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W. Meyer
Tierarztliche Hochschule Hannover

K. Neurand
Tierarztliche Hochschule Hannover

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A COMPARATIVE SCANNING ELECTRON MICROSCOPIC VIEW OF THE INTEGUMENT OF DOMESTIC MAMMALS

W. Meyer* and K. Neurand**

*Institut fur Zoologie, **Anatomisches Institut
Tierarztliche Hochschule Hannover
Bischofsholer Damm 15
D-3000 Hannover 1, F.R. Germany

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Abstract

Scanning electron microscopy (SEM) demonstrates efficiently species-specific differences of hairy skin (integumentum commune) of domestic mammals (pig, cat, dog, horse, cattle, sheep, goat). This technique is very helpful in characterizing the typical structural features of the epidermal layers, the arrangement of the collagen fibre bundles and the elastic fibre network in the dermis, the external and internal construction of hair follicles and hair shafts, and the functional development of skin glands. It is also possible to observe certain domestication effects, especially where the hair coat is concerned.

SEM supplements the knowledge about the integument as available from conventional transmission electron microscopy, light microscopy or histochemistry. Thus, comparative morphology can be the basis for the development of specific functional models of the different integumentary layers and derivatives or their tissues involved.

Key Words: Comparative scanning electron microscopy, hairy skin, domestic mammals, epidermis, dermis, skin glands, hair follicles.

*Address for correspondence:
For reprints and other information contact W. Meyer at the address above.
Phone no.: (0511) 856 8746

Introduction

Comparative vertebrate morphology provides evidence for the process and product of organic evolution. This thesis is also generally true of the integument and its derivatives. Furthermore, in skin morphology and other organ systems, the tendency towards a functional interpretation of structures receives increasing importance. Thus, morphology approaches an understanding of the development of these structures in the realm of phylogeny (6). Nevertheless, the skin of mammals still remains a matter of question in this connection, considering that the origin of its most important feature - the hair follicle and its glandular appendages - is broadly unknown, so that pure speculation is dominating (17).

Until now, the evolutionary interpretations of integumentary structures for mammals were based mainly on skin morphology studies of domestic species. In view of the theory that domestication may, to a certain extent, serve as a model for evolution (7), it is pertinent to investigate the integument comparatively. Additionally, the evaluation of domestication effects could be the most valuable tool for understanding mammalian skin biology as a whole (14).

The present paper is exclusively concerned with the hairy skin (integumentum commune), which includes previous results mainly achieved using porcine skin (14,16,19,21,23) and is supplemented by several new observations on other domestic mammals. The paper is designed to emphasize that scanning electron microscopy (SEM) methods can be applied to supplement the knowledge about the integument available from conventional TEM and light microscopy as well as histochemistry.

Most of our findings are based on conventional SEM methods (8), and are completed by two additional methodical approaches: a) as already described earlier (16), the collagen fibre bundle network in the dermis, for example, was demonstrated after a treatment of formalin-fixed frozen sections with hyaluronidase for several hours at +37°C, followed by an incubation with bacterial crude α-amylase for 2-4 days at room temperature; the elastic fibre content and arrangement was observed best after the ground substance and collagen were removed by autoclaving fresh tissue samples at +110°C and 103 kPa for 6-15 h in a high pressure autoclave system (Keller, type S-ECZ). In this connection, the original method, as described for human skin (31), was modified

according to the animal species used.

b) Further SEM observations on integumentary tissue were achieved by combining the osmium-DMSO -osmium method with the rapid freezing/freez-fracture technique (28,30). The procedure can be briefly outlined as follows: fresh frozen skin specimens were transferred into and kept in 2% osmium tetroxide in acetone at -80°C for 24 h. After substitution of ice and warming to room temperature, the specimens were washed in acetone, rehydrated through an acetone series and immersed in 25% and 50% DMSO for 30 min. Subsequently these specimens and, additionally, formalin-fixed skin pieces, were cracked in liquid nitrogen; the cracked pieces were immediately placed in 50% DMSO, then rinsed in 0.15 M phosphate buffer and afterwards left in 0.1 % buffered osmium tetroxide for about 3 days at room temperature. The specimens were fixed again for 1 h in 0.1% osmium tetroxide, then treated with 2% tannic acid solution overnight, rinsed with the buffer solution and immersed again in 1% osmium solution for 1 h. After dehydration, the pieces were prepared for critical point drying and xylene vapour drying (16). Instruments used were critical point dryer with a CO_2 device (Balzers), the sputter coaters BAE 120-FN 102 (Balzers) and S 150A (Edwards), and JEOL JSM-35 L scanning electron microscope operated at 25 kV.

Epidermis

The mammalian epidermis has been predominantly of interest from its surface appearance as shown by comparative SEM. The epidermis is seen in densely haired carnivorous domestic mammals as the stratum corneum with its numerous transverse folds (Fig. 1a) (12,20). In sparsely haired species like the pig (Fig. 1b), the epidermis is seen as deep marked grooves in a triangular or rectangular arrangement (10,14). In general, the surface pattern of the skin shows regional variations, which are particularly conspicuous when comparing the dorsal with the abdominal part of the body. In the latter, the cleft appearance is somewhat enhanced by an additional dermal folding. The stratum corneum lamellae are relatively thick, especially in sparsely haired domestic mammals (Figs. 1c, 1d), and often surround, as a multilayered envelope, the bundles of secondary hairs emerging from the skin as in the cat and dog. Moreover, with the help of the osmium-DMSO - osmium method it was even possible to demonstrate the thickened zone of plasmalemma and marginal band of the inner stratum corneum cells (Fig. 1c).

