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Testicular Ultrastructure of Zebu Bulls in Costa Rica

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With 20 figures

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Summary

The testicular ultrastructure of sexually mature Zebu *(Bos indicus)* bulls from Costa Rica was studied in relation to their classification based on andrological examinations undertaken in the field. Based on the testicular consistency at palpation, each bull was assigned to one of three groups, ranging from normal (Group A) to markedly reduced testicular consistency (Group C). The ultrastructure of the clinically normal testicle (Group A) in the Zebu bull resembled that of *Bos taurus*. In testicles with a slight to moderate reduction of testicular consistency (Group B) degenerated primary spermatocytes and spermatids were common at all stages of the seminiferous cycle. Abnormalities in the condensation of the chromatin, acrosomal defects, and the presence of degenerated cells and cellular debris were the most common of disturbances found. In testicles from bulls with a marked reduction of testicular consistency (Group C) the seminiferous epithelium of most tubules showed degenerative changes, thereby making it impossible to classify the stage of the cycle. The changes were mainly restricted to spermatocytes and spermatids, whose relative numbers were reduced. The number of cells undergoing meiotic divisions was also greatly reduced. Changes in the spermatids were also found including the appearance of severe acrosomal defects, such as acrosomal pouches, the failure of nuclear elongation and clear defects in chromatin condensation. The present ultrastructural findings in *Bos indicus* do not differ from the picture of normal testicular structure and of testicular degeneration usually found in *Bos taurus* bulls.

Introduction

The degree to which male reproductive characteristics affect the overall fertility rate of domestic animals has traditionally been underestimated. In fact, reproductive disorders in bulls give rise to major losses in cattle production. When the disorder is caused by

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alterations in the testes or epididymides, a long period usually elapses before the problem is detected, leading to considerable losses in the reproductive efficiency of the affected herds.

Malfunctions of the seminiferous epithelium are common and can result in a reduction in the quality and/or quantity of sperm produced. A malfunction is considered degenerative when the changes develop in a gonad that earlier had achieved functional maturity (JUBB and KENNEDY, 1970), and heat stress is the most common causal factor (SKINNER and LOUW, 1966). In general, and elevated, non-lethal temperature in the testis, regardless of its origin, tends to adversely affect testicular function. Among domestic animals, the consequences can be reduced sperm production, enhanced production of abnormal sperm, decreased sperm motility — leading to a reduction of fertility, or even the complete inhibition of spermatogenesis (SETCHELL, 1978).

Seasonally high ambient temperatures, which can be enhanced by high humidity, provoke changes in body temperature which affect other body functions and may in turn affect the testis. As early as 1940, ERB and co-workers had reported a reduction in breeding efficiency in cattle during the hot months of the year, which has been further documented in other studies (rev. by COWLES, 1965; SALISBURY and VANDEMARK, 1961; VANDEMARK and FREE, 1970). Concurrently with low fertility, semen volume and sperm motility reached their lowest levels during the hot summer months (SEATH and STAPLES, 1941; BROWN, 1960), and the frequency of sperm abnormalities increased by 25 % (ERB et al., 1942) despite shading or air conditioning (JOHNSTON and BRANTON, 1953; PATRICK et al., 1959). Local cooling did, however, prevent seminal degeneration due to short periods of exposure to high temperature (OKAMOTO et al., 1959). Exposure of prepuberal as well as adult bulls to high ambient temperatures (36—40 °C) for 8—12 h/day had a marked detrimental effect on spermatogenesis, with damage peaking 4 weeks after exposure (DE ALBA and RIERA, 1966; SKINNER and LOUW, 1966). This effect is reportedly more severe in *Bos taurus* than in *Bos indicus* (SKINNER and LOUW, 1966; VALE-FILHO et al., 1980). Additional studies have shown that spermatogenesis in young bulls is impaired by continuous exposure to temperatures above 30 °C for periods longer than 5 weeks (CASADY et al., 1953; SKINNER and LOUW, 1966).

In Costa Rica, the importance of andrological evaluation was not recognized until recently. Furthermore, in andrological investigations carried out by the School of Veterinary Medicine only 70 % of the bulls evaluated were considered to be potentially satisfactory breeders. Testicular degeneration due to heat stress was the most common pathological finding in Costarican Zebu bulls (MÜLLER, 1990). The present classification of testicular degeneration is based on an evaluation of testicular consistency and sperm abnormalities. Nevertheless, no clear-cut prognosis can be made in most cases. For this reason, there is a need for detailed morphological research on both the normal and affected testicles to determine the magnitude of the lesions of the seminiferous epithelium and the relationship between the severity of the lesion and the clinical findings.

