1986) or hormones (Gigon-Depeiges & Dufaure, 1977; Haider & Rai, 1987). The present research, which examined the natural reproductive cycle of *T. itambere*, determined that there was synchronous development of the testes and epididymis and that maximum testes volume and epididymis diameter (especially secretory cell development) occurred from June to December. Dufaure *et al.* (1986) studied the annual weight variations of testes during the sexual cycle. confirming *in vivo* experimental evidence previously obtained that associated testosterone control of the secretory activity.

Contrary to *T. itambere*, the epididymis of *T. torquatus* presents a clear-cut annual cycle in which the epididymis lumen remains completely filled with spermatozoa In addition, there was little difference in the quantity of spermatozoa and epithelial cell development between seasons (wet-dry) (Vieira *et al.*, 2001). For *T. itambere* the occurrence of spermatozoa in the epididymis lumen was most prevalent from August to January.

The investigation by Znari & El Mouden (1997) on the annual reproductive and fat body cycles of *Agama impalearis* in the central Jbilet Mountains (Morocco) had similar findings to those of the current study with *T. itambere*. First, the development of the testes and epididymis was synchronous. Secondly, testes volume and epididymis diameter were at maximum from April to July with post-reproductive regression from late summer through autumn.

While Mesure *et al.* (1991) affirmed that degeneration of the epididymis epithelial cells occurs in *Lacerta vivipara* during its reproductive cycle, this was not observed, even with transmission electron microscopy, in *T. itambere*.

With the identification of different proteins and glycoproteins that influence sperm maturation, Dufaure & Saint-Girons (1984), Depeiges *et al.* (1985) and Ravet *et al.* (1987), studied the biochemical and histochemical characteristics of epididymal secretions in lizards. They observed an increase of epididymal secretory activity during the period of

reduced reproductive activity which was thought to contribute to the maturation of the future germ cells. Based on variations in epididymal maturation, Dufaure *et al.*(1986) correlated cyclic development of the testis with that of the epididymis and its secretions.

Mesure *et al.* (1991) postulated a degenerative process leading to the destruction of the epithelium. However, the routine fixation methodology he used made confirmation of this impossible.

Gigon-Depeiges & Dufaure (1977) suggested that this epididymis in lizards, which secretes large granules, could be used as a model for the role played by epididymal secretions in the maturation of spermatozoa.

Seasonal variation in reproduction is common among lizards of the temperate zones (Fitch, 1980). Temperature and photoperiod are determinative factors for the reproductive activity of lizards in temperate zones and extreme conditions can determine the hibernation period for many species (Bartholomeu, 1953; Mayhew, 1964; Licht, 1973). In contrast, temperature and photoperiod under go less variation in the tropics and the dry period is comparable to the cold of temperate zones (Tinkle, 1969; Pianka, 1970). Precipitation has been demonstrated as the main factor influencing the reproductive cycle of some lizard species (Licht & Gorman, 1970; Sexton *et al.*, 1971; Ruibal *et al.*, 1972). The rains are important as a regulating factor of the reproductive cycle with the higher humidity preventing egg desiccation and guaranteeing the availability of animal and plant resources for the hatchings (Vallejo & Vallejo, 1981, Rocha, 1992).

CONCLUSIONS

The observed reproductive season for male *T. itambere* was determined to be very similar to that described by Van Sluys (1993). In fact, the well defined climatic changes in Brazil and other regions of the world did not dictate the male's reproductive cycle. While Van Sluys (1993) found that the *T. itambere* males in Atlantic Forest domain appear to

have the potential of reproducing during the first reproductive season, electron microscopy in this study found spermatozoa were degenerated from February to April.

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REFERENCES

ANDREWS, R. M. 1988.- Patterns of growth in reptiles. In: Biology of the Reptilia: 273-320. Gans, C. & F. H., Pough. (Eds). New York, Academic Press.

BARTHOLOMEU JR., G. C. 1953.- The modification by temperature of the photoperiodic control of gonadal development in the lizard *Xantusia vigilis*. Copeia, **1953**: 44-50.

CLERK, R. B. & R. A., ALFORD. 1993.- Reproductive biology of four species of tropical Australian lizards and comments on the factors regulating lizard reproductive cycles. J. Herpetol. **27**: 400-406.

CRUZ, F. B. 1997.- Reproductive activity in *Tropidurus etheridgei* in the semiarid Chaco of Salta, Argentina. J. Herpetol. **31**: 444-450.

DANIEL, P. M. 1960.- Growth and cyclic behavior in the West African lizard, *Agama agama africans*. Copeia **1960**: 94-97.

DEPEIGES, A.; G., BETAIL; M., COULET & J. P., DUFAURE. 1985.- Histochemical study of epididymal secretions in the lizard, *Lacerta vivipara*: localization of lectin-binding sites. Cell Tissue Res. **239**: 463-466.

DUFAURE, J. P. & H., SAINT-GIRONS. 1984.- Histologie comparée de l'epididyme et de ses secretions chez les reptiles (lezards et serpents). Arch. d'Anat. Microsc. **73**: 15-26.

DUFAURE, J. P.; Y., COURTY; A., DEPEIGES; M., MESURE & M., CHEVALIER. 1986.- Evolution and testosterone content of the epididymis during the annual cycle of the lizard *Lacerta vivipara*. Biol. Reprod. **35**: 667-675.

DUVALL, D.; L. J., GUILLETTE JR. & R. E., JONES. 1982.- Enviromental control of reptilian reproductive cycles. In: Biology of the Reptilia: 273-320. Gans, C. & F. H., Pough (Eds). New York, Academic Press.

FERREIRA, A.; I. A., LAURA & H., DOLDER. 2002.- Reproductive cycle of male green iguanas, *Iguana iguana* (Reptilia: Sauria: Iguanidae), in the Pantanal region of Brazil. Braz. J. Morphol. Sc. **19**: 23-28.

FITCH, H. S. 1970.- Reproductive cycles in lizards and snakes. Univ. Kansas Mus. Nat. Hist., Misc. Publ. **52**: 1-247.

GIGON-DEPEIGES, A. & J. P., DUFAURE. 1977.- Secretory activity of the lizard epididymis and its control by testosterone. Gen. Comp. Endoc. **33**: 473-479.

GOLDBERG, S. R. & O. O., RODRIGUES. 1986.- Reproductive cycles of two iguanid lizards from northern Chile, *Tropidurus quadrivittatus* and *T. theresioides*. J. Arid Environ. **10**: 147-151.

HAIDER, S. & U., RAI. 1986.- Effects of cyproterone acetate and flutamide on the testis and epididymis of the Indian wall lizard, *Hemidactylus flaviviridis* (Rüppell). Gen. Comp. Endoc. **64**: 321-329.

HAIDER, S. & U., RAI. 1987.- Epididymis of the Indian wall lizard (*Hemidactylus flaviviridis*) during the sexual cycle and in response to mammalian pituitary gonadotropins and testosterone. J. Morphol. **191**: 151-160.

HAIDER, S. 1985.- The effects of castration and testosterone replacement on the histology and histochemistry of the epididymis in the Indian wall lizard *Hemidactylus flaviviridis* (Rüppell). Monit. Zool. Ital. (N.S.) **19**: 189-195.

LICHT, P. & G. C., GORMAN. 1970.- Reproductive and fat cycles in Caribbean *Anolis* lizards. Univ. California Publ. Zool. **95**: 1-52.

LICHT, P. 1973.- Environmental influences on the testis cycles of the lizards *Dipsosaurus dorsales* and *Xantusia vigilis*. Comp. Biochem. Physiol. **45**: 7-20.

MAYHEW, W. W. 1964.- Photoperiodic responses in three species of the lizard genus *Uma*. Herpetologica **20**: 95-113.

MESURE, M.; M., CHEVALIER; A., DEPEIGES; J., FAURE & J. P., DUFAURE. 1991.- Structure and ultrastructure of the epididymis of the viviparous lizard during the annual hormonal cycle: changes of the epithelium related to secretory activity. J. Morphol. **210**: 133-145.

PIANKA, E. R. 1970.- Comparative autoecology of the lizard *Cnemidophorus tigris* in different parts of its geographic range. Ecology **51**: 703-720.

PRIETO, A. S.; J. R., LEON & O., LARA. 1976.- Reproduction in the tropical lizard, *Tropidurus hispidus* (Sauria: Iguanidae). Herpetologica **32**: 318-323.

RAVET, V.; Y., COURTY; A., DEPEIGES & J. P., DUFAURE 1987.- Changes in epididymal protein synthesis during the sexual cycle of the lizard, *Lacerta vivipara*. Biol. Reprod. **37**: 901-907.

ROCHA, C. F. D. 1992.- Reproductive and fat body cycles of the tropical sand lizard (*Liolaemus lutzae*) of the southeastern Brazil. J. Herpetol. **26**: 17-23.

RODRIGUES, M. T. 1987.- Sistemática, ecologia e zoogeografia dos *Tropidurus* do grupo *Torquatus* ao Sul do Rio Amazonas (Sauria: Iguanidae). Arq. Zool. SP **31**: 105-230.

RUIBAL, R.; R., PHILIBOSIAN & J. L., ADKINS 1972.- Reproductive cycle and growth in the lizard *Anolis acutus*. Copeia **1972**: 509-518.

SEIGEL, R. A. & N. B. FORD. 1991. Phenotypic plasticity in the reproductive characteristics of an oviparous snake, *Elaphe guttata*: implications for life history studies. Herpetologica **47**: 301-307.

SEXTON, O. J.; E. P., ORTLEB; L. M., HATHAWAY; R. E., BALLINGER & P., LICHT. 1971.- Reproductive cycles of three species of anoline lizards from the Isthmus of Panama. Ecology **52**: 201-215.

SHERBROOKE, W. C. 1975.- Reproductive cycle of a tropical teiid lizard, *Neusticurus ecpleopus* Cope in Peru. Biotropica **7**: 194-207

STEBBINS, R. C.; J. M., LOWENSTEIN & N. W., COHEN. 1967.- A field study of the lava lizard (*Tropidurus albemarlensis*) in the Galapagos Islands. Ecology **48**: 839-851.

TINKLE, D. W. 1969.- The concept of reproductive effort and its relation to the evolution of life histories of lizards. Amer. Natur. **103**: 501-516.

VALLEJO, L. R. & M. S., VALLEJO. 1981.- Contribuição ao estudo ecológico dos microartrópodos do "litter" na restinga de Maricá – RJ. Rev. Bras. Biol. **41**: 539-543.

VAN SLUYS, M. 1993.- The reproductive cycle of *Tropidurus itambere* (Sauria: Tropiduridae) in Southeastern Brazil. J. Herpetol. **27**: 28-32.

VIEIRA, G. H. C.; H. C., WIEDERHECKER; G. R., COLLI & S. N. BÁO. 2001.- Spermiogenesis and testicular cycle of the lizard *Tropidurus torquatus* (Squamata, Tropiduridae) in the Cerrado of central Brazil. Amphibia-Reptilia **22**: 217-233.

VITT, L. J. & S. R., GOLDBERG. 1983.- Reproductive ecology of two tropical lizards: *Tropidurus torquatus* and *Platynotus semitaeniatus*. Copeia **1983**: 131-141.

VITT, L. J. 1992.- Diversity of reproductive strategies among Brazilian lizards and snakes: the significance of lineage and adaptation. In: Reproductive biology of South American vertebrates: 135-149. Hamlett, W. C. (Ed.). New York, Springer-Verlag.

VITT, L. J. 1993.- Ecology of isolated open-formation *Tropidurus* (Reptilia: Tropiduridae) in Amazonian lowland rain forest. Can. J. Zool. **71**: 2370-2390.

VRCIBRADIC, D. & C. F. D., ROCHA. 1998.- Reproductive cycle and life-history traits of the viviparous skinks *Mabuya frenata* in southeastern Brazil. Copeia **1998**: 612-619.

WERNER, D. I. 1978.- On the biology of *Tropidurus delanonis*, Baur (Iguanidae). Z. Tierpsychol. **47**: 337-395.

WIEDERHECKER, H. C.; A. C. S., PINTO & G. R., COLLI, G. R. 2002.- Reproductive ecology of *Tropidurus torquatus* (Squamata: Tropiduridae) in highly seasonal Cerrado biome of central Brazil. J. Herpetology **36**: 82-91.

ZNARI, M. & E. H., EL MOUDEN. 1997.- Sexual dimorphism, reproductive and fat body cycles in Bibron's agama (*Agama impalearis*, Boettger, 1874) (Sauria: Agamidae). Herpetologica **53**: 411-422.

Figure Legends

Figure 1: **A-D:** Light Microscopy of the seminiferous tubule in the testis. HE. X400. **A.** Feburary-April: Seminiferous tubule with recognizable spermatogonia (g) and spermatocytes (c). The lumen is occluded with spermatids (t). The interstitial tissue (it) is thicker, with well-developed cells. **B.** April to June: Note the diameter increase of the seminiferous tubule with a regular layer of spermatocytes (c) and spermatids (t) and the appearance of the lumen. The interstitial tissue (it) has undergone initiated reduction. **C.** June-October: Germ cells possess a layered organization in the interior of the seminiferous tubule, where spermatocytes (c) are basal, followed by spermatids (t) and in the lumen there are numerous spermatozoa (z). The interstitial tissue (it) is extremely reduced. **D.** October-December: Period of maximum spermatogenesis, where all types of germ cells are numerous and some Sertoli cells (s) are observed. **E-F:** Transmission Electron Microscopy. X4000. **E.** Month of February where lumen occlusion occurs. Numerous spermatocytes (c) and a few spermatids (t), can be recognised among a great amount of degenerating cells (curved arrows). **F.** Period of maximum spermatogenesis, showing numerous early rounded spermatids (et) with a large nucleus, late elongating spermatids (lt), still with abundant cytoplasm around the heads (zh) and flagella (zf) of mature spermatozoa.

Figure 2: Light Microscopy of the epididymis. HE. X400. In all figures, notice the two epithelial cell types: basal cells (b), secretory cells (s). The epididymis tubules are

surrounded by connective tissue (ct), which does not vary during the annual reproductive cycle. **A.** April, the secretory cells present intense stratification and the cytoplasm is voluminous. **B.** June, the secretion produced by the secretory cells becomes very evident and intensely stained (arrow). **C.** August, a lot of secretory cells and some spermatozoa (z) are seen in the lumen. **D.** October, the cytoplasm of secretory cells begins to reduce. **E.** December, a lot of spermatozoa (z) dominates the lumen, while secretory cells possess reduced cytoplasm. **F.** February, period of reduced reproductive activity, with no spermatozoa in the lumen, and secretory cells initiating stratification, however with reduced cytoplasm.

Figure 3: Transmission Electron Microscopy of the epididymis. X2000. In the all figures, the arrows indicate secretion granules. Notice the gradual decrease in the amount of these granules (fig.A-F). The secretory cells present a basal nucleus and are columnar with stratification (figs. A-E) up to the period of reduction in reproductive activity when they become cubical and not stratified (fig. F). **A.** April. **B.** June. **C.** August. **D.** October. **E.** December. **F.** February.

Figure 1

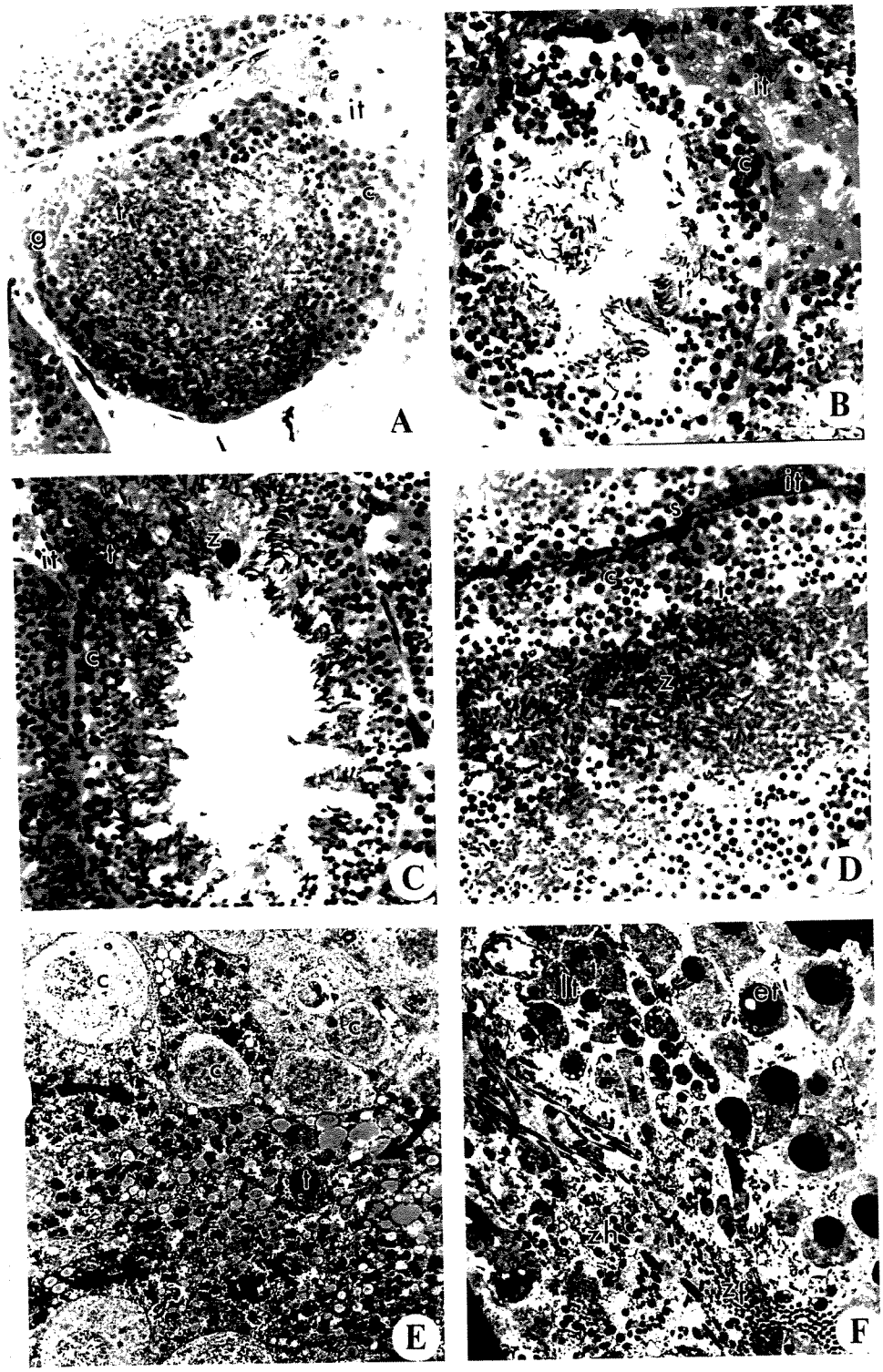


Figure 2

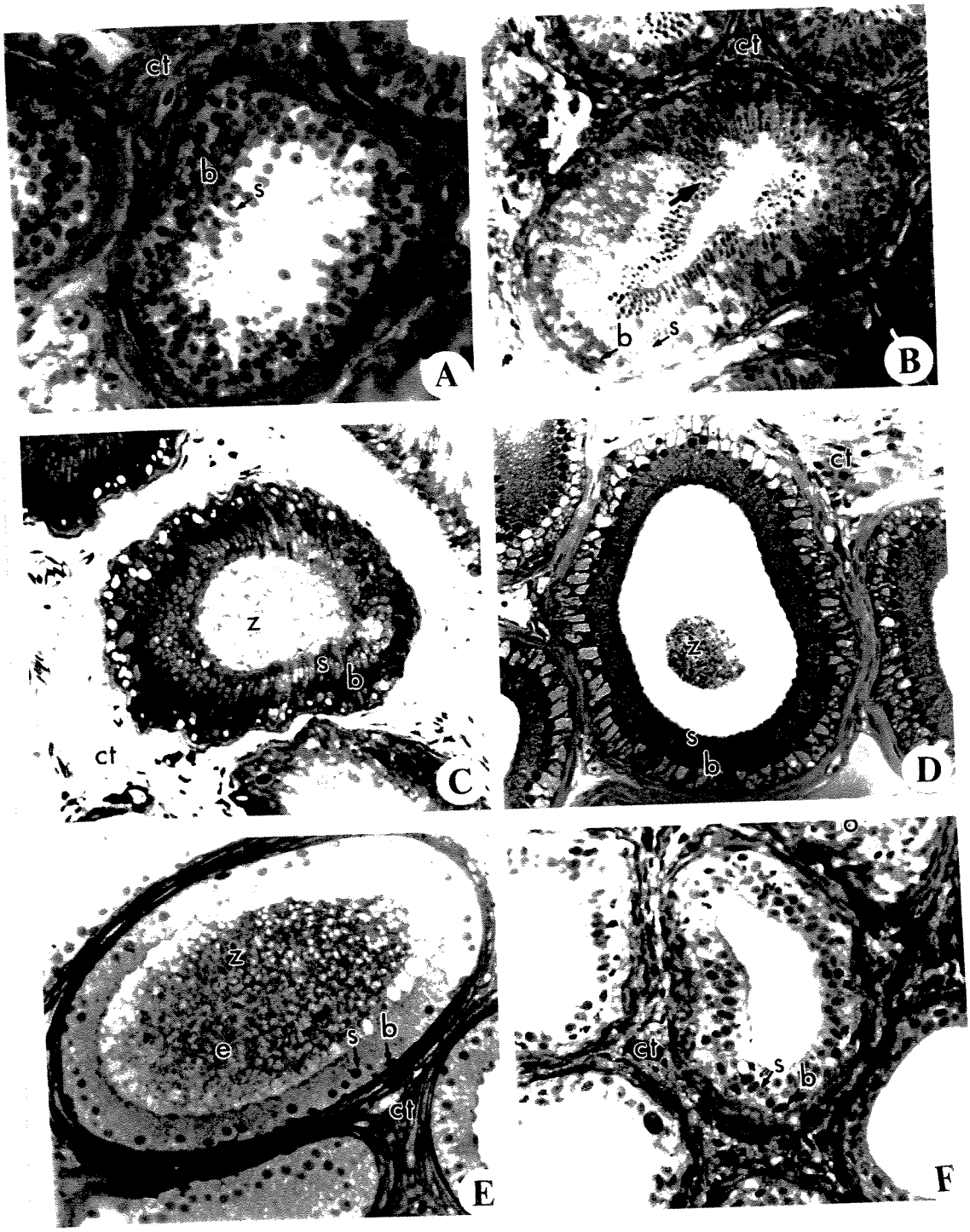
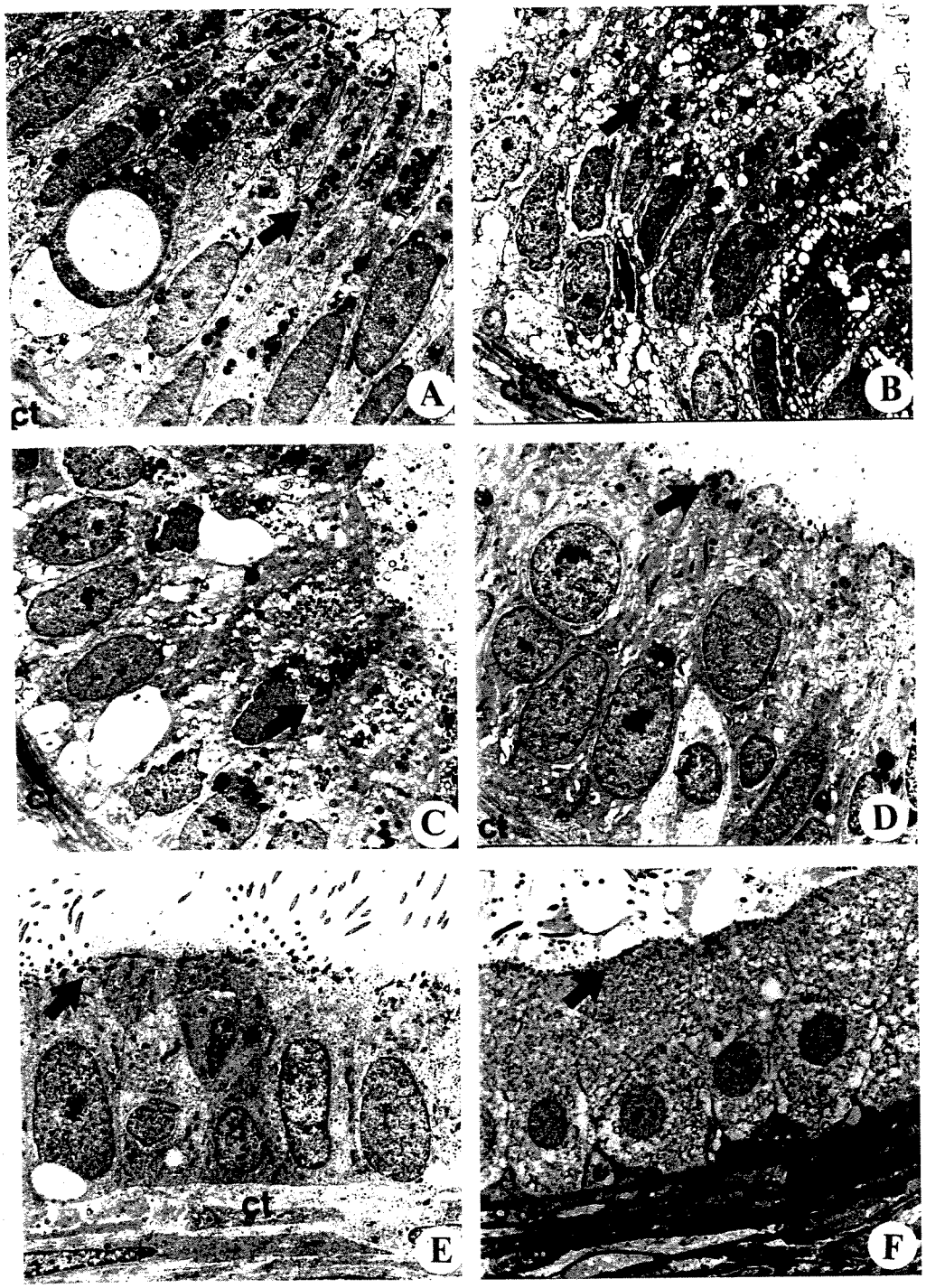
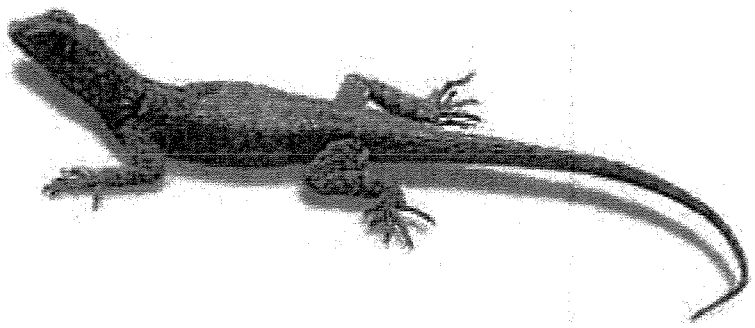


Figure 3



6.2. Sperm ultrastructure and spermatogenesis in the lizard, *Tropidurus itambere*



ABSTRACT

Spermatogenesis, with emphasis on spermiogenesis, is described for the lizard, *Tropidurus itambere*, using light microscopy, phase contrast and epifluorescence, as well as scanning and transmission electron microscopy. Cellular differentiation involves events of chromatin condensation, nuclear elongation and the formation of structural complexes, such as the acrosomal and axonemal ones. Other new characteristics, exclusive for this species, include various aspects of the subacrosomal granule, the insertion of the pro-acrosomal vesicle and the development of these structures to participate in the acrosomal complex. Radial projections occur just above the nuclear shoulders, which have been recognized already from the beginning of cellular elongation. The development of the midpiece, the dense bodies, formation of the flagellum and elimination of residual cytoplasm result in the final characterization of the mature spermatozoon. Comparisons between Tropiduridae and other lizard families are made.

