HISTOLOGICAL EXAMINATION OF THE FLORIDA MANATEE (*Trichecus manatus latirostris*) INTEGUMENT

By

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For my mother, Phyllis Graham, and my father, John Graham Sr. You have supported and inspired me to become who I am today. Thank you for always believing in me. I dedicate this to you. I love you.

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A baseline normal of the manatee skin was needed for a histological atlas being developed of the manatee. The first system that needed to be defined was the integumentary system. Samples of normal manatee skin were collected at necropsy from 25 sites of the body. Through several routine and special histological stains, the samples were examined. The skin of the manatee is unique for a marine mammal, being exceptionally thick throughout the dermis and epidermis, especially the stratum spinosum and stratum corneum. In most mammals, several characteristics of the manatee skin would be considered abnormal, such as the thickness of the stratum spinosum and stratum corneum, the thickness of the dermis, lack of a stratum granulosum, lack of glands throughout the skin, only having blood sinus hair follicles present on the postcranial body, and the presence of melanocytes in the dermis. The structure and properties of the manatee skin are most likely because of the environment the manatee inhabits.

The manatee's closest living terrestrial relative is the elephant. These two species are related through similar wrist bone morphology, horizontal tooth replacement, and experiments with amino acid sequencing of proteins all have proven the manatee and elephant are related. Sites of the elephant analogous to those of the manatee were sampled from Asian elephants and analyzed histologically. There were several similarities between the skin of both species, but overall the manatee skin was thicker with most of the epidermal thickness due to an extremely thick stratum spinosum and stratum corneum. Pacinian corpuscle were present in the same areas of the manatee and elephant, both most numerous in the nostril. Both animals lack glands throughout their skin, while the only gland present in the elephant was interdigital glands located near the nail of the foot. The manatee lacked a stratum granulosum throughout the epidermis of all regions of the body, whereas the elephant had a stratum granulosum present in areas that are exposed to friction and would lack a stratum granulosum in other areas. Most of these differences are probably due to the aquatic versus terrestrial environments these two species inhabit.

Manatees frequently suffer from horrendous wounds sustained from boat strikes. The manatee wound healing process has not been studied and requires more understanding to better assist these injured manatees. Preliminary results from pathological assessment and immunohistochemical localization, of the wounds in this study, show that there is a potential difference in the timeline of the wound-healing process of the manatee compared to other species.

CHAPTER 1
INTRODUCTION

As the outermost boundary of an organism, the integument is expected to have significant adaptations in response to pressures from the surrounding environment. While most mammals can swim, representatives of many orders are adapted to varying degrees for an aquatic existence; whether it be freshwater, marine, or both. So much is known about the skin of domesticated and many exotic species. Marine mammal skin has not been studied to the same extent. A description of the normal integument is fundamental to understanding subsequent cutaneous disease and injury. Many Florida manatees are identified by the scars they bear from non-fatal encounters with boats. For over two decades, the Manatee Individual Photo-identification System (MIPS) has been used to maintain data and images of individual manatees (Beck, 2002). This system is crucial to the manatee population dynamics. MIPS can tell biologists the survival among different age classes, reproduction success, survival rate in relation to major environmental events, and can be used in assessment, rate of acquisition, and healing dynamics of non-fatal boat strikes (Beck, 2002). It is the latter reason for the second part of this research. Manatees are frequently injured from boat strikes. The wounds they acquire can be severe, regardless; they can heal in the wild with no assistance. For this reason, a histological study of the wound healing process in the manatee is essential to be able to help those manatees that do need assistance to rehabilitate from injury.

Form and Function

The skin is more than just an external covering of the body; it is the largest organ of the body and has many vital functions. The skin acts as a barrier between the internal and external environment. This barrier prevents the loss of water, electrolytes, and macromolecules to the external environment, and also prevents the invasion of chemical, physical, and microbial agents. The skin also serves in temperature regulation. The cutaneous vasculature, subcutaneous fat, hair coat, and, in some animals, the secretory products of tubular glands, are all mediators of temperature regulation (Elsner, 1999). The skin is involved in calcium homeostasis through the conversion of 7-dehydrocholecalciferol to cholecalciferol by ultraviolet light. The pigmentation of the skin functions to protect against ultraviolet radiation damage, provide coat and skin color, as well as aids in heat absorption (Haake, 2001). The skin plays a role in communication, in both sensory and immunologic ways. General somatic afferent modalities, including pain, pressure, and temperature, as well as special somatic afferent information from the eyes and ears function to integrate the organism within its surrounding external environment (Banks, 1988).

Layers of the Skin

The skin is comprised of several layers, which are divided into three main parts; the epidermis, dermis, and hypodermis. The epidermis is the outermost layer of the skin and has tight conjunction with the dermis. These layers work together to form appendages such as hairs, nails, glands, etc. The interaction of the epidermis, dermis, and hypodermis are important in development and for maintenance of homeostasis (Haake, 2001).

Epidermis

The epidermis consists of a stratified, continually renewing epithelium that undergoes keratinization in a basal to superficial direction. Five layers comprise the epidermis: stratum basal, stratum spinosum, stratum granulosum, stratum lucidum, and the stratum corneum. The most basal layer is the stratum basale, cells is the region are cuboidal in shape to columnar. The stratum basale is overlain by the stratum spinosum, which varies in thickness throughout the skin of the body and gets its name from the "spiny" processes that form intercellular bridges. When pigment is present it extends throughout this zone and into the transition of the next region of the epidermis. The stratum granulosum is made up of flattened rhomboidal or squamous cells that produce keratohyalin granules. The stratum lucidum consists of one to several layers of translucent, squamous cells. This zone of the epidermis is limited to, and very prominent in thick, epidermal regions of the body such as the footpads and the nose. The stratum corneum consists of several to many layers of anucleated, squamous, cornified cells. It is in this layer of the epidermis that the superficial-most portion of dead cells is sloughed.

Process of Keratinization

Once keratinocytes migrate out of the basal layer they generally lose their ability to divide and begin the process of differentiation. It is through this process that the epidermal layers become delineated. The process of keratinization involves many changes, the first being the loss of the ability to proliferate. Another change that occurs is the synthesis of new structural proteins and modification of existing ones. The aggregation of keratin filaments by the protein filaggrin is one example. The appearance of new organelles, such as keratohyalin granules, and the eventual loss of all organelles

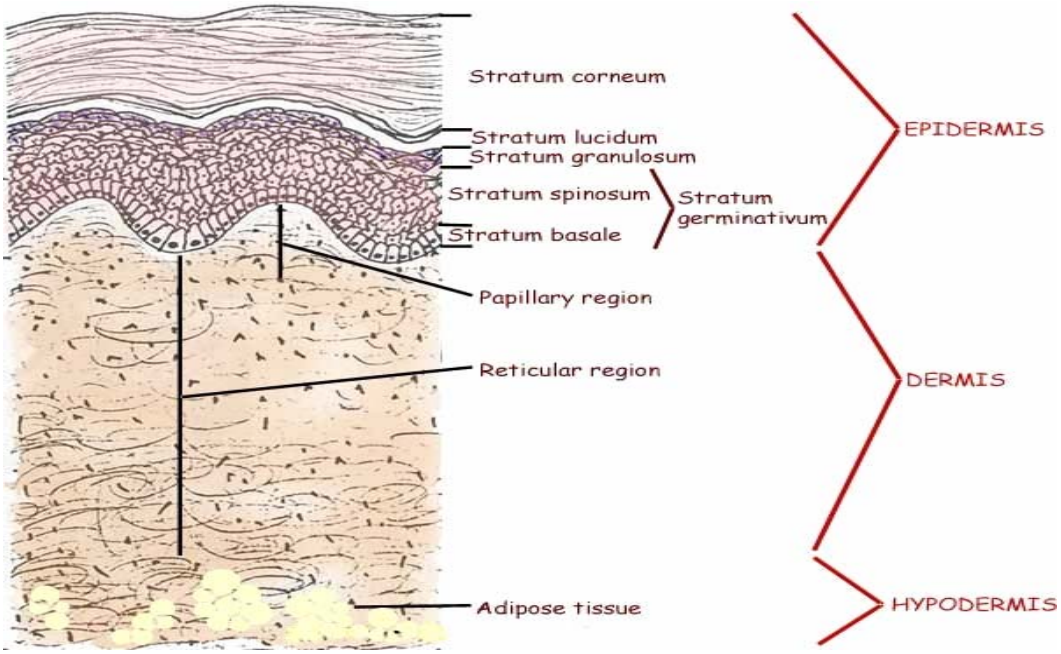

Figure1-1. Normal skin layers.

from the cell is yet another change involved in keratinization. While the cell continues to differentiate, an increase in its size and concominant flattening of its shape occurs. A thick envelope-containing protein called keratolinin forms beneath the cell membrane. This envelope acts as a barrier against chemicals and microbial agents, and provides a framework for the insertion of keratin filaments. Within the stratum corneum intercellular spaces are filled with lipid bilayer containing ceramides, cholesterol, and free fatty acids. This lipid bilayer is important to prevent water loss. The final change of keratinization is dehydration and cornification of the cell, to produce the anuclear flattened cells, sometimes called corneocytes, which make up the outermost layer of the skin.

Cells of the Epidermis

The epidermis has multiple cell types. The major cell type is the keratinocyte comprising approximately 90-95% of epidermal cells (Haake, 2001). These arise from superficial ectoderm of the embryo during the first few weeks of development.

Keratinocytes are involved in the process of keratinization, as previously discussed, and contain enzymes responsible for cell differentiation, and cytokines important for immune response and wound healing. While keratinocytes exist in every layer of the epidermis, they vary in structure, ranging from columnar in shape in the basal layer to polyhedral in shape in the stratum spinosum, and flattened as they differentiate within the granular layer. Finally, in the stratum lucidum and stratum corneum the keratinocytes are completely flattened and compact. Other cells of the epidermis include melanocytes, Langerhans cells, and Merkel cells. Melanocytes are the main dendritic cell of the epidermis and have a neural crest origin (Lever, 1975). They are primarily located in the basal layer of the epidermis and produce melanin. Each melanocyte has long dendritic processes and communicates with nearby keratinocytes to transfer melanosomes. Langerhans cells are also dendritic, originating from bone marrow. They are located in the basal and spinous layers and provide immune surveillance and antigen presentation and processing. Merkel cells can be found in the epidermis and also in the dermis, the origin is unclear but thought to originate from the neural crest or from young developing keratinocytes (Lever, 1975). Merkel cells are associated with the nervous system, being able to function as mechanoreceptors and also produce nerve growth factor (Haake, 2001).

Dermis

The dermis is a complex matrix of ground substance, fibers, and cells. This connective tissue portion of the skin provides tensile strength, pliability, and elasticity. The dermis functions to protect the body from mechanical injury, aids in thermal regulation, supports and nourishes the epidermis, as well as closely interacts with the epidermis during many processes including morphogenesis, wound repair, and

remodeling (Fitzpatrick, 1987). The dermis is divided into two zones: the papillary dermis and the reticular dermis. The papillary dermis generally conforms to the contours of the stratum basale and consists of loose connective tissue. This region of the dermis contains dermal papillae, which are finger-like projections that extend into the epidermis from the dermis. The dermal papillae serve to increase contact with the epidermis. Not all skin is papillated, such as hairy skin. The reticular layer of the dermis is a unique network of dense connective tissue. The dermis is considerably less cellular than the epidermis, being primarily composed of different fibers through which vessels and nerves run.

Fibers of the dermis

Collagen fibers make up 90% of the dermis (Montagna, 1974). Collagen adds tensile strength to the dermis and, when abundant and appropriately recognized, the tensile strength of this principal component of the extracellular matrix can be enormous. The most prevalent form of collagen found in adult mammal skin is collagen type I, which comprises about 80-85% of collagen found in the skin. The majority of the remaining collagen is type III, which is about 8-12% of the dermis (Haake, 2001). Collagen is resistant to most proteases, being degraded primarily by the enzyme collagenase. Each type of collagen has its own specific collagenase that is produced by fibroblasts, neutrophils and macrophages. Elastic fibers, derived from fibroblasts, found in the dermis contain microfibrils and elastin, a binding protein that holds the microfibrils together. The turnover of elastic fibers is relatively slow in the dermis, but can be accelerated by inflammation and ultraviolet light (Starcher et al., 2005). Reticular fibers, produced by fibroblasts comprise a protein of the collagen type III, being found surrounding the epidermal appendages, nerves, and vessels.

Cells of the dermis

Lining the epidermis, the dermis possesses a variety of cell types with the highest concentration of cells located in the papillary dermis. The most common cell of the dermis is the fibroblast which is a mesenchymally-derived cell responsible for the synthesis and degradation of fibrous and non-fibrous connective tissue matrix proteins. Fibroblasts are important in both normal tissue and wound healing, due to not only synthesis and degradation capabilities of the extracellular matrix, but also because of the production of a glycoprotein called fibronectin, and the response that fibroblasts have to immune mediators.

Mast cells are found in the dermis around vessels and contain metachromatic granules. These granules contain heparin, histamine, and proteolytic enzymes, that when stimulated are released. The mast cells are tightly coupled with the immune system and are activated most often when the dermis is exposed to foreign matter, including potential pathogens. The release of the granules promotes edema and attracts cells of defense.

Monocytes and macrophages make up the mononuclear phagocytic system of the skin. Tissue monocytes when activated, become macrophages and are called histiocytes. Both these cells and mast cells are generally found around vessels. Dermal macrophages are derived from bone marrow where they differentiate into monocytes and enter the blood stream, migrating to the dermis where they either actively become involved in defense or remain in a quiescent state as a histiocyte. Macrophages process and present antigen to immunocompetent lymphoid cells. They are microbicidal, tumoricidal, secrete growth factors and cytokines, and are involved in coagulation, wound healing, and remodeling of tissue (Haake, 2001).

Hypodermis

The hypodermis is primarily composed of adipose tissue. The fat cells, or adipocytes, are grouped into lobules surrounded by connective tissue containing blood vessels and nerves. This third and innermost layer of the skin functions to insulate heat, store lipid for reserve energy supply, give body contour, and allow for the movement of the skin over underlying structures. In some animals, such as the horse, dog, and cat, the fat in the foot pads acts as shock absorbers.

Hair, Nail, Innervation, and Blood Supply

The evolution of keratin-bearing cells has facilitated a variety of functions in animals, such as locomotion in aquatic species, flight in birds, and a protective function in mammals described below.

Hair

The evolution of this cell has resulted in the development of hair which protects the skin, provides insulation, conveys threatening behavior or fright by becoming erect, and provides specialized sensation (Freinkel and Haake, 2001). There are four different types of hairs found in the skin. Primary and secondary hairs are the most common in humans and hairy animals. Primary hairs, which are large coarse hairs, are also called guard hairs. Secondary hairs which are fine hairs, are normally found as the undercoat of most dog breeds. Lanugo hairs are normal in gestating fetuses and are also found to grow when there is a nutritional deficiency of fat; these hairs are fine and non-medullated (Freinkel, 2001). The final type of hair is the sinus or tactile hair. These hairs have a well developed connective tissue sheath in which there is an endothelial lined blood sinus that is abundantly supplied by nerves. These specialized hairs are important in the sensation of touch. In most animals tactile hairs are located on the muzzle and above the eye.

Nail

The nail is an epidermal appendage that consists of densely packed, fully keratinized, multilayer lamellae of cornified cells. The nail plate is composed of keratinized cells that originate in the nail matrix. According to Zaias (1970), these cells keratinize without the formation of keratohyalin granules. The proximal nail fold forms the nail cuticle, which keratinizes with the formation of keratohyalin granules. Unlike elsewhere in the skin, the rete ridges of the nail bed are oriented as parallel longitudinal ridges.

Innervation

The skin is an immense and important sensory organ. The majority of nerves in the epidermis and dermis are generally somatic afferent fibers with some efferent autonomic nerve branches. The afferent system contains sensory receptors for temperature, touch, pain, itch, and other various physical and chemical stimuli. The nerves in the skin arise from the spinal nerves, and develop cutaneous branches that run through the layers of the skin. Sensory nerves of the skin have both specialized and non-specialized free nerve terminals. Free nerve endings that are predominantly located in hairy skin consist of the penicilliate fibers, which function as rapidly adapting receptors, and the papillary endings, which function as receptors for cold sensations. Specialized receptors that predominant in glabrous skin include Merkel's disk, Meissner's corpuscles, Pacinian corpuscles, and pilo-Ruffini corpuscles. The Merkel's disk is composed of modified free nerve endings that are associated with epidermal cells and function as mechano-receptors for pain. Meissner's corpuscles are found within the dermal papillae of digital skin and in the junction between haired skin and mucous membranes. Pacinian corpuscles are distributed throughout the dermis and subcutis. The characteristic multilaminar structure resembles an onion and contains an unmyelinated axon in the center (Haake, 2001).

These corpuscles are the principal cutaneous receptor for mechanical perception and transmission. Pilo-Ruffini corpuscles encircle hair follicles and are located just below the sebaceous duct. In addition, there are the peri-follicular nerve endings that are associated with the hair follicle and may be slow-adapting mechanoreceptors that respond to the bending of hairs. It has also been suggested that they are heat receptors (Haake, 2001).