Until now, the living epidermis has been poorly represented by SEM. The osmium -DMSO- osmium method as modified by the application of rapid freezing (28) enabled us, however, to show some aspects of the cellular components of this skin layer. Nevertheless, it was difficult to discern specific cell organelles, because the cytoplasm of the epidermal cells is normally dominated by high amounts of filamentous proteins (prekeratins) (14), so that the fractured cells exhibited a somewhat compact and uniform appearance as revealed by SEM (Fig. 1e). Best results were achieved with fresh or shortly fixed material. In general, the latter method promises to produce rather satisfying results in the future study of epidermal structure.

Dermis

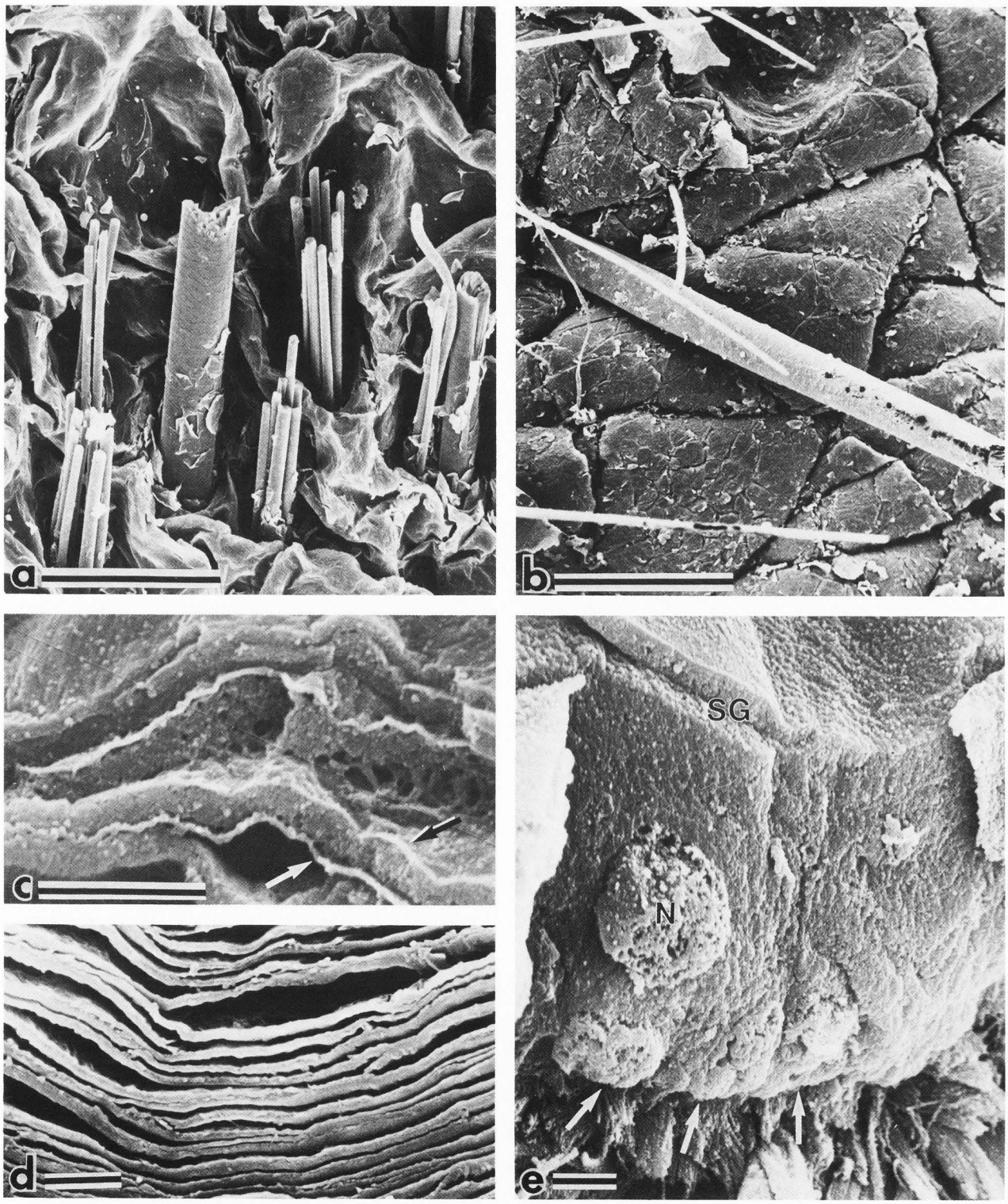
In the mammalian integument, collagen is the most abundant structural constituent of the dermis, comprising about three quarters of the dry weight of this part of the skin (13). Thus, dermal thickness of hairy skin is normally correlated with the amount and diameter of collagen fibre bundles. The application of SEM confirms that for domestic mammals fibre bundles in the reticular layer of the dermis, which is composed of a mid and deep layer, cross each other in two main directions and that single fibres or smaller fibre bundles often emerge from one bundle and pass into another one, in this way closely interweaving the dense network. A small number of thick collagen fibres and fibre bundles are also horizontally orientated, but only in mid and deep dermal zones. Finer fibres and fibre bundles in a horizontal arrangement are confined to the relatively flat stratum papillare (upper dermis) and are found around the pilosebaceous units, the apocrine glands and the blood vessels. Pig, horse, and cattle normally show a relatively coarse and solid texture of the dermis (Figs. 2a-c) as compared to the cat, dog, sheep, goat, and rabbit.