The present investigation was therefore undertaken to describe the fine structure of the testes and epididymides of Zebu bulls with field clinical diagnosis of different degrees of testicular alterations in Costa Rica.

Material and Methods

The animal population studied comprised a total of 35 adult, extensively reared Zebu *(Bos indicus)* bulls from the dry Pacific region in the northwestern part of Costa Rica. The animals belonged either to a group of commercially slaughtered bulls (GISA slaughterhouse at Liberia, Costa Rica, n = 10) or to a group of bulls that were clinically examined in the field three times at 2 to 3 month intervals (n = 25) prior to slaughter, between February and December 1990. At each clinical examination, semen was collected by electroejaculation, and immediate and mediate determinations of the relative amount of sperm abnormalities (according to LEIDL, 1986, modified by MÜLLER, 1990) were made. In both cases, the bulls were clinically examined antemortem and found to be healthy and in good body condition. The testicles and epididymides were carefully palpated and the bulls allotted to one of the following testicular consistency groups (a field-score system routinely used in Costa Rica):

Group A: normal testicular consistency
Group B: slight to moderate reduction of testicular consistency
Group C: marked reduction of testicular consistency

Testes and epididymides were collected immediately after slaughter of the bulls belonging to groups A (n = 13, 6 GISA and 7 field bulls), B (n = 14, field bulls) and B (n = 8, 4 GISA and 4 field bulls), and any macroscopic morphological abnormalities noted. The testicular artery was cannulated with a blunt needle, and after clearing the vascular bed with isotonic NaCl solution (0.9 %), the organs were fixed by manual vascular perfusion. The fixative was a 5 % solution of glutaraldehyde in 0.067 M cacodylate buffer (pH 7.2, 500 mOsm). Small (1-mm-thick) tissue samples were excised from the testis (proximal, medial and distal regions) at the ad-epididymal side and promptly immersed into the fixative for further storage at 4 °C. Spermatozoa were also pipetted out from the cauda epididymides and, together with pieces of cauda epididymides, immersion-fixed in the same fixative. Thereafter, the specimens were rinsed in cacodylate buffer at 4 °C, trimmed into smaller (1 mm^3) selected pieces and post-treated in 2 % osmium tetroxide. The tissue blocks were then dehydrated by exposure to graded concentrations of ethanol and propylene oxide, and embedded into Agar 100® plastic resin. Semi-thin sections (1 µm) for light microscopy were cut on a LKB Ultratome® and stained with buffered toluidine blue. Ultra-thin sections for transmission electron microscopy were cut from selected areas. The ultra-thin sections were picked up onto uncoated copper grids, counterstained with uranyl acetate and lead citrate, and examined in a Philips EM 201 electron microscope at 60—80 kV.

Results

Sperm motility for most of the bulls included in the study (n = 25) was low. The percentage of spermatozoa with primary abnormalities increased as testicular consistency decreased, although differences between groups were not significant [25.2 ± 11.0 in Group A ($n = 7$), 43.3 ± 13.5 in Group B ($n = 14$), 66.4 ± 8.5 in Group C ($n = 4$), means ± SD].