Blood Supply

The microvasculature of the skin consists of arterioles, capillaries, and venules. The cutaneous blood vessels are comprised of plexuses from which arterioles ascend into the dermis and are interconnected. Some arterioles lead into a subpapillary capillary plexus from which individual capillary loops branch. Each dermal papilla possesses one capillary loop that has an ascending arterial limb and a descending venous limb (Lever, 1980). Drainage occurs through the venules that drain into the venous plexus that is associated with the arterial plexus. The cutaneous vasculature serves a critical nutritional function to the skin and forms a conduit for elements of the immune system, playing a vital role in immune surveillance of the skin (Haake, 2001). Another critical function of the vasculature is thermal regulation, which involves fluctuations of blood flow through vasodilation or vasoconstriction (Haake, 2001). The endothelial cells of the vasculature play an important role in wound healing, control of homeostasis, and modulation of inflammation in the skin.

Marine Mammal Skin

In marine mammals the integument is different from terrestrial mammals with regard to both structure and function. This is due mostly to adaptations to aquatic specialization as the orders of marine mammals evolved from divergent ancestorial terrestrial mammals.

In marine mammals the integument not only acts as a barrier to the environment, but also prevents dehydration, reduces drag, provides buoyancy control, and regulates temperature. Modifications to the skin for reproductive and feeding purposes exist as well. Marine mammals have diverged down two paths from their terrestrial ancestors, one path being semi-aquatic, and the other completely aquatic. The semi-aquatic marine mammals have a dense pelage, while the completely aquatic marine mammals have a sparse pelage. In most marine mammals the epidermal and dermal thicknesses vary from one region of the body to another, whereas among terrestrial mammals the thickest areas of the skin are typically located in the nasal planum, foot pads or hooves, and scrotal region. Many marine mammals have interdigitating dermal papillae and epidermal pegs, with the latter, in some instances, having characteristic branches. This pattern, termed rete ridges, is only seen normally in terrestrial mammals in the three areas previously stated. For marine mammals, rete ridges occur normally throughout the entire body due to external pressures from the aquatic environment. Their presence provides better support and connection between the epidermis and dermis. Heat exchange in water is approximately 27 times greater than in still air of the same temperature (Ling, 1974). Marine mammals are generally exposed to water temperatures lower than that of their internal body temperatures. Regulating heat loss to the surrounding water is, therefore, critical. The rete ridges in marine mammals increases the surface area which allows for increased vascularization to the skin surface, facilitating thermoregulation. Vascularization of the skin and presence of a hypodermis full of adipose are the main regulating factors in thermoregulation for marine mammals so most marine mammals lack sebaceous and sweat glands. The vascularization of the skin allows for insulation as

well as a means of heat loss. Marine mammals release excess heat through areas either naked or thinly covered with hair such as the flippers, flukes, and fins. Due to the counter-current heat exchange system marine mammals can retain and lose heat as needed (Reynolds, 1999).

Cetaceans

Cetacean integument does not contain the typical five layers of the epidermis. They have a stratum corneum, stratum spinosum, and stratum basale, but lack a stratum lucidum and stratum granulosum (Sokolov, 1982). The epidermis has a large surface area connection with the dermis due to the formation of large epidermal pegs and dermal papillae. The resultant rete ridges can be so pronounced that in many areas of the cetacean skin these invaginations comprise more than fifty percent of the thickness of the epidermis. The surface of cetacean skin is smooth and has a very high turnover rate, replacing layers as often as every two hours in some subspecies, allowing for a self-cleaning surface that most likely reduces the amount of fouling organisms to settle and attach to the skin surface (Hicks et al. 1985). The cetacean epidermis tends to be very thick, ranging from 1.5 mm in *Megaptera* to 9 mm in *Delphinapterus* (Ling, 1974). The stratum corneum is not usually fully cornified (Sokolov, 1982). The retention of pyknotic nuclei in the cornified cells resembles parakeratosis in man and also in the normal epidermis of the kangaroo pouch (Sokolov, 1982). Cetaceans do not have sebaceous or sweat glands present in their integument. Hair and vibrissae are absent in most cetaceans except for a small area on the head. The dermis is composed of a dense collagen network that ranges from a dense compact plexus to a loosely compact plexus; and it is highly vascularized and innervated. Cetaceans have a significant adipose filled hypodermis. The adipocytes are not only concentrated in the hypodermis but can be seen throughout

the entire dermis up to the epidermis in most cetaceans. In a detailed study of an adult finback whale, the epidermis was found to be between 2.5 mm to 3 mm, being thickest over the ventral surface. The understructure had rete ridges oriented to the craniocaudad body axis, and dermal papillae that were long and pointed. The sensory cutaneous nerve endings found in the skin consisted of small Pacinian corpuscles situated in the higher level of the dermis and there were no hair follicles, sebaceous or sweat glands (Giacometti, 1970).

Sirenians

Most accounts of sirenian skin originate from the early 1900's, and are limited. In 1915, Dosch published a study on sirenian integument structure and development. Most of the samples he used were embryos of African manatees and dugongs, with sizes ranging from 13.6 cm to 151 cm. General skin structure was detailed, but much of the terminology has changed. Dosch noted epidermal depressions throughout the skin, as well as sparse hairs. Blood sinus hair follicles were described in the manatee, and dugong. The skin of the dugong and *Trichechus inungius* were studied by Sokolov (1982) in two young animals. Sokolov described the epidermis as thick and formed by three layers: stratum basale, stratum spinosum, and stratum corneum. The dermal papillae were found to rise between 410 microns to 543 microns and the epidermal pegs were measured at a maximum of 840 microns (Sokolov, 1982). The stratum malpighii, a term used to describe the stratum basale and stratum spinosum as one layer, is 20-30 cells deep. The dermis is pronounced, formed by a dermal papillae and subpapillary layer. In the dermal papillae, thin collagen fibers follow the longitudinal axis of the papillae and contain capillaries (Sokolov, 1982). The subpapillary region is formed by a dense collagen bundle plexus with a subcutaneous fat layer on its boundary. There are no sweat or

sebaceous glands, and there is a lack of arrectores pilorum muscles. Sokolov also described the skin of sirenians as having little pelage, though there are vibrissae on the lips, body, and flippers. He also stated that the structure of the roots and sheaths is typical of the vibrissae in land mammals. The most recent studies on the hairs and vibrissae of the manatee have shown that the hair on the face of the manatee is considerably denser than the rest of the body and that perioral bristles, found mainly on the snout, are modified vibrissae (Reep, 1998). In the postcranial body of the manatee there are 1500 hairs per side and characteristically all these hairs contain a blood sinus; there are more hairs present dorsally and each hair has an independent domain of movement (Reep et al., 2002). These hairs have been thought to represent an underwater tactile system, such as the lateral line of the fish, capable of conveying detailed information from the environment (Reep et al., 2002).

Land Mammal with Skin Similar to the Manatee

Some land animals that are either related to, or have skin morphology similar to, the manatee are the elephant and hippopotamus. While the environment that these species live in is different than that of the manatee, elephant skin is very similar to that of the manatee. The manatee's closest living terrestrial relative is the elephant. Elephants have been known to be attracted to the water; even to depths well over their heads. Fossil evidence and molecular data have provided the association between these seemingly unrelated orders of mammals. The specialized, unique morphology of the wrist bones organized serially, their horizontal tooth replacement, and experiments with amino acid sequencing of proteins all have proven the manatee and elephant are related. The skin is very thick and consists primarily of three layers; stratum basale, stratum spinosum, and stratum corneum. The skin varies in thickness throughout the body (11 mm on the trunk,

and 22 mm on the feet) (Sokolov, 1982). Epidermal pegs interdigitate with dermal papillae forming a well developed junction between the epidermis and dermis. According to Sokolov (1982), the dermis is not divided into the papillary and reticular regions. The collagen bundles are mainly horizontal and form a compact plexus. There are sparse hairs throughout the elephant's body, and vibrissae present on the trunk (Hill, 1953). The skin of the elephant lacks sebaceous and sweat glands, but does have a temporal gland near the eye and an interdigital gland located on the feet (Lamps, 2001). The anatomy of the elephant skin is said to allow for significant water loss by evaporative cooling (Mikota, 1975). The skin surface absorbs water and facilitates movement of water over the skin. Hydration of the skin promotes heat loss and helps to maintain heat balance. Elephants frequently bath, wallow in mud, or take dust baths to keep the epidermis hydrated and prevent overheating (Mikota, 1975).

The skin of the hippopotamus is also similar to the manatee. This is likely because the hippo spends most of its time in the water. According to Luck and Wight (1963), the hippopotamus skin is very similar to the elephant, white rhinoceros, and the walrus. The skin is smooth and almost hairless, having varying amounts of short fine hairs over the body and numerous bristles on the muzzle and tail (Luck and Wight, 1963). Skin thickness of the hippopotamus ranges from 12 mm to 35 mm. The epidermis consists of the stratum basale, stratum spinosum, and stratum corneum. Neither the stratum lucidum nor the stratum granulosum are clearly defined. The stratum corneum is compact and formed by ten to twenty clearly distinguishable layers (Luck and Wight, 1963). The dermis is composed of numerous bundles of collagen of a matted but patterned distribution. The blood vessels found in the dermis form a coarse meshwork. The dermal-

epidermal junction is characterized by deep epidermal ridges with dermal papillae, with 7-9 dermal papillae found per millimeter. There are no sebaceous glands, but the hippopotamus does have sweat glands. The subcutaneous fat layer is very thin and there are no adipose cells found in the dermis (Luck and Wight, 1963).

Pathologies of the Skin

Clinically the skin is an important organ, as it can reflect a variety of external and internal disease processes including, ectoparasitism, immune-mediated disease, endoparasitism, endocrine disorders, trauma, and nutritional problems.

Epidermal Changes

Epidermal hyperplasia, also called acanthosis, is an increase in the number of nucleated cells. Normal epidermis of most mammals often contains no more than two layers of nucleated cells (Yager, 1994). When the stratum spinosum increases in width, it causes the epidermis to create folds, that penetrate into the papillary dermis. These rete ridges are normal in hairless skin, such as the nose or footpads of the dog, cat, and other mammals. There are four types of hyperplasia; irregular, regular, papillated and psuedocarcinomatous (Yager, 1994). Irregular hyperplasia refers to hyperplastic changes that cause the rete ridges to form unevenly in height and shape. Regular hyperplasia is characterized by rete ridges of even width and depth, can be club-shaped and is unusual. Papillated hyperplasia refers to digitate projections of the epidermis, such as in papillomas and warts. Pseudocarcinomatous hyperplasia is distinguished by extreme, thin, irregular, branched, and fused rete ridges (Yager, 1994). Epidermal hyperplasia does not necessarily indicate chronicity, the epidermis responds rapidly to injury, and can induce a mitotic burst in cell population causing hyperplasia (Yager, 1994).

Hyperkeratosis occurs when the keratin layer is thickened but otherwise histologically normal. There are two types: orthokeratotic hyperkeratosis and parakeratotic hyperkeratosis. The former is a thickening of the stratum corneum, indicating an increase in the production of keratin or a decrease in the normal friction of the cornified layer. This type of hyperkeratosis can be morphologically classified three ways; basket-weave, compact, and laminated. The compact type is the most protective and occurs when the skin surface is subject to chronic low-grade trauma, such as licking or abrasion on hard, rough surfaces (Wheater, 2002). Parakeratotic hyperkeratosis is classified by a thickened stratum corneum with keratinocytes in which the nuclei are retained. This abnormality normally occurs due to an excess production of abnormally keratinized stratum corneum, which shows that there is a failure of normal differentiation (Yager, 1994).

Crusts on the skin indicate a previous episode of exudation and crusts usually consist of clotted plasma proteins, leukocytes, erythrocytes, epithelial cells, and often microorganisms. Microorganisms are normally found in crusts because the serum-rich exudate serves as a great environment for bacteria and fungi to grow. Exocytosis is the process through which leukocytes and erythrocytes migrate into the epidermis. Neutrophils are dominant in exocytotic reactions, and epidermis that is infested with ectoparasites normally contains eosinophils and lymphocytes. Epidermal necrosis normally occurs as a secondary lesion due to self-inflicted trauma such as itching, and physical damage from chemicals, burns or freezing, that form crusts and can cause the necrotic epidermis to be replaced by a layer of leukocytic debris (Yager, 1994). Apoptosis is yet another pathological process of the epidermis, being a specific type of

cell death, that is a common mechanism in skin turnover. Apoptosis is mainly seen in the basal layer of the epidermis, but can occur at any level. It is a process induced by T cells and occurs through an energy-dependent sequence of events that eventually leads to the self-destruction of individual cells without damaging the surrounding tissue and without inducing inflammation (Haake and Polakowska, 1993).

Dermal Changes

One type of dermal changes is an increase in collagen. Granulation tissue is an example. In granulation tissue, fibroblasts and blood vessels increase in number. Blood vessels grow perpendicularly to the skin and the newly laid down collagen fibers and fibroblasts are orientated parallel to the skin surface. Fibrosis is another type of collagen increase that refers to replacement of normal collagen with increased connective tissue. The collagen bundles are usually smaller in diameter, densely packed, and there is an increased number of fibroblasts (Yager, 1994). Both granulation and fibrosis occur when there is substantial damage to the connective tissue framework. In many instances the damaged tissue lacks the ability to regenerate specialized cells; therefore some architectural distortion occurs (Stevens et al., 2002). The purpose of granulation and fibrosis is to restore structural intergrity to the dermis of damaged skin.

Skin Pathologies on the Manatee

There are two main abnormalities that have been documented in manatee skin. One is caused by chronic exposure to cold water, while the other is viral in nature. Cold stress syndrome (CSS) occurs when water temperatures drop below 20°C for extended periods of time. A cascade of clinical signs is initiated, one of them being skin lesions. Manatees have been found to have diffuse cutaneous lesions found primarily on the fluke, flippers, and head that can extend in a patchy pattern to dorsolateral body regions

(Bossart et al., 2002). The epidermis of these lesions is thickened and consists of raised, irregular, pale grey plaques measuring up to 40 cm in diameter (Bossart et al., 2002). Epidermal hyperplasia with associated orthokeratotic hyperkeratosis, keratinocyte vacuolar degeneration of the stratum intermedium, and contamination of the superficial epidermis by gram-negative coccobacilli, septate fungal hyphae, and a few unidentified metazoan parasites are commonly found in CSS cutaneous lesions (Bossart et al., 2002). It has been suggested by Bossart, that this contamination is an indicator of a dysfunction of the dermatological primary immune surveillance. The increase in epidermal thickness is thought to be an adaptive response to insulate against cold temperatures.

Cutaneous viral papilloma lesions have been described in the manatee (Bossart, 2002). This lesion has similar characteristics to the CSS lesion. Seven of nine examined captive manatees developed multiple, cutaneous, pedunculated papillomas. Three years later, four of the seven manatees developed sessile, solid papillomas that were clinically distinct from the pedunculated papillomas. The original lesions were raised, firm, whitish-gray, and contained many fine anastosoming fissures distributed over the anterior body including the pectoral flippers, upper lips, external nares, and periorbital regions. The later, sessile papillomas, were more diffuse and numerous, firm, white, and macular to papular in shape and found mainly along lines of scratch marks or trauma (Bossart, 2002). Microscopically the original lesions had extensive hyperplastic and occasional dysplastic keratinocytes raised above the skin surface. Thick dermal papillae containing capillaries separated the rete ridges. Moderate numbers of keratinocytes within the stratum spinosum were characterized by vacuolated cytoplasm and pleomorphic vesicular nuclei that were centrally located (Bossart, 2002). The sessile lesions contained

hyperplastic keratinocytes with broad rete ridge formation. It was determined by transmission electron microscopy that sparse numbers of keratinocytes contained viral plaques. In the sessile papillomas, it appears that the lesions developed in response to trauma, representing activation of latent infection (Bossart, 2002).

Wounding of the Skin of Marine Mammals

Shark attacks, infectious processes, fight or play between animals, vessel strike, and other activities damage the skin to varying extents. The marine mammal most frequently subjected to intense integumentary wounding is the manatee (O'Shea et al. 1995). The cause of most wounds to this animal has been boat strikes. Wounds by boat strikes occur for several different reasons with increased tourism and permanent population both resulting in an increase in boats in the state of Florida. Another contributing reason is the comparatively slow swimming speed that the manatees perform when inhabiting shallow waters, making it difficult for the manatee to move out of the way of oncoming boats.

It has been reported in the bottlenose dolphin *(Tursiops truncatus*) that large wounds from shark bites healed completely without any human interference. Several wild bottlenose dolphins with fresh shark bites, ranging from mild to severe, were observed to heal from six months to a year in the wild (Corkeron et al., 1987). One dolphin that was observed with a fresh shark bite wound had nursed a two-month-old calf, and throughout the healing process her calf remained in good health and grew normally. Therefore, despite the metabolic stress associated with lactation and the requirements for efficient wound healing, the dolphin healed within seven months (Corkeron et al., 1987). It has been proposed that in captivity the nature of the treated water in which captive dolphins are rehabilitated may affect the wound healing process.