The studies on the elastic fibre meshwork of the dermis usually revealed considerable amounts of elastic fibres independent of hair density, skin thickness or collagen fibre bundle architecture (Figs. 2d,e). This is in contrast to a former view, maintaining the elastic element to be extremely dominant in the skin of densely haired, furbearing mammals (4,20,25). Our opinion was first proven by the dense meshwork of elastic fibres demonstrated in the dermis of the pig, also anchoring the hair follicle complex, nerves and blood vessels in the skin (14,19), and it was confirmed by our latest results from the cat, dog, and sheep, taking into consideration that regional variations occurred in all species.

It is evident that the mechanical properties of the dermis do not completely depend on the rather rigid and stable network of its collagen component. This is especially true when considering that the longitudinal extensibility of collagen fibres and fibrils is not very great. It is the elastic component, i.e., the elastic fibres forming a wide-meshed sponge throughout the whole dermis, that causes the constant tension of the skin. Through its close interweaving with the collagen fibres, this extremely plastic element enables the dermis to sustain a higher stress than would be predicted from normal structure. Therefore, an elastic solidity can also be developed by relatively thick skin like the porcine integument (19).

Our SEM studies on dermis tissue were also able to demonstrate domestication effects, especially

Figure 1: Epidermis - a) cat, back, strat. corneum surface with folds; b) pig, abdomen, strat. corneum surface with deep grooves; c) cat, inner strat. corneum cells with marginal zone (arrows); d) pig, strat. corneum lamellae, sagittally sectioned; e) cat, living epidermis with strat. granulosum (SG) and nuclei (N) of strat. spinosum and strat. basale (arrows). c and e: freeze fracture/ osmium-DMSO-osmium method. Scale bars represent: 100 µm in Fig. 1a; 500 µm in Fig. 1b; 2 µm in Fig. 1c; 5 µm in Fig. 1d; 2 µm in Fig. 1e.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in the pig. Breeding influenced skin structure in such a way that the coarse dermis architecture in the wild boar (Sus scrofa L.) changed into a finer and more homogeneous, compact network of all fibre bundle types (14). In this way, the dermis of high-bred pig races obtained clear structural parallels with the human dermis, thus emphasizing - together with other parameters - that the domestic pig has some value for experimental research in human skin (14,18).

The osmium-DMSO-osmium method was additionally able to exhibit other aspects of the dermis, i.e., the free cells of connective tissue randomly scattered throughout the densely packed collagen and elastic fibres (Fig. 2f). In our study these cells were normally represented by numerous mast cells as identified by their intradermal distribution and cellular morphology compared to TEM and light microscopical findings.

Hair and hair follicle

The demonstration of the typical structural features of the mammalian hair cuticle has been one of the first and most widely used applications of SEM during the last twenty years (Fig. 3a). This was often done assuming that different mammalian species could be almost entirely identified on behalf of the shape and arrangement of hair cuticle scales (1,3). However, experience has shown that species classification is not possible in this way, because there may be relatively similar cuticle patterns between the different hair types of non-related species, and there are also pattern variations along the hair shaft, or differences between the body regions, as well as hair structure changes induced by the normal moulting cycle of wild animals or the often altered moulting cycle of domestic species (14,29).

The internal structure of the hair follicle has not been the subject of an intense SEM approach, although our results have shown that typical morphological aspects become obvious, in particular where the growing hair fibre is concerned (Figs. 3b-d). In contrast to other microscopical procedures, SEM exhibits the already early definite subdivision of the hair into cortex and medulla in the bulb matrix (Fig. 3e), as well as manifold connective tissue elements and the blood capillaries in the hair papilla (14). In addition, the generally extremely tight adherence of the cornified, elongated cortex cells and their columnar arrangement are as clearly visible (Fig. 3b) as are the varying structures of the medullary air spaces (interdiscoidal spaces) (2,22). The latter show typical, perforated walls of medullary cells, which shrink to a considerable extent during the formation of the hair medulla within the follicle (Fig. 3c)(22). SEM also demonstrates the close interaction of the cells of the hair cuticle and the inner root sheath for an effective anchorage of the hair within the hair canal (Fig. 3f). This is especially true of all mammals with regard to the retention of the resting telogen-stage follicle by brush-like keratin cells, in particular those forming helical keratin ridges around the club of long and thick hairs (Fig. 3d) (14).