Keywords: ultrastructure, sperm, spermatogenesis, lizard, Tropiduridae.

INTRODUCTION

The *Tropidurus itambere* (Squamata, Reptilia) is a species that occurs in the dense Atlantic forest domains, as well as open formations of the states of São Paulo and Minas Gerais, Brazil (Rodrigues, 1987). The adult male individuals present an annual reproductive cycle of the continuous type with variations in their reproductive activity. These animals are potentially fertile during every month of the year, except March, the month where a considerable reduction occurs in the testicular volume and consequently in the production of spermatozoa (Van Sluys, 1993). Ultrastructural studies of the mature spermatozoon are known for other lizards, as in Chamaleonidae (Jamieson, 1995; Oliver *et al.*, 1996); Polychrotidae (Furieri, 1974; Texeira *et al.*, 1999; Scheltinga *et al.*, 2001); Phrynosomatidae (Scheltinga *et al.*, 2000) and also in Tropiduridae (Furieri, 1974; Teixeira *et al.*, 1999). Some descriptions focus on phylogenetic relationships. Spermiogenesis is described for some Chamaleonidae lizards (Al-Hajj *et al.*, 1987; Dehlawi and Ismail, 1990;

Dehlawi *et al.*, 1992), a few representatives of Iguanidae (Saita *et al.*, 1988; Ferreira and Dolder, 2002), in Phrynosomatidae and Polychrotidae (Clark, 1967). Despite descriptions of spermiogenesis for the Iguania group, only *Tropidurus torquatus,* among the lizards of the Tropiduridae family has been detailed in the literature (Furieri, 1974; Cruz-Landim and Cruz-Höfling, 1977; Cruz-Höfling and Cruz-Landim, 1978; Vieira *et al.*, 2001). The *Tropidurus* lizards were considered as pertaining to the Iguanidae family up to a recent systematic revision, which constituted the Tropiduridae family (Rodrigues, 1987). The possibility of contributing to the studies of phylogenetic relationships justifies detailed comparisons between these families. Data on spermatogenesis is uncommon for the majority of vertebrates, and almost absent for lizards. Some articles entitled spermatogenesis in lizards (Cruz *et al.*, 1994; Martinage *et al.*, 1996; Ibarguengoytia and Cussac, 1999; Amat *et al.*, 2000) bring information only on a given structure during a particular phase of the reproductive cycle. Our objective is to confirm this data and to evaluate the ultrastructural particularities of spermatogenesis in *Tropidurus itambere*, as well as of the characteristics of its testicular spermatozoa.

MATERIAL AND METHODS

The adult *T. itambere* (N=16) used in this study was collected from the Valinhos region (23°00' S, 47°00' W), São Paulo state, Brazil.

Light microscopy

We fixed tissues overnight at 4° C in a solution containing 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2, dehydrated in acetone and embedded in LRWhite resin. The sections stained with hematoxilin and eosin, were observed with a photomicroscope. A sperm suspension was spread and fixed as above. After drying at room temperature, it was observed with phase contrast. Some of these preparations were then stained for 15 min with 0,2µg/ml 4,6-diamino-2-phenylindole (DAPI) in PBS buffer, washed, and mounted with Vectashield. They were examined with epifluorescence microscopy (Olympus, BX60), equipped with a BP 360-370nm excitation filter.

Scanning electron microscopy

Testis was fixed as above; subsequently, they were washed in buffer, imbebed in sucrose solution (0.5M-3M) and criofratured in liquid nitrogen. They were washed in buffer and post-fixed for 1 h in 1% osmium tetroxide in 0.1M sodium caccodylate buffer, pH 7.2. The testes were dehydrated in acetone, critical point dried and sputter-coated with gold. They were observed with a scanning electron microscope (Jeol JSM 5800LV).

Transmission electron microscopy

Testis were fixed and embedded in LRWhite resin, as above. The ultrathin sections were stained with uranyl acetate and lead citrate and observed with the transmission electron microscope (Zeiss, Leo 906).

RESULTS

During the peak reproductive period, all phases of spermatogenesis are observed (fig. 1A). The germ cells present a regular organization, with the basally located spermatogonia, together with spermatocytes, occupying half of the layer of the spermatogenic epithelium (figs. 1A, 1B). Next to the lumen a great amount of spermatids and spermatozoa are embedded in the cytoplasmic prolongations of the Sertoli cells (fig.1C). Spermatogonia present a large, central nucleus, with dense clumps of chromatin (fig.1D). Some meiosis figures can be found, however the most commonly observed phases are anaphase and telophase, where the nuclear material is still condensed in chromosomes and the spindle microtubules can be found (fig. 1D). Spermatocytes are rich in organelles, especially the Golgi complex. The nucleus is central, with loose chromatin (fig. 1E).

The differentiation of spermatids into spermatozoa involves the events of nuclear elongation, formation of the acrosomal and axonemal complexes, and elimination of residual cytoplasm. Early spermatids possess a central, rounded nucleus, with granular chromatin and numerous mitochondria (fig. 1F). The Golgi complex and the endoplasmic reticulum well are developed, while the endoplasmic reticulum aggregates in patches (fig. 2B).

To constitute the acrosomal complex, innumerable vesicles dispersed in the cytoplasm, formed in the Golgi complex, accumulate beside the nucleus, and fuse to form the pro-acrosomal vesicle, which is lodged in a large nuclear depression (figs. 1F, 2C, 2D). The vesicle begins to flatten, containing a loose clear material (fig. 2D). As the pro-acrosomal vesicle develops, a dense granule appears in its interior. This granule is initially attached to the vesicle membrane, in contact with the nucleus (fig. 2C). Between the nucleus and the vesicle, an electron dense layer is observed (fig. 2D, 2G). Between these layers, two other clear layers can be found that will form the epinuclear electron-lucent region and the subacrosomal clear zone (figs. 2D, 2G). Chromatin adjacent to the vesicle becomes more compact than the remaining nuclear content (fig. 2C).

The acrosomal complex begins to take on its final structure covering the initial narrowed portion of the nucleus (figs. 2G, 3A, 3B, 3C). Two main compartments constitute this complex: the acrosome and the perforatorium. In the acrosome, two compartments can be further identified: an external acrosomal vesicle and an internal subacrosomal cone (figs. 2G, 3C, 3F, 3G, 3H). The acrosomal vesicle is made up of a clear cortex and an electron dense matrix (figs. 3C, 3D). The subacrosomal cone is well developed, circular and homogenous in cross section (figs. 2G, 3F, 3G, 3H). Just above the nuclear tip and beneath the subacrosomal cone, is the epinuclear electron-lucent region (figs. 3C, 3F, 3H). The perforatorium is a short rod located between acrosome and nucleus (figs. 3D, 3E) partly surrounded by the subacrosomal cone. Separating the perforatorium and the vesicular acrosome is a clear layer identified as the subacrosomal clear zone (figs. 3C, 3D, 3E, 3F, 3G, 3H).

The nuclei of early spermatids present loose chromatin that is gradually compacted. During chromatin condensation, thick fibers are formed and twisted in a spiral arrangement then fused into a honeycomb arrangement of lamellae (figs. 2E, 2F, 2G). A microtubular structure, called the manchette, wraps helically around the nucleus in the beginning, straitening to a longitudinal arrangement with further nuclear condensation (figs. 2F, 2G, 3I, 3J, 3K). These microtubules also surround the acrosomal complex (figs. 3E, 3G). Completing its condensation, the nucleus becomes homogeneously electron dense (figs. 3C, 3H, 3I, 3J, 3K, 3L) arched and conical in its anterior portion (figs. 3A, 3B, 3C), where it is

inserted in the subacrosomal cone (fig. 3C). The nucleus is strongly fluorescent due to the specificity of the DAPI reaction, while the other structures fluoresce lightly, probably due to the fixative used (Fig. 3B). The transition between the conical and the cylindrical portion, called the nuclear shoulders, is abrupt and marks the posterior limit of the acrosome (fig. 3C). A layer of radial trabeculae is observed between the nucleus and the microtubules of the manchette at the nuclear shoulders (figs. 3I, 3J, 3K).

At the nuclear base, a deposit of electron dense material called the pericentriolar layer can be found (fig. 4A, 4B, 4C). This region is considered the "spermatozoon neck" and consists of two centrioles, a proximal and a distal one, wrapped in the electron dense pericentriolar material (figs. 2A, 2D, 4A, 4B, 4C). Nine triplets of microtubules constitute each centriole, associated to nine peripheral fibers and a central pair of microtubules, one of which has a dense fiber associated to it in the distal centriole (figs. 4A, 4E). The distal centriole is short and maintains an intermediate position, just above the axoneme (figs. 4A, 4B, 4C, 4D). These are surrounded by rings of rounded mitochondria, which alternate with dense bodies (figs. 4C, 4D, 4E, 4F). Dense bodies develop between the mitochondria in the midpiece (figs. 4D, 4E), forming inter-mitochondrial rings (fig. 4D). The midpiece is limited by a dense ring called the annulus (figs. 4A, 4B, 4C), which can be identified already in early spermatids (figs. 2A, 2D). The initial piece of the flagellum is formed by an axoneme constituted by nine microtubule doublets, a central pair and peripheral dense fibers, associated to each of the nine pairs of microtubules (figs. 4E, 4F). The principal piece of the flagellum consists in the axoneme (9+2), and vestigial peripheral dense fibers associated to pairs 3 and 8, all surrounded by the fibrous sheath and then the plasma membrane (fig. 4G). The fibrous sheath is formed by regular individual rings, observed, in longitudinal sections, as two columns (figs. 4D, 4F, 4G). The end piece of the flagellum is narrower and consists only of the axoneme, with peripheral dense fibers associated only to the 3rd and 8th pairs and covered by the plasma membrane (fig. 4H). The peripheral dense fibers diminish in diameter along the length the of axoneme, and remain attached only to pairs 3 and 8 of axoneme up to the flagellar end piece (figs. 4E, 4F, 4G, 4H).

DISCUSSION

Among the structures found during spermiogenesis of *T. itambere*, some are shared with other vertebrate groups. Other characteristics are exclusive of the Squamata. The close association between Sertoli cells and spermatozoa observed in *T. itambere* presents a curious similarity with the formation of loose cysts in the seminiferous tubules, as in Aves (Góes and Dolder, 2002) e Amphibians (Taboga and Dolder, 1998; Báo *et al.*, 1991).

The acrosome is an *organe*lle rich in enzymes that participate in the penetration of the spermatozoon in the ovule (Baccetti and Afzelius, 1976). In Reptiles, the spermatids and spermatozoa have a considerable variation in the size, form, complexity and degree of compartmentalization. The compartmentalization of the acrosome could be an adaptation to facilitate the sequential release of acrosomal enzymes (Talbot, 1991). The presence of acrosomal layers covering the nuclear tip is a common feature in Squamata. According to Cruz-Landim and Cruz-Höfling (1977), the lizard *Tropidurus torquatus* has a homogeneous electron-dense acrosome but this does not occur in *T. itambere*. The presence of two distinct layers may indicate variations according to the degree of cell maturation. Similar to the acrosome of *T. itambere* are those of Phrynosomatidae (Scheltinga *et al.*, 2000), Polychrotidae (Scheltinga *et al.*, 2001) and Chamaleonidae (All-Hajj *et al.*, 1987; Dehlawi *et al.*, 1992). Within the Tropiduridae family, acrosome and subacrosomal cone are always circular in transverse sections (Furieri, 1974; Teixeira *et al.*, 1999) and can present an oval transverse section at the tip, as in other Iguania (Scheltinga *et al.*, 2000; 2001) and Agamidae (Oliver *et al.*, 1996). The epinuclear electron-lucent zone is absent in Scincidae (Jamieson and Scheltinga, 1993) and Gekkonidae (Jamieson *et al.*, 1996). For the Squamata, in general, it is always present, varying from poorly developed, as in all Iguania (Oliver *et al.*, 1996; Scheltinga *et al.*, 2000; 2001) to very well developed as in Pygopodidae (Harding *et al.*, 1995; Jamieson *et al.*, 1996).

The origin of the perforatorium, during spermiogenesis, is unknown, but this structure has been described as being made up of a fibrous material, probably a cytoskeletal fibril (Baccetti and Afzelius, 1976). In terrestrial animals, the perforatorium consists of actin filaments which, during the acrosomal reaction, undergo conformational changes (Baccetti, 1986; Shiroya *et al.*, 1986). In invertebrates, it is important for sperm penetration

into the ovum (Baccetti et al., 1980), while in some birds it does not occur and therefore penetration cannot be attributed exclusively to the perforatorium. Therefore, Baccetti *et al.* (1980) suggested that it only supports the acrosome. In mammals, actin occurs only as a layer in the developing spermatids, where it contributes to nuclear and acrosome elongation, disappearing before final maturation (Baccetti *et al.*, 1980; Baccetti, 1986). In some birds (Nagano, 1962; Humphreys, 1975) and reptiles (Del Conte, 1976), the granule found in the pro-acrosomal vesicle, in contact with the nucleus, has been pointed out as the origin of the perforatorium. According to Del Conte (1976) the granule may be produced by the interaction between the pro acrosomal vesicle and the nucleus. In Sphenodontes (Healy and Jamieson, 1992), Crocodilians (Saita *et al.*, 1987; Jamieson *et al.*, 1997), Chelonia (Sprando and Russel, 1988), Amphibians (Jamieson *et al.*, 1993) and some Birds (Sprando and Russel, 1988) the perforatorium is located in the interior of intranuclear canals. In some Iguania, it enters the subacrosomal cone's apex below the perforatorium where an electron dense plate occurs, known as the perforatorial base plate (Scheltinga *et al.*, 2000; 2001). However this base does not exist in *T. itambere* and other Tropidurus lizards (Teixeira *et al.*, 1999; Vieira *et al.*, 2001). This is one of the few differences between this species and the Iguanidae.

The acrosomal vesicle is derived from vesicles produced by both the endoplasmic reticulum and the Golgi complex, as was verified by Carcupino *et al.* (1989). This clearly seems to be the case in spermatids of *T. itambere*, considering the large amount of vacuoles of the aggregated endoplasmic reticulum, which could deliver their contents to the Golgi complex.

The great electron density of the nucleus, resulting from the extreme chromatin condensation, favors mobility and protects the genome against physical and chemical alterations during transport and storage (Krause, 1996). This elongated shape is established during spermiogenesis by the manchette (Soley, 1997) and by the degree of DNA and protein aggregation (Fawcett *et al.*, 1971). All lizard spermatozoa are slender, as in *T. itambere*, except in *Eugongulus*, Scincidae (Jamieson and Scheltinga, 1993) that possess a larger diameter. Little has been said of the radial projections observed in the region described as the nuclear shoulders, observed in *T. itambere* and some Iguania (Al-Hajj *et*

al., 1987; Vieira *et al.*, 2001). Radial trabeculae are frequent in spermatids and spermatozoa of *T. itambere*. Cruz-Höfling and Cruz-Landim (1978) mentioned radial trabeculae of the nuclear envelope, but this aspect was not well discussed. We believe that these structures most likely establish connection between the microtubules of the manchette and the nucleus as defined by Butler and Gabri (1984) and Al-Hajj *et al.* (1987).

The centriole serves as a pattern for axoneme formation and this process is similar for all Squamata (Al-Hajj *et al.*, 1987; Phillips and Asa, 1993). Nine peripheral dense fibers follow parallel to the nine doublets, as well as a single dense fiber attached to one of the central pair of microtubules is characteristic of all Squamata. Also characteristic for Squamata is the presence of a short distal centriole. It is doe not extend along the entire midpiece, ending well above the annulus, within the layer of encircling mitochondria. In Sphenodontia and Chelonia the distal centriole is long (Healy and Jamieson, 1992; Jamieson and Healy, 1992; Oliver *et al.*, 1996). In Reptiles, the Crocodilia differ from the lizards, by the presence of a thick, dense fiber around one of the pair of central microtubules of the axoneme and the distal centriole (Saita *et al.*, 1987; Jamieson *et al.*, 1997). Among the Squamata, the spermatozoa almost always present linear mitochondrial cristae, inter-mitochondrial dense bodies, a short, distal centriole and the fibrous sheath beginning already in the midpiece. The peripheral fibers of the distal centriole and axoneme are characteristic for Reptiles (Jamieson *et al.*, 1997). These peripheral fibers apparently lend extra motor force, contributing to bending movements (Hamilton and Fawcett, 1968). Another function attributed to these peripheral fibers is a control mechanism for sperm movement (Anderson and Personne, 1969). These peripheral fibers also stabilize, longitudinally, the rings that make up the fibrous sheath (Austin, 1965). The fibrous sheath has elastic properties that also suggest a role in spermatozoon mobility (Fawcett, 1970).

The annulus occurs in most Tetrapoda and is also found in many Invertebrate groups (Baccetti and Afzelius, 1976). It consists of a set of closely associated filamentous subunits, adjoined and firmly adhering to the plasma membrane. Its function is to avoid the dislocation of mitochondria from the midpiece during flagellar movement (Fawcett, 1970). Differently from *T. itambere*, in Chelonia and Sphenodontia the mitochondria are sub-spherical, with concentric cristae and an intra-mitochondrial dense body (Furieri, 1970;

Healy and Jamieson, 1994). The dense bodies in *T. itambere* form rings that closely resembles those of the Iguania (Oliver *et al.*, 1996; Teixeira *et al.*, 1999; Scheltinga *et al.*, 2000; 2001). Other Squamata present dispersed or helically arranged dense bodies (Jamieson, 1995; Oliver *et al.*, 1996). The dense bodies are considered to originate from mitochondria and are homologous to the intra-mitochondrial dense bodies (Carcupino *et al.*, 1989; Healy and Jamieson, 1992). The pattern of the axoneme microtubules in the flagellar end piece of lizards varies greatly, where it may maintain the typical 9+2 arrangement (Scheltinga *et al.*, 2000, 2001) or disorganize this typical pattern, as in other Squamata (Jamieson *et al.*, 1996).

In conclusion, we present here the first detailed ultrastructural description of spermatogenesis in lizards. Although the initial cells are very similar to those of mammals, information on this phase is important. The observation of different phases of early spermatids has clarified the development of the structures described for the acrosomal complex. The mature acrosome, the presence of an electron dense medulla and a clear cortex is clearly estabilished, which is contrary to the existing descriptions of the Tropiduridae family. In the terminal tail portion, the vestigial peripheral dense fibers continue, associated to the 3rd and 8th pairs of axoneme microtubules, which was not observed in Iguanidae. The similarities observed between *T. itambere* (Tropiduridae), Iguanidae and Agamidae contribute toward the confirmation of a close phylogenetic relationship between these three families. The above characteristics may be used as evidences that these families should really be separated, as has been recently established (Frost, 1992).

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REFERENCES

AL-HAJJ H, JANAKAT S, MAHMOUD F (1987). Electron microscopic study of sperm head differentiation in the lizard *Agama stellio*. Can. J. Zool. 65: 2959-2968.

AMAT F, LLORENTE GA, CARRETERO MA (2000). Reproductive cycle of the sand lizard (*Lacerta agilis*) in its southwestern range. Amphibia-Reptilia 21: 463-476.

ANDERSON WA, PERSONNE P (1969). Structure and histochemistry of basal body derivative, neck and axoneme of spermatozoa of *Helix aspersa*. J. Microsc. 8: 87-96.

AUSTIN CR (1965) Fine structure of the snake sperm tail. J. Ultrastruc. Res. 12: 452-462.

BACCETTI B (1986). Evolutionary trends in sperm structure. Comp. Biochem. Physiol. 85: 29-36.

BACCETTI B, AFZELLIUS BA (1976). The biology of the sperm cell. Monogr. Dev. Biol. 10: 1-124.

BACCETTI B, BIGLIARDI E, BURRINI AG (1980). The morphogenesis of the vertebrate perforatorium. J. Ultrastruct. Res. 71: 272-287.

BAO SN, DALTON GC, OLIVEIRA SF (1991). Spermiogenesis in *Odontophrynus cultripes* (Amphibia, Anura, Leptodactylidae): ultrastructural and cytochemical studies of proteins using E-PTA. J. Morphol. 207: 303-314.

BUTLER RD, GABRI MS (1984). Structure and development of the sperm head in the lizard *Podarcis* (=*Lacerta*) *taurica*. J. Ultrastruct. Res. 88: 261-274.

CARCUPINO M, CORSO G, PALA M (1989). Spermiogenesis in *Chalcides ocellatus tiligugu* (Gmelin) (Squamata: Scincidae): an electron microscope study. Boll. Zool. 56: 119-124.

CLARK AW (1967). Some aspects of spermiogenesis in a lizard. Amer. J. Anat. 121: 369-400.

CRUZ MVS, MENDEZ DE LA CRUZ FR, PARRAGAMEZ L (1994). Spermatogenesis in the lizard *Sceloporus mucronatus* (Reptilia, Phrynosomatidae). Rev. Biol. Trop. 42: 289-296.

CRUZ HÖFLING MA, CRUZ LANDIM C (1978). The fine structure of nuclei during spermiogenesis in the lizard *Tropidurus torquatus* (Lacertilia). Cytologia 43: 61-68.

CRUZ LANDIM C, CRUZ HÖFLING MA (1977). Electron microscope study of lizard spermiogenesis in *Tropidurus torquatus* (Lacertilia). Caryologia 30: 151-162.

DEHLAWI GY, ISMAIL MF (1990). Studies on the ultrastructure of the spermiogenesis of Saudian Reptiles 1 – The sperm head differentiation in *Uromatyx philbyi*. Proc. Zool. Soc. Arab. Rep. Egyp. 21: 79-89.

DEHLAWI GY, ISMAIL MF, HAMDI SA, JAMJOOM MB (1992). Ultrastructure of spermiogenesis of Saudian Reptiles 6 – The sperm head differentiation in *Agama adramitana*. Arch. Androl. 28: 223-234.

DEL CONTE E (1976). The subacrosomal granule and its evolution during spermiogenesis in a lizard. Cell Tiss. Res. 171: 483-498.

FAWCETT DW (1970). A comparative view of sperm ultrastructure. Biol. Reprod. (Suppl.) 2: 90-127.

FAWCETT DW, PHILLIPS DM (1970). Recent observations on the ultrastructure and development of the mammalian spermatozoon. In: Comparative spermatology (Procceding of the International Symposium). B. Baccetti, Eds. Accademia Nazionale dei Lincei, Rome, pp. 13-28.

FAWCETT DW, ANDERSON WA, PHILLIPS DM (1971). Morphogenetic factors influencing the shape of the sperm head. Develop. Biol. 26: 220-251.

FERREIRA A, DOLDER H (2002). Ultrastructural spermiogenesis of *Iguana iguana* (Reptilia: Sauria: Iguanidae). Eur. J. Morphol. 40: 89-99.

FROST DR (1992). Phylogenetic analysis and taxonomy of the *Tropidurus* groups of lizards (Iguania: Tropiduridae). Am. Mus. Novitates 3033: 1-68.

FURIERI P (1970). Sperm morphology in some reptiles: Squamata and Chelonia. In: Comparative spermatology (Procceding of the International Symposium). B. Baccetti, Ed. Accademia Nazionale dei Lincei, Rome, pp. 115-143.

FURIERI P (1974). Spermi e spermatogenesi in alcuni iguanidi argentini. Riv. Biol. 67: 233-279.

GÓES RM, DOLDER H (2002). Cytological steps during spermiogenesis in the house sparrow (*Passer domesticus*, Linnaeus). Tissue and Cell 34: 265-274.

HAMILTON DW, FAWCETT DW (1968). Unusual features of the neck and middle-piece of snake spermatozoa. J. Ultrastruct. Res. 23: 81-97.

HARDING HR, APLIN KP, MAZUR M (1995). Ultrastructure of spermatozoa of Australian blindsnakes, *Ramphotyphlops* spp. (Typhlopidae, Squamata): first observations on the mature spermatozoon of Scolecophidian snakes. In: Advances in Spermatozoal Phylogeny and Taxonomy. BGM. Jamieson, J. Ausio, JL. Justine, Eds. Mém. Mus. Natn. Hist. Nat. 166: 385-396.

HEALY JM, JAMIESON BGM (1992). Ultrastructure of spermatozoon of the tuatara (*Sphenodon punctatus*) and is relevance to the relationships of the Sphenodontida. Phil. Trans. R. Soc. Lond. B. 335: 193-205.

HEALY JM, JAMIESON BGM (1994). The ultrastructure of spermatogenesis and epididymal spermatozoa of the tuatara *Sphenodon punctatus* (Sphenodontida: Amniota). Phil. Trans. R. Soc. Lond. B. 344: 187-199.

HUMPHREYS PN (1975). The differentiation of the acrosome in the spermatid of the budgerigar (*Melopsittacus undulatus*). Cell Tissue Res. 156: 411-416.

IBARGUENGOYTIA NR, CUSSAC VE (1999). Male response to low frequency of female reproduction in the viviparous lizard *Liolaemus* (Tropiduridae). Herpetol. J. 9: 111-117.

JAMIESON BGM (1995). The ultrastructure of spermatozoa of the Squamata (Reptilia) with phylogenetic considerations. In: Advances in spermatozoal phylogeny and Taxonomy. BGM. Jamieson, J. Ausio, JL. Justine, Eds. Édition du muséum national d'histoire naturelle, Paris, pp. 395-383.

JAMIESON BGM, HEALY JM (1992). The phylogenetic position of the tuatara, *Sphenodon* (Sphenodontida: Aminiota), as indicated by cladistic analysis of the ultrastructure of spermatozoa. Phil. Trans. R. Soc. Lond. B. 335: 207-219.

JAMIESON BGM, SCHELTINGA DM (1993). The ultrastructure of spermatozoa of *Nangura spinosa* (Scincidae, Reptilia). Mem. Queensland Mus. 34: 169-179.

JAMIESON BGM, OLIVER SC, SCHELTINGA DM (1996). The ultrastructure of the spermatozoa of Squamata. I. Scincidae, Gekkonidae and Pygopodidae (Reptilia). Acta Zool. (Stockholm) 77: 85-100.

JAMIESON BGM, SCHELTINGA DM, TUCKER AD (1997). The ultrastructure of spermatozoa of the Australian freshwater crocodile, *Crocodylus johnstoni* Krefft, 1873 (Crocodylidae, Reptilia). J. Submicr. Cytol. Pathol. 29: 265-274.

KRAUSE WJ (1996). Meiosis and male reproductive organs. In: Essentials of Human Histology. WJ. Krause, Ed. Little, Brown and Company, New York, pp. 331-352.

MARTINAGE A, DEPEIGES A, WOUTERS D, MOREL L, SAUTIERE P (1996). Spermatogenesis of the lizard *Lacerta vivipara*: Histological studies and amino acid sequence of protamine lacertine 1. Comp. Ren. Acad. Scien. III – Scien. Vie-Life Scien. 319: 511-516.

NAGANO T (1962). Observations on the fine structure of the developing spermatids in the domestic chicken. J. Cell Biol. 14: 193-205.

OLIVER SC, JAMIESON BGM, SCHELTINGA DM (1996). The ultrastructure of spermatozoa of Squamata. II. Agamidae, Varanidae, Colubridae, Elapidae and Boidae (Reptilia). Herpetologica 52: 216-241.

PHILLIPS DM, ASA CS (1993). Strategies for formation of the midpiece. In: Comparative spermatology 20 years after. B. Baccetti, Ed. Raven Press, New York, pp. 997-1000.

RODRIGUES MT (1987). Sistemática, ecologia e zoogeografia dos Tropidurus do grupo Torquatus ao sul do rio Amazonas (Sauria: Iguanidae). Arq. Zool. S.P. 31: 105-230.