Skin is a complex organ, which does not possess the power of complete regeneration, in that damaged tissue is replaced by a fibrous scar tissue. On the surface, the damaged integument is covered by regenerated and remodeled epithelium. Bruce – Allen and Geraci (1984) pointed out that a distinguished feature of healing in the dolphin is the absence of a scab in the traditional sense. Instead a buffer zone is created by degeneration of cells exposed to seawater. By osmotically damaging the exposed cells, the seawater actually initiates the formation of the buffer zone shielding the underlying tissue and thereby permitting repair to take place. Repair of the epithelium can be fast due to the extensive folding of the germinal layer since the wound will expose several dermal papillae. Another possible factor for facilitating repair could be the constant gentle irrigation of the wound as the dolphins move through the water.

Wound-Healing Process

Wound healing occurs initially from the depths of the wound and progresses towards the surface. The source of the repair cells are the perivascular tissues at the edges and depths of the wound. Angiogenesis, fibroplasia, and collagen deposition occur early at these sites. Wounds can be classified into two categories: partial or full thickness. Partial thickness is limited to the epidermis and superficial dermis with no damage to the dermal blood vessels. Full thickness wounds are injuries that involve loss of the epidermis and dermis and extend to deeper tissue layers, disrupting dermal blood vessels (Swaim, 1990).

Wound healing usually occurs in four sequential stages: the inflammatory stage, the debridement stage, the repair stage, and the maturation stage (Schult, 2000). The immediate reaction of the skin to an injury is skin retraction. Enlargement of the wound bed, due to retraction of the skin edge, begins immediately following a cutaneous insult.

Skin tension is the major force for retraction, with the force varying with the location of the body that is injured. In the first five to ten minutes after an injury, intense vasoconstriction occurs, limiting hemorrhage (Bertone, 1999). This activity is then followed by a series of events that begins with vasodilation and increased permeability of venules in response to the release of vasoactive substances from the injured tissue. Plasma proteins leak into the wound and react to form a fibrin plug that quickly impedes lymphatics and localizes the inflammatory response (Swaim, 1990). Within 30 minutes, platelets and leukocytes begin to adhere to the vessel walls at the injury site. Platelets function in blood coagulation, chemotaxis of leukocytes, and activation of fibroblast precursors. Diapedisis and active migration of leukocytes increase their numbers in the wound. Initially mononuclear cells and neutrophils arrive at the site in the same proportions as those in the blood. The short-lived neutrophils subsequently die, release intracellular enzymes and enhance the inflammatory process. As these neutrophils die, the macrophages begin to outnumber the neutrophil population. Macrophages phagocytize pathogenic organisms as well as other cell and matrix debris. Once activated, macrophages also release a battery of growth factors and cytokines at the wound site (Martin, 1997).

Recent studies have shown that neutrophils are also a source of pro-inflammatory cytokines that serve as some of the earliest signals to activate local fibroblasts and keratinocytes (Hubner, 1996). The plasma proteins that leak into the wound bed participate in the clotting cascade and form interlaced fibrin strands from fibrinogen. The formation of a fibrin clot develops, consisting of platelets embedded in a mesh of fibrin fibers. The clot serves as a reservoir of cytokines and growth factors that are released as

activated platelets degranulate. This early mix of growth factors will initiate the wound closure process by providing chemotactic cues to recruit circulatory inflammatory cells to the wound site, promote the tissue movements of re-epithelialization and connective tissue contraction, and stimulate the wound angiogenic response (Martin, 1997). The fibrin clot acts additionally as a hemostatic barrier, fills dead space, holds tissue together and provides the framework for further healing.

A variety of cells play individual roles in inflammation and wound healing, including the neutrophil, macrophage and fibroblast. The neutrophil functions to kill organisms, especially bacteria, and to facilitate breakdown of debris by extracellular release of their enzymes. The inflammation and debridement stage of wound healing begins as soon as neutrophils accumulate in the wound and begin to phagocytize debris. Healing cannot proceed successfully without the completion of this phase. Accumulation of neutrophils can delay healing as matrix metalloproteinases (MMPs), consisting of collagenases and proteases, continue to be released and disassemble connective tissue proteins. Various members of the MMP family, each of which cleaves a particular collagen, are also upregulated by wound-edge keratinocytes. For example, MMP-9 can dissemble collagen type IV and anchoring fibril collagen type VII (Sternlicht and Werb, 2001).

The macrophage serves an irreplaceable role in wound healing. It is a necrotic debris scavenger that stimulates the maturation and function of the fibroblast and, it is the fibroblast that produces repair tissue. Therefore, this phase of the wound healing process is critical.

The epidermis has the ability to rapidly proliferate and seal insults. The intact epidermis provides protection to the deeper tissues from trauma and infection and also acts as a barrier to fluid loss. It has been proposed that a mitotic inhibitor called chalone is normally produced by maturing squamous epithelial cells (Bertone, 1999). After an injury these cells are lacking and proliferation of epithelial cells begins due to the decrease in chalone production.

In full-thickness wounds for successful re-epithelialization to occur, epithelial migration and proliferation must occur. Cells begin to migrate along the wound bed, but stop before they lose contact with their adjacent epithelial cell. Contact guidance and inhibition determine the extent of migration of the cells. In the horse, proliferation and migration is not detected histologically until day five (Bertone, 1999). Cellular division is a high energy process and requires a good supply of oxygen and fluid. Epithelial migration can occur in an anaerobic environment with energy obtained from glycolysis alone, but a moist environment is necessary for the movement of the cells and the transport of glucose.

Within several (~1–7) days after occurrence of an injury, undifferentiated mesenchymal cells that surround the endothelium of vessels in the wound bed begin transformation and migration to the wound surface. This involves free migration of individual cells that does not require contact with similar cells as is the case with epithelial migration. It is not possible through histologic morphology to identify these cells as angioblasts or fibroblasts during the early migration phase. Normally by day seven, angiogenesis is a prominent histological feature of wound healing (Bertone, 1999). Vascular endothelial growth factor (VEGF) is released at the wound site, being induced

in wound-edge keratinocytes and macrophages, possibly in response to other growth factors. Angiogenesis continues until dead space is filled and the hypoxic gradient is eliminated. It has been shown that VEGF may promote healing. In a study involving genetically diabetic mice, VEGF is not expressed at the site of the wound and healing is impaired (Frank, 1995).

Granulation tissue formation is the process of early fibroplasia. Fibroblasts proliferate and begin to secrete a substance of proteoglycan and soluble collagen precursors by day two of healing. The proteoglycan comprises the amorphous substance matrix that contributes little to the wound tensile strength (Bertone, 1999). Collagen synthesis can begin as early as day two in the healing process, peaking between days five and seven. The wound tensile strength increases with collagen production and maturation. In early wound healing, the collagen and ground substance exist in a less organized arrangement, being seen as a pink, highly vascular, relatively fragile, and easily crushed granulation tissue (Swaim,1990). It is during this time that the fibroblasts can differentiate into either myofibrocytes, collagen-secreting fibrocytes, or elastin-secreting fibrocytes.

Contraction of the wound typically begins between days five and nine, but it has been noted in some wounds as early as day three. The onset of this process is species dependent (Bertone, 1999). The process of wound contraction reduces the size of the wound bed by centripetal movement of the full thickness of the skin edges. Fibroblasts with contractile ability, i.e., myofibroblasts, develop firm attachments to the wound bed and edges. The contraction of these cells pulls the wound edges closer together. Contraction will stop when the wound edges touch, a process called contact inhibition.

Contraction will also stop when there is either a lack of myofibroblasts or opposing tensile forces are equal to the contraction force (Bertone, 1999). Scar size and shape both depend on the amount of skin tension, skin laxity, shape of the wound, and the maturity of the wound. After the wound has closed through contraction, the collagen formation continues in the adjacent tissues and as a result relieves the skin tension that had developed. This process is referred to as intussusceptive growth (Bertone, 1999).

Typically after day seven, late fibroplasias will occur. An increase in the number of fibrocytes continues for approximately three weeks after injury (Bertone, 1999). The increase of fibrocyte number causes an increase in collagen content at this phase of the healing, not an increase due to collagen synthesis. As the fibrocytes mature they become smaller and more spindle-shaped. While secreting collagen they align themselves parallel with the wound surface and perpendicular to the new capillaries. This architecture has been observed by days 10 to 20 in the lower limb of the horse (Bertone, 1999). After three weeks, the population of fibrocytes is stable and participates in the balanced process of collagen synthesis and degradation. This stage of the healing process produces a more firm and paler granulation bed.

As the collagen matures and is remodeled, the amount and organization of collagen fibers increases, and ultimately produces a dense, firm scar. The realignment of the fibrocytes, described previously, causes the realignment of the collagen fibers. The increase in complexity of the physical weave of collagen along with the increase in the stable intramolecular and intermolecular cross-linking of collagen continues to increase wound strength for more than one year (Bertone, 1999). After three weeks collagen dynamics reaches steady state as collagenolysis balances collagen synthesis. Eventually

the immature or developmental collagen (Type III) is completely replaced by the more mature, stronger, collagen (Type I).

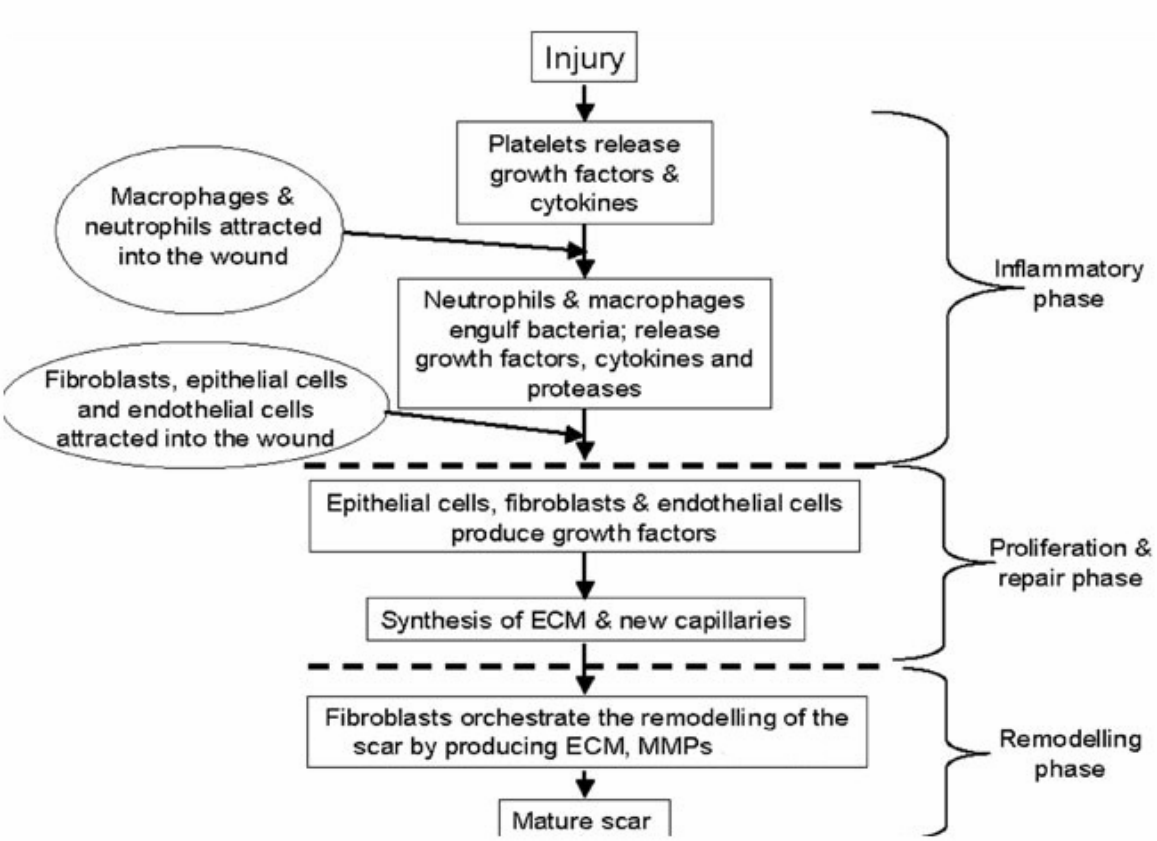

Figure1-2. Wound-healing process from injury to scar.

Some factors that affect the wound healing process can be systemic or local. Malnutrition, hormonal imbalances, and organ failures can disrupt this process and should be corrected or compensated to support effective healing. Acute and chronic viral infection has been shown in mice to impair healing, presumably from the alteration of macrophage function (Kenyon, 1983). Protein deficiencies, such as hypoproteinemia and dysproteinemia, delay wound healing mainly through a reduction in collagen and proteoglycan synthesis by the fibroblast, but also through a loss of plasma oncotic pressure resulting in increased tissue edema (Bertone, 1999). Alteration in the blood flow

through microcirculation at the wound that results in decreased oxygen exchange and tissue hypoxia impairs the healing process. Decreased tissue perfusion may produce local tissue dehydration or edema leading to impediment of epithelization (Peacock, 1984). Environmental temperature equivalent to body temperature supports more rapid wound healing than typical ambient temperature (Swaim, 1980). Increasing the environmental temperature increases the metabolic activity of the repair cells. It also increases the metabolic demands and the growth rate of bacteria.

Steroids impair wound healing by suppressing the inflammatory phase, angiogenesis, fibroplasias, collagen formation, and wound contraction (Peacock, 1984). Many pharmacologic agents impair wound healing. These agents are usually used to treat hypertrophic scar and keloids. For example, the lathyritic agents control fibrosis by blocking the biosynthesis and cross-linking of collagen. Lack of cross-linking increases the susceptibility of collagen digestion by collagenase (Jackson, 1979).

Local factors such as infection, motion, local oxygen gradients, bandaging, vitamin A, vitamin C, and zinc can all affect the healing process. Infection is dependent on the number of organisms inoculated, the organism virulence, and the host's defenses. Infection separates the wound edges, prolonging the debridement phase, and producing further tissue damage (Peacock, 1984). Excessive movement of the skin can disrupt the organizing capillary buds and fibroblastic cells. Movement can also increase the probability that infection may spread along lymphatics (Swaim, 1985). Bandaging should be done not to hinder blood flow or physically traumatize the wound surface. Vitamin A is essential for epithelial health, it has been shown to accelerate corneal and skin healing, therefore, a deficiency could hinder wound healing (Klein, 1975). Vitamin

C and zinc deficiencies could also hinder wound healing. Vitamin C is needed for the rate limiting step of collagen production and zinc can be excreted from an open wound can produce a zinc deficiency (Heughan, 1975).

Wound Care

There are four principles to wound care. The first is debridement; the removal of non-viable tissue. Debridement can be accomplished in four different ways; autolytic, biomechanical, mechanical, and sharp (Schultz, 2000). Autolytic debridement uses the body's own capacity to lyse and dissolve necrotic tissue. This process can be supported through the use of dressings that concentrate and encapsulate white blood cells and enzymes in the wound bed. Biomechanical debridement involves the use of enzymes to dissolve nonviable tissue. Mechanical debridement is accomplished by placing moist coarse-mesh gauze in the wound and allowing the dressing to dry. Necrotic tissue will adhere to the dressings when removed. This method can cause more harm; it can disrupt the granulation bed when the gauze is removed, and is painful. Sharp debridement dissects necrotic tissue from viable tissue with a scapel or scissors. It is the most rapid and effective method of debridement (Schultz, 2000). Cleansing is the removal of foreign debris. Cleansing can be done through vigorous or gentle techniques of flushing or by patting the surface of the wound. When a granulation bed is present, gentle flushing is preferred, and as the wound becomes cleaner, saline is the choice of flush. Chemical cleansers can be toxic to cells, especially fibroblasts. Saline and lactated Ringer's are non-toxic and will effectively remove contaminant from the wound surface when used in combination with some mechanical force that dislodges the foreign debris.

The second principle of wound care is maintaining a moist environment. This type of environment promotes re-epitheliazation and healing. Wet-Damp dressings

support autolytic debridement and absorb exudates and trap bacteria in the gauze which are all removed when the dressing is changed. Polyvinyl dressings such as Tegaderm©, are transparent, adhesive wound dressings that are semipermeable to oxygen and moisture and impermeable to bacteria and other contaminants (Frantz, 2001). Advantages to this type of dressing are that it maintains a moist environment and also concentrates the normal defenses of leukocytes, plasma, and fibrin in the wound bed. Another advantage is that it can be left on for several days. Absorptive dressings are not pre-moistened, but absorb moisture from exudate in the wound to maintain a moist environment. These dressings can absorb up to twenty times their weight. Calcium alginate dressings such as Sorbsan or Kalostat are two types of soft, fibrous, absorptive dressing that are derived from seaweed.

The third principle in wound healing is to prevent further injury. Elimination or reduction of the condition that allowed the wound to develop should be managed. In aquatic animals items such as flotation jackets have been used as a secondary protection layer to minimize the potential for further aggravation to the wound (Walsh, 2004).