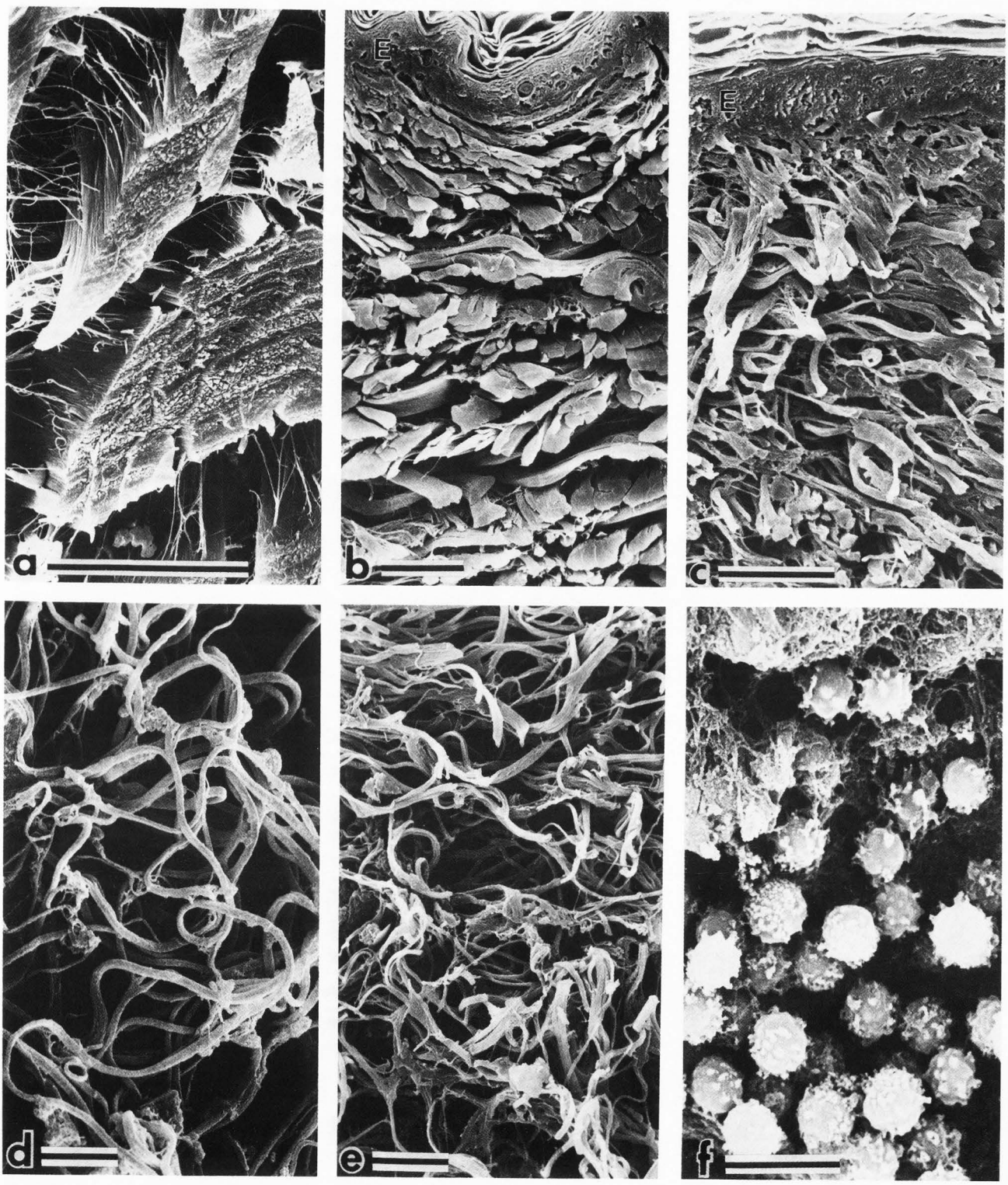
Skin glands

The presence of glands as appendages of the hair follicle contributes to the distinctive biology of mammals. In terms of evolution, pelage and associated skin glands constitute one of the most important steps from which the typical mammalian characteristics like endothermy and lactation naturally followed (17).

Complex, lobulated sebaceous glands occur mostly in pairs at the hair follicle of domestic mammals (Figs. 4a,b). Their size varies in relation to the length and thickness of the hair fibre produced, so that relatively large sebaceous glands can be found in the pig (14), horse and cattle, and smaller glands in the densely haired species (18,20). SEM reveals the typical structural features of their holocrine secretion mode, such as autolysis during their maturation by the accumulation of lipid droplets. In this connection, the cell walls normally show an increasing perforation as the cell structure disintegrates, like in the sebaceous glands of the pig, goat or carnivores (Figs. 4a,c,d). In the equine sebaceous glands, however, the autolysis is not complete, so that often parts of the cell walls and cytoplasm are found within the sebum. The sebaceous glands discharge into the hair canal via excretory ducts which are short in densely haired domestic mammals (Fig. 4b) and longer in the porcine integument (14). The excretory ducts of the porcine primary hair follicles are normally equipped with dilatations for sebum storage. These dilatations are effective in view of the robust bristles of the porcine pelage and the generally continuous secretory activity of sebaceous glands (5,14).

The second type of glands are simple tubular, branched tubular, and simple coiled tubular glands (14,18,20,25). In mammalian hairy skin, i.e., also in the skin of domestic mammals, these glands are normally of an apocrine secretion mode (Figs. 5a,b). The glands are primarily odoriferous in secretion function, which is demonstrated by the fact that the secretion produced is mixed on the skin surface with the sebum of the sebaceous glands. The secretion mixture has probably a slight fungicidal or bactericidal effect by establishing an acid pH milieu (14,23). By microbial disintegration of this heterogeneous material, weakly smelling volatile substances are released and add to the individual odour of the animal. This problem, however, should not be intermingled with the glandular functions of specific body regions, which are important for the scent marking behaviour of many mammalian species (23). Only in higher primates can eccrine glands as typical sweat glands be found in the common integument being involved in thermoregulation. In