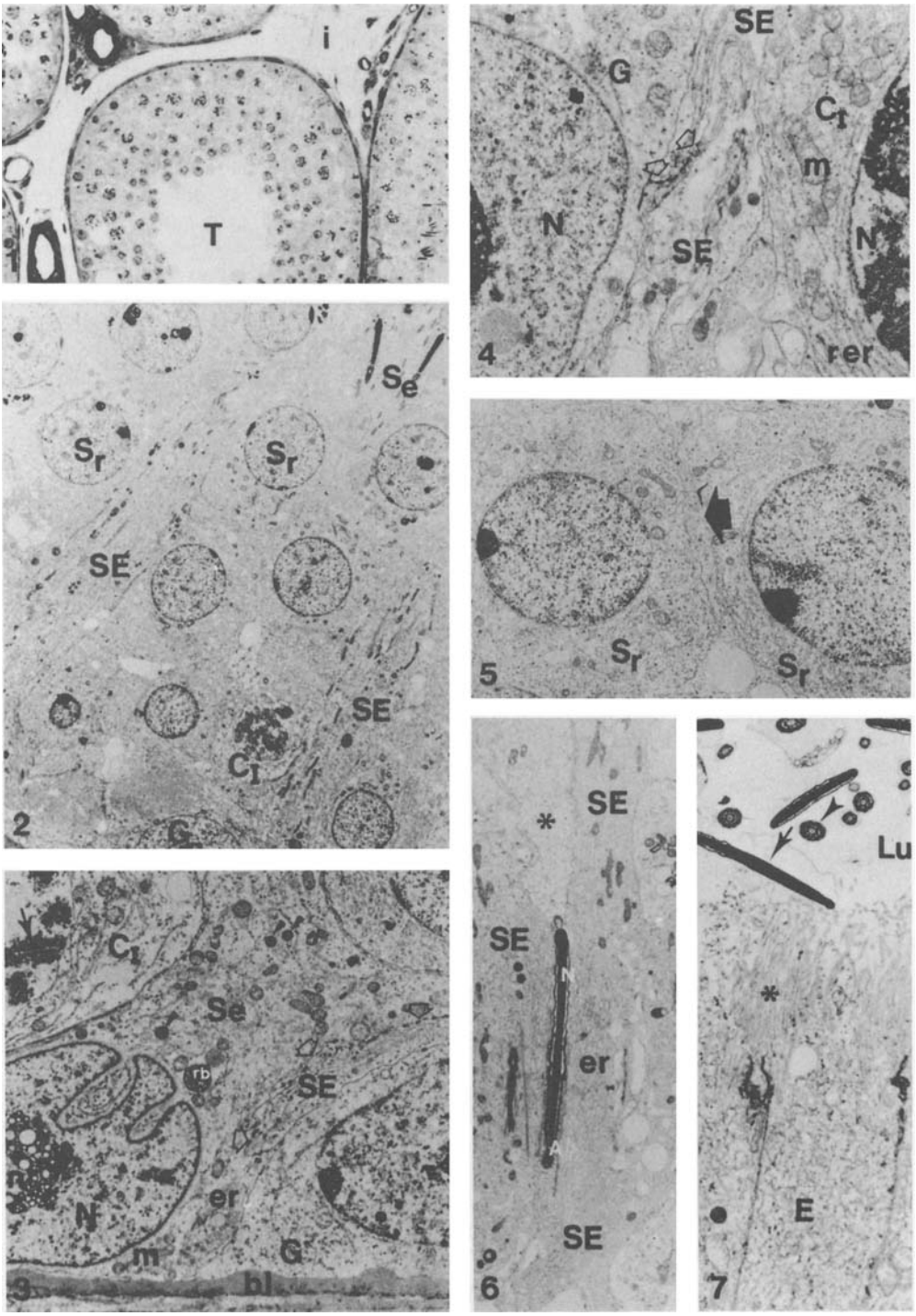
At slaughter, all testes and epididymides were macroscopically normal. The histological examination using light microscopy revealed the following findings:

Group A: normal spermatogenesis (n = 13),
Group B: diffuse tubular alterations (n = 8), otherwise normal spermatogenesis (n = 6) and,
Group C: clear testicular degeneration, with fibrosis and even atrophy in several areas (n = 8).

Electron microscopy confirmed the overall histology of the samples showing the following fine structure:

Group A (normal consistency): The testicular parenchyma was composed of seminiferous tubuli and interstitial tissue (Fig. 1). In some sections, parts of the intratesticular excurrent duct system were present. The seminiferous tubuli were surrounded by a multilayered basal lamina and several layers of myoid, peritubular contractile cells (Figs. 9, 10, 15). Most of the seminiferous tubuli had normal morphology, with all types of cells