The fourth principle for wound care is to provide substrates for healing. It is recognized that protein is essential for wound repair and regeneration. Amino acids are essential in supporting the immune response, angiogenesis, fibroblast proliferation, collagen synthesis, and scar remodeling. Adequate amounts of fats and carbohydrates are also needed to prevent amino acids from being oxidized for caloric need. Glucose is needed to meet the energy requirements of the cells involved in the wound healing process. Albumin is the most important indicator of malnutrition because it is sacrificed to provide essential amino acids in the event of protein deficiency. Since albumin has a

half-life of 20 days, low serum levels can indicate malnourishment. Necessary vitamin and mineral supplementation during wound healing can be with vitamin A, vitamin C, zinc, and iron. All of these are important in attaining most rapid physiological rates of healing.

Specific Aims

This research has two specific aims. The first is to describe the integument of the manatee histologically. The normal manatee integument has yet to be defined. A description of the normal integument is fundamental to understanding subsequent cutaneous disease and injury. The second aim of this research is to characterize the wound healing process of the manatee. Manatees are known to heal in the wild from severe wounds. Better knowledge of this healing process will assist rehabilitators care for injured manatees that have been rescued.

CHAPTER 2
MATERIALS AND METHODS

Sample Collection

The skin samples examined in this study were collected from 10 necropsied animals over a period of one and a half years at the Florida Fish & Wildlife Conservation Commission's (FWC) Research Institute's (FWRI) Marine Mammal Pathobiology Laboratory (MMPL) in St. Petersburg, Florida, as well as Sea World of Orlando in Florida. There were seven samples taken from living and recently deceased animals at Sea World of Orlando for the abnormal portion of the research. Samples were collected froom a total of seventeen Florida manatees (*Trichechus manatus latirostris)* of different ages, gender, and location. Normal skin samples were collected from 25 body sites (See Figure 2-1), including both dorsal and ventral regions, where applicable. Abnormal, wounded, and scarred skin samples were also collected when available. Samples were fixed for 24 hours in 10% neutral buffered formalin and then transferred to phosphate buffered saline solution. Samples taken for electron microscopy were fixed in 2% glutaraldehyde 0.1M sodium cacodylate buffer and stored in the refrigerator until they were processed.

Tissue Processing

Samples for light microscopy were dehydrated in the following sequence of alcohols: one change of 70%, one change of 80%, and two changes of 95%, and three changes of 100%. The tissue was then cleared with two changes of xylene. The tissues were infiltrated with two changes of Fisher Tissue Prep paraffin (Fisher Scientific,

Pittsburgh, Pennsylvania) at 57° Celsius. The tissues were then embedded using Fisher Tissue Prep T565 in metal molds and allowed to harden.[1]

Sectioning

Paraffin samples were cut using a Reichert-Jung 2030 microtome at 6 micron sections, except for elastin samples, which were cut at 10 microns. Tissue ribbons were floated out on an 80° Celsius water bath and then placed on Superfrost microscope slides, and Superfrost Plus slides (Fisher Scientific, Pittsburgh, Pennsylvania) with one section per slide. Each slide was labeled with the identification number of the animal, area where the sample was taken from, and number of the section cut. Sections were placed in a slide rack and into an oven at 60° Celsius overnight to dry. These samples were analyzed morphologically using a variety of stains including special stains for the extracellular matrix and immunohistochemical staining.

Electron Microscopy

Electron microscopy samples were post-fixed in 1% osmium tetroxide for one hour at room temperature, washed with buffer, dehydrated through a graded series of ethanol and finally into 100% acetone before being embedded in plastic (epon-Araldite mixture) (Freida, 1996).

Electron microscopy samples were cut on an ultramicrotome at 1 micron and stained with 1.0% toluidine blue; to identify specific areas of interest.[2] Ultrathin sections (80-90 nm thick) were cut and stained with Reynold's lead citrate and uranyl acetate and then ready to be examined under an electron microscope (AFIP, 1994).

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[1] for details on processing refer to Appendix A

[2] for details on processing refer to Appendix A

Staining

Several stains were used for the histological examination of the samples. Sections were deparaffinized with three changes of xylene for five minutes each, two changes of 100% ethanol alcohol, 95% ethanol alcohol for two minutes each in all stains. Hematoxylin and Eosin (H&E) was a traditional stain used for overall morphology, Periodic Acid Schiff (PAS) procedure was used to detect fungi, glycocalyx and mucin (McManus, 1948), and Masson's Trichrome stain to distinguish between muscle, keratin, and collagen (Masson, 1929). After examination using the traditional stains, some special stains were used for further examination of the samples. Several special stains were used to detect mast cells including the Luna's method for mast cells (Luna, 1968), Gaffney's one-hour giemsa (Sheehan, 1980), Wolbach's giemsa (Wolbach, 1922) and Toluidine Blue (Lillie, 1976). Not only does the Luna's method for mast cells stain for mast cells but it also stains elastin. Before this stain was used, the Verhoff's-Van Gieson stain was used to highlight elastin fibers (Mallory, 1942). Brown and Brenn gram stain was used for detection of gram-positive and gram-negative bacteria (Brown and Brenn, 1931).[3] When staining was completed, all slides where then coverslipped using synthetic toluene based mounting media.

Immunohistochemistry

Using VEGFR-1, VEGFR-2

Sections were deparaffinized with three changes of xylene for five minutes each, two changes of 100% ethanol alcohol, 95% ethanol alcohol, and distilled water for two minutes each. The slides were rehydrated with Tris buffered saline (TBS) solution. The

[3] for details on staining refer to Appendix A

sections were then quenched in 3% hydrogen peroxide for 20 minutes. Tris buffered saline (TBS) was used to wash the slides three times for two minutes each. Sections were blocked with 5% normal goat serum (53µL of normal goat serum and 1000µL of Dako antibody diluent, no. S0809, DakoCytomation, Carpinteria, California) for 20 minutes. The slides were washed three times in TBS for two minutes each. A primary antibody (Flt-1, polyclonal rabbit, catalog number SC316 or KDR, monoclonal mouse, no. SC251, both from Santa Cruz Biotechnology, Santa Cruz, California) was combined with the Dako antibody diluent at concentrations of 1:50, 1:100, and 1:200. The Flt-1, polyclonal rabbit primary antibody was used for the detection of VEGFR-1. The KDR, monoclonal mouse primary antibody was used for VEGFR-2 detection. The sections were incubated in a humidified chamber overnight at 10º Celsius. The procedure was completed with a Dako detection kit (no.K0673). After the sections were stained and rinsed twice with distilled water, twice with 95% ethanol alcohol, and three times with toluene, they were coverslipped using Richard-Allan Scientific mounting medium (Richard-Allan Scientific, Kalamazoo, Michigan). Due to variable results obtained in this section of immunohistochemistry further research is needed. No results will be discussed in this thesis.

Smooth-Muscle Actin

Sections were deparaffinized with three changes of xylene for two minutes each, two changes of 100% ethanol alcohol, 95% ethanol alcohol, 80% ethanol alcohol and distilled water for two minutes each. The slides were rehydrated with phosphate buffered saline (PBS) solution. The sections were then quenched in 3% hydrogen peroxide for 20 minutes. Phosphate buffered saline (PBS) was used to wash the slides three times for two minutes each. A primary antibody (Smooth Muscle Actin, Dako, monoclonal mouse,

catalog number M0851) was combined with the Dako antibody diluent at different concentrations of 1:50, 1:100, and 1:200. The sections were incubated in a humidified chamber overnight at 10º Celsius. The procedure was completed with a Dako detection kit (no.K0673) and Dako AEC substrate chromagen (no. K3464). After the sections were stained and rinsed twice with distilled water, twice with 95% ethanol alcohol, and three times with xylene, they were coverslipped using glycerol/gelatin mixture.

Matrix Metalloproteinase 2 and Matrix Metalloproteinase 9 (MMP-2, MMP-9)

Sections were deparaffinized with three changes of xylene for three minutes each, two changes of 100% ethanol alcohol, 95% ethanol alcohol, and 75% ethanol alcohol for two minutes each. All sections were rehydrated with distilled water for ten minutes. The sections were then quenched in 3% hydrogen peroxide for 20 minutes. Phosphate buffered saline PBS was used to wash the slides three times for two minutes each. Sections were blocked with 5% normal horse serum (3 drops of normal horse serum kit #SK-5100 and 10mL of PBS, pH 7.4) for 20 minutes. The slides were washed three times in PBS for two minutes each. A primary antibody (rhMMP-2, monoclonal mouse, or rhMMP-9, monoclonal mouse, both from R&D Systems, Minneapolis, MN) was combined with horse serum (kit no. SK-5100) at concentrations of 1:50, 1:100, and 1:200. The sections were incubated in a humidified chamber overnight at 10º Celsius. The procedure was completed with Vector Laboratories detection and chromagen kits (no.SK-5100 and SK-5200). MMP-2 slides were incubated with chromagen for 5-10 minutes in the light. MMP-9 slides were incubated for 5-10 minutes in the dark. After the sections were stained and rinsed with distilled water for ten minutes they were dehydrated through one bath of 75% ethanol alcohol, 95% ethanol alcohol, and 100% ethanol each for two minutes, and three times in xylene for two minutes each, they were

coverslipped using Richard-Allan Scientific mounting medium (Richard-Allan Scientific, Kalamazoo, Michigan).

Measurements

Measurements were taken on all collected skin samples using a Microline microscope (Longwood, Florida). Samples were measured in micrometers and converted into millimeters for the thickness of the epidermis and dermis. The undulating ridges, or peaks, and epidermal pegs were counted per linear 275μm (40X view) and the average was recorded. The distance between peaks was measured in micrometers and converted into millimeters. A minimum, maximum, and average distance between peaks was recorded per sample site on each manatee. Layers of the stratum corneum were counted individually. Areas of the stratum corneum that were too compact to count or fungi was present were noted and recorded in Appendix B by an asterick.[45]

[4] for detailed measurements on individual animals refer to Appendix B

[5] for details on minimum and maximum measurements refer to Appendix C

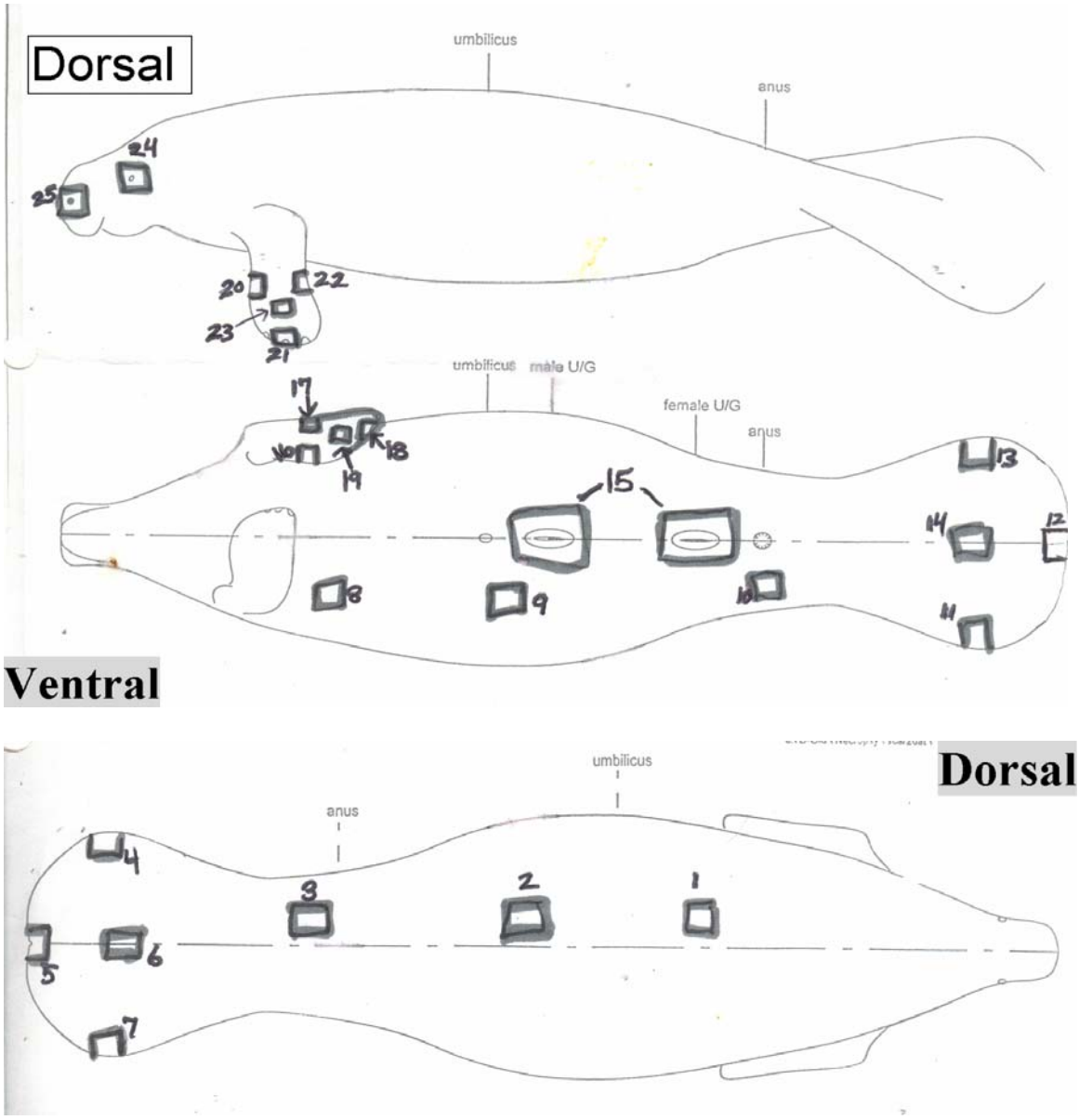

Figure 2-1. Sampling diagram: the 25 sites of collection for normal skin samples from necropsied manatees.

CHAPTER 3
RESULTS OF NORMAL HISTOLOGY

Individual measurements (Appendix B) were taken for each sample site on individual manatees.. Overall measurements and observations are compiled into Table 4-1 and Table 4-2.

Dorsal Skin (Samples Sites 1, 2, and 3).

The dorsal skin of the manatee is the thickest of the entire body. The epidermis in this region varies from 0.05– 0.8 mm in a neonate, and up to 2.6 mm deep in an adult male (Appendix B). The dermis in this area ranges from 5.4 mm in a neonate to 22.1 mm in a male adult. Based on the microscopic measurements, there are no notable differences in skin thickness between males and females. As the manatee ages, there is an increase in skin thickness with a slight increase in epidermal thickness and a more pronounced increase in the dermis. The epidermis consists of three layers; the stratum basale, stratum spinosum, and the stratum corneum. The manatee epidermis does not form a stratum granulosum and the stratum lucidum. The epidermis is marked by many long, invaginating dermal papillae and undulating ridges. The undulating ridges are responsible for the pitted appearance of the skin upon gross examination. In the neonate there are as many as 11 undulating ridges and 14 epidermal pegs per linear 275µm (40X field) (Figure 3-1). In the adult there are between 3–4 undulating ridges and anywhere from 9–16 epidermal pegs per linear 275µm (Figure 3-1). From anterior to posterior, the dermis thickness changes, being thickest at the mid-dorsal point of the back and thinnest when approaching the fluke. The network of collagen in these samples changes from neonate to

adult. As the manatee ages the collagen structure of the dermis becomes more organized (Figure 3-1 and 3-3). The network of collagen consists of thick dense collagen bundles that form a diagonal weave, with collagen fibers criss-crossing, resulting in a distinct pattern. The epidermis of the manatee has an unusually thick stratum corneum that in most animals would be considered hyperkeratotic. In the manatee, the hyperkeratotic appearance of the epidermis is normal (Figure 3-2–3-4). The stratum spinosum of the manatee skin is several cell layers thick (Figure 3-4). Pathologically this condition is called hyperplasia, yet it is normal for the manatee. The dermis is infiltrated with blood vessels, with the largest blood vessels located in the deepest part of the reticular dermis and the smallest arterioles and venules mainly found at the epidermal-dermal junction (Figure 3-5). There are nerves in this region of the skin, with most nerves being observed near blood vessels, at the connection of the dermis and epidermis, and in the dermal papillae. The elastin fibers in this region of the manatee skin vary from the epidermis to reticular dermis. Elastin fibers are most numerous and thickest in the deep reticular dermis and become fewer and thinner as they continue to the epidermis and extend into the dermal papillae (Figure 3-6 and 3-7).

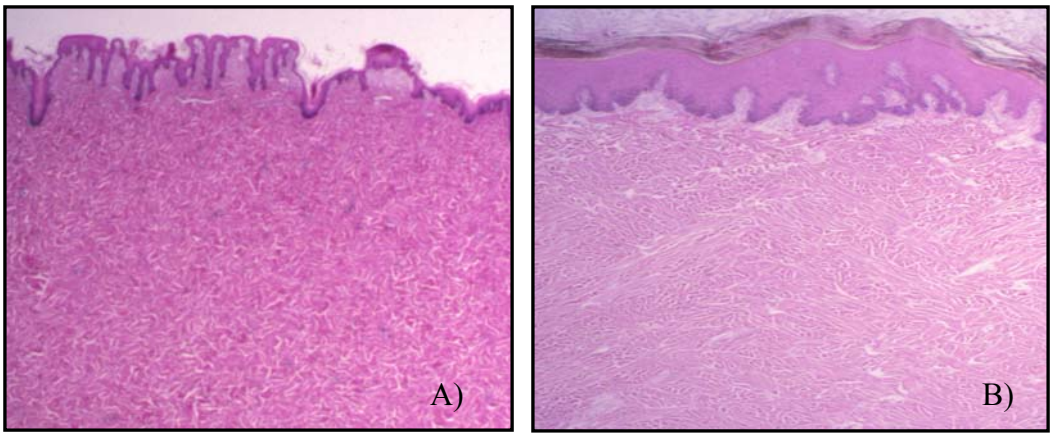

Figure 3-1. Comparison of the epidermis and dermis of a neonate and adult manatee. A) TM0311, neonate, site 1, H&E, 20X. B) MNW0342, adult female, H&E, 20X.