SAITA A, COMAZZI M, PERROTA E (1987). Electron microscope study of spermiogenesis in *Caimam crocodylus* L. Boll. Zool. 4: 307-318.

SAITA A, COMAZZI M, PERROTA E (1988). New data at the E. M. on the spermiogenesis of *Iguana delicatissima* (Laurent) involving comparative significance. Acta Embryol. Morphol. Exper. 9: 105-114.

SCHELTINGA DM, JAMIESON BGM, TRAUTH SE, MCALLISTER CT (2000). Morphology of the spermatozoa of the Iguanian lizards *Uta stanburiana* and *Urosaurus ornatus* (Squamata, Phrynosomatidae). J. Submicrosc. Cytol. Pathol. 32: 261-271.

SCHELTINGA DM, JAMIESON BGM, ESPINOZA RE, ORREL KS (2001). Descriptions of the mature spermatozoa of the lizards *Crotaphytus bicinctores*, *Gambelia wislizenii* (Crotaphytidae), and *Anolis carolinensis* (Polychrotidae) (Reptilia, Squamata, Iguania). J. Morphol. 247: 160-171.

SHIROYA Y, HOSOYA H, MABUCHI I, SAKAI YT (1986)- Actin filament bundle in the acrosome of Abalone spermatozoa. J. Exp. Zoo. 239: 105-115.

SOLEY JT (1994). Centriole development and formation of the flagellum during spermiogenesis in the ostrich (*Struthio camelus*). J. Anat. 185: 301-313.

SPRANDO RL, RUSSEL LD (1988). Spermiogenesis in red-ear turtle (*Pseudemys scripta*) and the domestic fowl (*Gallus domesticus*): a study of cytoplasmic elimination. J. Morphol. 198: 95-118.

TABOGA SR, DOLDER H (1998). Spermiogenesis of *Scinax ranki* (Amphibia, Anura, Hylidae): nuclear compactation and associated cytoplasmic events. Braz. J. Morphol. Sci. 15: 157-163.

TALBOT P (1991). Compartimentalization in the acrosome. In: Comparative spermatology 20 years after. B. Baccetti, Ed. Raven Press, New York, pp. 255-258.

TEIXEIRA RD, VIEIRA GHC, COLLI GR, BÁO SN (1999). Ultrastructural study of spermatozoa of the neotropical lizards, *Tropidurus semiaeniatus* and *Tropidurus torquatus* (Squamata: Tropiduridae). Tissue and Cell 31: 308-317.

VAN SLUYS M (1993). The reproductive cycle of *Tropidurus itambere* (Sauria: Tropiduridae) in Southeastern Brazil. J. Herpetol. 27: 28-32.

VIEIRA GHC, WIEDERHECKER HC, COLLI GR, BÁO SN (2001). Spermiogenesis and testicular cycle of the lizard *Tropidurus torquatus* (Squamata, Tropiduridae) in the Cerrado of central Brazil. Amphibia-Reptilia 22: 217-233.

FIGURE LEGENDS

Figure 1

A. Light microscopy. Seminiferous tubules showing the interstitial tissue (it), Sertoli cell (s), spermatogonia (g), spermatocytes (c), spermatids (t) and spermatozoa (z). Bar: 20 μm. **B.** and **C.** Scanning electron microscopy. **B.** Seminiferous tubules where germ cells are organized in tufts (*).Bar: 20 μm. **C.** Higher magnification of a group of spermatozoa (z) embedded in a Sertoli cell (s). Bar: 1.85 μm. **D.** – **F.** Transmission electron microscopy. **D.** Group of spermatocytes in various division stages (arrow). Bar: 1.92 μm. **E.** Groups of

spermatocytes, with a large nucleus and many organelles such as the Golgi complex (G) and mitochondria (m). Notice the presence of cytoplasmic bridges (arrow head). Bar: 1.78 μm. **F.** Early spermatids with more compact chromatin and cytoplasm rich in endoplasmic reticulum (er) and mitochondria (m). The pro-acrosomal vesicle is in an initial stage (av). Bar: 1.78 μm.

Figure 2

Transmission electron microscopy. **A.** Early spermatid, with a large nucleus and loose chromatin. The axonemic complex is in the first developmental steps, with the proximal centriole (pc), distal centriole (dc) and annulus (an) identified. Bar: 1 μm. **B.** Higher magnification of the organelles that contribute to the formation of the pro-acrosomal vesicle (av): Golgi complex (g) and endoplasmic reticulum (er). Bar: 1 μm. **C.** An early spermatid shows a large pro-acrosomal vesicle (av), with the acrosomal granule in its interior (ag). Notice a more evident chromatin condensation around the vesicle's depression into the nucleus (arrow heads). Bar: 1 μm. **D.** A more advanced early spermatid presents chromatin in initial condensation. Structures between nucleus (n) and pro acrosomal vesicle (av) will originate the acrosomal complex including an electron-dense region that will develop into the subacrosomal cone (sc), and a clear region, precursor of the epinuclear electron-lucent region (et). In the opposite cell pole, structures that will originate the axonemic complex, such as the proximal centriole (pc), the distal centriole (dc) and the annulus (an), can be found. Bar: 1 μm. **E. - F.** Stages in chromatin condensation. The curved arrows indicate the helical arrangement of the microtubules or the chromatin, respectively. Bar: 0.25 μm. **G.** Elongating spermatid, with compacting chromatin (curved arrow), surrounded by manchette microtubules (mt). The acrosomal complex now includes the subacrosomal cone (sc), a large subacrosomal clear zone (cz) and the external acrosome (a). Bar: 0.28 μm.

Figure 3

A. Phase contrast microscopy of the mature spermatozoon. The acrosome (a) corresponds to the darker, well-formed region at the nuclear apex (n). In the midpiece (m), it is possible to observe a density variation due to the dense bodies between the mitochondria. Flagellum,

(f). Bar: 2.5 μm. **B.** Epifluorescence microscopy shows the acrosome (a) corresponding to a less evident tip, the nucleus (n) to the most brilliant portion, followed by the midpiece (m). Bar: 5 μm. Notice in **A.** and **B.** the curvature of the head. **C – L.** Transmission electron microscopy. **C.** Longitudinal section of the spermatozoon head, with a compact, very electron-dense nucleus (n). Inserted over the apex of the nucleus, the acrosomal complex is made up of different regions. Bar: 1 μm. **D. - I.** Progressive transverse sections of the acrosomal complex. Bar: 0.30 μm. **J.** Longitudinal section of the transition region between the acrosomal complex and the nucleus (n). Notice radial trabeculae (rt). Bar: 0.33 μm. **K.** and **L.** Transverse sections of the nucleus (n) surrounded by radial trabeculae (rt) and the manchette (mt) Bar: 0.27 μm. In all figures: Acrosome cortex (co), acrosome medulla (me), epinuclear electron-lucent region (et), manchette (mt), microtubule embedded in dense material (*), nuclear shoulders (ns), plasma membrane (pm), perforatorium (p), subacrosomal clear zone (cz), subacrosomal cone (sc).

Figure 4

Transmission electron microscopy. **A. - D.** Longitudinal sections of the midpiece and flagellum regions. Notice the formation of structures such as the centriole pair, the aggregation of the pericentriolar material, the differentiation of the annulus, mitochondria and the dense bodies. Bar: 0.23 μm. **E.** Transverse section of the midpiece in the initial portion of the axoneme, with peripheral fibers associated to the doublets and one of the central microtubules. Bar: 0.10 μm. **F.** Transverse section of the end of the midpiece, where the axoneme presents vestigial peripheral fibers binding to the 3rd and 8th doublets and to the fibrous sheath (arrow head). Bar: 0.11 μm. **G. - H.** Transverse sections of the flagellum, in its principal piece (fig. G), formed by a 9+2 axoneme, connected to the fibrous sheath by the 3rd and 8th doublets (arrow head) and surrounded by the plasma membrane. The fibrous sheath begins to disappear (fig. H) in the end piece where the axoneme, still with the 9+2 pattern, retains dense fibers only on the 3rd and 8th doublets, connecting them to the plasma membrane. **G.** Bar: 0.10 μm, **H.** Bar: 0.08 μm. In all figures: Annulus (an), axoneme (ax), central singlet fiber (cf), dense bodies (db), distal centriole (dc), fibrous sheath (fs),

mitochondria (m), pericentriolar material (pcm), peripheral dense fibers (pf), plasma membrane (pm), proximal centriole (pc).

Figure 1

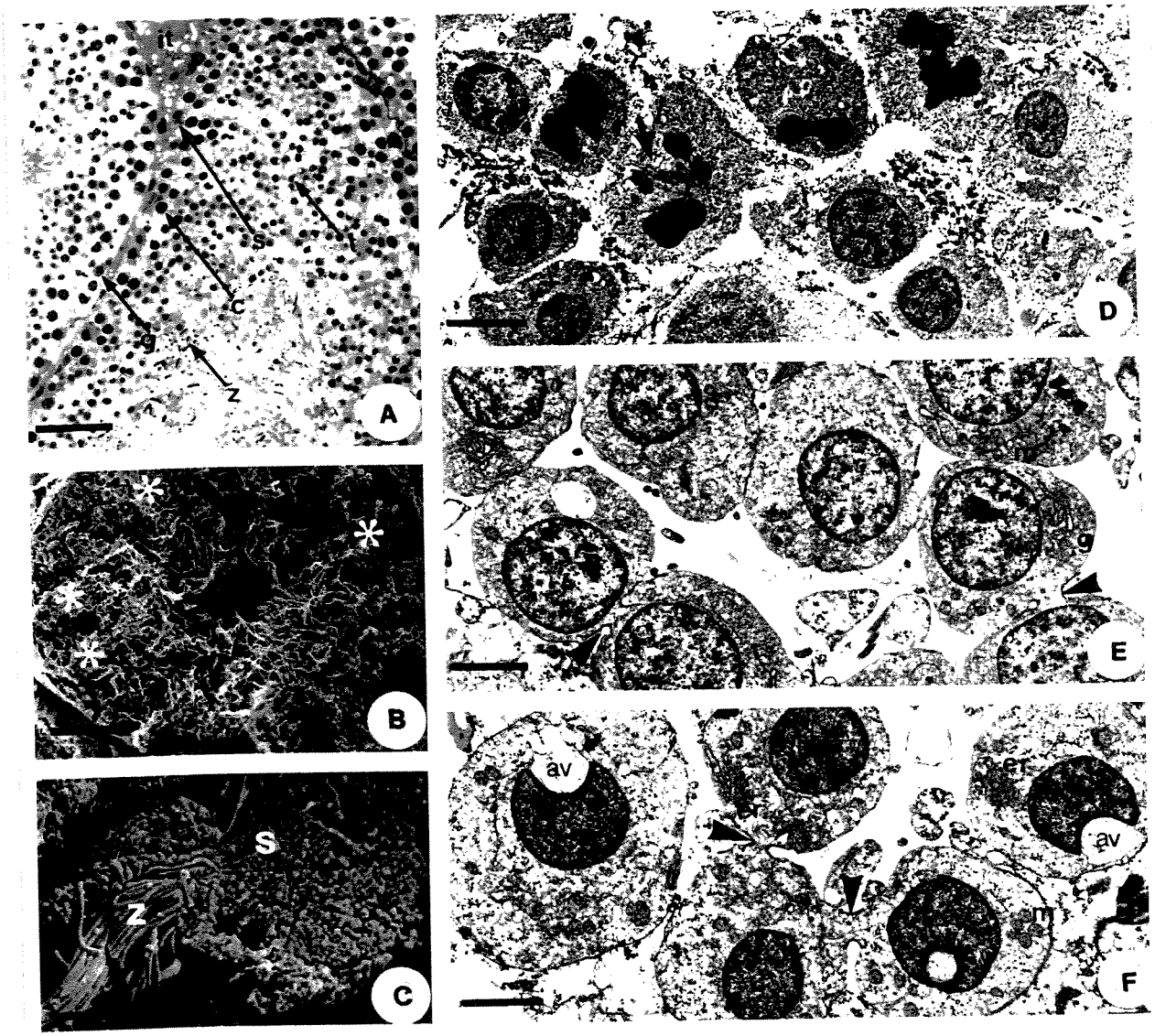

Figure 2

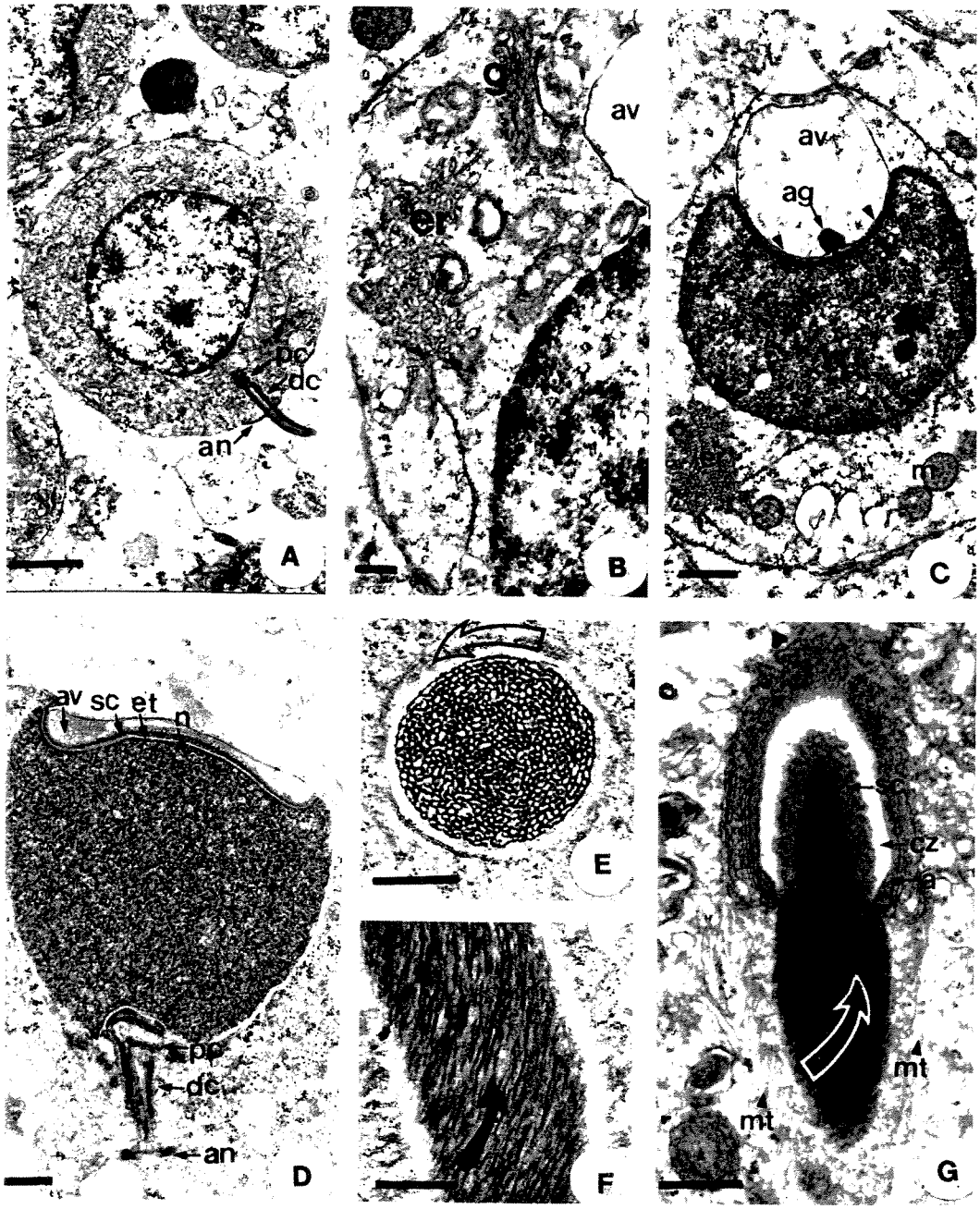

Figure 3

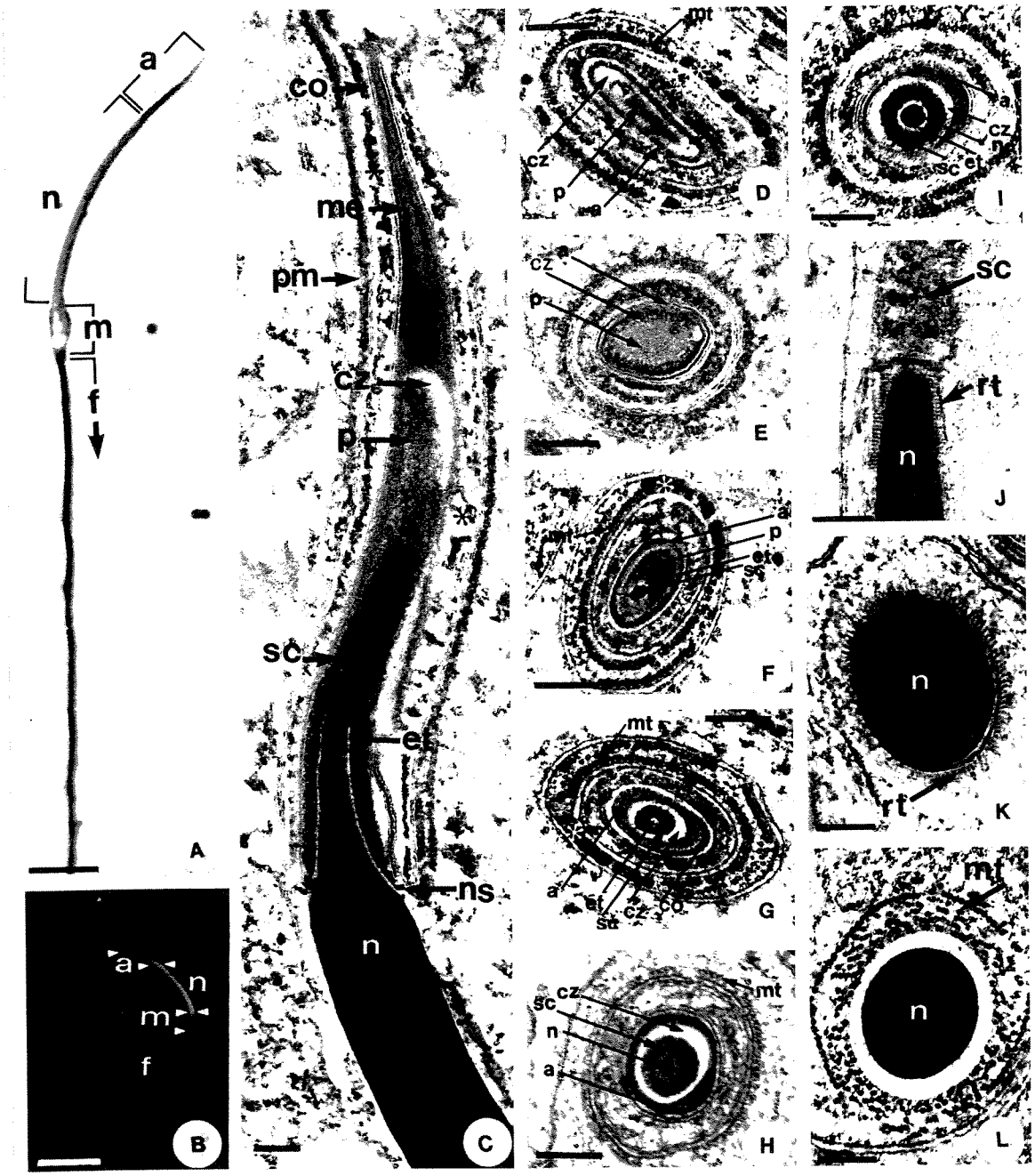
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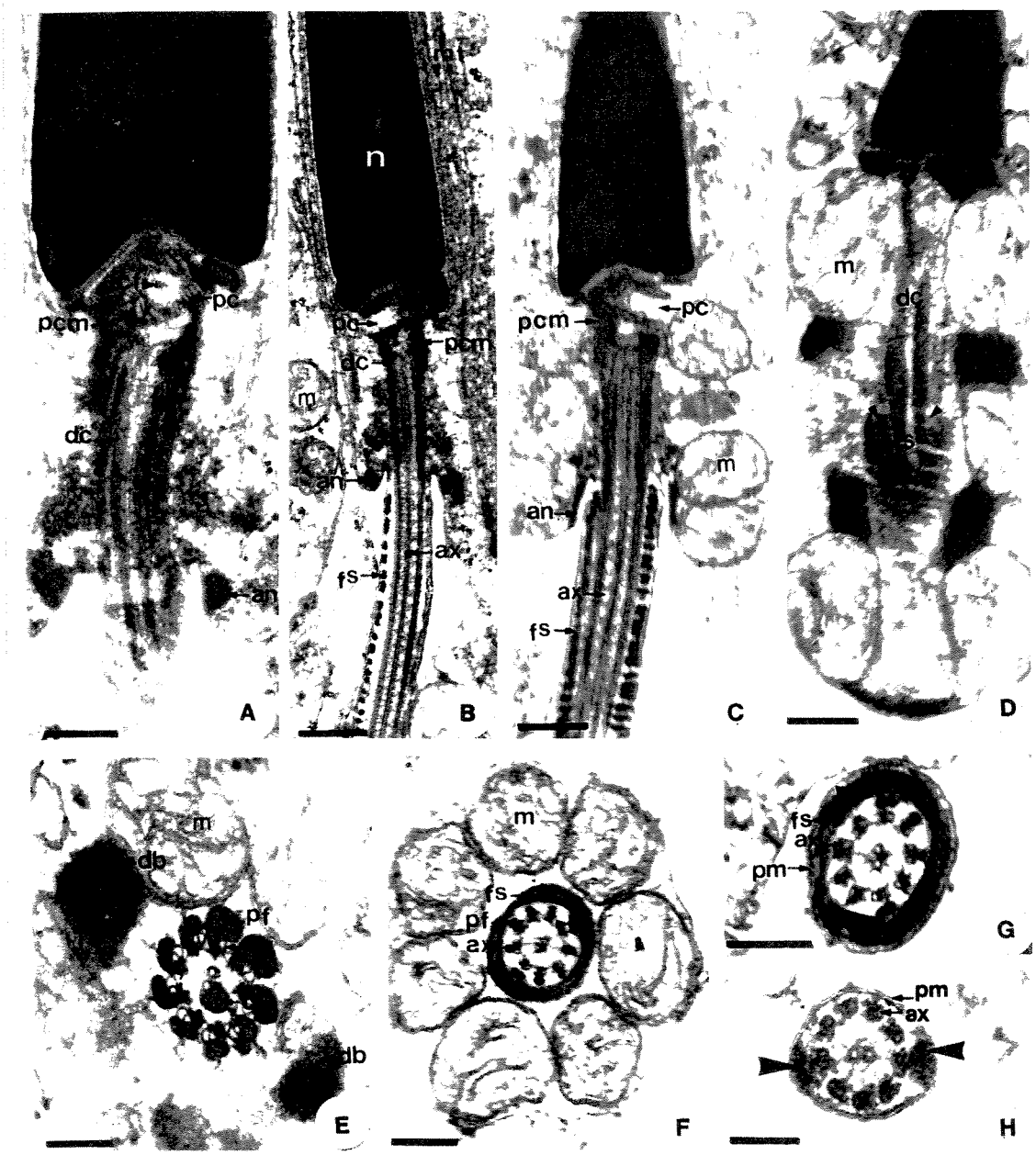
Figure 4



6.3. Histology, histochemistry and ultrastructure of the oviducts and seminal receptacle of *Tropidurus itambere* (Reptilia, Tropiduridae)

Summary. The present work shows new information regarding the histology, histochemistry and ultrastructure of the oviduct of *Tropidurus itambere*, an inhabitant of the Brazilian Atlantic forest. Three differentiated histological regions constitute the oviduct: the infundibulum has, as its main characteristic, many tubuloalveolar glands, the uterine region is remarkable for its many serous glands, just below the epithelium, and the vagina, next to the cloacal opening, is characterized by an epithelium with infolding borders and several crypts for sperm storage. The infundibulum and uterine regions were marked by the presence of acid glycosaminoglycans, demonstrated with Toluidine Blue (pH 4,0), and neutral polysaccharides, with the PAS reaction, which reacted strongly in the epithelium, and was absent in the functional units of the glands. In the sperm storage region, polysaccharides were not observed, occurring only in the epithelial unicellular glands. In all portions of the oviduct, a strong staining for proteins was obtained with Xylidine Ponceau. The ultrastructural study showed a more complex stratification of the epithelium than could be seen with light microscopy, as well as a large amount of fibers and smooth muscle in the lamina propria, that suggests flexibility. Some similarities were verified between the oviduct of the oviparous lizard, *T. itambere* and those observed in other lizards, snakes, turtles and birds. This research presents interesting differences in relation to lizards, data that may contribute to physiology studies.

Key words: oviduct, lizard, histology, histochemistry, ultrastructure, sperm storage.

Introduction

The morphological characterization of the oviducts and in particular the sperm storage structures have received the attention of many researchers. Considering reptiles (Cuellar 1966; Conner & Crews 1980; Adams & Cooper 1988; Kumari et al. 1990; Srinivas et al. 1995; Girling et al. 1997; Blackburn 1998) the characteristics of the oviducts of viviparous and oviparous lizards have been studied, and they possess some morphological similarities. Other reptiles also have been investigated in relation to sperm storage. Morphological aspects of turtle oviducts have been analyzed by Hattan & Gist (1975), Gist

& Congdon (1998), Gist et al. (2001), as well as in snakes by Hoffman & Wimsatt (1972), Halpert et al. (1982), Sever & Ryan (1999), and Sever et al. (2000). There is a consensus among the authors, that describe three distinct regions of the oviducts, including the infundibulum, next to the ovary, the uterus in the medial region and the vagina next to the cloacal opening. Morphological similarities are found in these groups, but there are differences in the distribution of the Sperm Storage Tubules (SSTs) in specific regions and the histochemical characteristics described for them. This study was undertaken to contribute to the understanding of the reproductive biology of the lizard, *Tropidurus itambere*, an inhabitant of the Atlantic forest in Southeastern Brazil. Correlations with other reptiles are made, with emphasis on other lizards, and we establish comparisons with other animal groups, such as fish, amphibians and birds. These other vertebrates, although phylogenetically distant from lizards, present various morphological characteristics of the SSTs that are very similar to those observed in *T. itambere*.

Material and methods

The adult females of *T. itambere* (N=16) used in this study were collected in the Valinhos region (23°00' S, 47°00' W), from the Atlantic forest in São Paulo state, Brazil. The animals were anesthetized by ethyl ether inhalation; the oviducts were removed and processed for the following techniques:

Histology

The tissues were fixed overnight at 4° C in a solution containing 2% glutaraldehyde, in 0.1M sodium caccodylate buffer, pH 7.2. They were dehydrated in acetone embedded in glycol methacrylate resin and the sections stained with hematoxylin and eosin. The samples were observed with a photomicroscope (Olympus, BX60) to evaluate general structure.

Histochemistry

For the histochemical study, the sections were processed as described previously and treated with: Periodic Acid Schiff for neutral polysaccharide and glycoprotein localization, Toluidine blue (pH 4.0) for glycosaminoglycan localization, and Xylidine Ponceau for proteins.

Scanning Electron Microscopy

The oviducts were fixed as above; subsequently, they were washed in buffer, embibed in (0.5M-3M) sucrose solution and fratured in liquid nitrogen. The fractured fragment were washed in buffer and post-fixed for 1 h in 1% osmium tetroxide in 0.1 M sodium caccodylate buffer, pH 7.2, followed by dehydration in acetone, critical point drying and sputter-coating with gold. They were observed with a scanning electron microscope (Jeol JSM 5800LV).

Transmission electron microscopy

The oviducts were fixed as above and embedded in LRWhite resin. The ultrathin sections were stained with uranyl acetate and lead citrate and observed with the transmission electron microscope (Zeiss, Leo 906).