Figs. 1 to 7. Morphology of the normal testis and epididymis of Zebu bulls (Group A). Fig. 1 (toluidine blue, 100×) presents topographical views of the seminiferous tubuli (*T*, stage 1) and testicular interstitium *(i)*. The electron micrograph in Fig. 2 (3,600×) shows an overview of the seminiferous epithelium with the general morphology of bovine Sertoli cells *(SE)* and their relationship to the different stages of the germ cells (*G:* spermatogonia, C_I: spermatocyte I, S_r: round spermatid, S_e: elongated spermatid). The basal area of the seminiferous epithelium is depicted in Fig. 3 (5,400×) showing a Sertoli cell *(SE)* and a spermatogonium *(G)* apposed to the basal lamina *(bl)* (*er:* endoplasmic reticulum, *m:* mitochondria, *rb:* residual body, *N:* nucleus, *n:* nucleolus, *arrow:* synaptonemal complex, *arrowheads:* lipid droplets). Inter-Sertoli cell junctions (*empty arrows* in Figs. 3, 5,400×, and 4, 7,200×) separate the basal compartment (*G:* spermatogonium) from the adluminal one where a primary spermatocyte (C_I, *m:* mitochondria, *rer:* rough endoplasmic reticulum) is located. Fig. 5 shows two sister round spermatids (S_r) connected through an intercellular bridge *(arrow)* (5,400×). A spermatid in maturation phase (*A:* acrosome, *N:* nucleus, **:* cytoplasm) appears surrounded by the apical cytoplasm of the Sertoli cell *(SE)* which contains accumulations of



regularly arranged cisterna of endoplasmic reticulum *(er)* (Fig. 6, 5,400×). Fig. 7 (5,400×) depicts the apical epithelium *(E)* of cauda epididymidis, provided with numerous stereocilia *(*)*, and spermatozoa cut longitudinally *(arrow)* and transversally *(arrowhead)* in the lumen *(L)*

present, and the normal chain of events of spermatogenesis (Figs. 1 to 7). The seminiferous epithelium consisted of two cell populations, the various stages of spermatogenic germ cells (Figs. 2 to 6) and the sessile, non-proliferating Sertoli cells (Figs. 2 and 3). The latter were tall, irregular cells extending from the lamina propria to the tubular lumen (Fig. 2). They were interconnected by specialized cell junctions, which divided the seminiferous epithelium into basal and adluminal compartments (Figs. 3 and 4). These cells typically had a conspicuous nucleolus composed of a large number of membrane-limited tubuli and vesicles of various sizes (Figs. 3 and 18) with a non-electron dense content sometimes flocculent within the strands of the nucleolonema. Sertoli cell cytoplasmic processes occupied the spaces among the various types of germ cells. Since all types of germ cells were visible, it was possible to classify the stages of bull spermatogenesis in almost all cases. Cells with degenerative changes were only found occasionally, and usually appeared during late stages of spermiogenesis. The morphology of the cauda epididymides appeared to be normal, and its lumen was well-filled with normally-looking spermatozoa (Fig. 7).

Group B (slight to moderate reduction of testicular consistency): Although the seminiferous epithelia of many tubules showed many intercellular cavities (Figs. 8 and 9) the germ and Sertoli cells present were of normal appearance (Fig. 10). The other tubules contained degenerated primary spermatocytes and spermatids (Figs. 11 and 12). Disturbances in the condensation of the chromatin (Fig. 12), acrosomal defects, degenerated cells (Fig. 12), and cellular debris in the cytoplasm of the Sertoli cells (Fig. 13) were most common among the affected tubuli (Fig. 11). The cauda epididymides were morphologically normal. Cross-sectioned caudal spermatozoa showed many abnormal acrosomes.

Group C (marked reduction of testicular consistency): Most tubules showed degenerative changes of the seminiferous epithelium that varied in intensity from slight (Fig. 14) to severe. In most cases, it was not even possible to classify the stage of the cycle of the seminiferous epithelium, sometimes leaving the tubuli empty from germ cells (Fig. 17). The changes were mainly restricted to spermatocytes and spermatids (Figs. 14 and 15), which showed reductions in their relative numbers. The number of cells undergoing meiotic divisions was greatly reduced (Fig. 15). Degenerative changes in the elongating spermatids were found mostly in the head region, and consisted mainly of acrosomal pouches and clear defects in the chromatin condensation (Figs. 19 and 20). Most Sertoli cells contained huge amounts of phagocytosed material (Figs. 16 and 18). The cauda epididymides appeared normal, but many caudal spermatozoa had abnormal nuclear shapes and acrosomal defects.

Discussion

The present ultrastructural description of the normal testis in Zebu bulls agrees closely with previous descriptions of the testicular morphology of taurine animals (WROBEL et al., 1979, 1981, 1982; BIELANSKA-OSUCHOWSKA and SYSA, 1981; EKSTEDT et al., 1986; SINOWATZ and AMSELGRUBER, 1988).