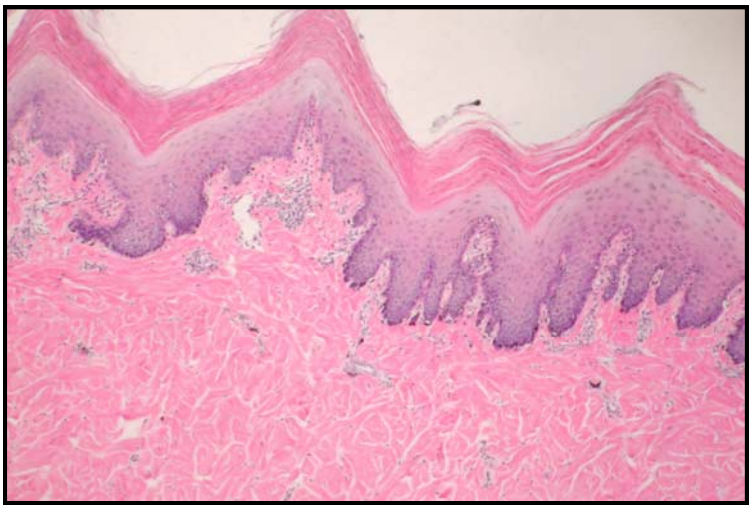

Figure 3-2. MSW03170, male calf, site 3, H&E, 40X, epidermis and dermis.

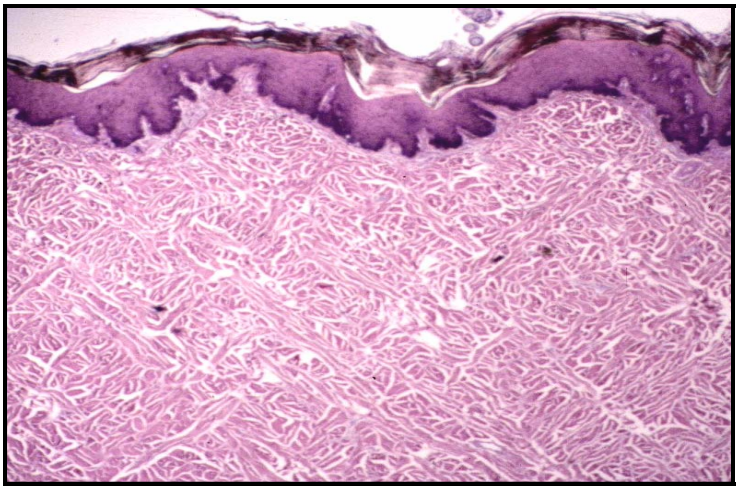

Figure 3-3. MEC0348, adult male, site 1, H&E, 20X, epidermis and dermis.

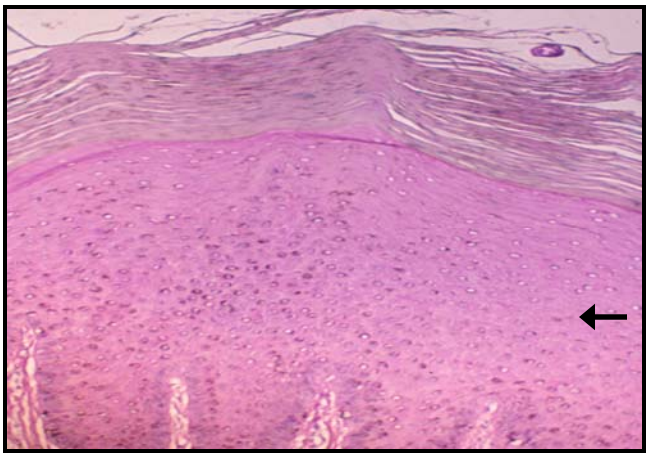

Figure 3-4. MNW0342, adult female, site 1, H&E, 100X, epidermis. Notice the thickness of the stratum spinosum(arrow) and the lack of stratum granulosum.

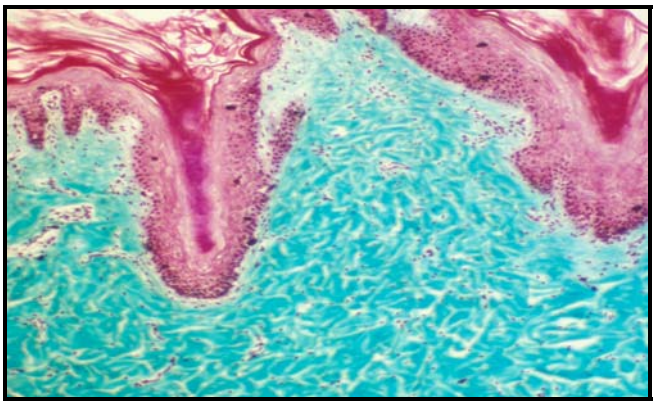

Figure 3-5. TM0311, neonate, site 1, trichrome, 100X, epidermal-dermal junction. The collagen is recognized in this stain by the green color and the keratin in red.

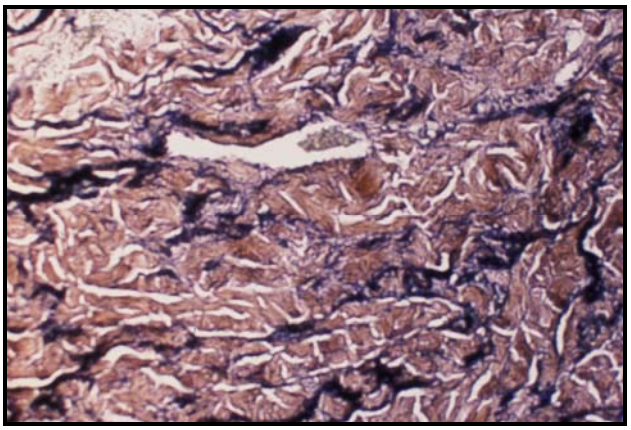

Figure 3-6. MEC0348, adult male, site 3, elastin in deep dermis, Verhoff-Van Geison, 100X. Note the thick black elastin fibers.

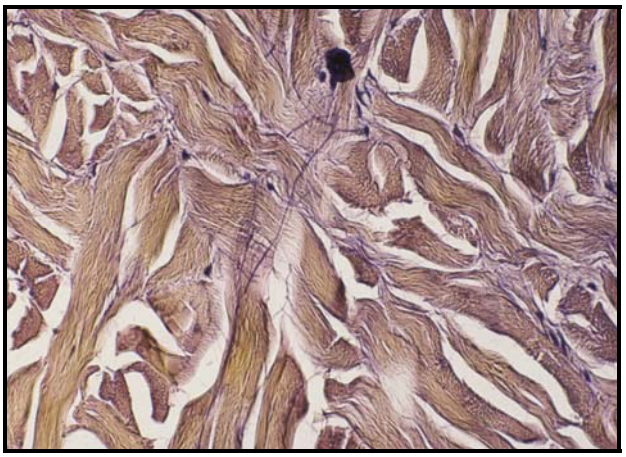

Figure 3-7. MEC0348, adult male, site 3, elastin in dermis, Verhoff-Van Geison, 200X. Elastin fibers are thin compared to the deeper dermis in Figure 3-6.

Dorsal Fluke Skin (Sites 4, 5, 6, and 7)

The fluke of the manatee is supported structurally by the skin. Muscle and bone are present at the center of the fluke. Nevetheless, the majority of the fluke is composed of dermis and epidermis. The only structure that separates the dorsal and ventral edges of the fluke is the caudal vascular bundle. Epidermal and dermal thicknesses vary from manatee to manatee as well as within each manatee throughout the fluke. The edges of the fluke are not identical in thickness. The left and right dorsal fluke edges (sites 4and7) vary slightly from one another with as much as a 1mm difference in epidermis thickness and a 2mm difference in the thickness of the dermis. The most caudal edge of the fluke (site 5) has a somewhat thinner epidermis than the left and right dorsal edges (Figure 3-10). The most central dorsal point of the fluke (site 6) has the thinnest epidermis, and the thickest dermis of the dorsal fluke sample sites (Figure 3-8). The fluke skin appears very rigid, has an exceptionally thick stratum spinosum and stratum corneum Overall the fluke is very vascular throughout the entire dermis regardless of the sample site (Figure 3-9, 3-13, and 3-14). Numerous elastin fibers are present in the fluke (Figure 3-17 and 3-18). Although they are observed throughout the dermis, the elastin fibers are most prevalent in

the superficial dermis, extending into the dermal papillae (Figure 3-16).

The epidermis of the fluke edge was found to be the thickest in a medium-sized adult female, measuring 3.9 mm. By comparison the epidermis is thinnest in a medium-sized male calf, having measured 0.4 mm. There are several pointed undulating ridges present in the fluke; the number of undulating ridges ranges from 3 peaks per linear 275µm in a medium adult female manatee to 6 peaks per linear 275µm in a small male calf and these ridges vary in height and thickness. There are numerous epidermal pegs present that vary in depth and thickness. They vary from 9 pegs per linear 275µm in a medium adult male to 17 pegs per linear 275µm in a medium male calf. The stratum corneum in the fluke is very thick and hard (Figure 3-13 and 3-14). This outermost layer of the epidermis was found to have as few as 15 cell layers in a small adult male to 170 cell layers thick in a medium adult female. There are melanocytes present in the stratum basale and in the papillary dermis. The dermis of the fluke is composed of a dense, intricate weave of collagen. The collagen is organized in a 90-degree criss-cross pattern (Figure 3-12), with the vertical collagen bundles having a perpendicular orientation to the surface of the skin, in site 6 the collagen is in a slightly more diagonal weave than the edges (Figure 3-8 and 3-11). At the very edge of the sample the dermis was as thin as 1.7 mm in a small male calf. Closer to the center of the sample the dermis was measured at 9.8 mm in a medium adult male; the thickest of all the dorsal fluke edge samples. The central dorsal site of the fluke (site 6) had the thinnest epidermis and thickest dermis of all the dorsal fluke samples (Figure 3-8). The epidermis not being as thick as the fluke edges, is the thinnest at 0.3 mm in a medium male calf, and thickest at 1.7 mm in a medium adult male. The dermis of this area ranged from 6.4 mm in a medium male calf

up to 10.9 mm in a medium adult female. In all areas of the dorsal fluke sampled there was no hypodermis. In the fluke edges there were some fat cells present in the deep dermis but not enough to label it a hypodermis. In the central dorsal site of the fluke (site 6), no hypodermis was present as was the case with the fluke edges. However, unlike at the edges, this area of the fluke underwent a transition directly from dermis to muscle (Figure 3-8).

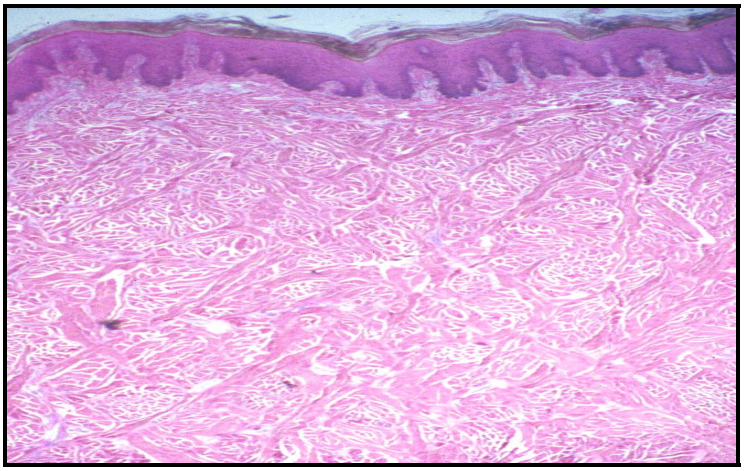

Figure 3-8. MEC0348, adult male, site 6, H&E, 20X, epidermis and dermis.

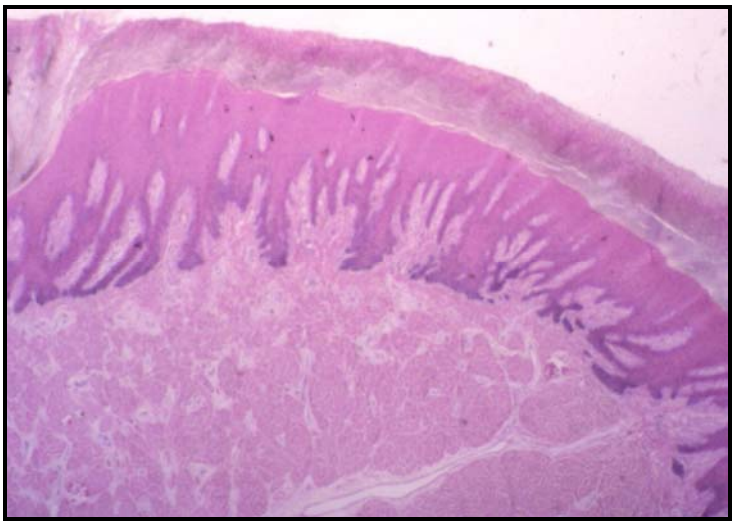

Figure 3-9. MNW0342, adult female, site 5, H&E, 20X, epidermis and dermis.

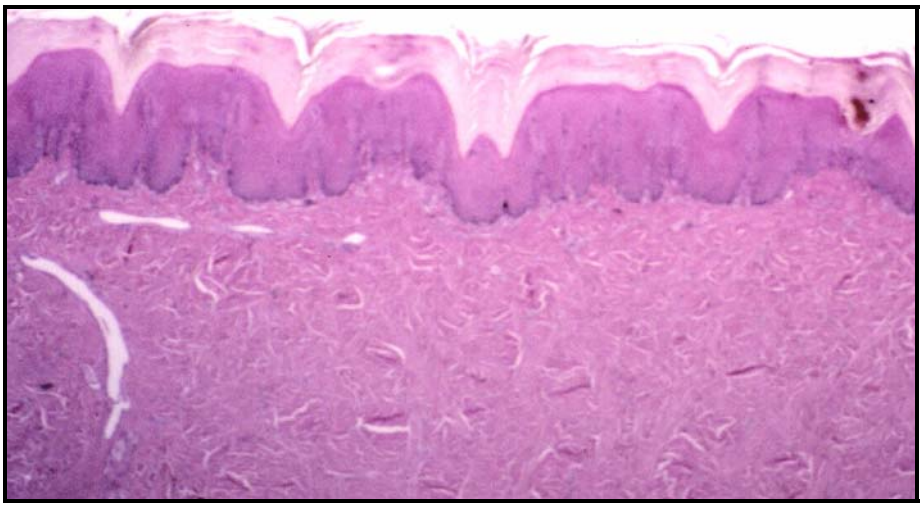

Figure 3-10. LPZ101820, male calf, site 7, H&E, 20X, epidermis and dermis.

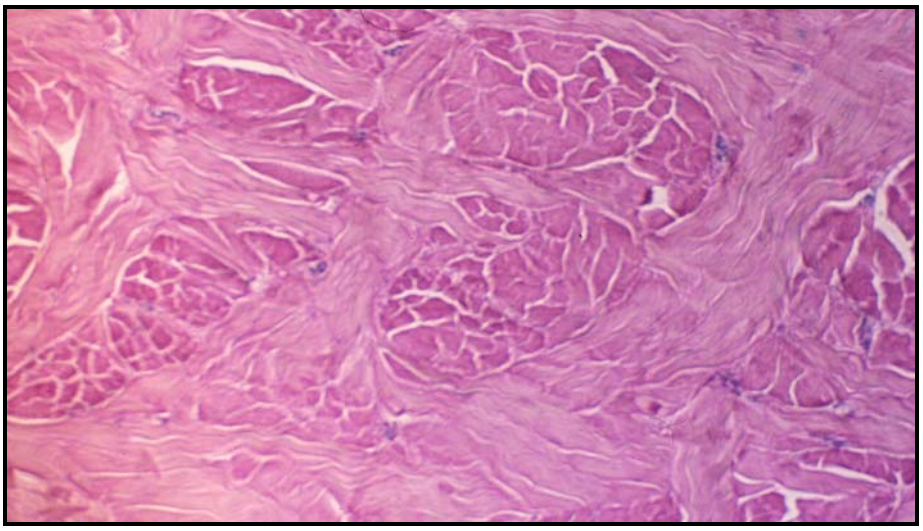

Figure 3-11. LPZ101820, male calf, site 6, H&E, 100X, dermis.