Figure 2: Dermis - a) pig, back, collagen fibre bundles of strat. reticulare; b) dog, back, strat. papillare and upper strat. reticulare; c) sheep, back, strat. papillare; d) pig, abdomen, elastic fibre network; e) sheep, abdomen, thicker elastic fibres around hair follicles; f) cattle, mast cell group below the epidermis, osmium-DMSO-osmium method. a - e sagittal sections (E= epidermis). Scale bars represent: 20 μm in Figs. 2a-c; 1 μm in Fig. 2d; 10 μm in Fig. 2e; 5 μm in Fig. 2f.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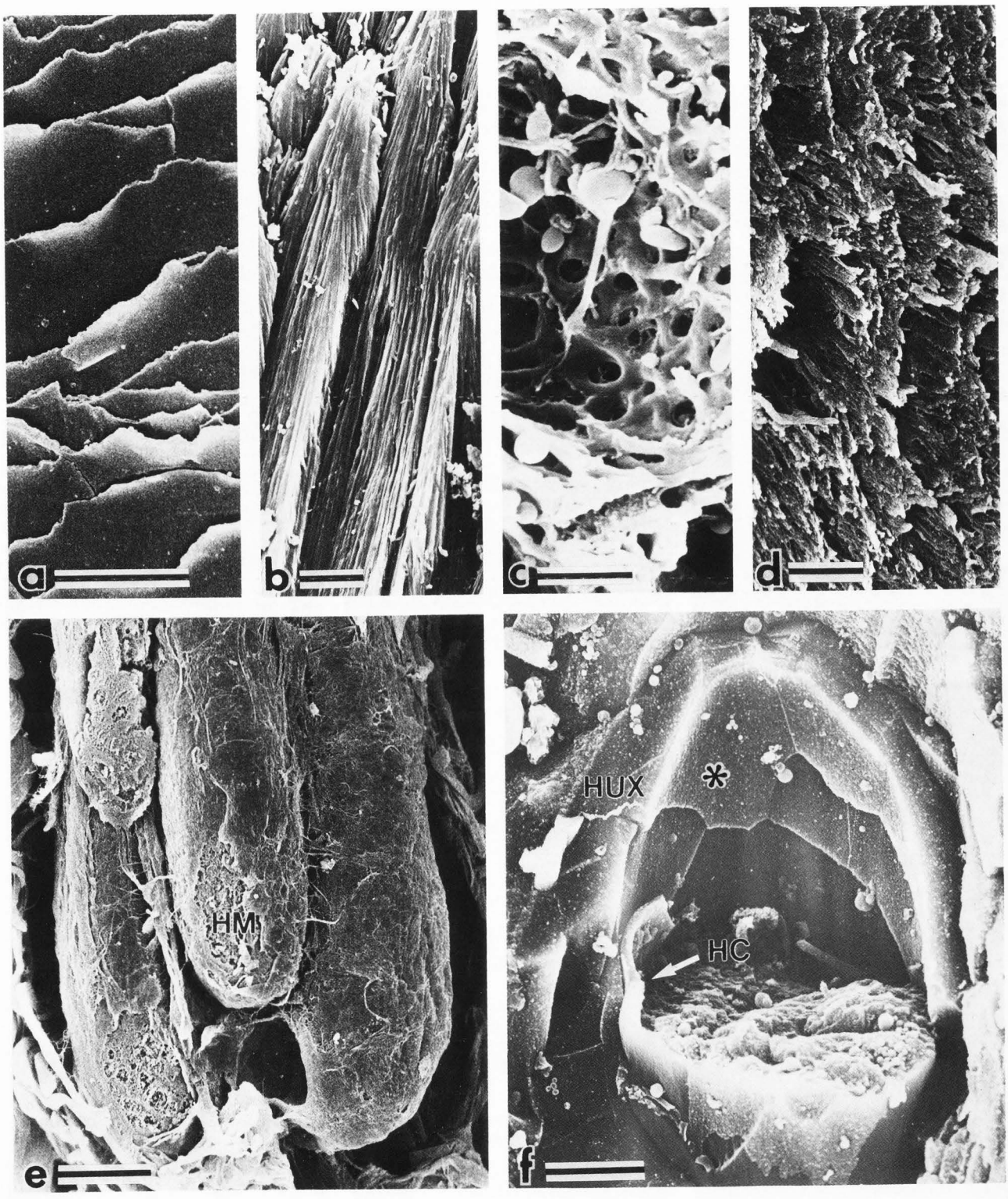

Figure 3: Hair and hair follicle - a) pig, hair cuticle scale pattern of primary hair; b) pig, hair cortex cells; c) horse, hair medulla cells; d) pig, keratin ridges of club hair base; e) cat, hair follicle bulb with definite hair medulla (HM); f) sheep, fracture of hair follicle with cuticle of inner root sheath (asterisk), hair cuticle (HC), and cornified Huxley layer (HUX). Scale bars represent: 5 μm in Figs. 3a,b; 1 μm in Fig. 3c; 10 μm in Fig. 3d; 20 μm in Fig. 3e; 5 μm in Fig. 3f.