Ultrastructural Cytochemistry

For basic protein detection, were block stained with a solution of 3% phosphotungstic acid in absolute ethanol for 12h at 4°C (Bloom & Aghajanian 1968).

Results

Lizard oviducts consist of three distinct regions: the infundibulum in the anterior region, (Figs. 1A-1D, 4A), the uterus in the medial region (Figs. 2A-2D, 4B) and the vagina in the posterior, next to the cloaca (Figs. 3A-3D, 4C).

The infundibulum region is characterized by the presence of a stratified epithelium, consists of two cell types, cuboidal and ciliated cuboidal cells, arranged in well defined patches. Supported by the lamina propria, which consists of fibroblast-rich connective tissue and a few blood vessels, and which are more abundant here than in other oviduct regions. Just above the lamina propria, there are tubuloalveolar glands, drained by the main excretory duct (Fig. 1A). The glands is supported by a thin layer of smooth muscle (Figs. 1B - 1C). Submitted to Toluidine blue staining (pH 4,0), the epithelium is strongly stained, specially the ducts and the basal region of the glands (Fig. 1B). When the PAS reaction was applied, the epithelium also stained strongly, however, the glands did not stain with the same intensity as observed with Toluidine blue (Fig. 1D).

The middle region of the oviduct, called the uterus, is a straight tube. The internal epithelium consists of two cell types, simple columnar and simple cuboidal secretory cells. The underlying lamina propria consists of the serous gland layer, and is limited, externally, by a narrow band of longitudinal smooth muscle and blood vessels (Figs. 2A, 2B). When stained with Toluidine Blue (pH 4,0), a strong reaction is found in the epithelial region, which is even more intense than in the infundibulum region (Fig. 2C). The same epithelial layer is stained by PAS, however, less intensely than in the infundibulum (Fig. 2D).

The vagina is located next to the cloaca opening. The lumen presents crypts, which the sperm storage tubules (SSTs) are located, and spermatozoa are inserted (Figs. 3A, 3B, 3C). Regarding its histological characteristics, the vagina can be divided in two sub-regions. The anterior region presents a stratified columnar epithelium, with groups of apical and basal ciliated cells. The epithelium, where sperm storage occurs, is supported by a small lamina propria, containing small blood vessels, and abundant smooth muscle fibers (Figs. 3D, 3E, 3F). The posterior region presents a pseudostratified columnar epithelium, which alternates with unicellular exocrine glands. Its lamina propria is not very abundant, but presents innumerable blood vessels and is supported by smooth muscle fibers (Figs. 3G, 3H). The vagina did not stain with Toluidine blue (pH4,0), however, when submitted to PAS, the reaction was intense in the unicellular glands of the posterior region (Fig. 3I).

All regions, submitted to Xylidine Ponceau showed a very homogeneous reaction, and all structures were equally stained, which demonstrates a high protein content in the oviduct and its secretions.

The ultrastructural study has corroborated some characteristics observed with light microscopy, such as, the presence of stratified epithelial cells in the infundibulum, as well as the presence of cilia and non-ciliated cells in patches (Figs. 4A). The uterine region is lined with cuboidal epithelial cells rich in superficial electron dense secretion vesicles; these epithelial cells are supported by a narrow lamina propria and collagenous fibers (Fig. 4B). The vagina presents pseudo-stratified columnar ciliated epithelium (Fig. 4C). This epithelium is supported by a lamina propria and a thick collagenous layer (Fig. 4B). The extracellular matrix, observed below the lamina propria, supported by a very large amount of smooth muscle (Fig. 4D). Innumerable spermatozoa are observed in the vaginal lumen,

which is surrounded by unicellular glands (Figs. 4C, 4F). In the region of the anterior vagina, vesicles rich in basic protein secretion can be found in the apical epithelium portion, as well as in the lumen, near the spermatozoa (Figs. 4E, 4F).

Discussion

The oviduct in Squamata has been investigated regarding its morphological and physiological characteristics. However, for the Tropiduridae family we have found no information in the literature. Considering the different lizard species, including viviparous (Girling et al. 1997; Blackburn 1998) and oviparous specimens (Conner & Crews 1980; Adams & Cooper 1988; Kumari et al. 1990; Srinivas et al. 1995) investigated up to the present, all of them present a noticeable morphological homogeneity.

A common feature in all the reptiles is that the oviduct can be divided, according to its histological characteristics, into three regions (Blackburn 1998). The most significant difference between lizards are the amount of glands, as well as the quantity of ciliated epithelial cells; these variations were also observed in *T. itambere*. One of the initial articles related to sperm storage, written by Cuellar (1966), suggests that mucous secretion occurs above the vaginal epithelium; he states that seminal receptacles only protect the sperm from mechanical damage and that their prolonged survival is assured by the mucous secretions of the vaginal epithelium. Kumari et al. (1990) observed an oviduct structure very similar to the one studied here and they also obtained similar positive results for proteins and mucopolysaccharide reactions in an agamid lizard. These observations were also made for the agamid *Psammophilus dorsalis* lizard studied by Srinivas et al. (1995), which is similar to what we observed.

According to Perkins & Palmer (1996), during the quiescent reproductive period, sperm are found in "crypt-like folds" in the anterior vagina lumen, which they called "sperm receptacles" and described as tubular exocrine glands. Sever & Hamlett (2002) observed the ultrastructural characteristics to verify the sperm-epithelial interaction during the period of sperm storage in the oviparous lizard *Anolis sagrei*. According to the above

authors, the SSTs present cuboidal cells and there are no crypts, which is also different from *T. itambere*.

Differences in the location and morphology of the SSTs among iguanid lizards and our species, *T. itambere*, can be an interesting contribution for taxonomic studies of the group. It is interesting to note that the *Tropidurus* genus was included in the Iguanidae family up to a few years ago, according to the revision of Rodrigues (1987) and Frost (1992).

In the viviparous lizards, changes in oviduct morphology occur after and during the vitellogenic period (Girling et al. 1997; Blackburn 1998), but in *T. itambere,* studied throughout the annual reproductive cycle, no changes occurred. According to Girling et al. (1997) in viviparous lizards, the infundibulum is covered by many ciliated epithelial cells with abundant secretion, even more abundant than observed for *T. itambere*. Our results showed that the apical epithelium and the basal portions of the glands produced acid glycosaminoglycans, while the epithelium also has a neutral glycoprotein content. According to Girling et al. (1997) the uterus presents mucous cells, which is also different from the observations made here. *T. itambere* possess a squamous secretory uterine epithelium similar to that which appears in the gestational period of viviparous lizards. This region has glandular cells, as observed with the electron microscopy, producing acid glycosaminoglycans and neutral glycoproteins according to histological evidence.

Considering the variety of glands observed in the oviduct passage, some conclusions may be made. The tubular glands release an albumen-like secretion (Blackburn 1998). Histological and histochemical research concluded that single cell oviduct glands produce a mucous substance that promotes an adequate medium or sperm sustenance (Blackburn 1998). This is in agreement with our observations.

The microvasculature, characteristic of *T. itambere*, was also observed in viviparous lizards, where it could furnish oxygen to the embryo (Girling et al. 1997; Blackburn 1998). While this interpretation appears appropriate for these lizards, we believe that the network in *T. itambere* could also contribute to the nutrition of sperm stored in the SSTs.

Retention of sperm with a fertilizing capacity has been demonstrated for periods ranging from 5 months in certain species, to nearly a year in fish (Fox 1977). Extreme

examples of storage for periods of 4 to 6 years were reported for turtles and snakes, but this seems to be exceptional among vertebrates (Fox 1977).

The reproductive cycle of the lizard *T. itambere* was studied in an open area near Campinas, Sao Paulo State, southeastern Brazil. Females had vitellogenic follicles or oviductal eggs only during the wet season, whereas males had large testes with spermatozoa throughout the year. Hatchlings were found from the height of the rainy season to its end (January to May). Mean growth rate was inversely related to snout-vent length. Juveniles from eggs laid at the beginning of the rainy season could potentially have reproduced in the first wet season after they hatched (Van Sluys 1993).

The similarity of reproductive characteristics in lizards and birds is perhaps another example of convergence due to similar functional adaptations and the design limitations for the vertebrate's oviduct and sperm (Sever et al. 2000). As with other morphological characters, the discernment of subsequent specializations, which are phyletically relevant from those that are homoplastic, will be a challenge (Sever & Hamlett 2002).

Variations can occur regarding the accurate localization of the SSTs in lizards, which may be at the junction of uterus and vagina (Conner & Crews 1980; Adams & Cooper 1988; Blackburn 1998) or in the anterior region of the vagina (Cuellar 1966; Kumari et al. 1990; Srinivas et al. 1995), as also observed in *T. itambere.*

Sperm storage in *T. itambere* is in agreement with observations in numerous Squamata where spermatozoa are stored in the vaginal region, in crypts among short ciliated tubules formed by epithelial folding (Blackburn 1998).

In snakes (Hoffman & Wimsatt 1972; Halpert et al. 1982; Sever & Ryan 1999) and certain gekkonid lizards (Girling et al. 1997; Blackburn 1998), other types of sperm receptacles are found in the vicinity of the posterior infundibulum. In snakes, there is a rather dense secretion in the SSTs (Sever & Ryan 1999; Sever et al. 2000), which does not occur in *T. itambere*. In turtles, all the morphological characteristics described for the oviducts are very similar to those of *T. itambere* (Gist et al. 2001).

Descriptions of seminal receptacles and sperm storage are known for other animal groups, such as amphibians (Sever & Brizzi 1998; Wake & Dickie 1998; Sever 2002), fish (Hamlett et al. 2002; Koya et al. 2002), and birds (Bakst 1998; Holm & Ridderstrale 2002).

In this literature, we find some similarities regarding the morphology and functionality, especially between our model, turtles and birds. Considering the amount of glands and the identification of polysaccharides, the oviducts of *T. itambere* are similar to Amphibia (Massood Parveez & Nadkarni 1991). Our cytochemical reactions in the seminal receptacle of *T. itambere* revealed large amounts of unicellular glands, strongly stained for neutral glycoproteins and basic proteins, as shown by PAS and phosphotungstic acid techniques.

From the male's perspective, sperm storage confers greater opportunities, as well as being economical. Whereas sperm storage usually is thought of as a female adaptation that separates copulation from nesting, it encourages sperm competition, or allows for cryptic female choice (Olsson et al. 1994).

The production of sperm that can survive long-term storage in females clearly benefits successful males by increasing the number of eggs that they may fertilize (Pearse & Avise 2001).

Hypotheses have associated this mechanism to a temporal separation between mating and fertilization, thus optimizing male and female reproductive cycles (Girling et al. 1997). Sperm storage is such an intrinsic part of the reproductive cycle of reptiles that the phenomenon deserves attention in any study on reptilian natural history (Sever & Hamlett 2002). The elaborate sequence of sperm transport and the specialized structures for sperm storage represent crucial functional adaptations of the female (Halpert et al. 1982), and may characterize important adaptations of a species to environmental conditions (Ferreira et al. 2002).

According to Conner & Crews (1980), the formation of the tubules by fusion of longitudinal folds in the storage tubules, suggests a mechanism for sperm transport into the tubules, that is, by traveling along the folds, sperm may be funneled directly into the tubules.

The intimate association of sperm with the receptacle cells, seen ultrastructurally, suggests a nutritional mechanism based on exocrine secretions. Most sperm heads, and some mid pieces as well, are in close contact with the receptacle cell surface and often indent the cells to varying degrees. Such a relationship is similar to observations made

between maturing spermatids and Sertoli cells in various vertebrate testes (Hoffman & Wimsatt 1972).

Sperm storage should be considered in experiments designed to determine male precedence in fertilization, so as to allow adequate time for sperm transport to the infundibulum (Olsson et al. 1994; Pearse & Avise 2001).

No chemical attraction seems to be involved, since the secretions of the lining and the glands are similar in the different regions of the oviduct (Sever & Ryan 1999), as was also found for *T. itambere*.

The receptacles not only offer a physical refuge for the sperm, but they could also be of physiological importance for their nutrition or chemical arrest of activity. The frequent occurrence of compact bundles of neatly arranged sperm is in agreement with the concept that concentrated sperm masses tend to display reduced activity with a consequent conservation of energy (Fox 1977).

Much more work combining morphological, ecological, and genetic data is necessary before we can elucidate the relationship between sperm storage and sperm competition in any reptile.

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References

Adams CS, Cooper WE (1988) Oviductal morphology and sperm storage in the keeled earless lizard, *Holbrookia propinqua*. Herpetologica 44: 190-197

Bakst MR (1998) Structure of the avian oviduct with emphasis on the sperm storage in Poultry. J Exp Zool 282: 618-626

Blackburn DG (1998) Structure, function, and evolution of the oviducts of squamate reptiles, with special reference to viviparity and placentation. J Exp Zool 282: 560-617

Bloom FE, Aghajanian GK (1968) Fine structural and cytochemical analysis of the staining of synaptic junctions with phosphotungstic acid. J Ultrastruct Res 22: 361-371

Conner J, Crews D (1980) Sperm transfer and storage in the lizard, *Anolis carolinensis*. J Morphol 163: 331-348

Cuellar O (1966) Oviducal anatomy and sperm storage structures in lizards. J Morphol 119: 7-20

Ferreira A, Laura IA, Dolder H (2002) Reproductive cycle of male green guanas, *Iguana iguana* (Reptilia, Sauria, Iguanidae), in the Pantanal region of Brazil. Braz J Morphol Sci 19: 23-28

Fox H (1977) The urogenital system of reptiles. In: Gans C (Ed) Biology of Reptilia, vol. 6. Academic Press, New York, pp. 329-491

Frost DR (1992) Phylogenetic analysis and taxonomy of the *Tropidurus* groups of lizards (Iguania: Tropiduridae). Am Mus Novitates 3033: 1-68

Girling JE, Cree A, Guillette LJ (1997) Oviductal structure in a viviparous New Zealand gecko, *Hoplodactylus maculatus*. J Morphol 234: 51-68

Gist DH, Congdon JD (1998) Oviductal sperm storage as a reproductive tactic of turtles. J Exp Zool 282: 526-534

Gist DH, Dawes SM, Turner TW, Sheldon S, Congdon JD (2001) Sperm storage in turtles: a male perspective. J Exp Zool 292: 180-186

Halpert AP, Garstka WR, Crews D (1982) Sperm transport and storage and its relation to the annual sexual cycle of the female red-sided garter snake, *Thamnophis sirtalis parietalis*. J Morphol 174: 149-159

Hamlett WC, Reardon M, Clark J, Walker TI (2002) Ultrastructure of sperm storage and male genital ducts in a male holocephalan, the elephant fish, *Callorhynchus milii*. J Exp Zool 292: 111-128

Hattan LR, Gist DH (1975) Seminal receptacles in the eastern box turtle, *Terrapene carolina*. Copeia 3: 505-510

Hoffman LH, Wimsatt WA (1972) Histochemical and electron microscopic observations on the sperm receptacles in the garter snake oviduct. Am J Anat 134: 71-96

Holm L, Ridderstrale Y (2002) Development of sperm storage tubules in the Quail during sexual maturation. J Exp Zool 292: 200-205

Koya Y, Munehara H, Takano K (2002) Sperm storage and motility in the ovary of the marine sculpin *Alcichthys alcicornis* (Teleostei: Scorpaeniformes), with internal gametic association. J Exp Zool 292: 145-155

Kumari TRS, Sarkar HBD, Shivanandappa T (1990) Histology and histochemistry of the oviductal sperm storage pockets of the agamid lizard *Calotes versicolor*. J Morphol 203: 97-106

Massood Parveez U, Nadkarni VB (1991) Morphological, histological, histochemistry and annual cycle of the oviduct in *Ichthyophis beddomei* (Amphibia: Gymnophiona). J Herpetol 25: 234-237

Olsson M, Gullberg A, Tegelström H (1994) Sperm competition in the sand lizard, *Lacerta agilis*. Anim. Behav. 48: 193-200

Pearse DE, Avise JC (2001) Turtle mating systems: behavior, sperm storage and genetic paternity. Am Gen Assoc 92: 206-211

Perkins MJ, Palmer BD (1996) Histology and functional morphology of the oviduct of an oviparous snake, *Diadophis punctatus*. J Morphol 217: 205-217

Rodrigues MT (1987) Sistemática, ecologia e zoogeografia dos Tropidurus do grupo Torquatus ao sul do rio Amazonas (Sauria: Iguanidae). Arq Zool SP 31: 105-230

Sever DM (2002) Female sperm storage in Amphibians. J. Exp. Zool. 292: 165-179

Sever DM, Brizzi R (1998) Comparative biology of sperm storage in female Salamanders. J Exp Zool 282: 460-476

Sever DM, Hamlett WC (2002) Female sperm storage in reptiles. J Exp Zool 292: 187-199

Sever DM, Ryan TJ (1999) Ultrastructure of the reproductive system of the black swamp snake (*Seminatrix pygaea*): Part I. Evidence for oviducal sperm storage. J Morphol 241: 1-18

Sever DM, Ryan TJ, Morris T, Patton D, Swafford S (2000) Ultrastructure of the reproductive system of the black swamp snake (*Seminatrix pygaea*). II. Anual oviductal cycle. J Morphol 245: 146-160

Srinivas SR, Shivanandappa T, Hedge SN, Sarkar HBD (1995) Sperm storage in the oviduct of the tropical rock lizard, *Psammophilus dorsalis*. J Morphol 224: 293-301

Van Sluys M (1993) The reproductive cycle of *Tropidurus itambere* (Sauria: Tropiduridae) in Southeastern Brazil. J Herpetol 27: 28-32

Wake MH, Dickie R (1998) Oviduct structure and function and reproductive modes in Amphibians. J Exp Zool 282: 477-506

Legends of the figures

Fig. 1. The infundibulum. **A.** Scanning electron microscopy shows tubuloalveolar glands and the secretion vesicles on the epithelium surface (s). Bar= 7.1μm. **B.** Light microscopy, with Toluidine blue (pH 4,0) intensely staining the epithelium and the basal portion of the glands. Bar= 25μm. **C.** Light microscopy, stained with Hematoxylin and Eosin. Bar= 12.5μm. **D.** Light microscopy, Periodic Acid Schiff reaction intensely stains the epithelial layer. Bar= 12.5μm. In all figures: Tubuloalveolar glands (tg), lamina propria (lp), epithelium (e), muscle (m), veins (v).

Fig. 2. The uterus. **A.** Scanning electron microscopy. **B.** Light microscopy, stained with Hematoxylin and Eosin. **C.** Light microscopy, stained with Toluidine blue (pH 4,0), to show the intense staining in the epithelium. **D.** Light microscopy, with Periodic Acid Schiff intensely stained the epithelium surface and folds (arrows). In all figures: Epithelium (e), serous glands (sg), muscle (m), veins (V). Bar= 12.5μm.

Fig. 3. The vagina. **A. - C.** Scanning electron microscopy. Observe the crypt-rich epithelium where sperm storage occurs (*). The vagina can be divided into two sub-regions, anterior and posterior, where the former is a seminal receptacle. The anterior, as well as the posterior region of the vagina, presents pseudo-stratified cuboidal ciliated

epithelium (arrow heads). The epithelium is supported by a small lamina propria and muscle fibers. **A.** Bar= 1μm. **B.** Bar= 8.3μm. **C.** Bar= 5μm. **D. - F.** Anterior region of the vagina presents many crypts and in this region spermatozoa are observed more frequently (*). **D.** Bar= 1μm. **E.** Bar= 25μm. **F.** Bar= 12.5μm. **G. - I.** Posterior region of the vagina presents many unicellular glands in the epithelium (arrows), which reacted positively to the PAS technique (fig. I). The lamina propria presents many blood vessels (v). **G.** Bar= 33μm. **H.** Bar= 12,5μm. **I.** Bar= 25μm. In all figures: epithelium (e), lamina propria (lp), muscle (m).

Fig. 4. Transmission electron microscopy. **A.** The infundibulum. Stratification of the epithelium is much more elaborate than was discernable with light microscopy. The epithelial cells (e) occur in patches of ciliated (arrows) or smooth surfaces (*).Bar= 3.3μm. **B.** The uterus. The apical region of the epithelial cells (e) is filled with large electron dense secretion vesicles (s). Lamina propria (lp) rich in collagens fibers and fibroblasts (f). Bar= 2.5μm. **C.** The vagina (or seminal receptacle). Pseudo-stratified cuboidal ciliated epithelium. Unicellular glands (s) occur between the epithelial cells; and many spermatozoa in the lumen (z). Bar= 2.5μm. **D.** Extracellular matrix of the all region, including an abundance of smooth muscle cells (M). Bar= 4..2μm. **E. - F.** The secretory vesicles (s) in the vaginal epithelium (e) and in the lumen are positive when stained by the ethanol-phosphotungstic acid technique for basic proteins. **E.** Bar= 2μm. **F.** Bar= 1μm.