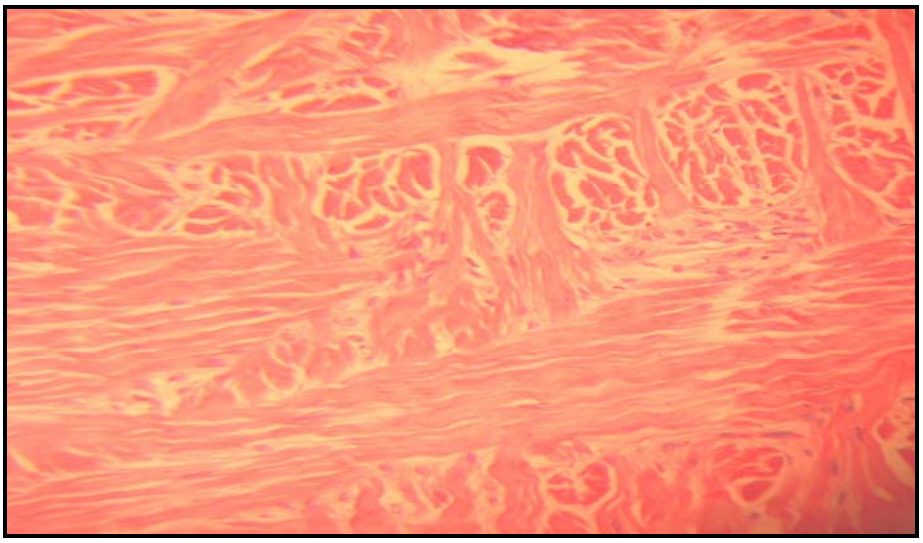

Figure 3-12. MNW0347, male calf, site 5, H&E, 100X, dermis.

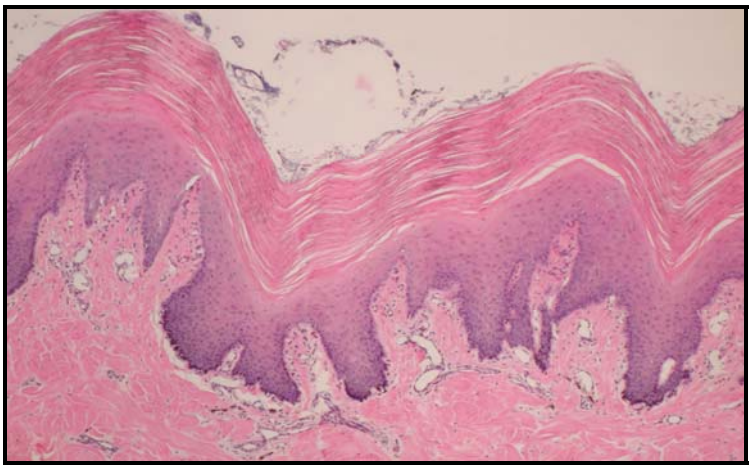

Figure 3-13. MSW03170, male calf, site 7, H&E, 40X, epidermis and dermis.

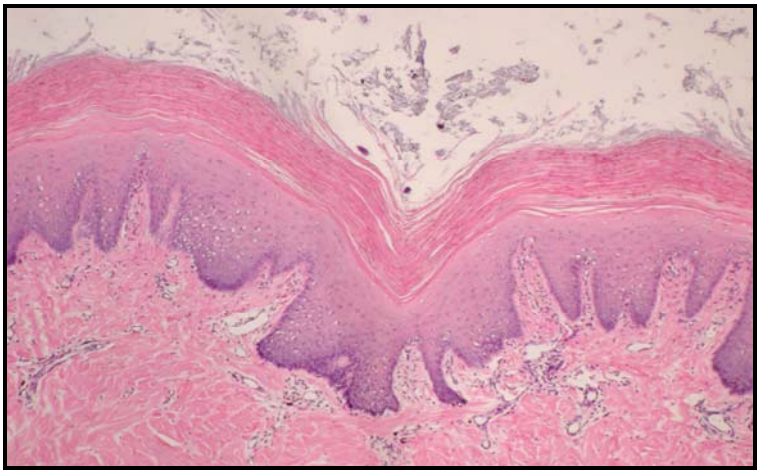

Figure 3-14. MSW03170, male calf, site 4, H&E, 40X, epidermis.

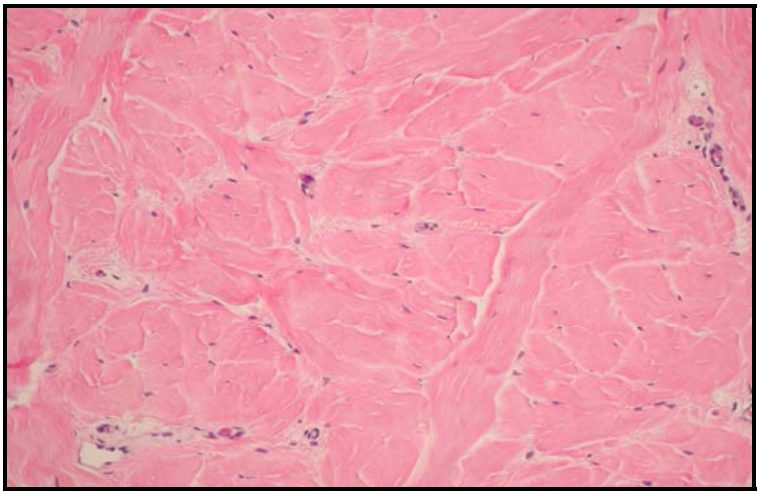

Figure 3-15. MSW03170, male calf, site 7, H&E, 100X, dermis.

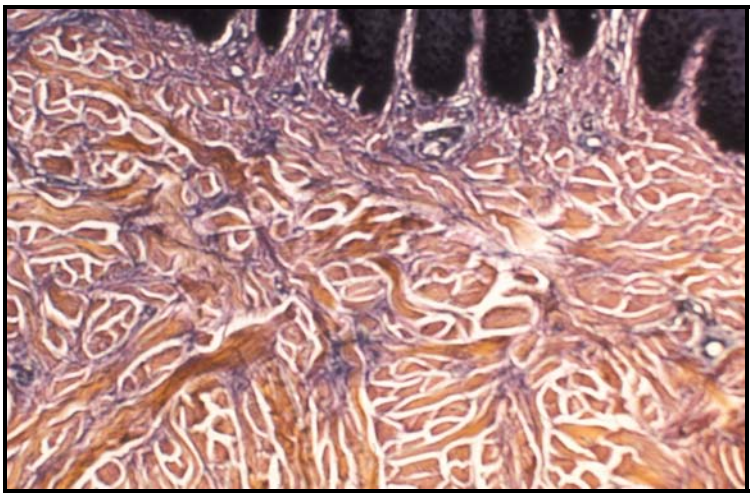

Figure 3-16. MEC0348, adult male, site 6, Verhoff-Van Geison, 100X, elastin fibers.

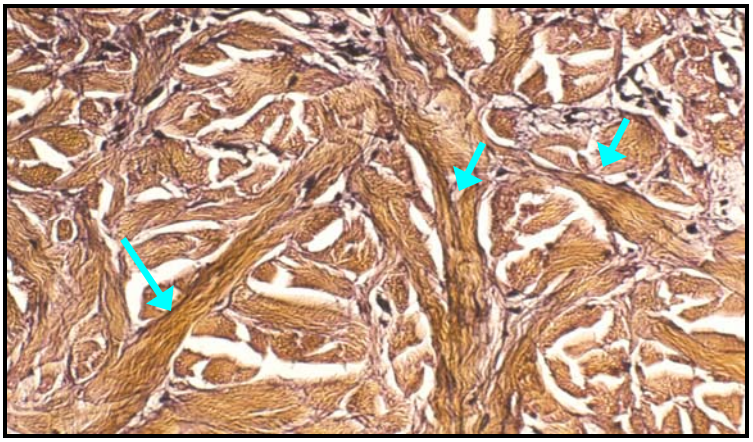

Figure 3-17. MEC0348, adult male, site 7, Verhoff-Van Geison, 200X. The arrows identify just a few of the several thin elastin fibers present in the dermis of the dorsal fluke edge skin.

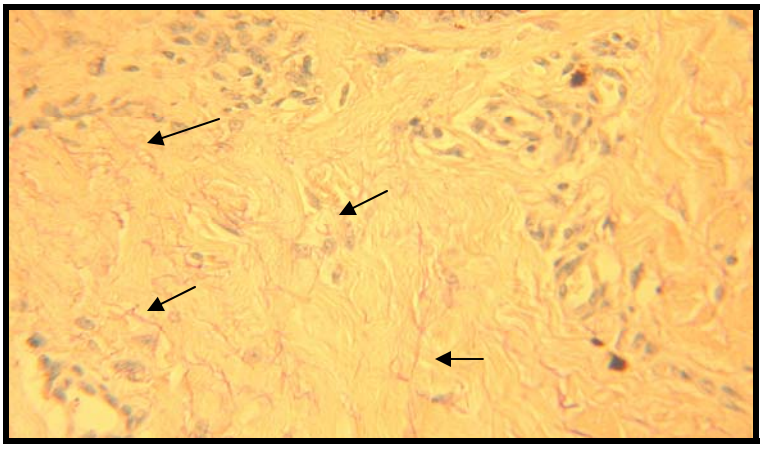

Figure 3-18. MSW03170, male calf, site 4, Luna's stain for mast cells, 250X, very thin elastin fibers (arrows) present in the dermis.

Dorsal Flipper Skin (Sites 20, 21, 22, and 23)

The flipper of the manatee is used for feeding, playing, and mating. The dorsal surface of the flipper has 3–4 nails at the caudal-most tip. The skin of the flipper, like all areas of the manatee skin, is rigid, has a very thick stratum spinosum, thick stratum corneum, and the epidermis is composed of three layers; the stratum basale, stratum spinosum, and stratum corneum (Figure 3-29). The dorsal flipper skin epidermis, like the dorsal fluke skin epidermis, has very similar thicknesses around the perimeter. The epidermis in the dorsal center of the flipper is the thinnest of all the dorsal flipper samples. The thinnest epidermis was measured at 0.2 mm in a medium male calf, and thickest at 2.8 mm in a small adult male. The stratum corneum exhibits a wide range of cell layers in the dorsal flipper. There are as few as nine cell layers in a medium male calf and as many as 109 in a medium adult female. In some areas the stratum corneum was too compact to clearly see the individual cell layers (Figure 3-28). The undulating ridges present in the dorsal flipper range from 2–6 per linear 275µm. The undulating ridges near the nail are not pronounced and the epidermis is practically flat, as you move further away from the nail toward the center of the flipper the undulating ridges begin to become pronounced. The epidermal pegs are abundant in the calf, 18 per linear 275µm, and fewer in the adult, 10 per linear 275µm. The epidermal pegs in the dorsal flipper vary in depth and thickness as do to undulating ridges. There are melanocytes present in the stratum basale and in the papillary dermis. The nail thickness was between 1.4mm, in a small adult male, to 4.6 mm, in a medium adult male. The dermis of the dorsal flipper is comparatively thin measuring 2.1–3.4 mm in thickness at the edges in a small male calf, with the thinnest dermis at the nail. The adult male dermis ranges from 2.6–8.3 mm at the edges with the thinnest dermis at the nail. The dorsal center skin of the flipper is thinner

than at the edges ranging from 1.9 mm (calf) to 7.5 mm (adult). The flipper, like the fluke, is very vascular with the largest arteries and vessels in the deep reticular dermis. There is no hypodermis in the flipper, having only sparse fat cells in the deep reticular dermis. The dermis of the flipper is attached to muscle in all parts except near the nail, where it is attached by connective tissue to the phalange. The elastin fibers of the flipper skin exist as very thin fibers ascending throughout the dermis and ending at the dermal papillae and epidermal pegs.

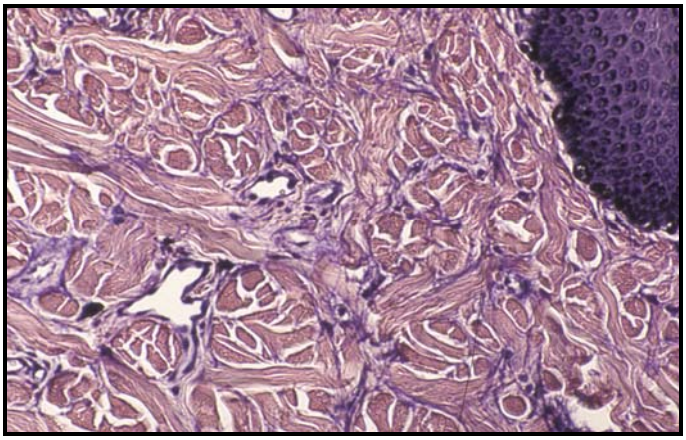

Figure 3-19. MEC0348, adult male, site 21, Verhoff-Van Geison, 200X.

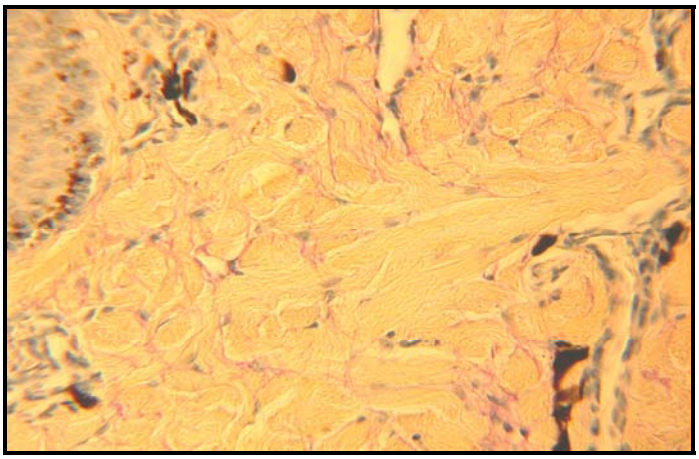

Figure 3-20. MSW03170, male calf, site 21, Luna's stain for mast cells, 250X, elastin fibers(violet) present in the papillary dermis.

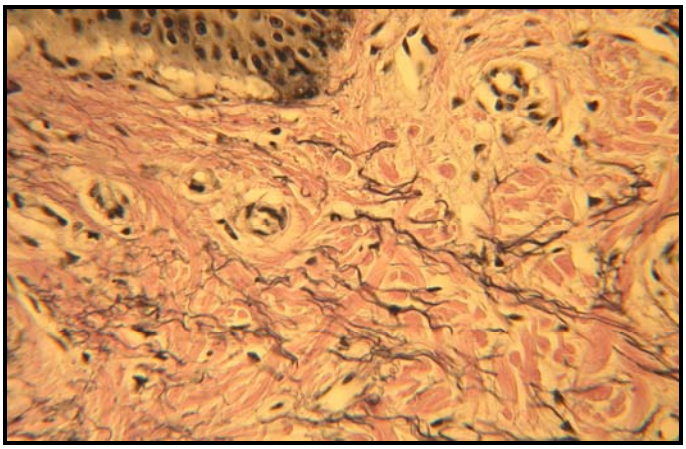

Figure 3-21. MNW0342, adult female, site 22, Verhoff-Van Geison, 250X, black elastin fibers present in the dermis.

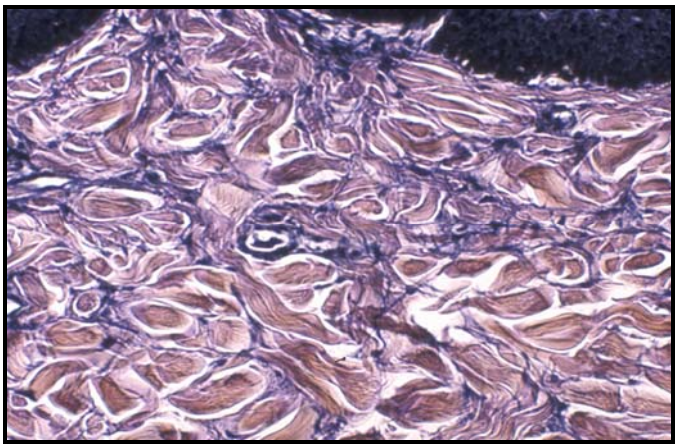

Figure 3-22. MEC0348, adult male, site 22, Verhoff-Van Geison, 200X, elastin fibers.

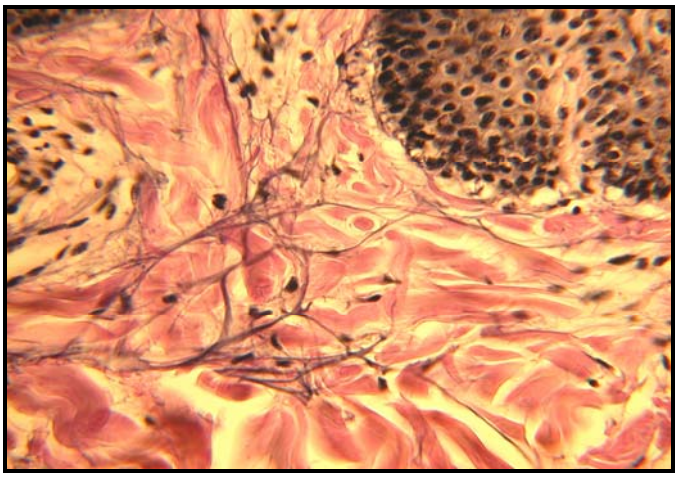

Figure 3-23. MNW0342, adult female, site 20, Verhoff-Van Geison, 250X, network of elastin fibers just below the epidermis.