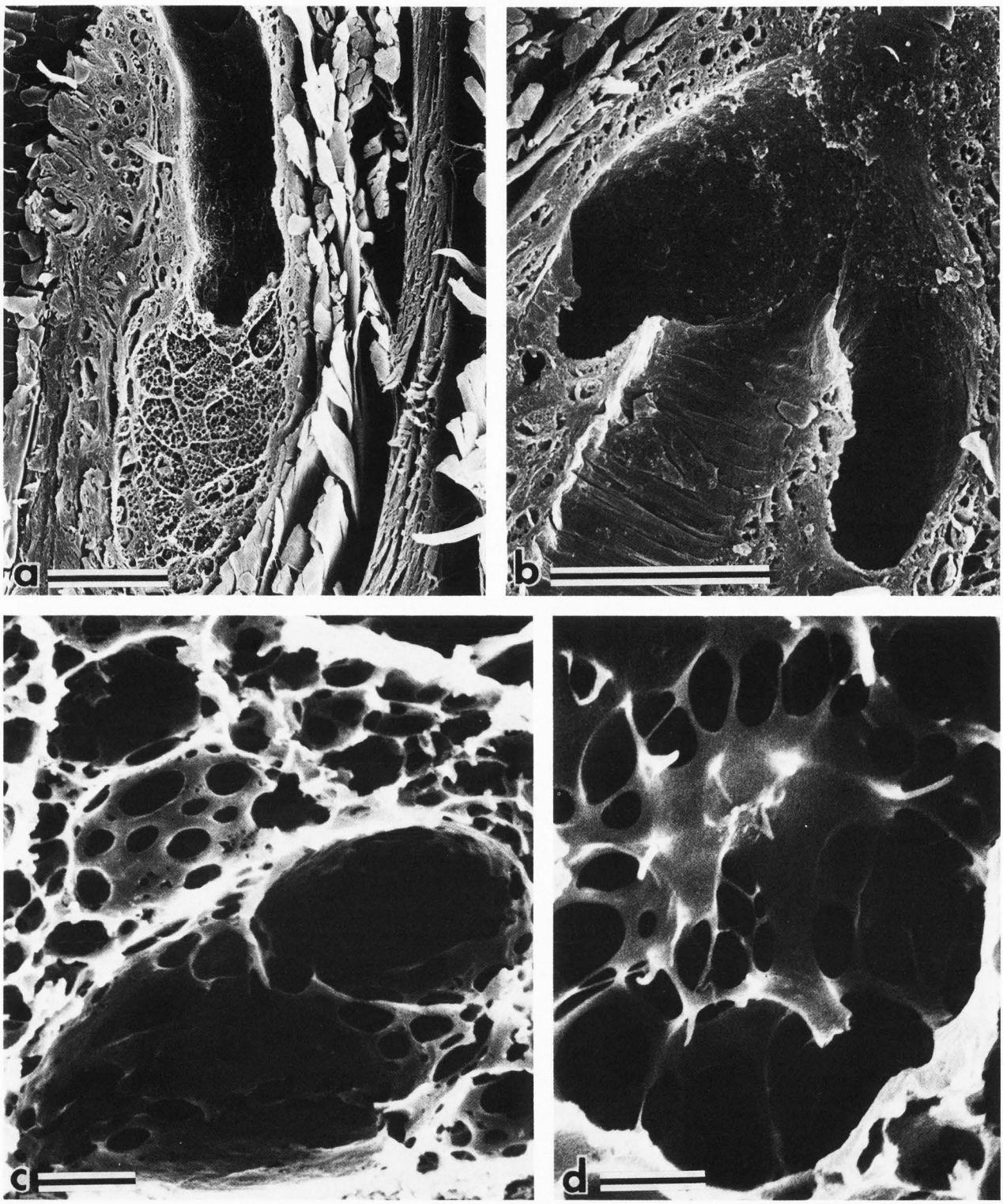

Figure 4: Sebaceous glands - a) cat, whole gland with excretory duct; b) dog, openings of two excretory ducts into the hair canal; c) goat, central glandular part with perforated cell walls; d) pig, disintegrated glandular centre. Scale bars represent: 20 μm in Figs. 4a,b; 2 μm in Figs. 4c,d.

all other mammals this gland type is confined to specific body regions such as the snout or the foot pads.

In domestic mammals each primary hair follicle is usually equipped with one apocrine gland except for rabbits and the rodent species. The gland is shorter, only slightly coiled in the carnivores (Fig. 5b) (18,20), or longer, branched and coiled, particularly in porcine (14), bovine, ovine and equine skin, sometimes forming a definite gland layer below the hair bulbs (Fig. 5c). In the pig, for example, the different stages of apocrine secretion mode are clearly demonstrable with the help of SEM technique (Fig. 5a) (14,23).

In the equine skin, the cells of the secretory epithelium of the voluminous apocrine glands only rarely exhibit the typical large apocrine protrusion at the cell apex. This may be connected with the type of sweating occurring in the horse, and partly in domestic ruminants (Figs. 5d,e). In these species one should consider the type of secretion and secretion function as a domestication effect, correlated with structural modifications of the secretion mode. Especially in the horse, and in contrast to most of the other domestic mammals, sweating occurs in high secretion rates as a profuse output of fluid (11). This functional feature obviously deeply influences the typical morphological appearance of the secretory cells as revealed by SEM. As visible also in light microscopy and TEM (26), the cytoplasm is dominated by numerous secretion storage vesicles, which are best demonstrated by applying the osmium-DMSO-osmium rapid freezing technique (Fig. 5e).

Conclusions

SEM demonstrates the variety of important integumentary structures which are indispensable for the normal functioning of the organ skin. The comparative view not only reveals several similarities but also a number of typical species-specific differences of the hairy skin of domestic mammals. These differences are generally related to the varying development of the pelage among the species investigated. The variations in hair coat structure may originate from the different evolutionary platforms of the mammalian species concerned, but could also be due to domestication influences (14). Furthermore, and as already emphasized in the Introduction, by completing the knowledge available from TEM, light microscopy or histochemistry, comparative morphology on SEM basis can be essential for the establishment of specific functional models of the different integumentary layers and derivatives.

In view of the recent modifications of the conventional SEM technique applied until now, and considering the relevant observations described in the present study, it is obvious that the comparative SEM approach to skin structure still has numerous additional investigative possibilities. This is especially true of the developing integument and has already been demonstrated to some extent for humans and laboratory animals (9,27). As for domestic mammals, the results show that functional interpretations seem to hold true here as in the adult animals (15,24).