Figure 1

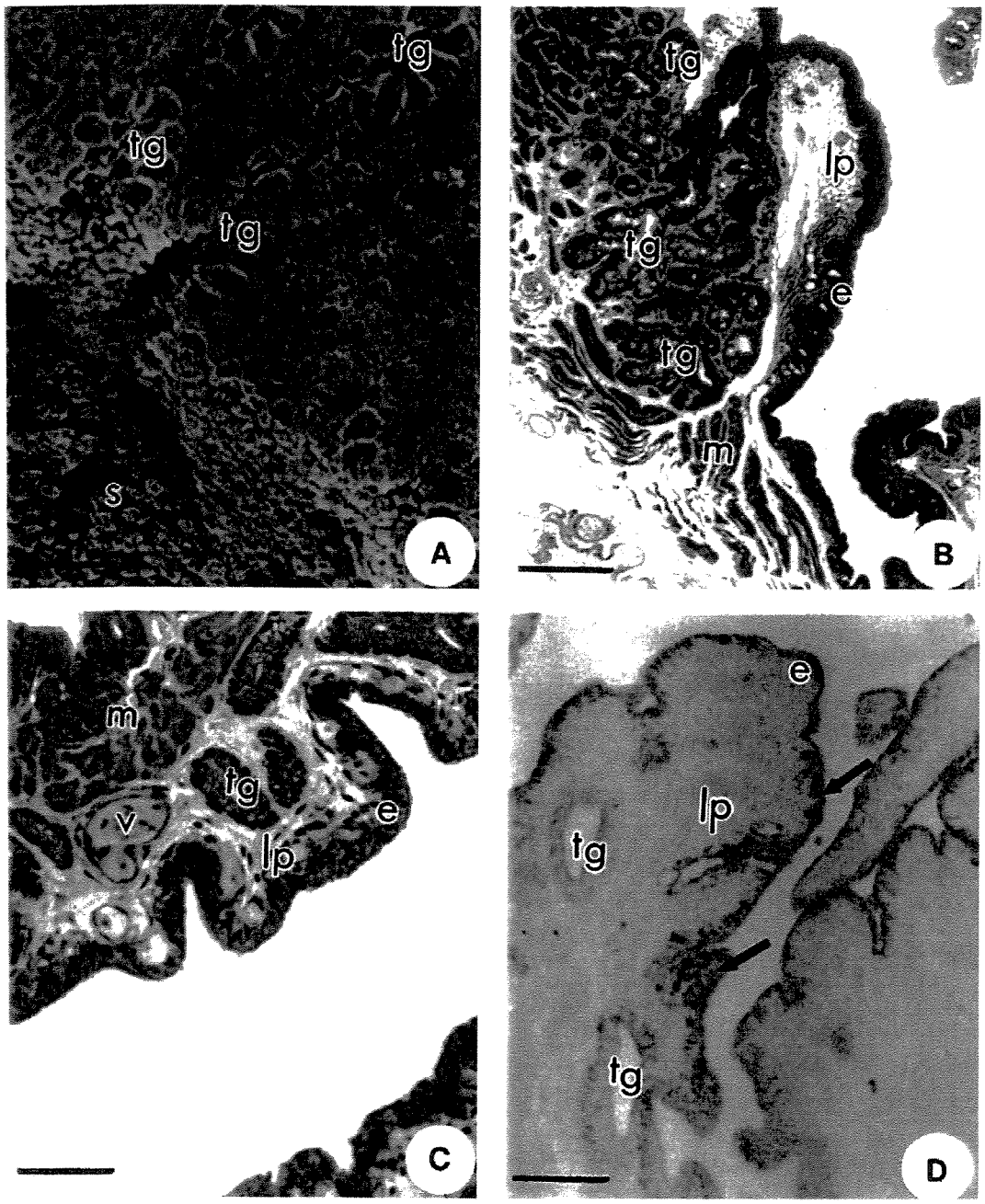


Figure 2

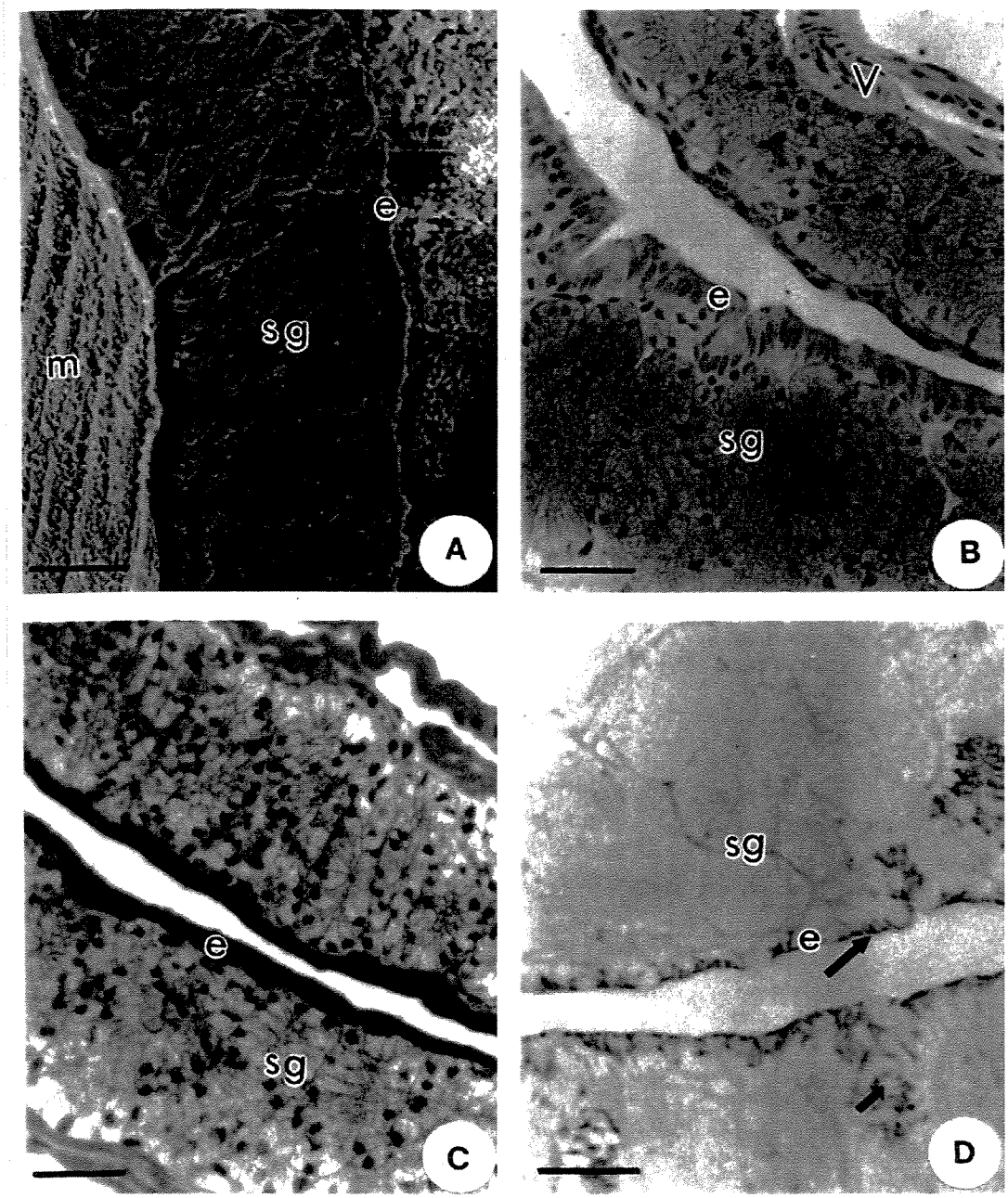


Figure 3

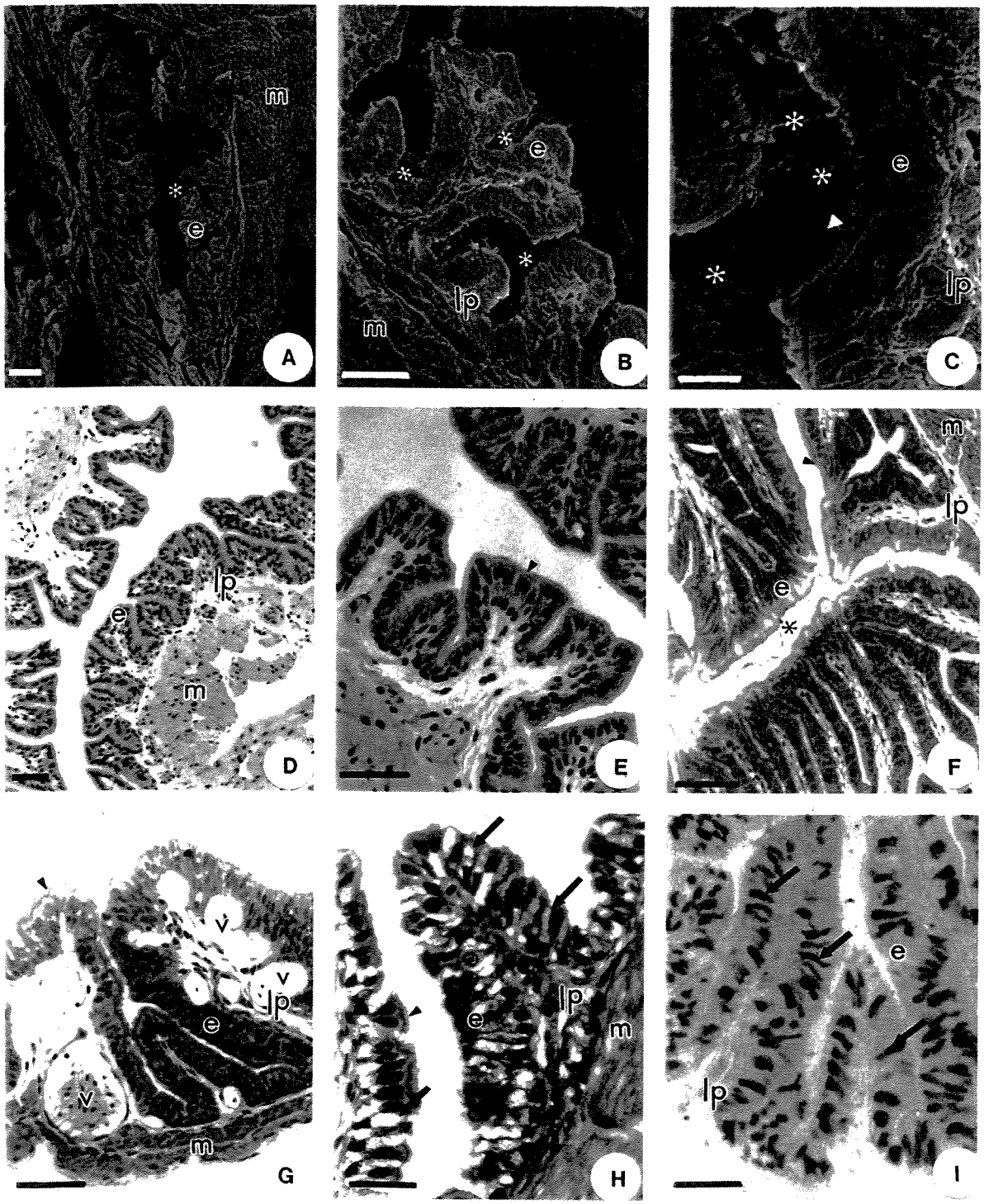
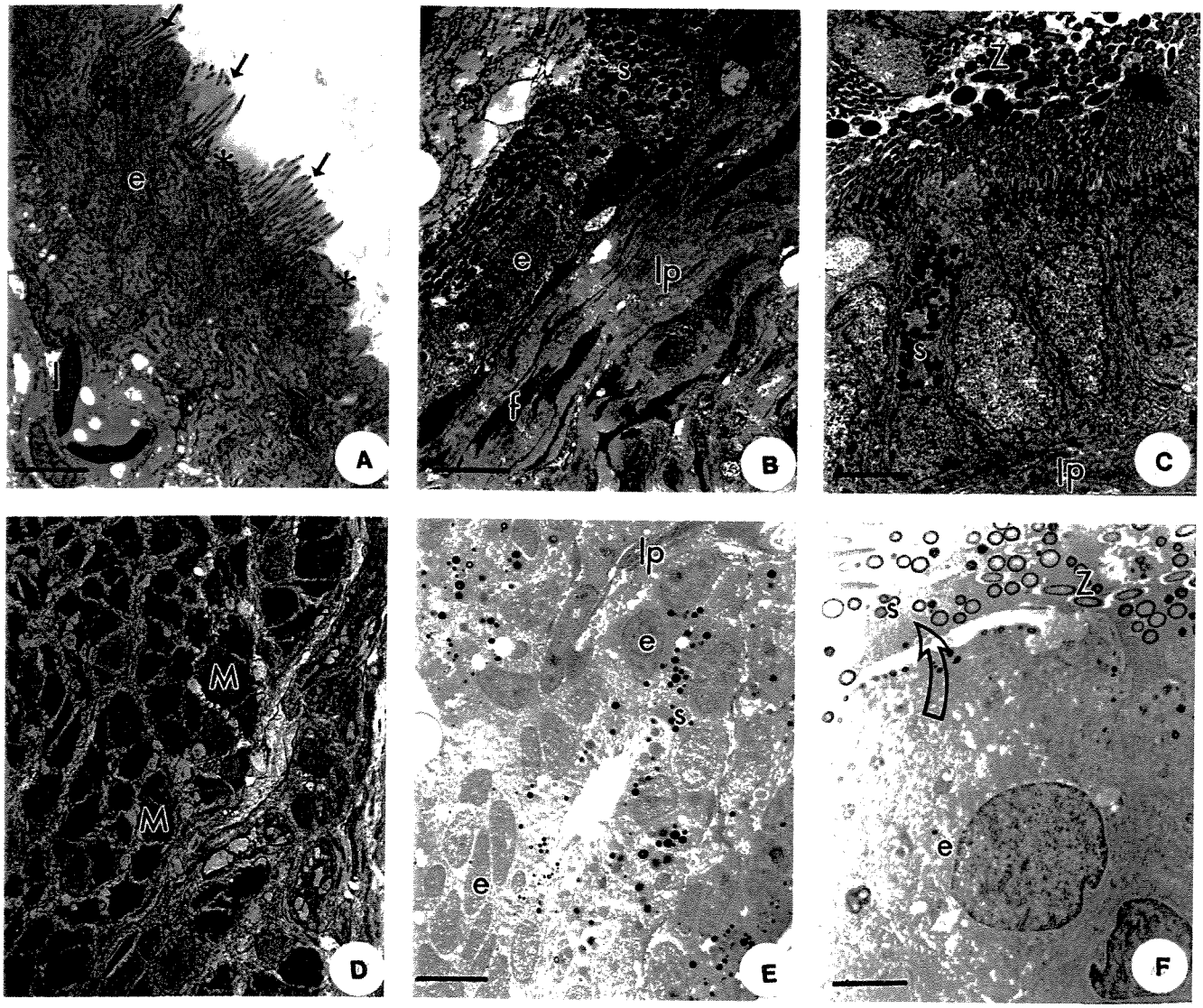
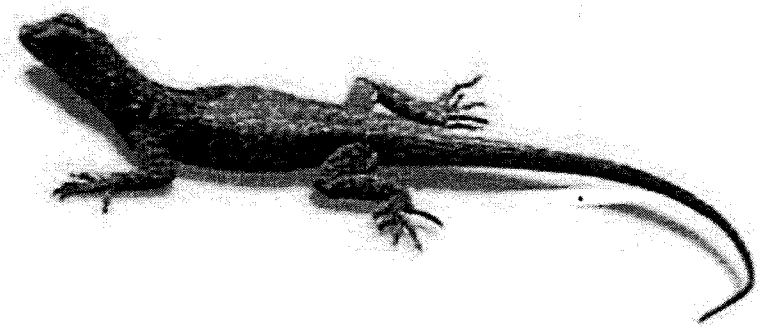


Figure 4



6.4. Cytochemical study of the spermiogenesis and mature spermatozoon of the lizard, *Tropidurus itambere* (Reptilia, Squamata)



Summary

The present study was undertaken to detect essential components of spermatozoa by ultrastructural and cytochemical analyses of testis and epididymis of the lizard *Tropidurus itambere* at different points in its annual reproductive cycle. Essential components are: 1) polysaccharides, identified by PAS staining, abundantly present in Sertoli cell elongations, in acrosomal vesicles and in the acrosome of sperm cells; 2) glycoconjugate variations, labeled by different lectins and used to investigate cell modifications during spermiogenesis and found in mature spermatozoa in the female's seminal receptacle; 3) basic proteins, present in large quantities in spermatozoa in the subacrosomal cone, the pericentriolar material, the midpiece dense bodies, the peripheral fibers of the axoneme, and the fibrous sheath of the flagellum; 4) the final reaction product of acid phosphatase reativity in several stages of acrosome development, specifically in the clear zone and in the epinuclear electron-lucent region of the spermatozoal acrosomes, as well as in very active lysosomes found during the quiescent period of the reproductive cycle; 5) lipids, abundantly present in the cytoplasm of Leydig cells during the quiescent period. The cytochemical methods show that the ultrastructurally complex acrosome is also biochemically different, with specific layers rich in glicoproteins, basic proteins or acid phosphatase. These different components may possibly act in sequence during sperm penetration into the ovule. Basic proteins are largely responsible for structures surrounding the axoneme which convey resistance to the flagellum. In the quiescent period, acid phosphatase was related to the elimination of unnecessary spermatic cells, while the lipids in Leydig cells are used in hormone production which begins at this time to stimulate a new reproductive cycle. Variations in lectin revealed glycoconjugates show that spermatozoa undergo post-testicular maturation up to their storage in the female. Cytochemical investigations of spermiogenesis have not been performed so for in Squamata and are scarce for the lower orders of vertebrates.

Keywords: cytochemistry - lectin - ultrastructure - spermiogenesis - spermatozoon - lizard

Introduction

Spermiogenesis in lizards undergoes an annual variation with active and quiescent periods, and these have been investigated with cytochemical methods. The process of spermiogenesis is essential for successful reproduction, and the lizard's spermatozoon is one of the most specialized animal cells envolved in fertilization (Fawcett et al., 1971). Spermiogenesis in lizards has been described by various authors (Clark, 1967; Butler and Gabri, 1984; Courtens and Depeiges, 1985; Al-Hajj et al., 1987; Ferreira and Dolder, 2003) and special attention has been given to the lizard *Tropidurus torquatus* (Cruz Landim and Cruz Höfling, 1977; Cruz Höfling and Cruz Landim, 1978; Vieira et al., 2001). Spermiogenesis in lizards includes processes such as nuclear elongation, chromatin condensation, formation of the acrosomal complex, and formation of the flagellum and accessory structures.

The spermatozoon is a highly specialized cell in terms of fertilization and transfer of genetic material and is formed in the interior of the testicles through the process of spermatogenesis. Afterwards, spermatozoon maturation still proceeds along the reproductive tract where new surface molecules are added.

In the case of Squamata, storage of spermatozoa takes place in a specialized organ known as the seminal receptacle (Cuellar, 1966; Blackburn, 1998). This organ has its counterpart in both sexes, being present in females in the lower and medium region of the oviducts (Cuellar, 1966; Conner and Crews, 1980; Adams and Cooper, 1988; Kumari et al., 1990; Srinivas et al., 1995; Blackburn, 1998). An important step in the capacitation of spermatozoa occurs at the molecular level in this organ. However, the mechanism is still not completely understood.

Storage of spermatozoa in the seminal receptacle has already been described in lizards, snakes (Hoffman and Wimsatt, 1972; Olsson et al., 1994), and turtles (Pearse and Avise, 2001; Pearse et al., 2002). Sperm storage is considered to be an important evolutionary adaptation (Olsson et al., 1994). Little is known about morphological and functional characteristics of spermatozoa and even less about changes in spermatozoa during their journey from the testis to seminal receptacle.

Although a certain variability has been found, spermatozoa of Squamata show a surprisingly constant ultrastructure in different families (Jamieson, 1995). This aspect warrants the study of germ cell components that are essential for fertilization. These compounds have not yet been described in lizards. Furthermore, specific sugars present in spermatids and spermatozoa in the interior of the testis and in the seminal receptacle have been identified in the lizard *Tropidurus itambere* in the present study.

Material and methods

Adult *Tropidurus itambere* (Reptilia, Squamata, Tropiduridae) lizards were collected in their natural habitat in the Atlantic Forest in the Valinhos region (23°00'S, 47°00'W) in São Paulo State, Brazil, at monthly intervals between June 2001 and June 2002. The animals were killed by ethyl-ether inhalation, and testis and epididymis of males as well as seminal receptacle of females were removed by dissection.

1) Light microscopy

The organs were fragmented and fixed overnight at 4° C in a solution containing 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Tissue fragments were dehydrated in acetone and embedded in LR White resin. Sections (0,5µm) were stained with hematoxilin-eosin and observed with a BX60 photomicroscope (Olympus, Tokyo, Japan) to evaluate general morphological structures.

2) Transmission electron microscopy

Tissue fragments were also fixed overnight for electron microscopical purposes at 4° C in a solution containing 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, washed in buffer, dehydrated in acetone, and embedded in LR White resin.

3) Structural and ultrastructural cytochemistry

For polysaccharide detection, thin sections (0,5µm) were stained with the periodic acid Schiff (PAS) technique. Ultrathin sections (60nm) were collected on gold grids, incubated in periodic acid and 1% thiosemicarbazide in 10% acetic acid, rinsed several times in 2-10% acetic acid and 1% silver proteinate (Fluka; Buchs, Switzerland), and observed without further staining of the sections (Thièrry, 1967).

For glycogen detection, ultrathin sections were collected on nickel grids, then stained with 1% tannic acid in distilled water and 4% uranyl acetate (Afzelius, 1992).

For detection of acid phosphatase activity, the testis were briefly fixed (15 min at 4°C) in 1% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, washed with buffer, and incubated for 1 h at 37°C in 0.1 M Tris maleate buffer, pH 5.0, 7 mM cytidine-5-monophosphate, 2 mM cerium chloride, and 5% sucrose (Pino et al., 1981).

For basic protein detection, tissue fragments of testes were stained with a solution of 3% phosphotungstic acid in absolute ethanol for 12 h at 4°C (Bloom and Aghajanian, 1968).

For lipid detection, testes were fixed for 48 h at 4°C in a solution of 2% osmium tetroxide in distilled water. Tissue fragments were stained for 2 h at room temp. with 2% uranyl acetate (Hayat, 1993). Other testis fragments were fixed for 30 min in a solution of 2% glutaraldehyde in 0.1 M imidazole buffer, rinsed in the same buffer, and postfixed in 1% osmium tetroxide also in the same buffer (Angermuller and Fahimi, 1984).

For detection of carbohydrates, testes and seminal receptacles were fixed for 3 h at 4°C in a solution of 0.5% glutaraldehyde, 4% paraformaldehyde, 0.2% picric acid, 3.5% sucrose, and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer at pH 7.2. After several rinses in the same buffer, free aldehyde groups were quenched with 50 mM ammonium chloride in 0.1 M sodium cacodylate buffer for 1 h. After dehydration in acetone and embedding in LR White resin, the blocks were polymerized during 72 h in closed vials at 37°C. Ultrathin sections were collected on nickel grids, pre-incubated in phosphate buffered saline (PBS) containing 1.5% bovine serum albumin (PBS-BSA) and 0.01% Tween 20, and subsequently incubated for 1 h at room temp. in the presence of gold-labeled lectin in PBS-BSA, pH 8.0. The dilutions of the gold labeled lectins were 1:10 for HPA and 1:5 for GS-I, PNA, WGA, UEA (Table 1). After incubation, grids were washed first with PBS, then in distilled water, and finally stained with uranyl acetate and lead citrate. The lectins (Sigma; St. Louis, Mo., USA) were labeled with colloidal gold particles (8-10 nm) according to Roth (1983). Controls incubations were in the presence of 200 mM of the corresponding monosaccharides in the incubation media

All materials used for cytochemical analysis were embedded and sectioned as described above; sections were observed without further staining. All observations were performed using a Leo 906 transmission electron microscope (Zeiss, Oberkocher, Germany).

Results

The arrangement of germ cells in seminiferous tubules varied considerably throughout the annual reproductive cycle. During the reproductive period, innumerable spermatogonia were present in the basal region and were topped by a layer of spermatocytes and then by layers of spermatids and spermatozoa in the lumen (Fig. 1a). Cells penetrated deep foldings of cytoplasmic elongations of Sertoli cells (Fig.1a). During the reproductive period, these elongations extended from the basal region to the lumen and were rich in polysaccharides, probably glycoproteins (Fig. 1b). During the quiescent period, all cell types were present as detected with light microscopy. However, they appeared to be present in a loose and disorganized manner in the lumen of seminiferous tubules (Fig. 1c). Some cells were still embedded in cytoplasmic elongations of Sertoli cells but in a more dispersed arrangement (Fig. 1d). Polysaccharides of cytoplasmic elongations of Sertoli cells appeared to be glycoconjugates (Fig. 1e) and glycogen (Fig. 1f). Glycoproteins were present in pro-acrosomal vesicles (Fig. 1g), and glycogen in cortical regions of the perforatorium in the acrosomal complex (Fig. 1h).

Testicular spermatids and spermatozoa contained binding sites for D-galactose, N-acetyl-D-glucosamine, L-fucose, α-galactose/α-galactosamine and N-acetyl-α-galactosamine. The acrosomal complex was the most frequently marked structure. Labeling was also found in the nucleus, midpiece, and flagellum. Results are summarized in Table 1. In spermatids, HPA staining was found in the pro-acrosomal vesicle only (Fig. 2a-e) and GS-I in the periphery of the nucleus during chromatin compaction (Fig. 3a). In testicular spermatozoa, parts of the acrosome were intensely stained by HPA (Fig. 2f) in a similar way as the spermatozoal acrosome in seminal receptacles of females (Fig. 2g, h), demonstrated by the presence of N-acetyl-α-galactosamine residues. Large amounts of

these residues were also found in secretions involved in storage of spermatozoa in seminal receptacles (Fig. 2h). In the nuclear periphery of spermatids, on microtubules forming the so-called "manchette" and in the nucleus and implantation fossa of testicular spermatozoa, α-galactose/α-galactosamine residues were stained with GS-I (Fig. 3b, g). D-galactose was stained with PNA and was present in large amounts in the nucleus of spermatids as well as in spermatozoa of testes and seminal receptacles (Fig. 3c-f). The neck region of spermatozoa, a region rich in sugars, peripheral proteins of the centriole and dense bodies were also intensely stained (Fig. 3c). Flagella of spermatozoa in all regions of the male and female tract showed residues of L-fucose (UEA-I), N-acetyl D-glucosamine (WGA) in the fibrous sheath region (Fig. 3h, i).

Surprisingly, basic proteins were found in early spermatids with heavy staining of the dense granule located in the pro-acrosomal vesicle and with lighter staining of the loose chromatin (Fig. 4a). Basic proteins were also detected in cortical regions of the acrosome (Fig. 4c) of spermatozoa, and they were intensely stained in the subacrosomal cone (Fig. 4f, g). In the midpiece, these proteins were accumulated in pericentriolar material in a central dense fiber and in 9 peripheral dense fibers surrounding the axoneme. The strongly stained dense bodies were modified mitochondria appearing between normal mitochondria, which do not stain (Fig. 4b, d, e). At the end of the midpiece, the annulus was also stained strongly for basic proteins (Fig. 4b). In the flagellum, the initial portion of the main piece contained vestigial peripheral dense fibers attached to the axoneme's peripheral microtubules, as was revealed by staining of basic proteins (Fig. 4h). The fibrous sheath, present in the entire extension of the flagellum, was clearly made of basic proteins (Fig. 4i).

Acid phosphatase activity was present in the cortical region of the pro-acrosomal vesicle, in early spermatids in which chromatin has begun to condense (Fig. 5a, b). In spermatozoa, acid phosphatase activity was found in central and peripheral regions of the acrosomal complex (Fig. 5c-f). During the quiescent period, acid phosphatase activity was high in residual bodies indicating that they are active lysosomes (Fig. 5g).

Leydig cells are not directly involved in spermiogenesis, but they are important in regulation of this process. During the quiescent period, Leydig cells increase in volume (Fig. 6a). Lipid vesicles (Fig. 6b) were present in the cytoplasm of Leydig cells as detected

with osmium tetroxide and imidazole. It was also possible to show the higher amounts of lipids in the Golgi apparatus and on the surface of pro-acrosomal vesicles in comparison with other membranes (Fig. 6c, d).

Discussion

During spermiogenesis, germ cells are remodeled and biochemical changes occur throughout the process. *In situ* detection of chemical components in the cells help us to understand functioning of germ cells by relating function to subcellular compartments.

It is well known that Leydig cells and Sertoli cells are key cells in testicular function, responding to climate-induced endocrine changes (Muñoz et al., 1997). Therefore, an important objective of the present study was to detect cytochemically changes particularly in glycoproteins in Sertoli cells during the reproductive cycle of *T. itambere*, to determine ultrastructurally parameters that are sensitive to hormonal fluctuations.

Among reptiles, observations on Sertoli cells have been primarily incidental in studies of spermatogenesis, and are only light microscopical (Pearson and Licht, 1990). Seasonal histological changes in these cells have been studied in the lizard *T. torquatus* (Vieira et al., 2001), and some ultrastructural features of the Sertoli cells have been described for lizards (Dufaure, 1971; Baccetti et al., 1983). However, cytochemical studies of changes occurring during the cell cycle have not been performed yet. Using a light microscopal method, we found glycoprotein secretion in the cytoplasmic extensions of these cells, that are present consistently throughout the reproductive period as well as the quiescent period. During the entire reproductive cycle, Sertoli cells supported all stages of germ cells throughout the active period but they were disorganized during the quiescent period. Assuming that glycogen and glycoconjugates are associated with germ cell nutrition, the higher organization of Sertoli cells is expected during active spermatogenesis. Sertoli cell organization appears to be much more complex in reptilians than in fish and amphibians. It is closer to that of birds, but simpler than in mammals (Baccetti et al., 1983). In addition to the supportive, nutritive and phagocytic roles in relation to the germ cells, Sertoli cells are the source of a great number of proteins that are secreted (Griswold, 1988).

Sertoli cells also secrete macromolecules at their abluminal side. Approximately 10% of the total amount of synthesized proteins the cultured Sertoli cells are secreted glycoproteins (Wilson and Griswold, 1979). It seems likely that glycoproteins secreted by Sertoli cells *in vivo* have direct regulatory roles in germ cell development and other reproduction related processes (Griswold, 1988). Dufaure (1971) reports one of the first cytochemical studies of Sertoli cells of the lizard, using the technique described by Thièrry (1967). He detected glycogen in cytoplasmic extensions and stated that glycogen has an important function in energy supply of spermatids and spermatozoa.

Glycoconjugates has been reported to be particularly significant for the specificity of gamete recognition and fusion (Yanagimachi, 1988). The acrosomal complex is a structure located in the anterior head region, and it contains hydrolytic enzymes stored in the form of pro-enzymes. Carbohydrates were observed in association with the acrosomal complex in spermatozoa of *T. itambere* as was observed by Craveiro and Báo (1995). The positive staining of carbohydrates in this organelle was expected since the pro-enzymes known to occur in the acrosome are usually glycoproteins.

The head described for Iguanidae spermatozoon is similar to that of *T. itambere* and consists of a nucleus and an acrosomal complex, which is a layered structure with an external acrosome, a clear zone, and a subacrosomal cone separated by an epinuclear lucent region from the perforatorium (Ferreira and Dolder, 2003). It was possible to verify that the acrosomal granule of spermatids is rich in basic proteins as was found in the subacrosomal cone in the spermatozoon of *T. itambere*. This fact is not in line with studies of Nagano (1962), Humphreys (1975), and Del Conte (1976), who postulated that the acrosomal granule is responsible for the formation of the perforatorium.

In many animals, the midpiece region of sperm cells contains an accumulation of basic proteins. These proteins have been interpreted as histones, which are removed from the nucleus during chromatin condensation and remain in the midpiece region to participate in the rapid nuclear reorganization after sperm penetration into the egg (Werner, 1975). Apparently, the peripheral fibers generate extra motor force for flagellar movements (Hamilton and Fawcett, 1968) or contribute as a control mechanism to sperm movement

(Anderson and Personne, 1969). The fibrous sheath has elastic properties, which suggests a role for this structure in mobility of spermatozoon (Fawcett, 1970).

Although the identity of the intervening underlying factors and the nature of the changes involved in maturation are still unknown, there is evidence showing that many modifications occur on the sperm plasma membrane during epididymal transit, such as the acquisition of surface antigens (Hunter, 1969; Barker and Amann, 1971; Johnson and Hunter, 1972) and negatively-charged particles (Cooper and Bedford, 1971). These events are considered to reflect the replacement of sperm-coating antigens of testis origin with new ones produced by the epididymis (Kohane et al., 1980). The epididymis is involved in sperm maturation, an event that is greatly dependent on the local environment, especially on factors in the epididymal fluid (Orgebin-Crist and Fournier Delpech, 1982). The main components of the epididymal fluid are specific proteins and glycoproteins, which are synthesized and secreted by the epididymis itself (Devine and Carrol, 1985). This can help explain why the cytochemical reactions in mature spermatozoa in the epididymis are more complex than in the testis.

Spermiogenesis involves various enzymes, including acid phosphatase, which participates in phosphate metabolism. This enzyme has been located in the pro-acrosomal vesicle (Anderson, 1968; Souza et al., 1988; Báo et al., 1989; Furtado and Báo, 1996), in the acrosome (Allison and Hartree, 1970; Báo et al., 1989; Fernandes and Báo, 1998), and in the acrosomal complex in lizards. It is interesting to remember that some of the acrosomal layers identified in other lizards are similar to those of the acrosomal complex of Squamata. Acid phosphatase activity was often found to be diffuse, making it difficult to determine the precise activity site within the acrosomal complex.

Leydig cells in the testis of some lizards have been studied using histochemistry. Studies report that innumerable lipid droplets are present in the cytoplasm of Leydig cells, as in *T. itambere*. Dufaure (1968) discussed this in the light of the function of these cells in the production of steroids. Thus, the cytoplasm and endoplasmic reticulum are well developed with many mitochondria and lipid inclusions called "liposomes". Upadhyay and Guraya (1972) verified that the diffuse lipids that are present in these cells consists of phospholipids, triglycerides, and/or cholesterol. All agree that these lipid characteristics of

Leydig cells are directly related to the production of steroids controlling spermatogenesis. The abundant diffuse lipoproteins, as demonstrated histochemically, may also serve as a reservoir for the storage of hormone precursors (Guraya, 1968). According to Upadhyay and Guraya (1972), the anatomical relationship between lipid inclusion bodies and cytoplasmic membranous components (or diffuse lipoproteins) favors possible interactions between these components, resulting in steroid biosynthesis. The accumulation of lipid droplets (rich in cholesterol or cholesterol esters or both) in the interstitial gland cells of the lizard's testis, which show little spermatogenetic activity, suggests that the cells may function as storage reservoirs for a hormone precursor that is necessary to stimulate the next active reproductive stage. It is interesting that Leydig cells are reduced in size and appear to be less active in testis undergoing spermiogenesis.