Testicular function can be adversely affected by several factors, i.e. malnutrition, genetic predisposition, infections and environmental stress. In the latter cases, heat stress is the most common source of injury, affecting most categories of postpuberal mammals, despite their nutritional status. The bulls included in the present study had a good

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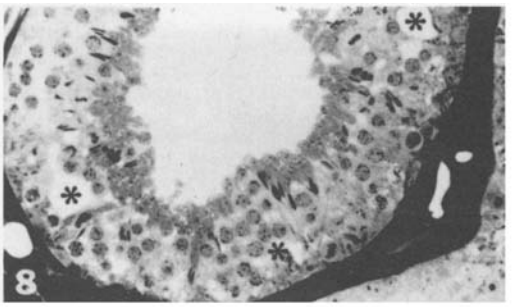
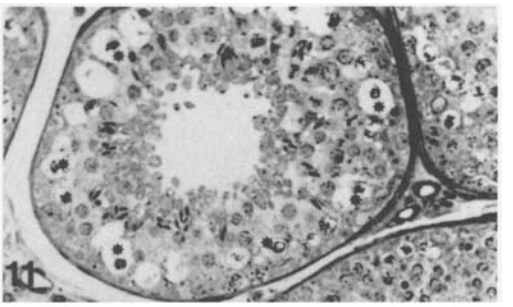
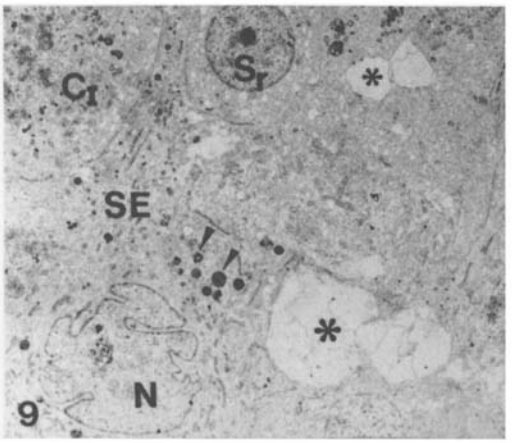
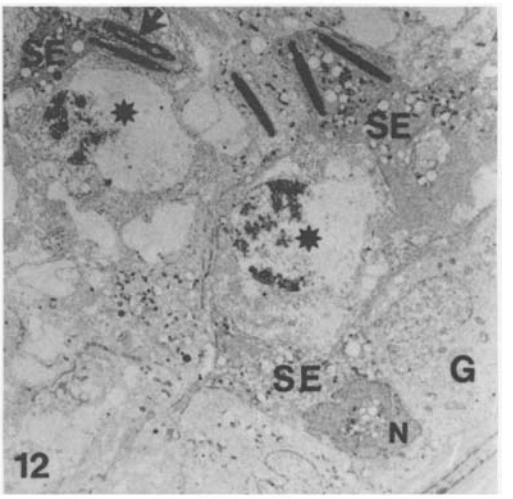
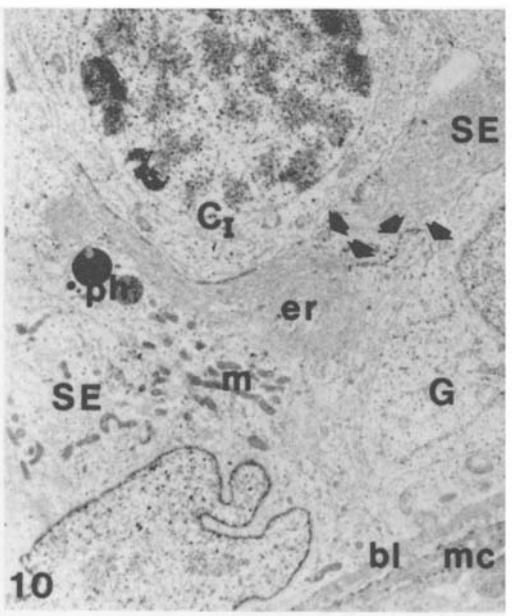
Figs. 8 to 13. Morphology of the testes of Zebu bulls with a slightly to moderately decreased consistency at palpation (Group B). The minor alterations (hollow areas, *) seen in this group of animals are shown in a transversely cut tubulus (Fig. 8, toluidine blue, 100×) and in a low-magnification electron micrograph (Fig. 9, 3,600×) among the Sertoli cells (*SE,* [*arrowheads:* lipid droplets, *N:* nucleus]) and germinal cells (C_I and S_r). A well preserved morphology is present in Fig. 10 (5,400×) showing the basal area of a tubulus with Sertoli cells *(SE)* separating with cell junctions *(arrows)* a spermatogonium *(G)* from a primary spermatocyte in leptotene (C_I) *(er:* endoplasmic