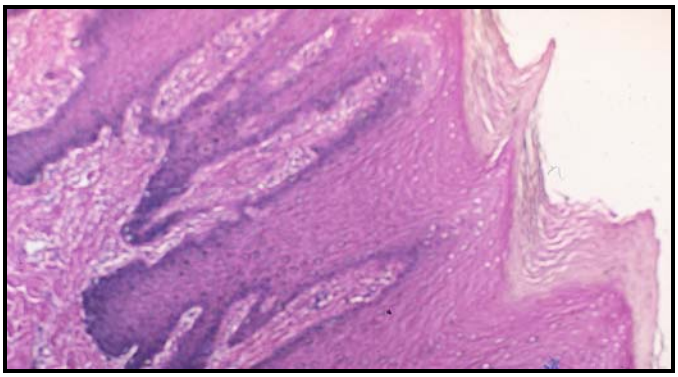

Figure 3-24. MEC0348, adult male, site 21, H&E, 100X, epidermis.

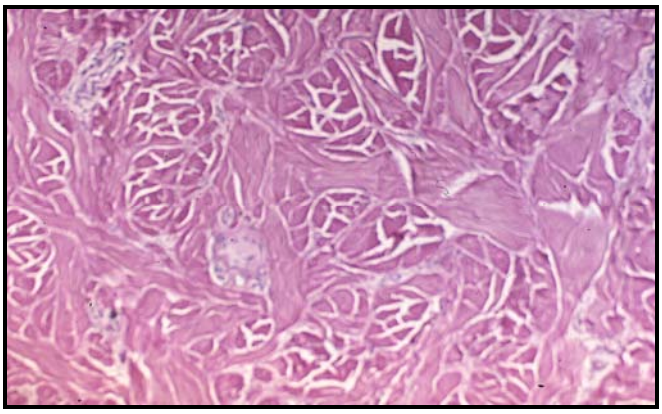

Figure 3-25. MEC0348, adult male, site 21, H&E, 100X, dermis.

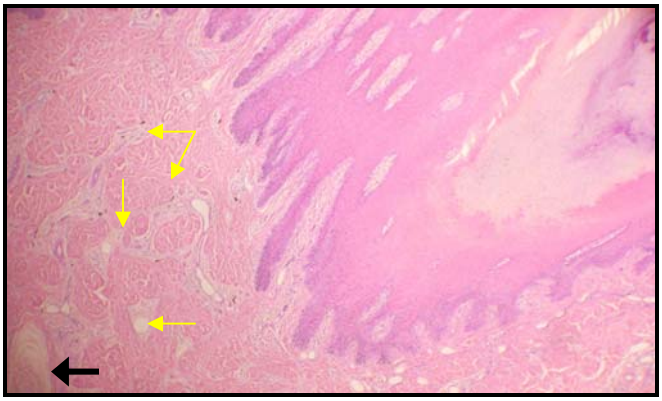

Figure 3-26. MNW0342, adult female, site 21, H&E, 40X, nail bed. Notice the exceptionally thick epidermis, large vessels (yellow arrows), and Pacian corpuscle present (black arrow).

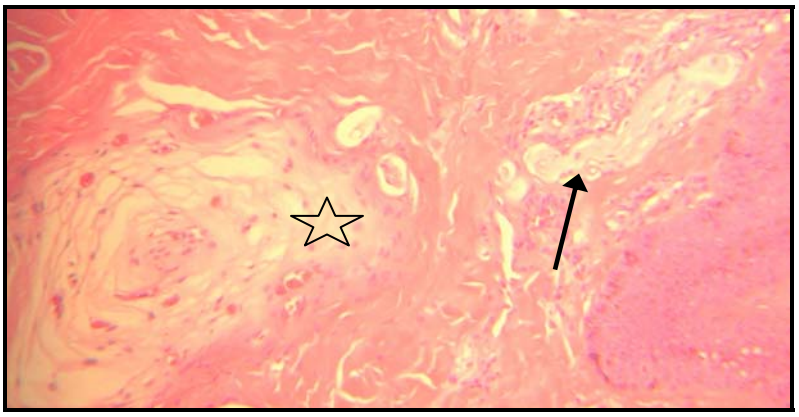

Figure 3-27. MSW03169, sub-adult male, site 21, H&E, 100X, a large pacinian corpuscle present in the left corner of the picture (star), as well as a Meissner corpuscle(arrow) present ascending into a dermal papillae.

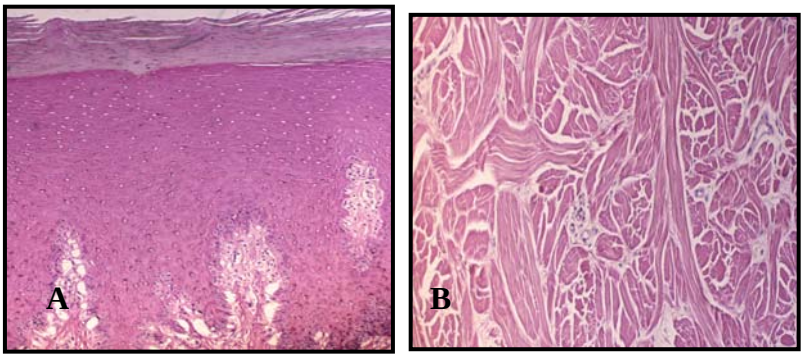

Figure 3-28. MNW0342, adult female, site 22, H&E, 100X. A) Epidermis B) Dermis.

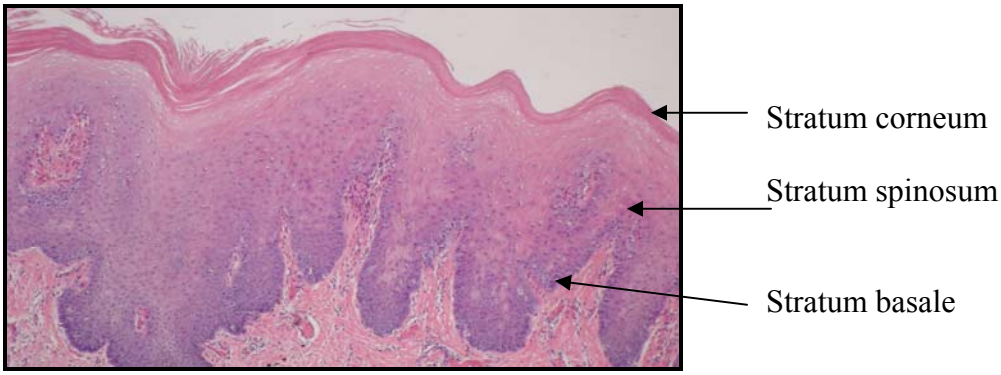

Figure 3-29. MSW03170, male calf, site 20, H&E, 40X, epidermis.

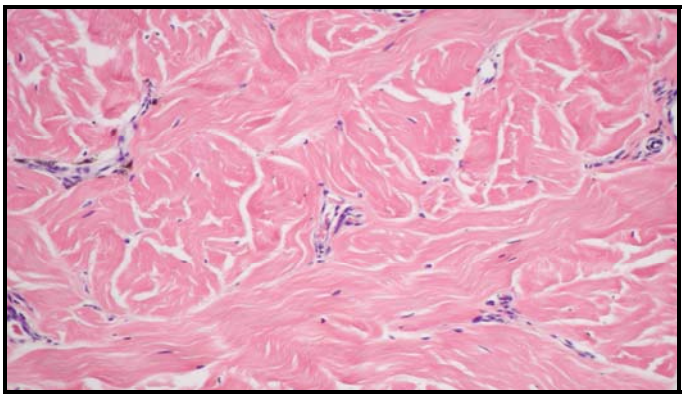

Figure 3-30. MSW03170, male calf, site 20, H&E, 100X, dermis.

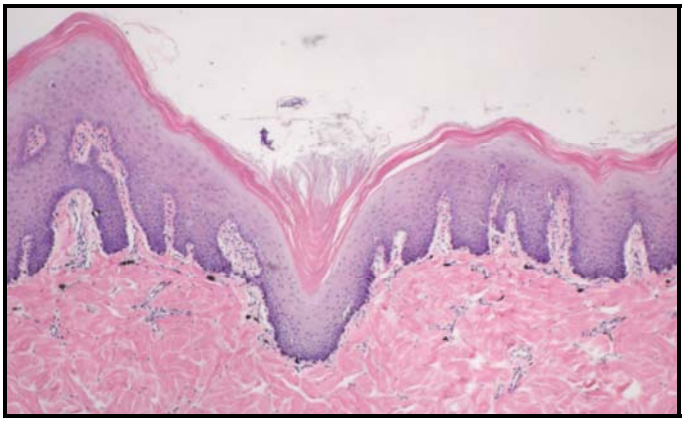

Figure 3-31. MSW03170, male calf, site 22, H&E, 40X, epidermis and papillary dermis. Notice the lack of the stratum granulosum and also the presence of melanocytes (arrows) that have dropped below the stratum basale into the papillary dermis.

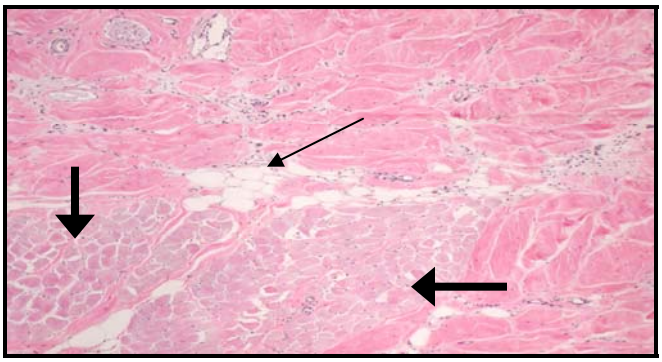

Figure 3-32. MSW03170, male calf, site 22, H&E, 40X, dermis connecting to muscle. The larger arrows are pointing to the skeletal muscle, and the smaller arrow is pointing to adipose cells. Notice how the dermis transitions to the muscle in the flipper with no hypodermis.

Ventral Skin (Sample Sites 8, 9, and 10)

The epidermis in this region varied in thickness from 0.2 mm in a male calf to 3.8 mm in an adult male (Appendix B). The dermis in this area ranges from 6.1 mm in a male calf to 19.6 mm in a male adult. Based on the microscopic measurements, there are no notable differences in skin thickness between males and females. As the manatee ages, there is an overall increase in ventral skin thickness. There is a slight increase in epidermal thickness and a more pronounced increase in the dermis. The epidermis consists of three layers; the stratum basale, stratum spinosum, and the stratum corneum, lacking the stratum granulosum and the stratum lucidum.

The epidermis is marked by many long, invaginating dermal papillae and undulating ridges, which give the skin its pitted appearance upon gross examination. In the calf there are as many as 6 undulating ridges and 16 epidermal pegs per linear 275μm. In the adult there are between 3–5 undulating ridges and anywhere from 9–16 epidermal pegs per linear 275μm. The epidermal pegs in the dorsal flipper vary in depth and thickness as do to undulating ridges. Going from cranial to caudal, the dermis thickness changes, being thickest in the middle and thinnest as you get closer to the fluke. The network of collagen in these samples changes from calf to adult. As the manatee ages, the collagen structure of the dermis becomes more organized. The network of collagen consists of thick dense collagen bundles that form a diagonal weave, with collagen fibers criss-crossing, forming a distinct pattern. The epidermis of the manatee has an unusually thick stratum corneum, in most animals this would be considered hyperkeratotic. In the manatee, the hyperkeratotic condition of the epidermis is normal. The stratum spinosum of the manatee skin is several cell layers thick. There are melanocytes present in the

stratum basale and in the papillary dermis. The melanin dispersed from these melanocytes within the stratum basale can be seen forming caps on the keratinocytes in the stratum spinosum and sometimes carry all the way into the stratum corneum (Figure 3-38). The dermis is infiltrated with blood vessels, the largest blood vessels being located in the deepest part of the reticular dermis, and small arterioles and venules are mainly found at the epidermal-dermal junction. Nerves are present in this region of the skin, mostly observed near blood vessels, at the connection of the dermis and epidermis, and in the dermal papillae. The elastin fibers in this region of the manatee skin vary from the epidermis to reticular dermis. Elastin fibers are most numerous and thickest in the deep reticular dermis and become fewer and thinner as they continue to the epidermis and extend into the dermal papillae (Figure 3-39 and 3-40).

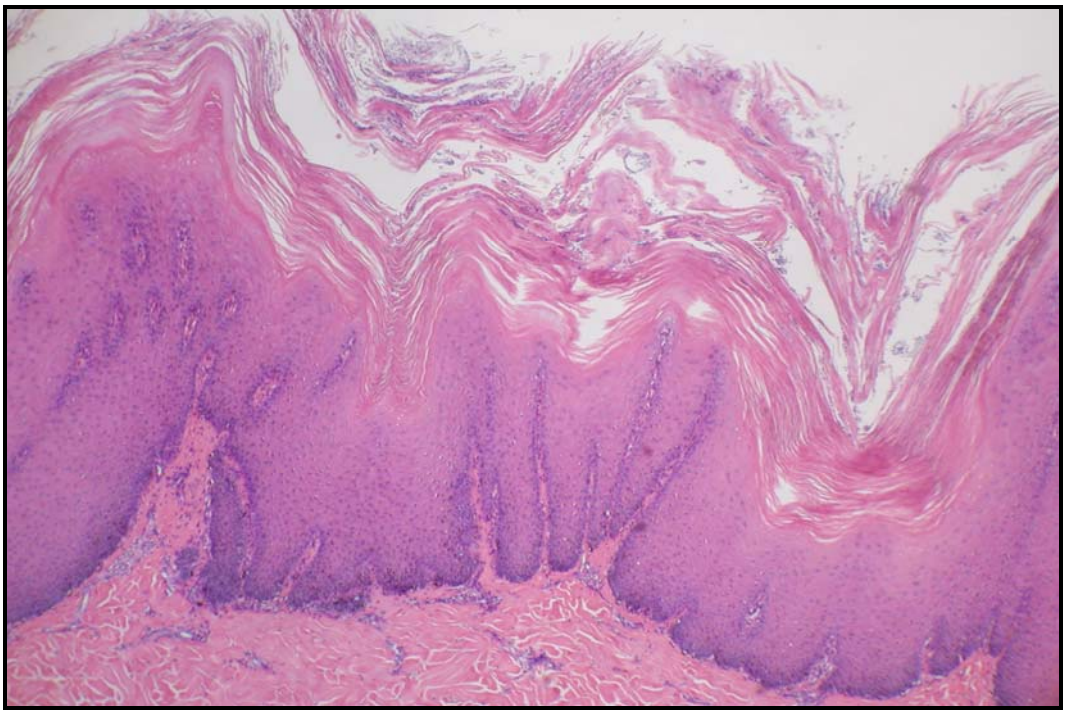

Figure 3-33. TM0406, adult male, site 9, H&E, 40X, epidermis and papillary dermis.

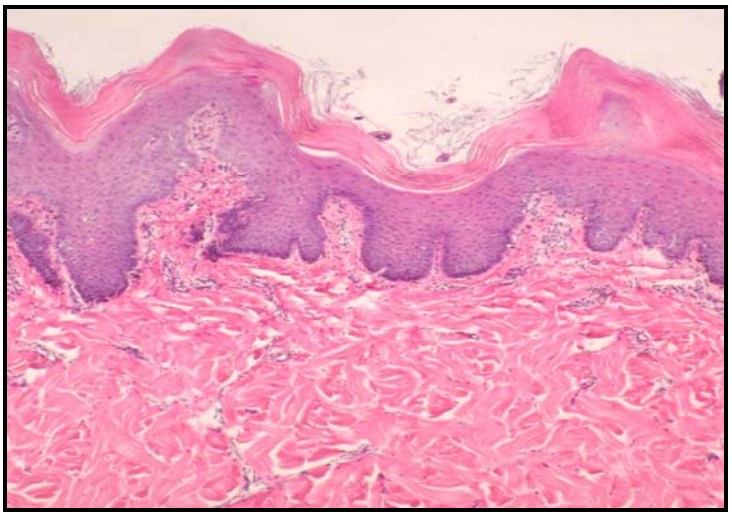

Figure 3-34. MSW03170, male calf, site 9, H&E, 40X, epidermis and dermis.

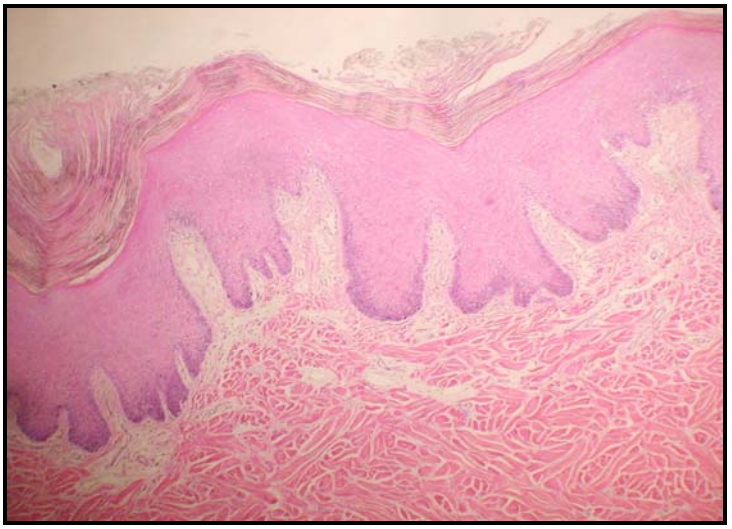

Figure 3-35. MNW0342, adult female, site 9, H&E, 40X, epidermis and dermis.