Lectins belong to a protein group that can bind selectively to specific sugars. Due to this property, they have been used for the identification and localization of carbohydrates in cells. They also permit detection of variations in glycoconjugate expression dependent on the functional state of cells or on their differentiation grade (Sharon and Lis, 1989, 1993).

Studies to localize intracellular carbohydrates do not exist for lizard spermatozoa. The cell surfaces contain various sugars as part of polysaccharides, glycoproteins, and glycolipids. These glycoprotein complexes in the plasma membrane are directly involved in cellular recognition, adhesion, and regulation of proliferation (Báo et al., 2001). The glycoproteins have a particular role in the specificity of recognition and gamete fusion (Mavcek and Shur, 1988). The residues of N-acetyl-α-galactosamine and D-mannose in the acrosomal complex indicate participation of these carbohydrates in recognition of gametes and enzymatic activity of the acrosome, as reported by Báo et al. (2001), to occur in Anuran amphibians. Clear labeling was not observed on the plasma membrane of spermatozoa of *T. itambere*, suggesting that these cells do not have an extensive cell coat. The residues of N-acetyl-D-glucosamine and L-fucose in the flagellum and particularly in the fibrous sheath may be directly related to its elastic properties and its function in flagellum mobility, as was considered by Fawcett (1970).

Few studies have been conducted to analyze carbohydrate distribution patterns in lower vertebrate spermatozoa as has been done in mammals (Ahluwalia et al., 1990; Bains

et al., 1992; De Cerezo et al., 1996). Labate and Desantis (1995) detected with light microscopy residues of specific sugars in the testis and epididymis (Labate et al., 1997) of the lizard *Podarcis s. campestris*. In the present study, testes showed different markers to be expressed on spermatogonia (Con A, WGA) and spermatocytes (GS-I, SEA, UEA-I). In spermatids, only the head region was positive with RCA and PNA staining. Depeiges et al. (1985) identified the presence of D-glucose, N-acetyl-glucosamine and N-acetyl-α-galactosamine in the central core and peripheral vacuoles of secretory granules of epididymis cells of the lizard *Lacerta vivipara* using lectins in light microscopy. With the same methods, Desantis et al. (2002) found regional differences in carbohydrate components of epididymis epithelium of the lizard *Lacerta vivipara.*

The glycoconjugates found in secretory granules of epididymis cells are the same found in abundance in the spermatozoon head in the seminal receptacle of *T. itambere* females. We did not study sugars present in the epididymis, but when N-acetyl-α-galactosamine is present in *T. itambere*, as described for *Lacerta vivipara*, post-testicular addition of this sugar to spermatozoa is masked by its presence in the acrosome of testicular spermatids and spermatozoa, even when the spermatozoa in the seminal receptacle showed a much heavier labeling.

Results obtained in other lizard species are difficult to compare with those found in *T. itambere*, because these were obtained only with light microscopy which does not permit a detailed localization of the labeled lectins. Thus, staining of D-galactose as described for the sperm head of *Podarcis A. campestris* could be staining of extracellular material as was found for *T. itambere*. The presence of α-β-glucosamine in spermatocytes of *Podaris A. campestis* was detected in *T. itambere* where this sugar was found in nuclei and manchette. However, localization of L-fucose cannot be compared because it is present in the fibrous sheath of spermatozoa whereas this structure does not exist in spermatocytes, which were the cells labeled in the study of Labate and Desantis (1995).

The fact that fewer sugar types were identified in spermatozoa in comparison to spermatids may be due to the higher condensation of protein structures during sperm development so that some sugars may no longer be available to bind with labeled lectins.

Fertilization in reptiles is always internal. During sexual reproduction in Squamata, sperm is deposited directly in the female's cloacae by a hemipenis found in one of the two pockets of the cloaca's posterior wall, which is located at the tail base of males. In contrast to most vertebrates, reptile sperm can survive for months or years in an inactive form in the female, which can result in delayed fertilization (Cuellar, 1966; Conner and Crews, 1980; Adams and Cooper, 1988; Olsson et al., 1994).

The organ for sperm storage has been called the seminal receptacle, and it has been found in turtles (Palmer and Guillette Jr, 1988), snakes (Sever and Ryan, 1999), and lizards (Cuellar, 1966; Kumari et al., 1990; Blackburn, 1998). Sperm storage in this organ was recently explored in turtles (Pearse and Avise, 2001; Pearse et al., 2002) and lizards (Conner and Crews, 1980; Adams and Cooper, 1988; Kumari et al., 1990; Srinivas et al., 1995). However, little is known about the reasons for this storage, or physiological conditions that may affect capacitation of spermatozoa for fertilization. Certainly, these characteristics are important for reproductive success of a species as well as for an evolutionary adaptation to competition among males of the same species. The production of sperm that can survive long-term storage in females clearly benefits males by increasing the number of eggs they fertilize (Pearse et al., 2000).

It is unlikely that morphological changes occur after spermatozoa have left the testis, but it is known that this cell is not yet completely mature, and changes at the molecular level occur during its passage of the reproductive tract (Kohane et al., 1980; Ravet et al., 1987), such as the addition of specific sugars. Although the identity of the underlying mechanisms and the nature of changes involved in maturation are still unknown, there is evidence that many modifications occur in the sperm plasma membrane during epididymal transition, such as acquisition of surface antigens (Hunter, 1969; Johnson and Hunter, 1972) and negatively charged particles (Cooper and Bedford, 1971). In *T. itambere*, the epididymis may play a role in the fact that lectin labeling in mature spermatozoa from the seminal receptacle is restricted to only 2 sugar types found in 2 structures.

In the present study, we have used histochemistry at the light and electron microscopical level to improve our understanding of spermiogenesis in male lizards and the long periods of sperm storage in female lizards. Besides the addition and loss of specific

sugars that we have shown here, other modifications may well occur in spermatozoa during maturation which need further studies.

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References

Adams CS, and Cooper WE (1998) Oviductal morphology and sperm storage in the keeled earless lizard, *Holbrookia propinqua*. Herpetologica 44:190-197

Afzelius BA (1992) Section staining for electron microscopy using tannic acid as a mordant: a simple method for visualization of glycogen and collagen. Microsc Res Techn **21**: 65-72

Ahluwalia B, Farshori P, Jamuar M, Baccetti B, and Anderson WA (1990) Specific localization of lectins in boar and bull spermatozoa. J Submicrosc Cytol Pathol **22**: 53-62

Al-Hajj H, Janakat S, and Mahmoud F (1987) Electron microscopic study of sperm head differentiation in the lizard *Agama stellio*. Can J Zool **65**: 2959-2968

Allison AC, and Hartree EF (1970) Lysosomal enzymes in the acrosome and their possible role in fertilization. J Reprod Fert **21**: 501-515

Anderson WA, and Personne P (1969) Structure and histochemistry of basal body derivative, neck and axoneme of spermatozoa of *Helix aspersa*. J Microsc **8**: 87-96

Anderson WA (1968) Cytochemistry of sea urchin gametes. III. Acid and alkaline phosphatase activity of spermatozoa and fertilization. J Ultrast Res **25**: 1-14

Angermuller SL, and Fahimi DH (1984) A new cerium-based method for cytochemical localization of thiamine pyrophosphatase in the Golgi complex of rat hepatocytes. Histochemistry **80**: 107-111

Baccetti B, Bigliardi E, Talluri MV, and Burrini AG (1983) The Sertoli cell in lizards. J Ultrast Res **85**: 11-23

Bains HK, Sehgal S, and Bawa SR (1992) Human sperm surface mapping with lectins. Acta Anat **145**: 207-211

Báo SN, Quagio-Grassioto I, and Dolder H (1989) Acrosome formation in *Ceratis capitata* (Diptera, Tephritidae). Cytobios **58**: 93-100

Báo SN, Vieira GHC, Fernandes AP (2001) Spermiogenesis in *Melanophryniscus cambaraensis* (Amphibia, Anura, Bufonidae): ultrastructural and cytochemical studies of carbohydrates using lectins. Cytobios **106**: 203-216

Barker LDS, and Amann RP (1971) Epididymal physiology. II. Immunofluorescent analyses of epithelial secretion and absorption and of bovine sperm maturation. J Reprod Fertil **26**: 319-332

Blackburn DG (1998) Structure, function, and evolution of the oviducts of squamate reptiles, with special reference to viviparity and placentation. J Exp Zool **282**: 560-617

Bloom FE, and Aghajanian GK (1968) Fine structural and cytochemical analysis of the staining of synaptic junctions with phosphotungstic acid. J Ultrastruct Res **22**: 361-371

Butler RD, and Gabri MS (1984) Structure and development of the sperm head in the lizard *Podarcis* (=*Lacerta*) *taurica*. J Ultrastruct Res **88**: 261-274

Clark AW (1967) Some aspects of spermiogenesis in a lizard. Am J Anat **121**: 369-400

Conner J, and Crews D (1980) Sperm transfer and storage in the lizard, *Anolis carolinensis*. J Morphol **163**: 331-348

Cooper GW, and Bedford JM (1971) Acquisition of surface charge by the plasma membrane of mammalian spermatozoa during epididymal maturation. Anat Rec **169**: 300-301

Courtens JL, and Depeiges A (1985) Spermiogenesis of *Lacerta vivipara*. J Ultrast Mol Struct Res **90**: 203-220

Craveiro D, and Báo SN (1995) Localization of carbohydrates in spermatids of three *Chrysomelid beetles* (Coleoptera, Chrysomelidae). Biocell **19**: 195-202

Cruz Höfling MA, and Cruz Landim C (1978) The fine structure of nuclei during spermiogenesis in the lizard *Tropidurus torquatus* (Lacertilia). Cytologia **43**: 61-68

Cruz Landim C, and Cruz Höfling MA (1977) Electron microscope study of lizard spermiogenesis in *Tropidurus torquatus* (Lacertilia). Caryologia **30**: 151-162

Cuellar O (1966) Oviductal anatomy and sperm storage structures in lizards. J Morphol **119**: 7-20

De Cerezo JMS, Marquínez AC, Sarchi MI, and Cerezo AS (1996) Fucosylated glycoconjugates of the human spermatozoon. Comparison of the domains of these glycoconjugates with the α-fucosyl binding sites, and with lactosaminic glycoconjugates and β-D-galactosyl binding sites domains. Biocell **20**: 11-20

Del Conte E (1976) The subacrosomal granule and its evolution during spermiogenesis in a lizard. Cell Tissue Res **171**: 483-498

Depeiges A, Betail G, Coulet M, and Dufaure JP (1985) Histochemistry study of epididymal secretions in the lizard, *Lacerta vivipara*. Localization of lectin binding sites. Cell and Tissue Res **239**: 463-466

Dessantis S, Labate M, Labate GM, and Cirillo F (2002) Evidence of regional differences in the lectin histochemistry along the ductus epididymis of the lizard *Podarcis sicula* Raf. Histochem J **34**: 123-130

Devine KM, and Carrol J (1985) Peptide synthesis by the murine epididymis. Biochem Soc Trans **13**: 506-507

Dufaure JP (1968) L'ultrastructure des cellules interstitielles du testicule adulte chez deux Reptiles Lacertilies: le lézard vivipare (*Lacerta vivipara* Jacquin) et l'orvet (*Anguis fragilis* L.). C R Acad Sc Paris **267**: 883-885

Dufaure JP (1971). L'ultrastructure du testicule de lézard vivipare (Reptile, Lacertilien). II. Les cellules de Sertoli. Etude du glycogène. Z Zellforsch **115**: 565-578

Fawcett DW (1970) A comparative view of sperm ultrastructure. Biol Reprod (Suppl.) **2**: 90-127

Fawcett DW, Anderson WA, and Phillips DM (1971) Morphogenetic factors influencing the shape of the sperm head. Developm Biol **26**: 220-251

Fernandes AP, and Báo SN (1998) Cytochemical localization of phosphatases in the germ and Sertoli cells of *Odontophrynus cultripes* (Amphibia, Anura, Leptodactylidae). Biocell **22**: 93-101

Ferreira A, and Dolder H (2003) Sperm ultrastructure and spermatogenesis in the lizard, *Tropidurus itambere*. Biocell: ***in press***

Furtado DA, and Báo SN (1996) Ultrastructural localization of phosphatase in sperm cells of the beetle *Diabrotica speciosa* (Coleoptera, Chrysomelidae). Cytobios **88**: 83-93

Griswold MD (1988) Protein secretions of Sertoli cells. Int Rev Cytol **110**: 133-156

Guraya SS (1968) A histochemical study of lipids in goat testis. Acta Morphol Neerl Scand **7**: 15-27

Hamilton DW, and Fawcett DW (1968) Unusual features of the neck and middle-piece of snake spermatozoa. J Ultrastruct Res **23**: 81-97

Hayat MA (1993) Stains and cytochemical methods. 1st Ed. Plenum. New York NY, USA

Hoffman LH, and Wimsatt WA (1972) Histochemical and electron microscopic observations on the sperm receptacles in the garter snake oviduct. Am J Anat **134**: 71-96

Humphreys PN (1975) The differentiation of the acrosome in the spermatid of the budgerigar (*Melopsittacus undulatus*). Cell Tissue Res **156**: 411-416

Hunter AG (1969) Differentiation of the rabbit sperm antigens from those of seminal plasma. J Reprod Fertil **36**: 221-214

Jamieson BGM (1995) The ultrastructure of spermatozoa of the Squamata (Reptilia) with phylogenetic considerations. In: Jamieson BGM, Ausio J, Justine JL (Eds) Advances in spermatozoal phylogeny and taxonomy. Édition du Muséum National d'Histoire Naturelle, Paris, pp 395-383

Johnson WL, and Hunter AG (1972) Immunofluorescent evaluation of the male rabbit reproductive tract for sites of secretion and absorption of seminal antigens. Biol Reprod **6**: 13-22

Kohane AC, González-Echeverría FMC, Piñeiro L, and Blaquier JA (1980) Interaction of proteins of epididymal origin with spermatozoa. Biol Reprod **23**: 737-742

Kumari TRS, Sarkar HBD, and Shivanandappa T (1990) Histology and histochemistry of the oviductal sperm storage pockets of the agamid lizard *Calotes versicolor*. J Morphol **203**: 97-106

Labate M, and Desantis S (1995) Histochemical analysis of lizard testicular glycoconjugates during the annual spermatogenetic cycle. Eur J Histochem **39**: 201-212.

Labate M, Desantis S, and Corriero A (1997) Glycoconjugates during the annual sexual cycle in lizard epididymal ductuli efferentes: A histochemical study. Eur J Histochem **41**: 47-56

Mavcek MB, and Shur DB (1988) Protein in carbohydrates complementary in mammalian gametes recogniton. Gamete Res **20**: 93-109

Muñoz E, Fogal T, Dominguez S, Scardapane L, Guzmán J, and Piezzi RS (1997) Seasonal changes of the Leydig cells of viscacha (*Lagostomus maximus maximus*). A light and electron microscopy. Tissue Cell **29**: 119-128

Nagano T (1962) Observations on the fine structure of the developing spermatids in the domestic chicken. J Cell Biol **14**: 193-205

Olsson M, Gullberg A, and Tegelström H (1994) Sperm competition in the sand lizard, *Lacerta agilis*. Anim Behav **48**: 193-200

Orgebin-Crist MC, and Fournier Delpech S (1982) Sperm-egg interaction: evidence for maturation changes during epididymal transit. J Androl **3**: 429-433

Palmer BD, and Guillette Jr LJ (1988) Histology and functional morphology of the female reproductive tract of the tortoise *Gopherus polyphemus*. Am J Anat **183**: 200-211

Pearse DE, Janzen FJ, and Avise JC (2000) Genetic markers substantiate long-term storage and utilization of sperm by female painted turtles. Heredity **86**: 378-384

Pearse DE and Avise JC (2001) Turtle mating systems: behavior, sperm storage and genetic paternity. Am Gen Assoc **92**: 206-211

Pearse DE, Janzen FJ, and Avise JC (2002) Multiple paternity, sperm storage, and reproductive sucess of female and male painted turtles (*Chrysemys picta*) in nature. Behav Ecol Sociobiol **51**: 164-171

Pearson AK, and Licht P (1990) The double nucleus of the Sertoli cell in the lizard *Anolis carolinensis*. Tissue Cell **22**: 221-229

Pino RM, Pino LC, and Bankston PW (1981) The relationships between the Golgi apparatus. GERL, and lysosomes of fetal rat live Kupffer cells examined by ultrastructural phosphatase cytochemistry. J Histochem Cytochem **29**: 1061-1070

Ravet V, Courty Y, Depeiges A, and Dufaure JP (1987) Changes in epididymal protein synthesis during the sexual cycle of the lizard, *Lacerta vivipara*. Biol Reprod **37**: 901-907

Sever DM, and Ryan TJ (1999) Ultrastructure of the reproductive system of the black swamp snake (*Seminatrix pygaea*): Part I. Evidence for oviductal sperm storage. J Morphol **241**: 1-18

Sharon N, and Lis H (1989) Lectins as cell recogniton molecules. Science **246**: 227-246

Sharon N, and Lis H (1993) Carbohydrates in cell recognition. Scient Am **268**: 74-81

Souza M, Moradas Ferreira P, Amorin A, and Azevedo C (1988) Starfish acrosomal acid phosphatase: a cytochemical and biochemical study. Biol Cell **63**: 101-104

Srinivas SR, Shivanandappa T, Hedge SN, and Sarkar HBD (1995) Sperm storage in the oviduct of the tropical rock lizard, *Psammophilus dorsalis*. J Morphol **224**: 293-301

Thièrry JP (1967) Mise en evidence des polysaccharides sur coupes fines en microscopie electronique. J Microsc **6**: 987-1018

Upadhyay SN, and Guraya SS (1972) Histochemical observations on the interstitial gland (or Leydig) cells of the lizard testis. Gen Comp Endocrin **19**: 88-95

Vieira GHC, Wiederhecker HC, Colli GR, and Báo SN (2001) Spermiogenesis and testicular cycle of the lizard *Tropidurus torquatus* (Squamata, Tropiduridae) in the Cerrado of central Brazil. Amphibia-Reptilia **22**: 217-233

Werner G (1975) Changes in the spermatozoon during fertilization in *Triturus helveticus* Raz. In: Afzelius BA (Ed) The functional anatomy of the spermatozoon. Pergamon Press, Oxford, pp 47-56

Wilson RM, and Griswold MP (1979) Secreted proteins from rat Sertoli cells. Exp Cell Res **123**: 127-135

Yanagimachi R (1988) Mammalian fertilization. In: Knobil E, and Neill JD (Eds) The physiology of reproduction. Raven Press, New York NY, USA, pp 135-185

Table 1.

Lectins used as histochemical probes in the present studs and labeling patterns obtained in spermatids and spermatozoa in testes and seminal receptacles.

Sources of lectin	Sugar specificity	Labeling pattern
Arachys hypogaea (PNA)	D-galactose	Nucleus, dense bodies, central and peripheral dense fibers of centrioles in the midpiece region
Griffonia simplicifolia (GS-I)	α-Galactose/α-galactosamine	Chromatin and manchette (during spermiogenesis), and nucleus of mature spermatozoa
Helix pomatia (HPA)	N-acetyl-α-galactosamine	Pro-acrosomal vesicle and acrosome during entire spermiogenesis and the external region of the acrosome in mature spermatozoa. Secretions involving mature spermatozoa in the lumen of the seminal receptacles of the female
Triticum vulgaris (WGA)	N-acetyl-D-glucosamine	Fibrous sheath of the flagellum of late spermatids and mature spermatozoa
Ulex europaeus (UEA-I)	L-fucose	Fibrous sheath of flagellum of late spermatids and mature spermatozoa

Figure legends

Fig. 1. a and **b.** Seminiferous tubule during the peak reproductive period. The organization of tubules is as follows: spermatocytes (c) in the basal region, spermatids (t) and innumerable spermatozoa (z) in lumen, grouped by Sertoli cell extensions (sc). Hematoxilin-eosin staining. **b.** PAS staining result is strong positivity of cytoplasmic elongations of Sertoli cells from the tubule base to the lumen (arrow heads). **c** and **d.** Seminiferous tubule during the quiescent period. **c.** The typical germ cell organization is lost with some spermatocytes (c) and spermatozoa (z) being present in the lumen. Hematoxilin-eosin staining. **d.** PAS method resulted in staining of cytoplasmic elongations of Sertoli cells but these are dispersed throughout the seminiferous tubule (arrow heads). **e-h.** Transmission electron microscopical micrographs of seminiferous tubules during the reproductive period; arrow heads indicate positive reactions for glycoproteins and glycogen. Polysaccharides were stained according to Thièrry (1997) in e and g, and glicogen according to Afzelius (1992) in f and h. **e** and **f.** Strong positivity is present in cytoplasmic elongations of Sertoli cells (sc) and light staining in spermatocytes (c). **g.** Early spermatid with positivity in the pro-acrosomal vesicle (av) inserted in the nucleus (n). **h.** Transverse section of the acrosome's apical region with positivity in the perforatorium (p), the acrosome (a), and the clear zone (cz). Thièrry (1967) technique in **e.** and **g.** and Afzelius (1992) technique in figures **f.** and **h.** Bars, a-d, 40μm; e, 2μm; f, 1.5μm; g, 0.5μm; h, 0.2μm.

Figure 2 - Ultrastructural localization of carbohydrates using lectins during spermiogenesis and in the seminal receptacle of the female. The results are summarized in table 1. In all the figures the arrowheads point out gold labeled lectins. The structures observed are: **a.** Pro-acrosomal vesicle (av) stained with HPA and inserted in the nucleus of an early spermatid. **b.** Transverse sections of the acrosomal vesicle during formation of the complex: acrosome (a) stained with HPA, future perforatorium (p), and nucleus in condensation (n). **c.** Longitudinal section of early spermatid undergoing nuclear elongation (n), with the future perforatorium (p), and the acrosome (a) intensely stained with HPA. **d. - e.** Spermatids are intensely stained with HPA on the acrosome layer. **f.** Testicular spermatozoa showing the

strongly stained acrosomal complex. Notice apical sections on the left and a basal profile on the right. **g. - h.** Spermatozoa in the seminal receptacle of the female reproductive tract. Observe the same staining in the acrosomal complex with HPA, and in the secretions surrounding the spermatozoa. Bars, a, 0.4μm; b, 0.2μm; c, 0.3μm; d, 0.3μm; e-f, 0.5μm; g, 0.2μm; h, 0.7μm.

Figure 3 –The ultrastructural localization of carbohydrates using lectins. **a.** Longitudinal section of the nucleus of an early spermatid where condensing chromatin is stained with GS-I. **b.** Transverse section of the spermatid head with encircling microtubules, that make up the structure called manchette (mt), are stained with GS-I. **c.** Residues of D-galactose were also stained with PNA in the midpiece region, more specifically in pericentriolar material (pcm), in central (cf) and peripheral dense fibers of the centriole (pf), and in dense bodies (db). **d. - f.** Transverse section of a mature spermatozoon with a compacted nucleus in the testicles (**d.**) and seminal receptacles (**e.** and **f.**), stained with PNA. **g.** Longitudinal sections of the nucleus in testicular spermatozoa stained with GS-I. **h. – i.** UEA-I and WGA staining occurs the rings of the fibrous sheath (fs) surrounding the axoneme (ax) in the testicle (**h.**) and seminal receptacle (**i.**). Bars, a, 0.2μm; b, 0.3μm; c, 0.4μm; d-i, 0.3μm.

Figure 4 -Ultrastructural localization of basic proteins with ethanolic phosphotungstic acid (E-PTA). **a.** Early spermatids show a lightly stained nucleus (n), strongly stained pro-acrosomal vesicle (av), and acrosomal granule (ag). **b. - i.** Mature spermatozoa. **b.** Longitudinal section of the midpiece: the pericentriolar material (pcm), central dense fiber of the proximal centriole (cf), peripheral dense fibers of the distal centriole and axoneme (pf), dense bodies (db), fibrous sheath (fs), and annulus (an) are strongly positive. **c.** Longitudinal sections of the acrosomal complex in which the acrosome (a) shows strong staining **d.** Transverse section of the midpiece where the stained structures include dense bodies (db) and central (cf) and peripheral dense fibers (pf). **e.** Longitudinal section of the midpiece: pericentriolar material (pcm), peripheral dense fibers of the centriole and axoneme (pf), and dense bodies (db) are positive. **f. - g.** Transverse sections of the acrosomal complex: stained structures are the periphery of the acrosome (a), the

perforatorium (p) and the subacrosomal cone (sc). Unstained structures are the clear zone (cz) and epinuclear lucent region (et). **h.** Transverse section of the flagellum in the initial region: peripheral dense fibers (pf) and fibrous sheath (fs) are strongly positive. **i.** Longitudinal and tangential sections of flagella: fibrous sheath (fs) is well stained. In all figures the arrow heads indicate a high concentration of basic proteins. Bars, a, 0.5μm; b, 0.2μm; c, 0.4μm; d, 0.2μm; e, 0.3μm; f-h, 0.1μm; i, 0,4μm.

Figure 5 - Ultrastructural localization of acid phosphatase. In all the figures the arrowheads show positive reaction for acid phosphatase. **a.** Longitudinal section of early spermatid, with nucleus undergoing condensation of its chromatin (curved arrow). Notice a positive reaction on the acrosomal vesicle surface. **b.** Transverse section of early spermatid with a condensing nucleus (n) with positive staining located on the acrosomal vesicle surface. **c.**, **e.**, and **f.** Cross sections of the acrosomal complex of spermatozoa, where positivity is observed in layers of the acrosomal complex interpreted as the acrosome, the subacrosomic region, and the perforatorium. **d.** Tangential section of the acrosomal complex, where the clear region corresponds to the epinuclear electron lucent region (et) and is covered by the strongly stained subacrosomal complex (sc) observed between this structure and the acrosome (a). **g.** Luminal region of a seminiferous tubule in the quiescent period of the reproductive cycle. The asterisks (*) show the strong reaction in degenerating structures. Bars, a-b, 0.6μm; c, e-f, 0.5μm; d, 0.6μm; g, 1.5μm.

Figure 6 - a. Conventional light microscopy of the testis during quiescent period of the reproductive cycle. In the interstitial tissue, observe voluminous Leydig cells with a central nucleus and cytoplasmic vacuoles (lines). Hematoxilin-eosin staining. **b.** Transmission electron microscopy of the Leydig cell. The cytoplasm is filled with small lipid vesicles (arrows) stained with the osmium tetroxide and imidazole technique. **c. - d.** Transmission electron microscopy of early spermatid, showing the Golgi complex (arrow) and membranes of the acrosomal vesicle (av) strongly stained by the osmium tetroxide impregnation technique. Bars, a, 20μm; b, 1.5μm; c, 0.4μm; d, 0.5μm.