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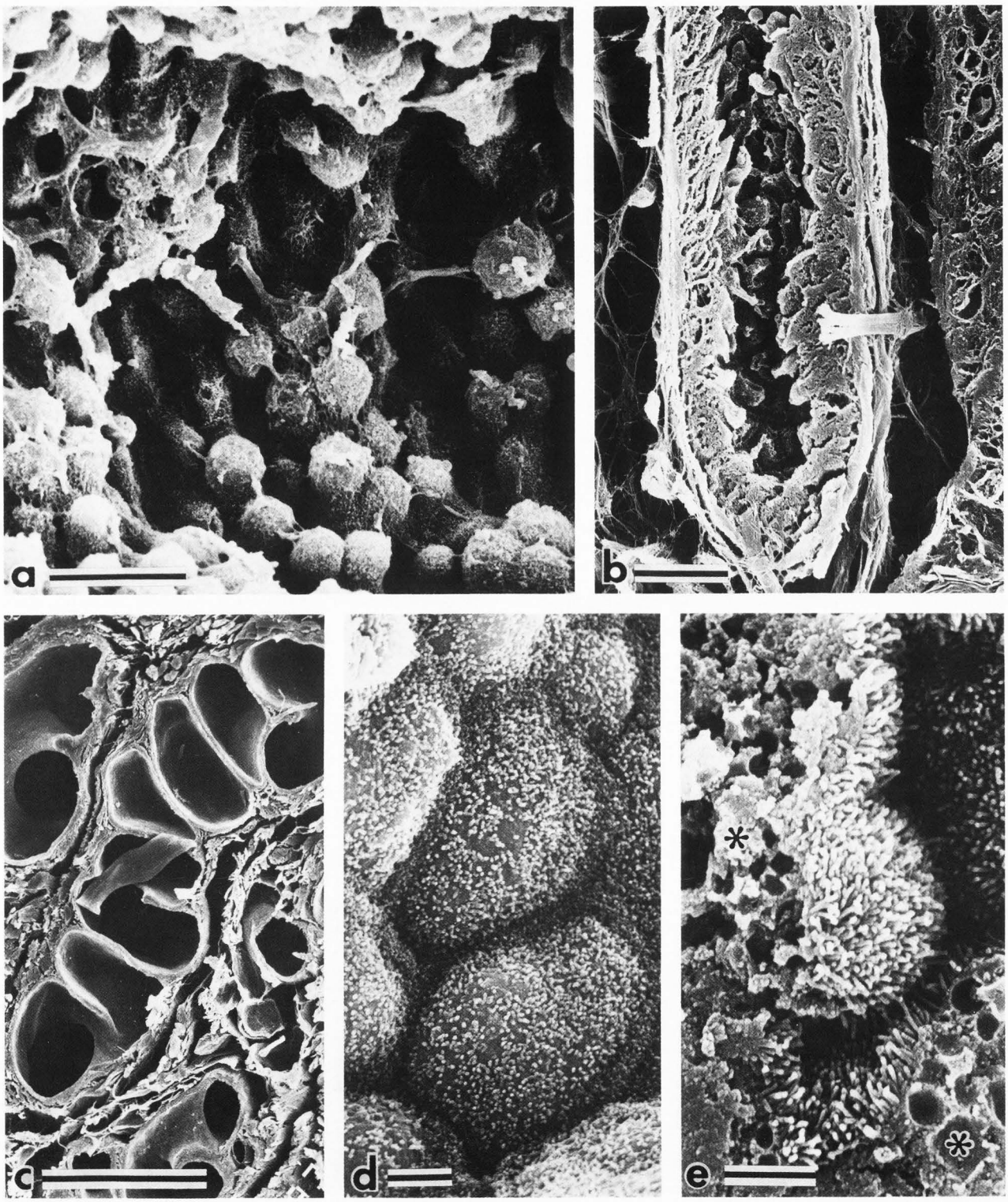
Figure 5: Apocrine glands - a) pig, secretory portion with apocrine protrusions of highly active epithelial cells; b) cat, longitudinal section of active secretory portion; c) sheep, horizontal section through glandular layer; d) cattle, surface of inactive secretory cells; e) horse, freeze fracture of active secretory cells with secretion storage vesicles (asterisks), osmium-DMSO-osmium method. Scale bars represent: 10 μm in Figs. 5a,b; 100 μm in Fig. 5c; 2 μm in Figs. 5d,e.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Discussion with Reviewers

C.J. Doillon: Can you define in more detail some regional variations of the surface pattern of the epidermis?

Authors: The cleft appearance of the epidermal surface is enhanced by an additional irregular dermal folding pattern, especially in the axillary, inguinal and the whole abdominal body regions.

C.J. Doillon: By using the osmium-DMSO-osmium method, have you observed differences or similarities in the cellular components of the epidermis of different domestic mammals?

Authors: We did not observe any clear difference in the appearance of cellular components in the epidermal cells of the animals investigated. This may be connected with the fact that in all species the epidermal cells are filled with large amounts of cytofilaments (prekeratins). Therefore, single cells or cell layers appeared relatively compact and uniform in the scanning electron microscope.

B. Forslind: In the paper it is stated that the type of apocrine sweating occurring in the equine skin and in cattle is an effect of domestication. I suppose that this proposal is supported by comparative studies of wild species (horses, zebra etc.).

Authors: In wild species related to the horse or cattle (Wild donkey, zebra, bison), the apocrine glands, though affected by seasonal influences, in our preparation show no clear features of a higher secretional activity. In the wild animal, thermoregulation is normally carried out through effective insulation by a dense hair coat, higher rates of peripheral blood circulation, as well as panting activities, but not by evaporative cooling. However, in many of the domestic species concerned, evaporative cooling has been developed by a reduction of hair density and changes in apocrine gland shapes or secretion composition and secretion discharge rates. Most changes are definitely race-specific and in cattle often obscured by the multiple cross-breeding of European and Asian races (see e.g., Lyhs L (ed.) (1971), Der Warmehaushalt landwirtschaftlicher Nutztiere (The Heat Balance of Agricultural Domestic Animals), G. Fischer Verlag, Jena, 37-186; Sokolov VE (1982), Mammal Skin, Univ. California Press, Berkeley, 462-572).