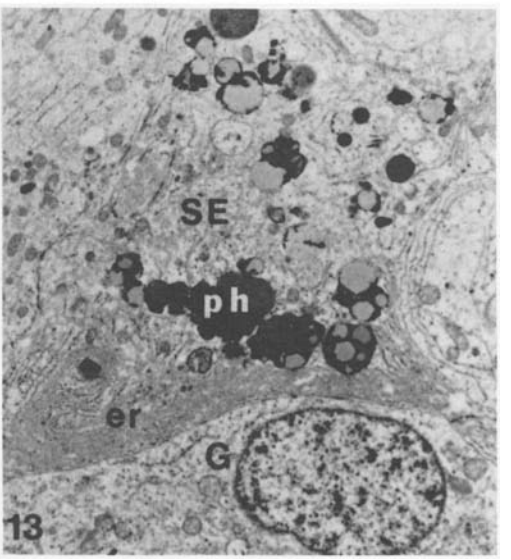




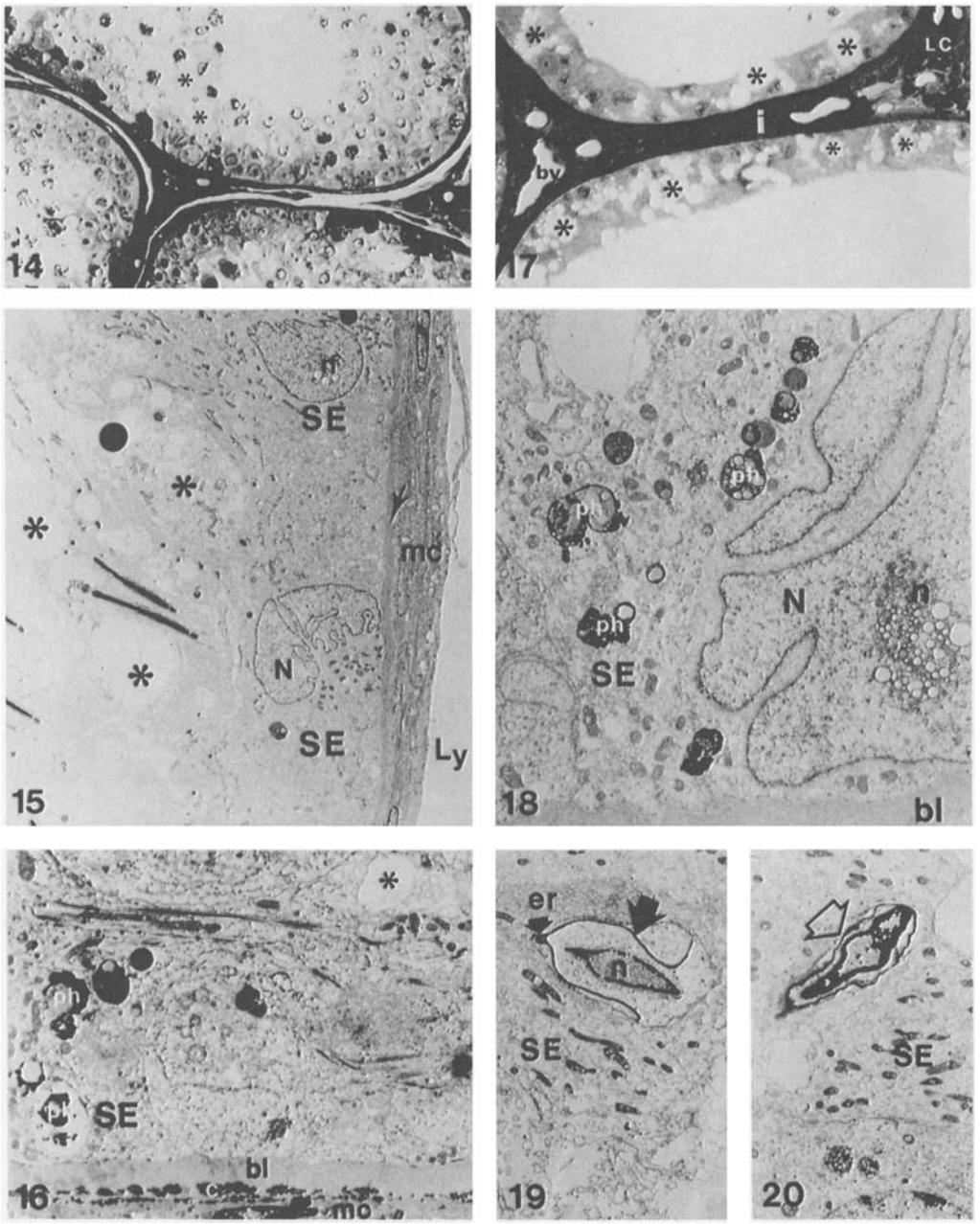








reticulum, *m:* mitochondira, *ph:* phagosomes, *bl:* basal lamina, *mc:* myoid cells). More severe changes are shown in Fig. 11 (toluidine blue, 100×) and 12 (3,600×) with vacuolation and piknosis of the spermatocytes *(stars),* malformations of the spermatids *(arrow)* and hollow areas among the Sertoli cells *(SE)* (*G:* spermatogonia, *N:* nucleus). The basal cytoplasm of the Sertoli cells contains numerous phagosomes *(ph)* as well as lipid and pigment deposits (Fig. 13, 7,200×)



Figs. 14 to 20. Morphology of the testes of Zebu bulls assigned to Group C (i. e. with a marked decreased consistency at palpation). Morphological alterations in the tubuli varied in intensity (Figs. 14, 100×; and 17, 160×, toluidine blue) from hollowed areas *(*)* and absence of pachytene spermatocytes (Fig. 15, *SE:* Sertoli cell, *N:* nucleus, *arrow:* basal lamina, *mc:* myoid cells, *ly:* lymphatic space, 3,600×) to absence of most germinal cells (Fig. 17, *bv:* blood vessel, *i:* interstitium, *LC:* Leydig cells). Most Sertoli cells (16 and 18, 7,200×) contained numerous phagosomes *(ph)*, lipid and pigment deposits, and even phagocytosed cell fragments (*bl:* basal lamina, *c:* collagen, *mc:* myoid cell, *N:* nucleus, *n:* nucleolus). Severely malformed spermatids are depicted in Figs. 19 (*er:* endoplasmic reticulum, *n:* condensing nucleus, *small arrow:* acrosome protusion, *big arrow:* acrosome vesicle, *SE:* Sertoli cell, 5,400×) and 20 (*empty arrow:* degenerated acrosome, 5,400×)

nutritional status without a known genetic handicap. Testicular function is impaired not only by factors causing a rise in body temperature, but also by heat applied directly or by insulation of the scrotum, as shown in a large number of experimental studies (see SETCHELL, 1978). In many of the early studies on the effects