Figure 1

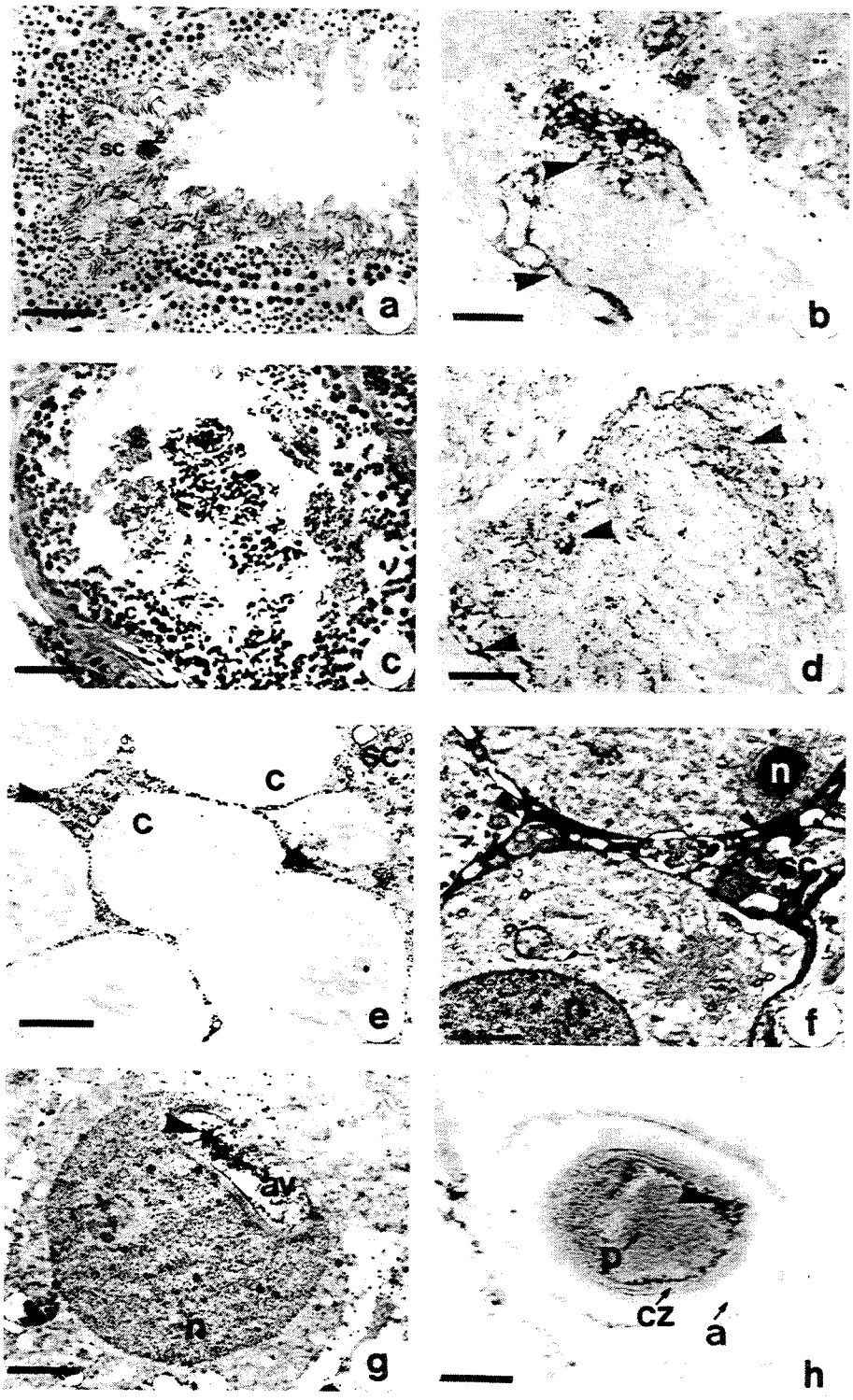


Figure 2

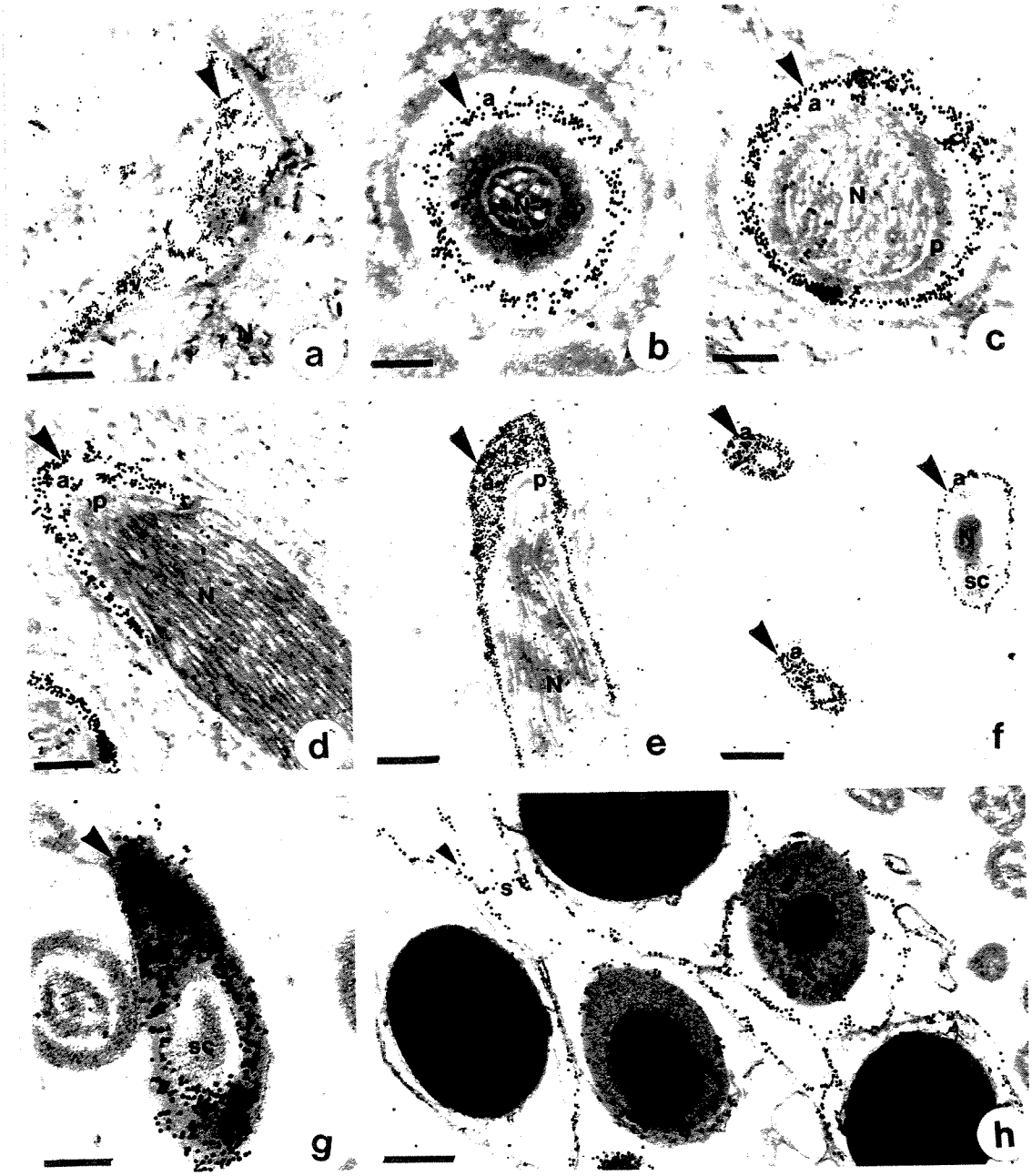
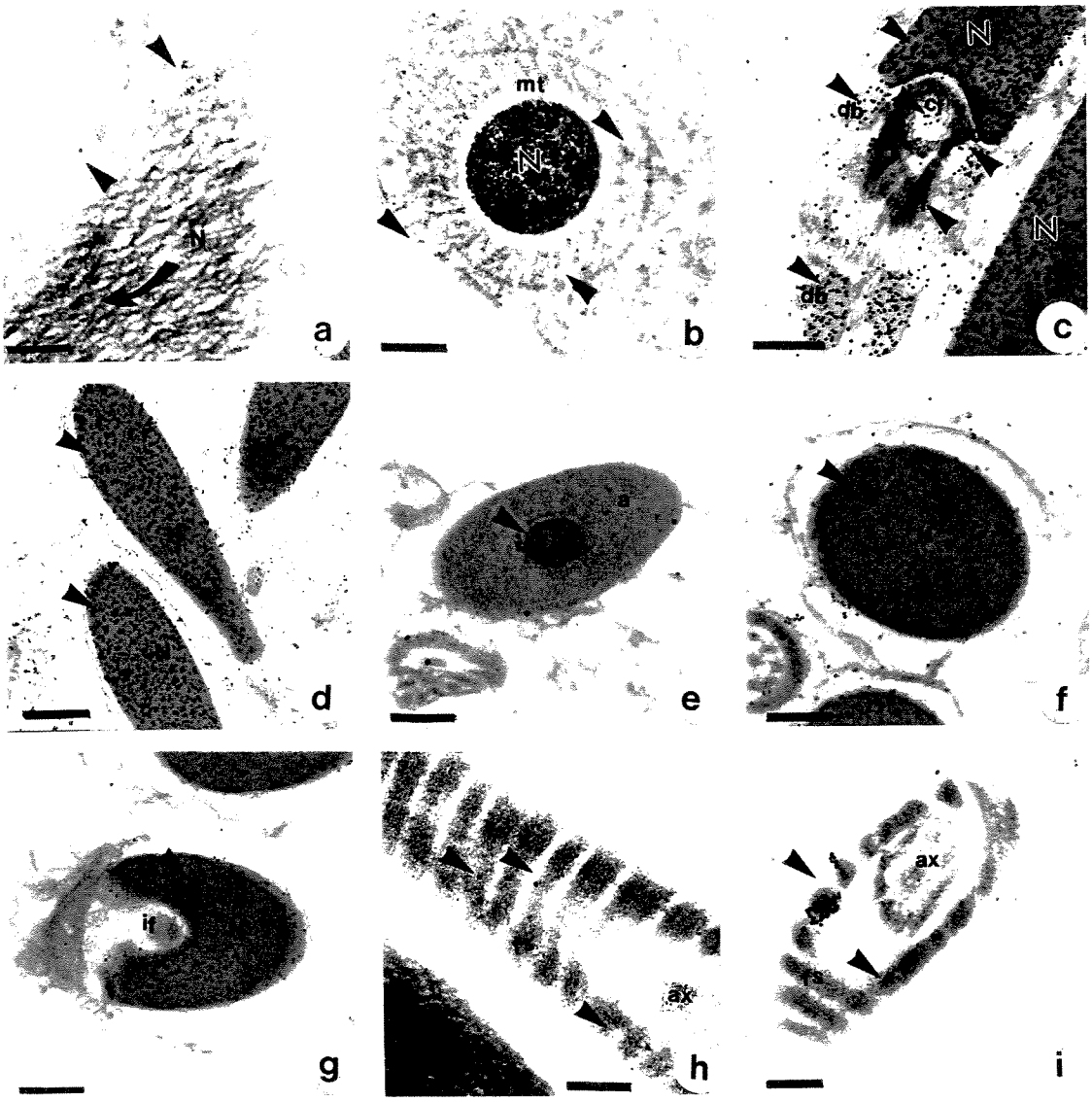


Figure 3



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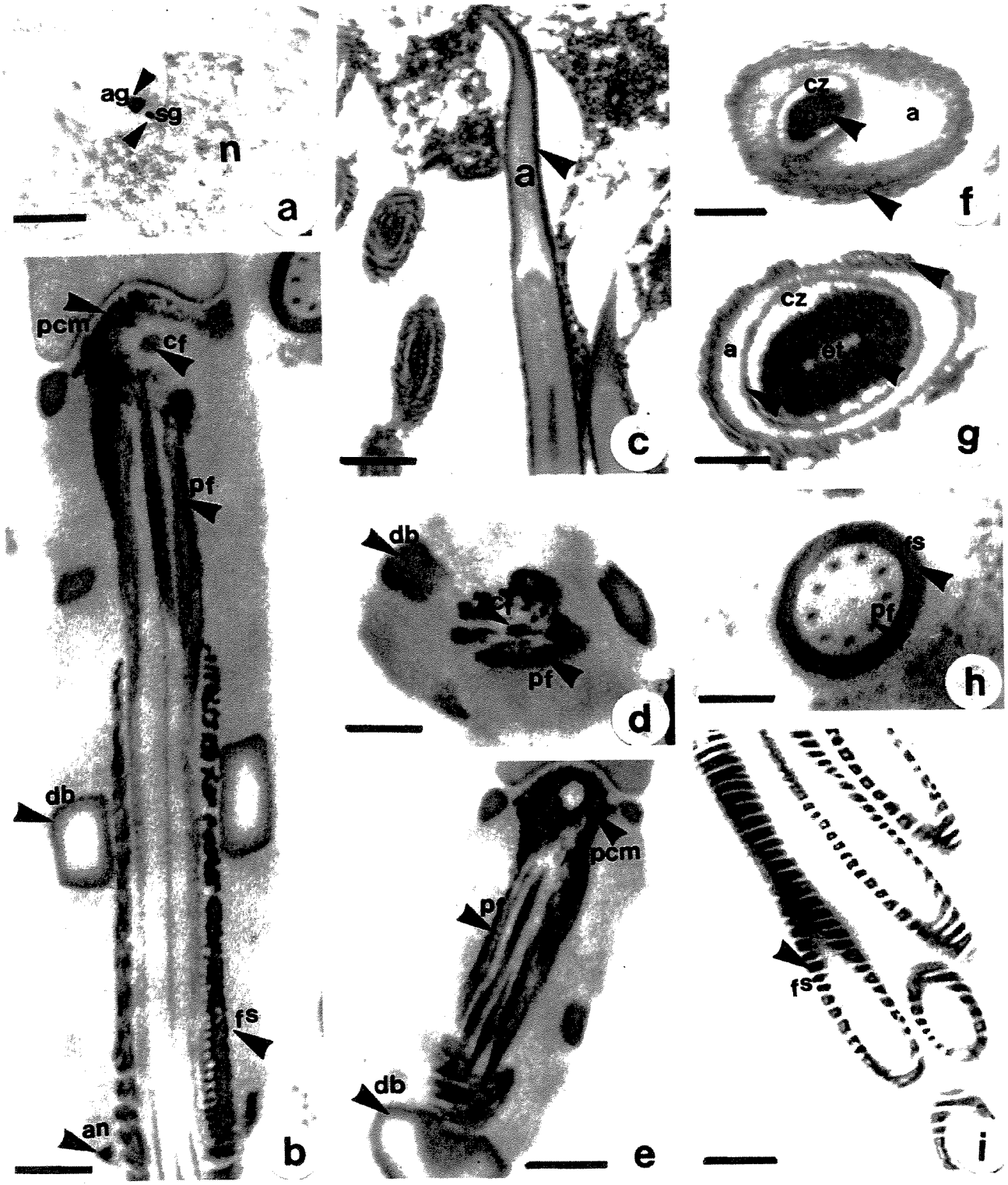
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Figure 5

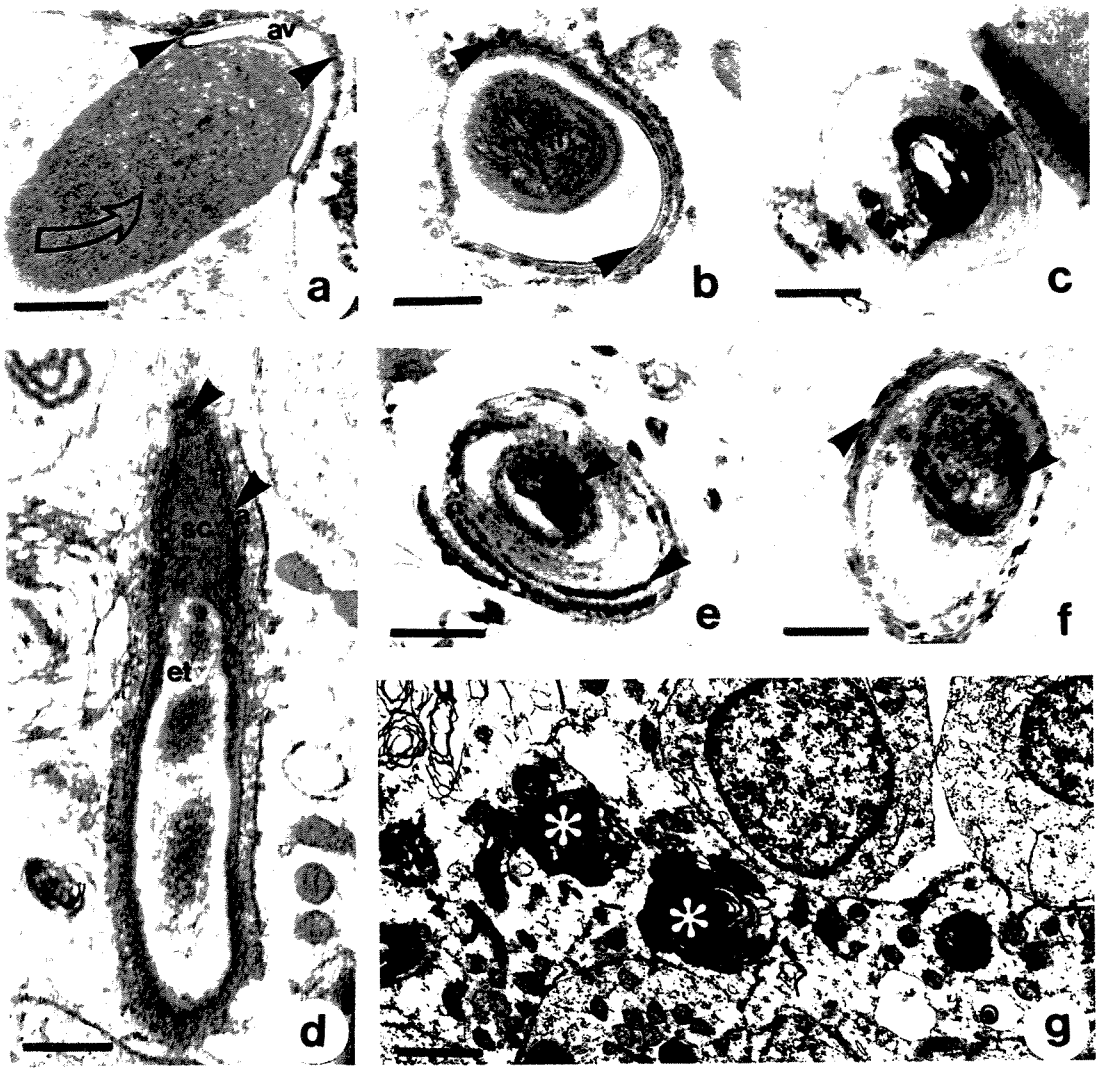
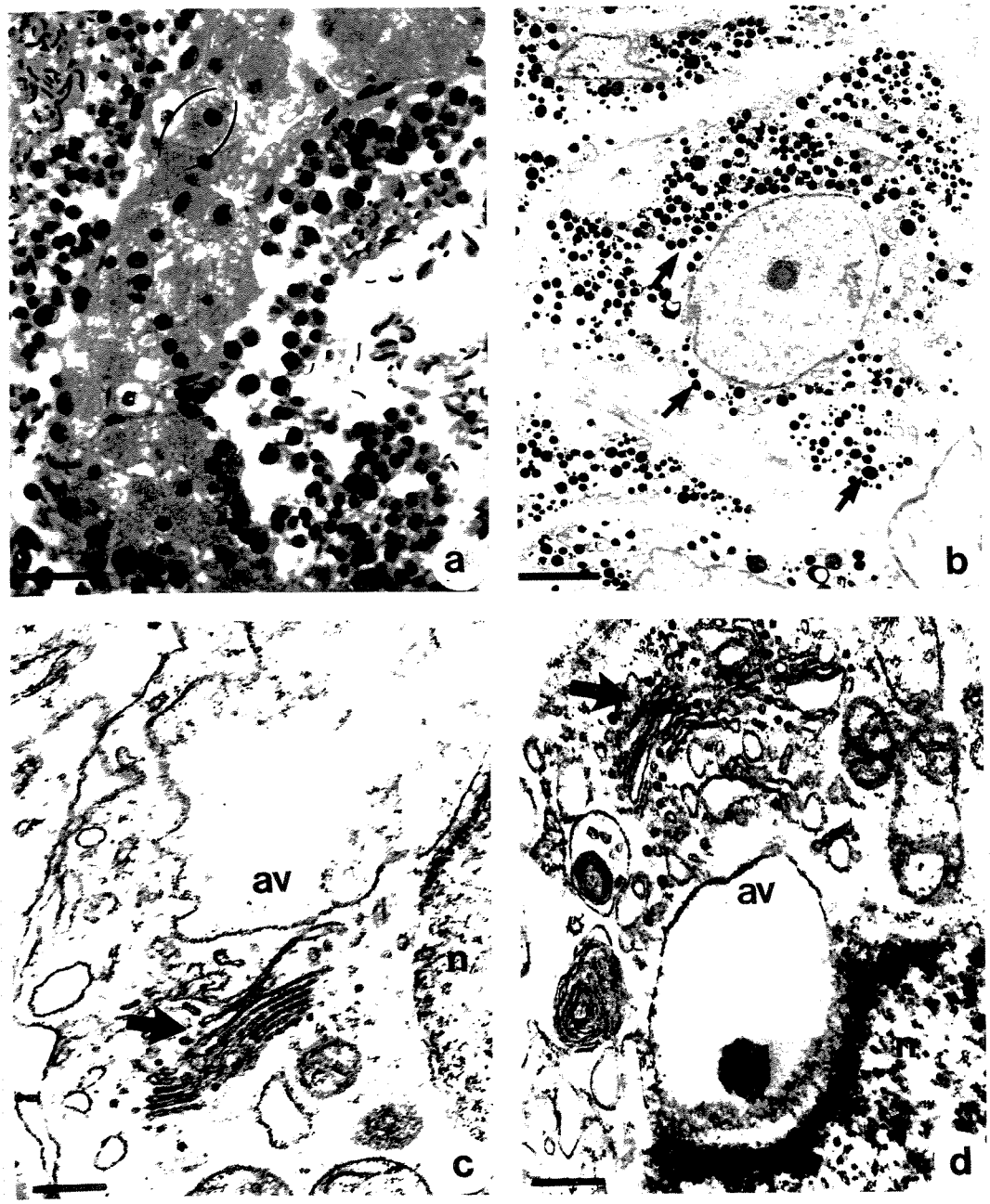
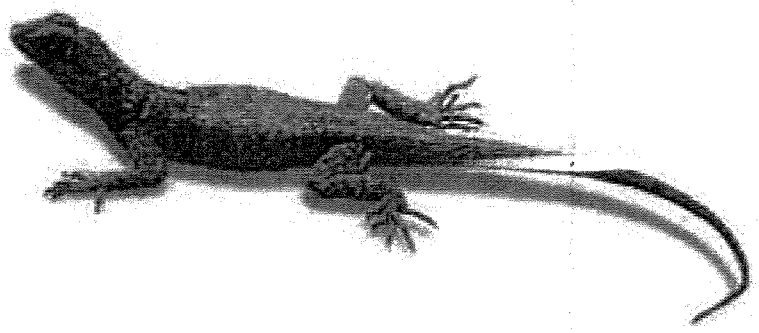


Figure 6



6.5. Ultrastructural immunocytochemical evidence for actin in the acrosomal complex during spermiogenesis of the lizard *Tropidurus itambere* (Reptilia, Squamata)



Abstract - In lizards little has been discussed as to the origin of structures that contribute to the acrosomal complex and some discord exists in relation to the origin of the perforatorium and the subacrosomal cone. I this work early stages of development of the head of the spermatozoon are shown, including the nuclear and the acrosomal complex in formation. This data has led to new suggestions as to the origin of these structures during spermiogenesis of the *Tropidurus itambere* lizard and consequently of other lizards. We also show actin detection in the perforatorium and subacrosomal cone, using ultrastructural immunocytochemistry. These results endorse the view that in lizards, the perforatorium and subacrosomal cone are homologous, in composition and probably also in function.

Key words: spermiogenesis, lizard, acrosome, perforatorium, subacrosomal cone, actin, immunocytochemistry.

INTRODUCTION

Among the events that occur during the differentiation process of spermiogenesis in lizards, the more important ones are nuclear elongation, chromatin condensation, acrosome formation, intensive cytoplasm elimination, and flagellar development. The acrosome of lizards is made up of a set of structures known as the subacrosomal cone, the epinuclear clear zone, the perforatorium, the clear middle zone and the acrosome cap (CRUZ-HÖFLING and CRUZ LANDIM 1978; TEIXEIRA *et al.* 1999; VIEIRA *et al* 2001).

Herein we describe ultrastructural modifications in the head of spermatids and spermatozoa, and the immunocytochemical localization of actin in the acrosomal complex, of the neotropical lizard, *Tropidurus itambere* (RODRIGUES 1987). This species commonly occurs in open, sometimes rocky areas in the Atlantic Forest domain, in central and southeastern Brazil (VAN SLUYS 1993).

Presently, it is well known that the perforatorium presents a great amount of actin, evidenced by immunocytochemical techniques in spermatids and spermatozoa of the rabbit (COURTENS *et al.* 1991), hamster (FOUQUET *et al.* 1991), rat and mouse (FOUQUET *et al.*

1992; PARANKO *et al.* 1994), and bivalves (GUERRA *et al.* 1994). However, no information exists for the acrosomal complex in lizards.

Most lizards possess an acrosomal complex containing a perforatorium and an acrosomal cone (BUTLER and GABRI 1984; AL-HAJJ *et al.* 1987; TEIXEIRA *et al.* 1999). However little is known as to the functions to these organelles during the acrosomal reaction and penetration into the ovule, as well as to the differences in composition, and the possible relations of homology between these two organelles (GARDA *et al.* 2002).

MATERIAL AND METHODS

Adult lizards, *Tropidurus itambere* (Reptilia, Squamata, Tropiduridae), were collected in their natural habitat, from the Atlantic forest in Valinhos region (23°00'S, 47°00'W), São Paulo state, Brazil, at monthly intervals between June 2001 and June 2002. The animals were killed by ethyl-ether inhalation, the testis removed by dissection.

Transmission electron microscopy

The organs were cut into fragments and fixed by immersion for 5 hours at 4° C in a solution containing 2% glutaraldehyde in 0.1M sodium caccodylate buffer, pH 7.2. The tissue fragments were washed in buffer, dehydrated in acetone (30-90%), and embedded in LRWhite resin. The ultrathin sections were colleted on nickel grids and stained with uranyl acetate and lead citrate and observed with a Zeiss, Leo 906 Transmission Electron Microscope.

Ultrastructural immunocytochemistry

Ultrathin sections collected on nickel grids, pre-incubated in 0.05M Tris-HCl buffer pH7.6, containing 0.05% glycin and 1.0% bovine albumin for 10 minutes at room temperature. After several rinses in the same buffer, unspecific binding was blocked with 0.05M Tris-HCl containing 1% bovine albumin and 0.05% Tween 20 for 30 minutes. Subsequently incubated for 1h with an antibody against α-smooth-muscle-actin (mono clone α-SMA), diluted 1:100 (Novocastra Laboratories Ltd. - England). After washing with 0.05M Tris-HCl, containing 0.5% bovine albumin, the grids were incubated for 1h with the respective labeled secondary antibody - RAM (Rabbit Anti-mouse - IgG- Au- conjugated

10nm) at a dilution of 1:50 (Sigma-Aldrich Co. - USA). After incubation, the grids were washed with 0.05M Tris-HCl and distilled water. The preparations were observed without staining.

RESULTS

The spermiogenesis process of *T. itambere* involves, among others events, the formation of an acrosomal complex located at the nuclear apex. The term, acrosomal complex, has been implemented due to the many layers representing different structures observed even in the initial spermiogenesis phases. Another important event for head formation of the lizard spermatozoon is the extreme compacting of the chromatin and consequently the nucleus.