B. Forslind: What are the principal changes in the morphological expression of the sweating that allows you to infer that the sweating is an effect of the domestication and no other factor? Further, what would be the biological (dis-) advantage of the proposed change in the pattern of sweating?

Authors: The principal changes are normally the relatively larger gland shapes in domestic species compared with the wild relatives (there are, however, no true, living direct wild ancestors of the

domesticated horse and cattle!). Higher sweat discharge rates are a biological advantage only for the acclimatization of Asian cattle races in hot areas. The higher amounts of proteins in the horse sweat, for example, seem to be a clear biological disadvantage for the long term endurance capacity of the animal (also for other biological aspects see above). It must be emphasized that it is the long domestication period of at least 8000 years that has caused important alterations in structure and function, not only in the integument but also in other organ systems.

B. Persky: What specific morphological changes as seen with SEM are noted in the arrangement of collagen fibres after domestication?
Authors: Comparing the wild boar with domestic pig races, for example, the whole dermis pattern of collagen fibre bundle arrangement is definitely finer and more compact in the domestic forms than in the wild animal.

B. Persky: Could morphological differences in the epidermis and the dermis which are attributed to domestication also be explained as normal regional variations of skin?
Authors: Clear morphological differences due to domestication could be discerned independent of regional variations of the skin. This was best visible, for example, using several domestic pig races, as well as by comparing structures only within the body regions concerned.

B. Persky: Have SEM studies been done on the integument of non-domestic mammals? If yes, how do your findings compare?
Authors: Until now, we have done SEM studies on the wild boar (Sus scrofa L.), the European wild cat (Felis silvestris Schreber) and the European wolf (Canis lupus L.). Clear differences in skin structure were only visible in the porcine integument.

Reviewer V: References 10 and 12, the latter in particular, show that the SEM appearance of the integument prepared by freeze-drying is significantly different from that prepared by critical point drying (CPD) (which removes sebum and outer cornified layers). Would the authors like to comment on this difference and on why their review only considers CPD preparation methods?
Authors: We did not intend to consider exclusively papers using the CPD method. It is obvious, however, that until now most of the SEM publications cited, and concerning the skin of domestic mammals, rely on CPD rather than on freeze-drying or freeze substitution. The removal of sebum by CPD preparations, moreover, sometimes gives a better overall presentation of specific histological details, for example, typical aspects of the holocrine secretion mode of sebaceous glands.

Reviewer V: It is stated that the apocrine glands "...are primarily odoriferous in secretion function...". What odoriferous components of the sweat are known? If it is only after mixture with sebum components or as a consequence of bacterial degradation that odour is produced, is it not misleading to describe the primary function of these sweat glands as odoriferous?
Authors: The volatile odorous substances released from the surface of the hairy skin are normally not the result of a single process, i.e., they would derive from only one gland type with one typical secretion or part of it, respectively. The odour produced has always to be regarded as the result of microbial degradation of substance masses of different secretional origin. Odour variations are possible as related to the different species or some endogenic factors (e.g., sex, age, cyclus stage, etc.). The fact that the secretion of apocrine glands may contain a certain, more or less great amount of water, does not generally imply, in our opinion, that a discharge of such a secretion is predominantly and biologically associated with thermoregulatory sweating. It could only be regarded as a good prerequisite for sweating development in some domestic mammals.

Reviewer V: It is stated that, "Only in higher primates can eccrine glands as typical sweat glands be found in the common integument being involved in thermoregulation". It is well known that the apocrine glands of horses and cattle are involved in thermoregulation. Nor is this a consequence of domestication; years ago Robertshaw and Taylor found that the high sweat output in an African antelope is comparable to the output rate in cattle. Please comment.
Authors: The paper of Robertshaw and Taylor (A comparison of sweat gland activity in eight species of East African bovids. J. Physiol. 203, 135-143, 1969), relies on results obtained from shaved skin areas, and does not consider the conditions within the intact hairy skin. The methodical approach, therefore, must be regarded as a somewhat artificial one (including pharmaceutical stimulation), which does not take into consideration the normal biological conditions, for example the insulatory effect of the fur (e.g., about 3,500-5,000 hairs per cm^2 in the dorsal region of some Antilopinae) or the problems involved in the humido-arid climate of East Africa (a warmer, humid period followed by a somewhat cooler, arid period). Could it be imagined what a higher evaporative water loss, as predictable from experimental results, would really mean to the animals during a hot and arid season?

Reviewer V: Fig. 5a purports to show different stages in the apocrine secretion mode. How was the active state induced in the glands examined? How does this state differ in appearance from the inactive gland?
Authors: The active secretional state of the apocrine gland showed in Fig. 5a was not induced by artificial handling. It is normal for the pig, but also for some of the other domestic species, that highly active states, as well as less active, flat states with no prominent macroapocrine protrusions, are found at the same time independently from each other in neighbouring glands. This was not only visible in SEM preparations but also, for example, in unfixed or fixed freeze sections (see e.g., text ref. 14).

Reviewer V: The authors find that comparable amounts of elastic fibres are present in the skin of sparsely and densely haired species. Autoclaving is well known to produce gross tissue shrinkage. Can they confirm that differential shrinkage was not involved? What are the shrinkage factors for Figs. 2d and 2e?

Authors: We know that the method used includes tissue shrinkage, but it should be noted that first of all we wanted to show typical three-dimensional details of the elastic fibre network. The amount of elastic fibres as well as shrinkage problems were controlled, if necessary, with classical histological techniques using freeze sections (for literature see e.g., text ref. 14).