of direct scrotal heating, temperatures between 45 and 50 °C were used (review: VANDEMARK and FREE, 1970), which resulted in severe degenerative changes. The direct application of water at elevated temperatures, prevents evaporative cooling, the normal mechanism by which scrotal temperature is regulated. This leads to a rapid increase in testicular temperature. By contrast, under field conditions, high environmental temperatures, including warm winds, lead to a much slower — although long-lasting — increase in intratesticular temperature. Although changes in body temperature are not as effective as the application of local heat in raising the temperature of the testis (HARRISON and HARRIS, 1956), high fever for few days causes seminal degeneration to various degrees (SINGLETON, 1968).

Little is known about the relative sensitivity of different cell types to heat, except that certain stages of the long meiotic prophase of primary spermatocytes have been found to have a high thermal sensitivity in all species so far examined (NELSON, 1951). This could explain why primary spermatocytes were degenerated and reduced in number in both groups of our bulls with clinically diagnosed reduced testicular consistency. Primary spermatocytes are the first cells to die following excessive heating, as shown in rodents (cf VANDEMARK and FREE, 1970). The pachytene spermatocytes of the ram and boar pass through a thermally sensitive period towards the end of stage 7 (WAITES and ORTAVANT, 1967, 1968; MAZZARRI et al., 1970; MALMGREN and LARSSON, 1989). Diplotene spermatocytes are considerably reduced in affected boar testes (MAZZARRI et al., 1970; MALMGREN and LARSSON, 1989). Dividing spermatocytes are also damaged by heat (ATTAL and COUROT, 1963; WAITES and ORTAVANT, 1967, 1968). Young spermatids are also very heat sensitive whereas older ones are apparently more resistant (MALMGREN and LARSSON, 1989).