In early spermatids, the Golgi complex produces innumerable vesicles; these vesicles are fused, forming a large vesicle, called the pro-acrosomal vesicle that fits into a nuclear cavity (figs. 1a, 1b). In the region where the vesicle is annexed onto the nucleus, other structures are formed, such as an electron dense granule made up of two parts, the acrosomal granule inside the acrosomal vesicle and a small subacrosomal granule just outside this membrane (fig. 1a). At the nuclear membrane an electron-dense layer develops below the pro-acrosomal vesicle, which has been called the subacrosomal material or layer (fig. 1a). This layer is separated from the nuclear envelope by a clear layer (fig. 1b). During chromatin condensation and nuclear elongation, it is possible to observe the formation of thick filaments arranged longitudinally and undergoing a light twist around the central axis (fig. 1c). In cross section, the chromatin filaments fuse to make a network arrangement of interconnecting filaments (fig. 1d).

Intermediate spermatids can be identified by the more closely compacted chromatin (figs. 1e, 1f). The acrosomal complex follows around the nuclear tip in layers in which the epinuclear clear zone, the perforatorium, the subacrosomal cone, the clear middle zone and the acrosome cap can be identified (fig. 1e, 1f). Since the head of these spermatids is sickle-shaped and the nucleus in figure 1e is tangentially sectioned, the subacrosomal cone, which covers only the nuclear tip, is not shown. These cells enter into an intimate association with

the Sertoli cell, and membranes of this cell appear parallel to the acrosomal complex (fig. 1e). Also marking the limit of this complex are the electron-dense expansions of the nucleus, called the nuclear shoulders (fig. 1f).

Late spermatids are elongated, as is the acrosomal complex, that here presents clearly defined regions, such as the subacrosomal cone, the less dense perforatorium, located at the nuclear apex, and more externally, the acrosomal cap. These structures are separated by clear regions, such as the epinuclear clear zone, between the subacrosomal cone and the perforatorium, and by the clear middle zone, located between the perforatorium and the acrosomal cap (fig. 2a, 2b, 2c). Chromatin is totally compacted (fig. 2d), and this extreme condensation is associated with a layer of microtubules, called the manchette, that surrounds the nucleus (fig. 2e).

Intense immunocytochemical marking identified actin in two regions of the acrosomal complex, the subacrosomal cone and the perforatorium (figs. 2f, 2g).

DISCUSSION

FAWCETT *et al.* (1971) furnished the classical description, elucidating the factors that influence in the establishment of the spermatozoon head shape, among which the manchette appears to exert the strongest influence. Moreover a nuclear ring was observed, at the contact of the nuclear membrane with the acrosomal complex, similar to an annulus. In our study, this structure has been described as the nuclear shoulders, in accordance with more recent publications of TEIXEIRA *et al.* (1999) and VIEIRA *et al.* (2001). This would be the attachment point of the manchette, for the establishment and maintenance of shape of the spermatid head in formation. Another external factor involved in the molding of the nucleus is the influence exerted by the Sertoli cell (FAWCETT *et al.* 1971).

It is impossible to attribute the shape of the nucleus entirely to external forces applied by the microtubules. Intrinsic forces must also determine nuclear configuration. The factors determining acrosome shape appear to be generated from within rather than occurring as a consequence of externally applied forces. FAWCETT *et al.* (1971) concluded that there is a redistribution of cytoplasm that takes place during spermatid elongation, but

this is probably not directly involved in nuclear shaping. The different patterns, which can be observed in condensing spermatid nuclei of various species, probably depend on the synthesis of specific arginine-rich histones that control the shape and aggregation mode of the DNA-histone complexes formed during nuclear condensation. Therefore, the condensation would be the result of the elimination of certain nuclear components, including water and non-histonic proteins. This is, at least, partially in agreement with CRUZ-HÖFLING and CRUZ LANDIM (1978) who assumed that the process of chromatin condensation would be a result of the dehydration of the nucleoprotein as the nucleoplasm is eliminated.

The perforatorium, according to HUMPHREYS (1975), is derived from a subacrosomal granule interposed between the acrosomal vesicle and the nucleus, in birds. This was also accepted by DEL CONTE (1976) for reptiles, and even for mammals this concept has been generally accepted. DEL CONTE (1976) suggested that the granule is a product of acrosome and nucleus interaction.

Synchronous with the nuclear changes, the acrosomal vesicle and granule, originating from the Golgi complex, make up the anterior acrosomal cap. The subacrosomal space becomes dilated and its material diffuses, originating the subacrosomal cone, according to VIEIRA *et al.* (2001).

Our results have leaded us to disagree with this hypothesis. The acrosomal granule appears to be the responsible for the formation of the perforatorium, together with the subacrosomal granule located just below the acrosomal granule. However, beside it is the pro-acrosomal vesicle, which is responsible for the formation of the subacrosomal cone. This conclusion, is based as much on the location of the structures during spermiogenesis, as well as on the density observed with electron microscopy, and cytochemical properties such as the amount of basic protein which is very similar in the acrosomal and subacrosomal granule, perforatorium and subacrosomal cone (FERREIRA, *personal observations*). This assumption coincides with what was ultrastructurally demonstrated by BUTLER and GABRI (1984) in the *Podarcis taurica* lizard.

The formation of the spermatozoon head of *T. itambere* occurs in a manner very similar to the lacertilian. It also presents many similarities with the spermiogenesis of birds,

in accordance with the description of SOLEY (1997) in *Struthio camelus*, but with the difference that in this case chromatin condensation occurs in tufts and not in filaments, and the manchette in lizards is not so well defined.

Some relations of homology between structures of the acrosomal complex have already been observed. In descriptions of spermatozoon ultrastructure of some Anura, the subacrosomal cone was not considered homologous to the conical perforatorium (BURGOS and FAWCETT 1956; JAMIESON *et al.* 1993) mainly based on differences in electron density. However, more recent work (JAMIESON 1999; GARDA *et al.* 2002) defines these organelles as homologous. If a comparison is made of this data related to the subacrosomal cone and perforatorium of Anura with *T. itambere* lizard, it can be noticed that these structures have differences in electron density, but immunocytochemical methods demonstrate that, at least in regard to actin composition, and therefore possibly to function, these organelles can be considered homologous.

According to BACCETTI *et al.* (1980) a spherical invagination of the nuclear envelope appears in its center. This progressively increases in length as the acrosome elongates and is, step by step, transformed into the perforatorium. These steps were not observed for *T. itambere*, although a similar location of the subacrosomal layer was found.

AL-HAJJ *et al.* (1987) when studying the *Agama stellio* lizard found other differences in the initial stages of the acrosomal complex, such as the formation of two pro-acrosomal vesicles, which in *T. itambere* does not occur. Also, they believed that the subacrosomal cone, located between the pro-acrosomal vesicle and the nucleus of spermatids would be responsible for the future perforatorium.

During differentiation, the subacrosomal material does not diffuse homogeneously, leading to the formation of a lateral electron-lucent layer between the acrosomal vesicle and the subacrosomal cone (or unilateral ridge) and a slender electron-lucent channel above the nucleus, the epinuclear clear zone. The subacrosomal granule has been reported in other lizard species (DEL CONTE 1976; BUTLER AND GABRI 1984; AL-HAJJ *et al.* 1987) and has been considered responsible for perforatorium formation.

According to BACCETTI *et al.* (1980) the perforatorium is made up of an oriented bundle of actin filaments, which would maintain the sperm shape and is conserved in the

ejaculated sperm until penetration of the egg; the same role cannot be attributed to the developing perforatorium during spermiogenesis, because the organelle is too small to sustain nuclear shape. Therefore, according to these authors, this organelle in Sauropsids seems to be a residual one, destined to be lost during evolution.

Actin was detected in the subacrosomal space of spermatids during the greater part of spermiogenesis in the rat, hamster, monkey and man (FOUQUET *et al.* 1989). We also identified immunocytochemically the presence of actin in the perforatorium and in the subacrosomal cone of the lizard *T. itambere*.

The role of actin from late spermatids onwards remains an unsolved question, subacrosomal actin is a constant feature during spermiogenesis verified so far, which suggests that this cytoskeletal protein may have a role in spermatid differentiation (FOUQUET *et al.* 1989).

The subacrosomal layer of spermatids in hamster contains filamentous actin during the greater part of spermiogenesis (FOUQUET *et al.* 1992). However, in early spermatids of *T. itambere*, markings with actin antibody were not sufficiently specific to determine the presence of this element in the region of the subacrosomal material, nor in the subacrosomal and acrosomal granules.

According to FOUQUET *et al.* (1992) the filamentous actin of the subacrosomal layer appears as transitory scaffolding that might be involved in the assembly of other proteins at the perinuclear cytoskeleton and indirectly in nuclear shaping.

One of the explanations for the presence of actin in the structures that compose the acrosomal complex is the fact that during spermatozoon approach to the egg a sequence of events takes place. First, the acrosomal reaction occurs when the spermatozoon is in the vicinity of the egg envelope, then the perforatorium suffers an enlargement and becomes associated to the oolema. Finally, the perforatorium material contracts to bring the whole spermatozoon in contact with the egg (GUERRA *et al.* 1994). This has been described for spermatozoa of mammals, however in lizards, the subacrosomal cone has been suggested to possess the same function as that of the perforatorium (JAMIESON 1995).

Another interesting explanation is that actin filaments may be involved in Golgi vesicle transport during acrosome formation; however, there is no indication that

subacrosomal actin (FOUQUET *et al.* 1989). Subacrosomal actin also may have a structural and binding function and may serve to anchor the acrosome to the nucleus in order to harmonize their shaping through mutual interactions during the elongation phase of spermatids. In addition, actin of the subacrosomal space may give a certain structural rigidity to the acrosome of the spermatid, and even of the spermatozoon, if a modified actin remains present at this location (FOUQUET *et al.* 1989).

Therefore, we present in this study some suggestions as to the origin of the structures that compose the acrosomal complex in lizards. Many similarities exist between the localization of actin in the different animal groups, and the purpose of the perforatorium and the subacrosomal cone, which may be important for fertilization.

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REFERENCES

AL-HAJJ H., JANAKAT S. and MAHMOUD F., 1987 - *Electron microscopic study of sperm head differentiation in the lizard Agama stellio.* Can. J. Zool., 65: 2959-2968.

BACCETTI B., BIGLIARDI E. and BURRINI A.G., 1980 - *The morphogenesis of the vertebrate perforatorium.* J. Ultrastruct. Res., 71: 272-287.

BURGOS M.H. and FAWCETT D.W., 1956 - *An electron microscope study of spermatid differentiation in the toad, Bufo arenarum Hensel.* J. Biophys. Biochem. Cytol., 2: 223-239.

BUTLER R.D. and GABRI M.S., 1984 - *Structure and development of the sperm head in the lizard Podarcis (=Lacerta) taurica.* J. Ultrastruct. Res., 88: 261-274.

COURTENS J.L., BIGGIOGERA M., GABBIANI G. and FAKAN S., 1991 - *The striated body: a new structure in the sperm head of the rabbit spermatozoon.* J. Exp. Zool., 259: 128-137.

CRUZ HÖFLING M.A. and CRUZ LANDIM C., 1978 - *The fine structure of nuclei during spermiogenesis in the lizard Tropidurus torquatus (Lacertilia).* Cytologia, 43: 61-68.

DEL CONTE E., 1976 - *The subacrosomal granule and its evolution during spermiogenesis in a lizard.* Cell Tiss. Res., 171: 483-498.

FAWCETT D.W., ANDERSON W.A. and PHILLIPS D.M., 1971 - *Morphogenetic factors influencing the shape of the sperm head.* Develop. Biol., 26: 220-251.

FOUQUET J.P., KANN M.L. and DADOUNE J.P., 1989 - *Immunogold distribution of actin during spermiogenesis in the rat, hamster, monkey, and human.* Anat. Rec., 223: 35-42.

FOUQUET J.P., FRAILE B. and KANN M.L., 1991 - *Sperm actin and calmodulin during fertilization in the hamster: an immune electron microscopic study.* Anat. Rec., 231: 316-323.

FOUQUET J.P., VALENTIN A. and KANN M.L., 1992 - *Perinuclear cytoskeleton of acrosome less spermatids in the blind sterile mutant mouse.* Tissue & Cell, 24: 655-665.

GARDA A.A., COLLI G.R., AGUIAR-JUNIOR O., RECCO-PIMENTEL S.M. and BAO S.N., 2002 - *The ultrastructure of the spermatozoa of Epipedobates flavopictus (Amphibia, Anura, Dendrobatidae), with comments on its evolutionary significance.* Tissue & Cell, 34: 356-364.

GUERRA R., CAMPOS B. and ESPONDA P., 1994 - *Analysis of the spermatozoa of four bivalves with particular reference to the acrosome and plasma membrane glycoproteins.* J. Submicrosc. Cytol. Pathol., 26: 489-495.

HUMPHREYS P.N., 1975 - *The differentiation of the acrosome in the spermatid of the budgerigar (Melopsittacus undulatus).* Cell Tissue Res., 156: 411-416.

JAMIESON B.G.M., LEE M.S.Y. and LONG K., 1993 - *Ultrastructure of the spermatozoon of the internally fertilizing frog Ascaphus truei (Ascaphidae, Anura, Amphibia) with phylogenetic considerations.* Herpetologica, 49: 52-65.

JAMIESON B.G.M., 1995 - *The ultrastructure of spermatozoa of the Squamata (Reptilia) with phylogenetic considerations.* In: Jamieson B.G.M., Ausio J., Justine J.L. (Eds.), "Advances in spermatozoal phylogeny and Taxonomy", pp. 395-383. Édition du muséum national d'histoire naturelle, Paris.

JAMIESON B.G.M., 1999 - *Spermatozoal phylogeny of the vertebrata.* In: Gagnon C. (Ed.), "The Male Gamete: From Basic Science to clinical Applications", pp. 303-331. Cache River Press, Vienna.

PARANKO J, YAGI A and KUUSISTO M, 1994 - *Immunocytochemical detection of actin and 53 kDa polypeptide in the epididymal spermatozoa of rat and mouse.* Ana. Rec., 240: 516-527.

RODRIGUES M.T., 1987 - *Sistemática, ecologia e zoogeografia dos Tropidurus do grupo Torquatus ao Sul do Rio Amazonas (Sauria: Iguanidae).* Arq. Zool. S.P., 31: 105-230.

SOLEY J.T., 1994 - *Centriole development and formation of the flagellum during spermiogenesis in the ostrich (Struthio camelus).* J. Anat., 185: 301-313.

TEIXEIRA R.D., VIEIRA G.H.C., COLLI G.R. and BAO S.N., 1999 - *Ultrastructural study of spermatozoa of the neotropical lizards, Tropidurus semiaeniatus and Tropidurus torquatus (Squamata: Tropiduridae).* Tissue & Cell, 31: 308-317.

VAN SLUYS M., 1993 - *The reproductive cycle of Tropidurus itambere (Sauria: Tropiduridae) in Southeastern Brazil.* J. Herpetol., 27: 28-32.

VIEIRA G.H.C., WIEDERHECKER H.C., COLLI G.R. and BAO S.N., 2001 - *Spermiogenesis and testicular cycle of the lizard Tropidurus torquatus (Squamata, Tropiduridae) in the Cerrado of central Brazil.* Amphibia-Reptilia, 22: 217-233.

FIGURE LEGENDS

Fig. 1 - **a.** and **b.** - Early spermatids. **a.** - Pro-acrosomal vesicle (av) in close contact with the nucleus. Observe the acrosomal granule (ag) inside the vesicle and the subacrosomal granule (sg) outside this vesicle. Notice also the electron dense layer below the pro-acrosomal vesicle, and in contact with the nucleus, which has been called the subacrosomal material (sm). Bar = 0,46μm. **b.** - Pro-acrosomal vesicle, showing the subacrosomal material (sm) and the initial formation of one of the clear layers of the acrosomal complex, apparently the epinuclear clear zone (e) in the contact with the nucleus (n). Bar = 0,11μm. **c.-f.** - Intermediate spermatids. **c.** - A longitudinal section shows the compacting of chromatin in thick filaments that are twisted (curved arrows), in a process of molding and

elongation of the nucleus. Bar = 0,64μm. **d.** - Transverse section of an intermediate spermatid. Notice the network of condensing chromatin filaments. Bar = 0,16μm. **e.** and **f.** - Longitudinal or slightly tangential views of the acrosomal complex in formation. It is possible to distinguish the different layers, such as, acrosome cap (a), clear middle zone (c), perforatorium (p), epinuclear clear zone (e) and subacrosomal cone (s). External to the acrosomal complex, membranes of the Sertoli cell (SC) can be seen. At the limit between the acrosomal complex and the nucleus, observe the nuclear shoulders (ns). **e.** Bar= 0,12μm. **f.** Bar= 0,46μm.

Fig. 2 - Late spermatids. **a.** - Longitudinal section of the acrosomal complex. This structure is divided into compartments: the subacrosomal cone (s), epinuclear clear zone (e) perforatorium (p), clear middle zone (c) and acrosomal cap (a). Observe the membranes of the surrounding Sertoli cell (SC). The hatched lines indicate the probable regions of the transverse sections in figures **b.** and **c.** Bar = 0,49μm. **b.** - The apical region of the acrosomal complex. Perforatorium (p), clear middle zone (c) and acrosome cap (a). Bar = 0,14μm. **c.** - Transverse section of the basal region of the acrosomal complex. Subacrosomal cone (s), perforatorium (p) and acrosome cap (a). Bar = 0,18μm. **d.** - Transverse section of the nucleus (n) showing complete chromatin condensation. Bar = 0,27μm. **e.** - Longitudinal section. Observe the longitudinally (arrow) arranged microtubules (t) around the nucleus (n). Bar = 0,13μm. **f.** - Transverse section of the apical region of the acrosomal complex, similar to the section in figure 2b. Observe immunogold labeling of the perforatorium (p) and its absence in the acrosome (a). Bar = 0,18μm. **g.** - Longitudinal section of an intermediate spermatid. The acrosomal complex is sectioned near the region shown in figure 1f. Observe intense immunogold labeling in the region of the perforatorium (p) and the subacrosomal cone (s) and its absence in the intermediate (epinulear clear zone) and the external region (acrosome cap), nucleus (n). Bar = 0,23μm.

Figure 1

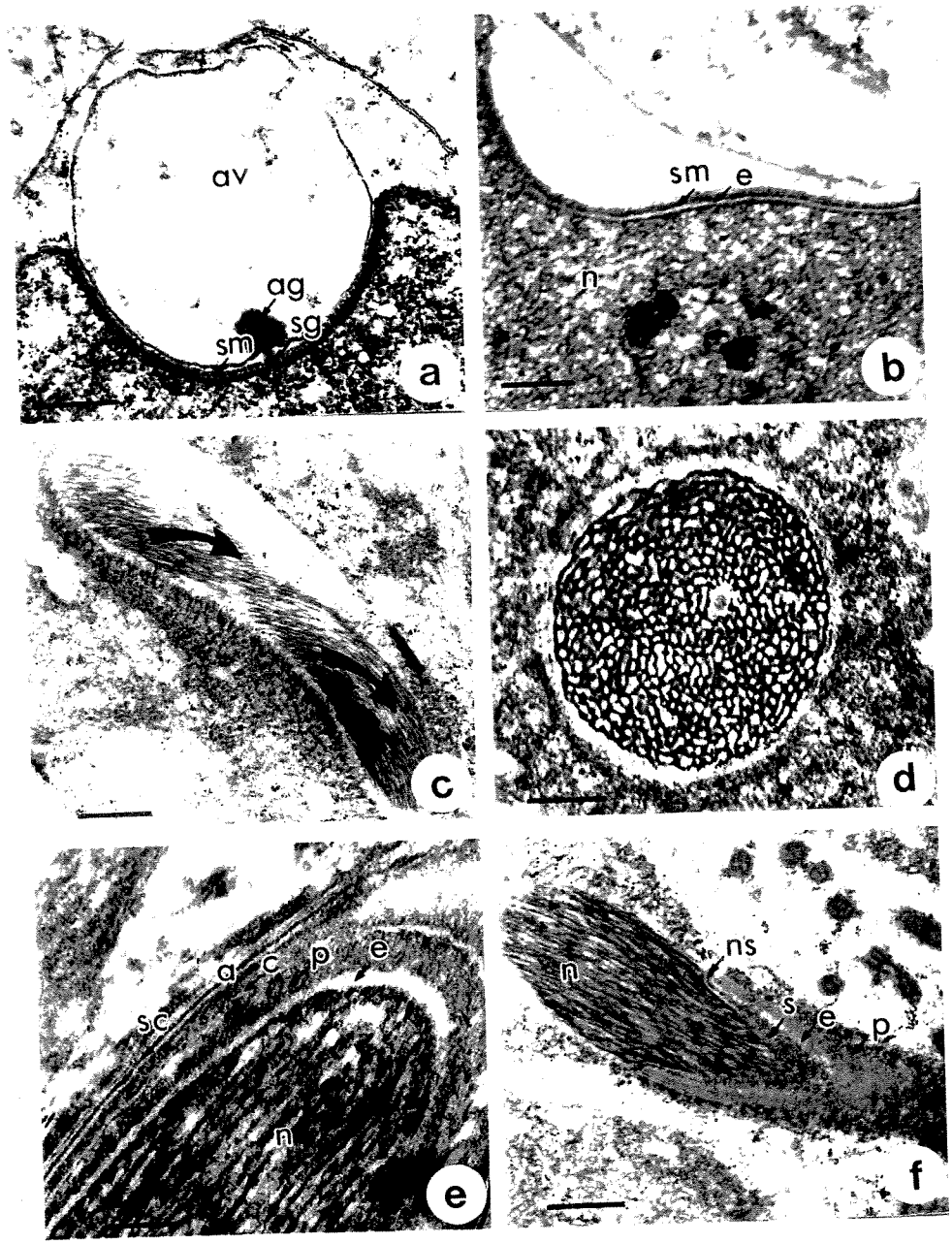
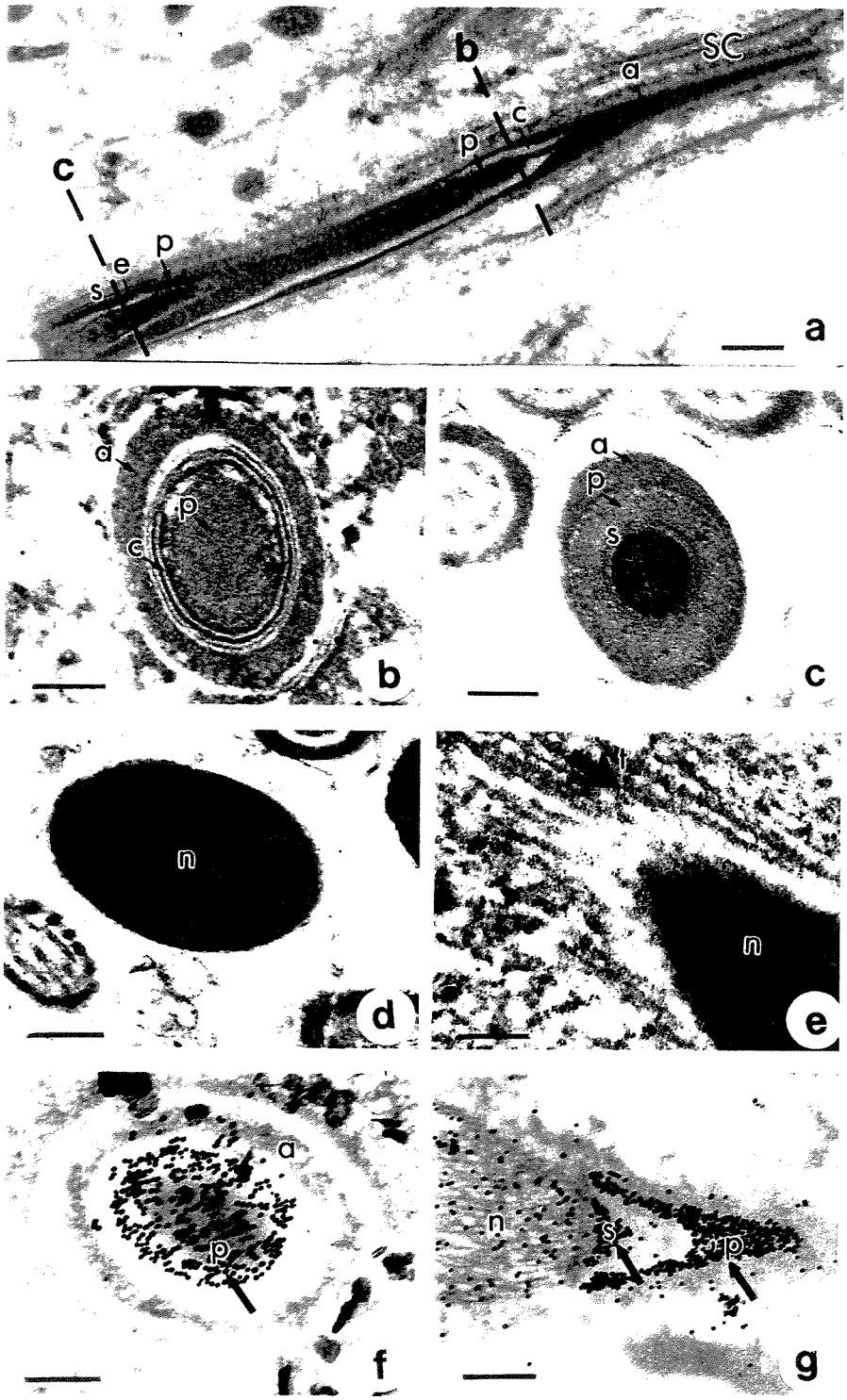


Figure 2



7. Conclusões

- ✓ O ciclo reprodutivo testicular do lagarto *T. itambere* é do tipo anual, com um breve período de descontinuidade (três meses), onde são observadas células em degeneração no lúmen dos túbulos seminíferos.

- ✓ O ciclo epididimário apresenta uma variação durante o ano, com aumento da altura do epitélio e da produção de secreções, que coincide com o período de redução de espermatogênese.

- ✓ As variações morfológicas durante o ano estão associadas às variações sazonais, onde a redução da espermatogênese coincide com a redução das temperaturas e pluviosidade.

- ✓ A espermatogênese apresenta muitas semelhanças com o processo descrito para os demais vertebrados amniotas.

- ✓ A espermiogênese envolve eventos de alongamento nuclear e compactação cromatínica, formação de complexos estruturais como o complexo acrosomal e o complexo axonêmico, e uma íntima associação entre espermátides e célula de Sertoli.

- ✓ Os dados sobre a ultra-estrutura do espermatozóide de *T. itambere* são consistentes para reforçar a separação das famílias Tropiduridae e Iguanidae, de acordo com revisão sistemática recente.

- ✓ Através de histoquímica e citoquímica ultra-estrutural foram evidenciadas diversas características da espermatogênese:

- Evidência de polissacarídeos e glicogênio que ocorre no interior da célula de Sertoli, permitindo a demonstração da organização desta célula em fases distintas do ciclo. Demonstra ainda a presença destes componentes na vesícula pró-acrosomal e no complexo acrosomal de espermátides jovens e adiantadas.
- Evidência de glicoconjugados específicos em diversas regiões de espermátides mostrando-se coincidentes com estruturas do espermatozóide no complexo acrosomal, peça intermediária e flagelo. Também evidenciaram a riqueza de glicoconjugados em secreções dos ovidutos e receptáculo seminal das fêmas.
- Evidência de proteínas básicas, durante a espermiogênese, na formação das organelas dos espermatozóides.
- O produto da reação da fosfatase ácida nas áreas claras do complexo acrosomal e células em degeneração no período quiescente.
- Gotas de lipídios evidenciados no citoplasma da célula de Leydig no período de quiescência. E concentração de lipídio na formação da vesícula pró-acrosomal de espermátides jovens.

✓ Através de imunocitoquímica ultra-estrutural observamos sítios de localização de actina em duas regiões do complexo acrosomal em espermátides, o cone subacrosomal e o perforatório. Assim, estas estruturas seriam homólogas na sua constituição e função no processo de fertilização.