No damage to spermatogonia was found in the animals examined in this study, in accordance with the literature. The sensitivity of the spermatogonia seems to vary between species. Moderate temperature elevation has no apparent effect on the spermatogonia in rodents and pigs (MAZZARRI et al., 1970; VANDEMARK and FREE, 1970; MALMGREN and LARSSON, 1989) nor does it influence the A-spermatogonia in rams (WAITES and ORTAVANT, 1967, 1968). In B-type spermatogonia a temperature elevation can lead to an increase in their mitotic index (WAITES and ORTAVANT, 1967, 1968). A similar effect was reported in bulls exposed to high air temperatures (SKINNER and LOUW, 1966). The early stages of spermiogenesis were affected adversely by heat and immediate losses of round spermatids have been observed soon after temperature elevation in rams (WAITES and ORTAVANT, 1967, 1968), boars (MAZZARRI et al., 1970; MALMGREN and LARSSON, 1989), and bulls (VANDEMARK and FREE, 1970). The alterations found in the present study among Zebu bulls with a marked lack of testicular tonicity (Group C) indicate that the cells from the preleptotene stage were affected throughout spermatogenesis. Contrary to previous reports (SKINNER and LOUW, 1966) on the same species, spermatogonia seemed unaffected in the examined animals.

Results of the clinical examinations agreed well with the corresponding testicular morphology. Palpation of the testis is an easy examination and should be undertaken on all breeding bulls. The increase in the percentage of primary sperm abnormalities, however, was not associated with either the decrease in testicular consistency or the morphological changes found, although electroejaculates from bulls in Group A had the lowest percentage of those considered primary sperm abnormalities, and those from bulls in Group C had the highest. The percentages of sperm abnormalities found in the present study, would be considered high by any standard method used. Particularly for bulls in Group A, no testicular abnormalities were consistently present that could be associated to the high percentage of primary sperm abnormalities found. It is possible that multiple electroejaculates are necessary to obtain a correct estimate of primary sperm abnormalities, and

estimate the repeatability of the occurrence of primary sperm abnormalities in consecutive electroejaculates from the same bull. Elevated temperatures in the testis damage germ cells during spermiogenesis or in the proximal part of the ductus epididymides (SETCHELL, 1978). The long-term exposure of bulls to high temperatures under tropical field conditions could therefore explain why large numbers of affected spermatozoa were present in the electroejaculates obtained from the bulls examined in the present study.

Blood flow through the testes should vary considerably depending on the temperature applied to the scrotum. Local hypoxia occurs in areas receiving less than the normal blood flow (STEINBERGER, 1970). At temperatures above 37 °C, the testis enters into a state of self-induced hypoxia (WAITES and SETCHELL, 1964; SETCHELL, 1978). At above-normal body temperatures, a reduction in the amount of testicular fluid produced by the Sertoli cells can lead to damage of the germinal cells, which obtain their nutrients from fluid secreted into the lumen (SETCHELL, 1978).

Metabolic changes in the testis may reflect qualitative and quantitative deleterious changes in cell populations, i. e. the loss of germinal cells implies that the remaining Sertoli cells undergo a change in their metabolism. This metabolic change could explain the accumulation of lipids as well as the changes in lipid morphology reported in cases of seminal degeneration (LYNCH and SCOTT, 1951), which were also observed in our Group C bulls.

Despite the deleterious effect of heat on the germinal epithelium, the interstitial tissue has been found to be gonadotropin-responsive, as indicated by the fact that males with degenerated testicles maintained their libido (VANDEMARK and FREE, 1970). No evidence has yet been found to indicate that heat-induced degenerative changes in the testicles can influence the accessory sexual glands of the bull (SKINNER and ROWSON, 1967). Heat stress might be detrimental to androgen production; however, the effects of altered androgen levels on gonadotropin hormones have yet to be established. Experiments aimed at determining the degree of testicular responsiveness to GnRH challenge in affected Zebu bulls are currently being performed under field conditions in Costa Rica.

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