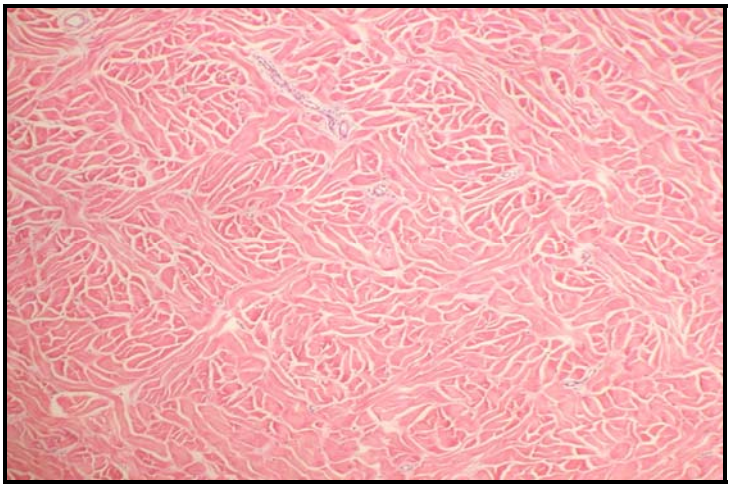

Figure 3-36. MNW0342, adult female, site 9, H&E, 40X, dermis.

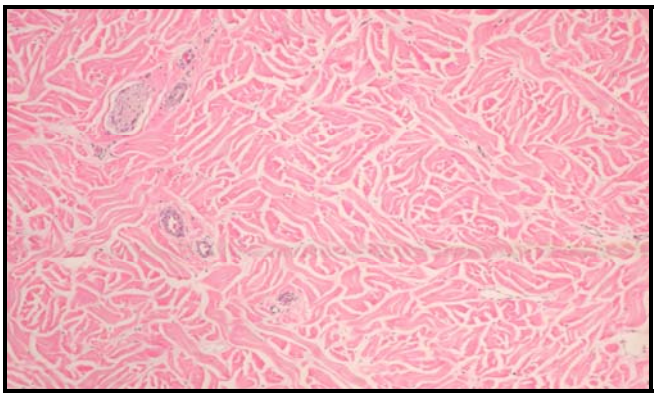

Figure 3-37. MSW03170, male calf, site 8, H&E, 40X, dermis.

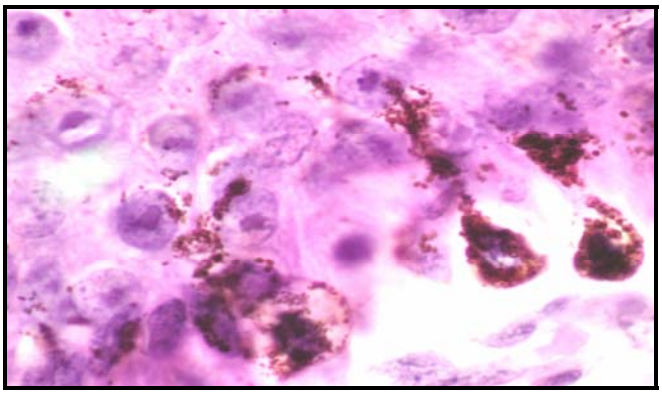

Figure 3-38. MSW03170, male calf, site 9, H&E, 1000X. Melanocytes in the stratum basale, distributing melanin to adjacent keratinocytes.

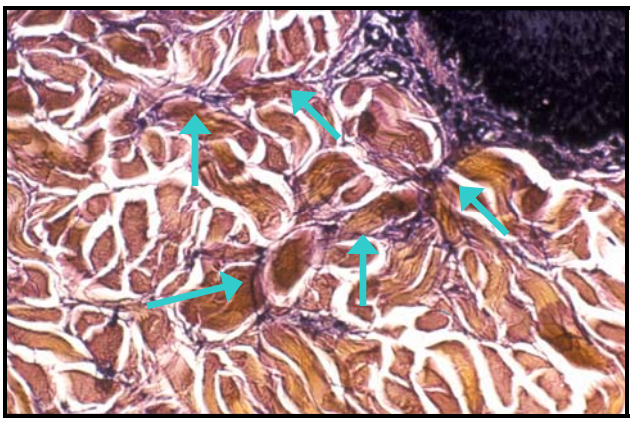

Figure 3-39. MEC0348, adult male, site 8, Verhoff-Van Geison, 200X, papillary dermis. Elastin fibers (arrows) show up black with this stain. They are very fine and numerous as they project towards the epidermis.

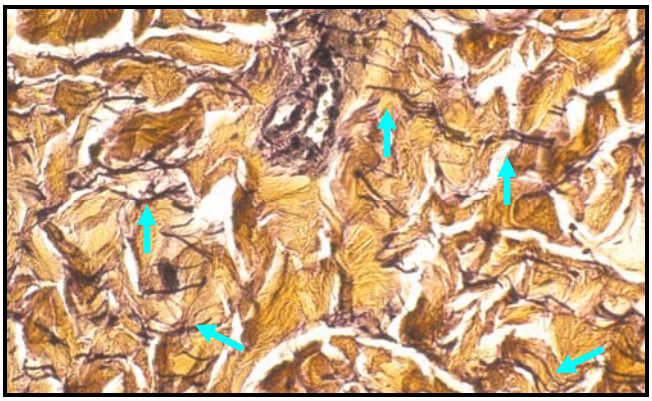

Figure 3-40. MEC0348, adult male, site 9, Verhoff-Van Gieson, 200X, reticular dermis. Notice how the elastin fibers are thicker than in the papillary dermis, and are more numerous.

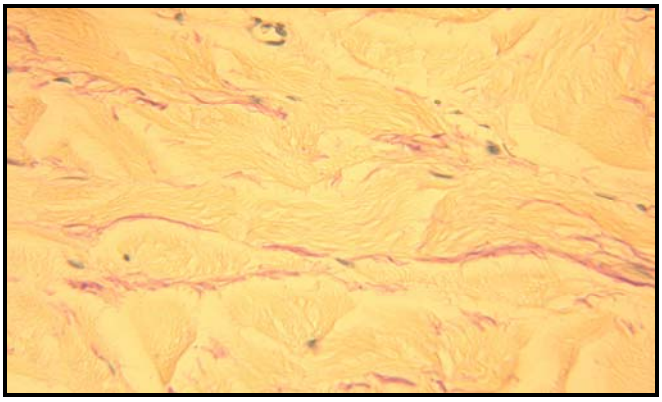

Figure 3-41. MSW03170, male calf, site 9, Luna's stain for mast cells, 250X, elastin fibers (violet) present in the deep dermis.

Ventral Fluke Skin (Sites 11, 12, 13, and 14)

The majority of the fluke is composed of dermis and epidermis, but towards the center of the fluke there is muscle and bone. The dorsal and ventral edges of the fluke are separated by the caudal vascular bundle. Epidermal and dermal thicknesses vary from manatee to manatee. These measurements also vary in each manatee throughout the fluke as seen dorsally. The left and right ventral fluke edges do not have the exact same measurement, varying slightly from one another, having at the most a 1.2 mm difference in epidermis thickness, and a 4 mm difference in the thickness of the dermis (Figure 3-43). The most central ventral point of the fluke (site 14) has the thinnest epidermis, and

the thickest dermis of the ventral fluke sample sites (Figure 3-47). The fluke skin is very rigid, with an extremely thick stratum spinosum and stratum corneum. Overall the fluke is very vascular throughout the entire dermis regardless of the sample site. There is a high density of elastin fibers in the fluke. They are observed throughout the dermis, but are most prevalent in the papillary dermis, extending into the dermal papillae (Figure 3-49). The epidermis of the fluke edge is the thickest in the medium adult male, measuring 5.1 mm. The epidermis is 0.3 mm in a medium male calf, being the thinnest of all the samples.

There are several pointed undulating ridges present in the fluke; the number of undulating ridges range from 3 peaks per linear 275µm in a medium adult female manatee to 6 peaks per linear 275µm in a small male calf and are different in height and thickness. There are numerous epidermal pegs present that vary in depth and thickness, from 7 pegs per linear 275µm in a medium adult male to 17 pegs per linear 275µm in a medium male calf. The stratum corneum in the fluke is very thick and hard. This outermost layer of the epidermis is found to have as few as 16 cell layers in a small adult male to 156 cell layers thick in a medium adult female. There are melanocytes present in the stratum basale and in the papillary dermis. The dermis of the fluke is composed of a dense, intricate weave of collagen, being organized in a 90-degree criss-cross pattern, with the vertical collagen bundles having a perpendicular orientation to the surface of the skin (Figure 3-42). In site 14 the collagen is in a slightly more diagonal weave than the edges. At the very edge of the sample the dermis narrows to 2.0 mm in a medium male calf. Towards the center of the sample, the dermis measures at 15.2 mm in a small adult male, being the thickest of all the ventral fluke edge samples. The central ventral site of

the fluke (site 14) has the thinnest epidermis and thickest dermis of all the ventral fluke samples. The epidermis is not as thick as the fluke edges. The thinnest measurement of the epidermis is 0.4 mm in a medium male calf, and the thickest is 1.9 mm in a medium adult male. The dermis of this area ranges from 4.1 mm in a medium male calf and up to 11.8 mm in a medium adult female. In all areas of the ventral fluke samples there is no hypodermis. In the fluke edges there are some fat cells present in the deep dermis but nothing substantial enough to be referred to as hypodermis. In the central ventral site of the fluke (site 14) no hypodermis was present, as was the case with the fluke edges. Instead of being separated into dorsal and ventral dermis of the fluke by the vascular bundle, like in the fluke edges, this area of the fluke changes from dermis to skeletal muscle.

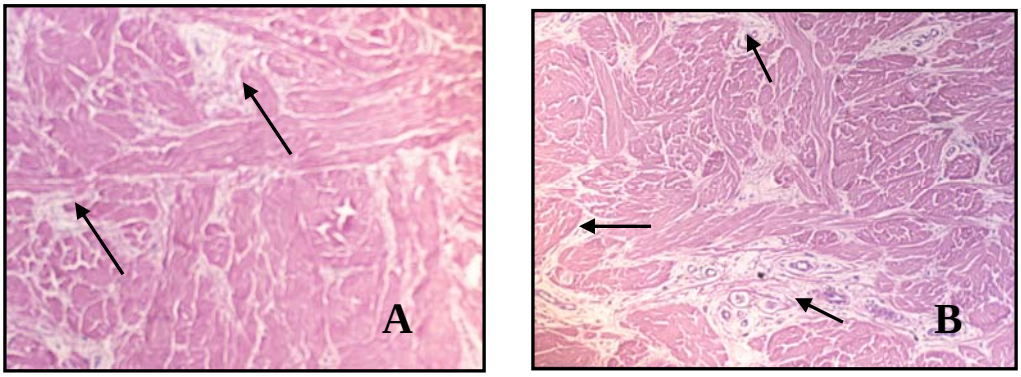

Figure 3- 42. Dermis of the ventral fluke. A) MEC0348, adult male, site 12, H&E, 100X, dermis. B) MNW0342, adult female, site 13, H&E, 100X, dermis. Blood vessel are identified by the arrows.

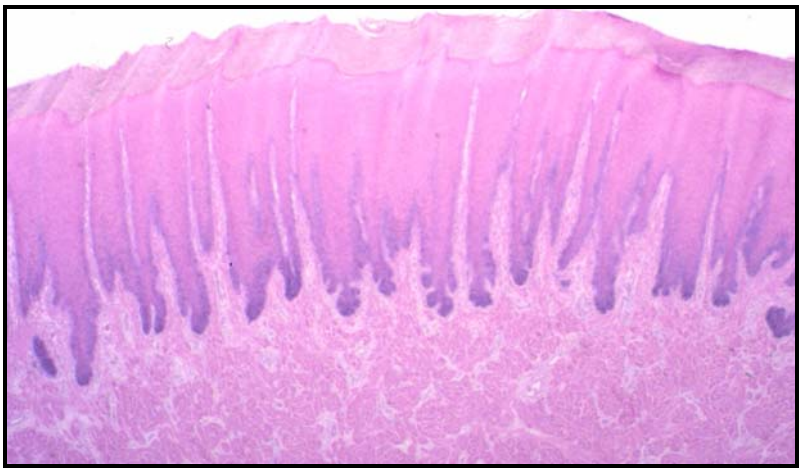

Figure 3-43. MNW0342, adult female, site 13, H&E, 20X, epidermis and dermis.

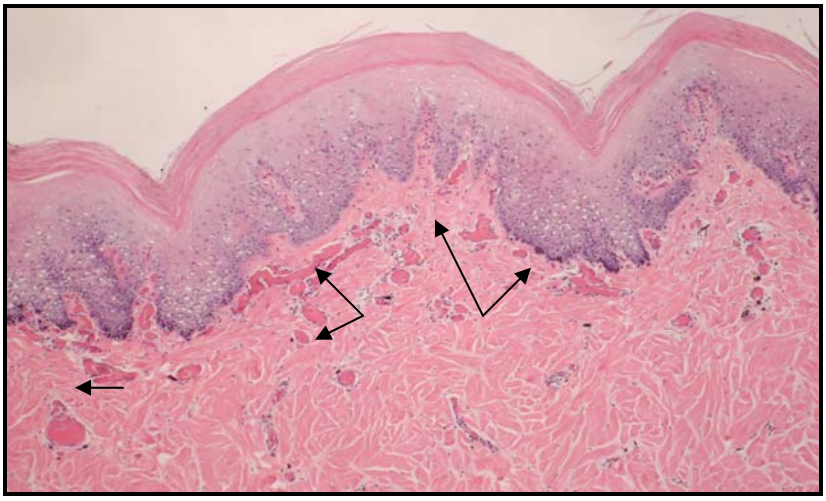

Figure 3-44. MSW03170, male calf, site 13, H&E, 40X, epidermis and dermis. Blood vessels are highlighted by arrows.

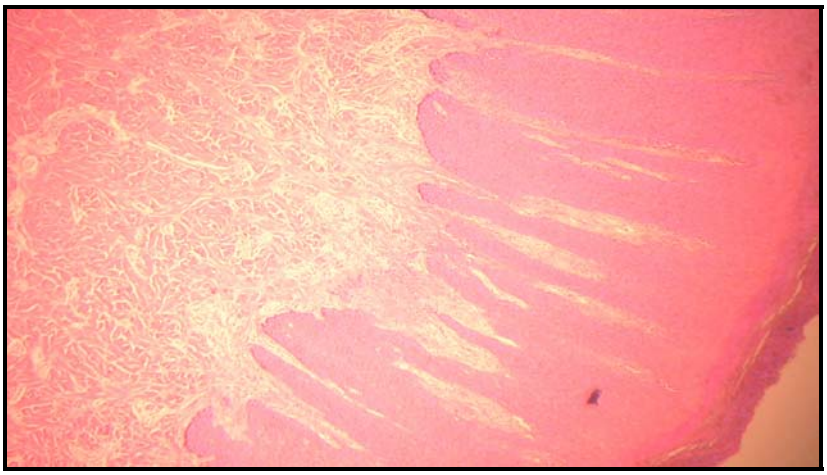

Figure 3-45. MSW03169, sub-adult male, site 11, H&E, 20X, epidermis and dermis.

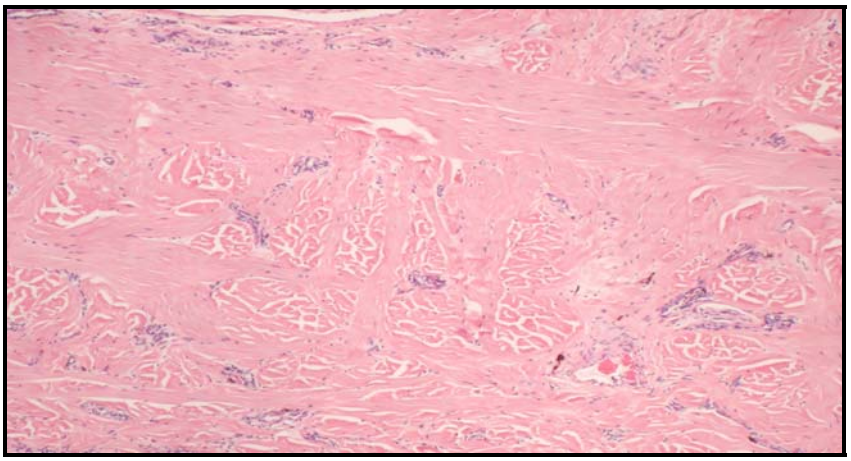

Figure 3-46. MSW03170, male calf, site 13, H&E, 40X, dermis.

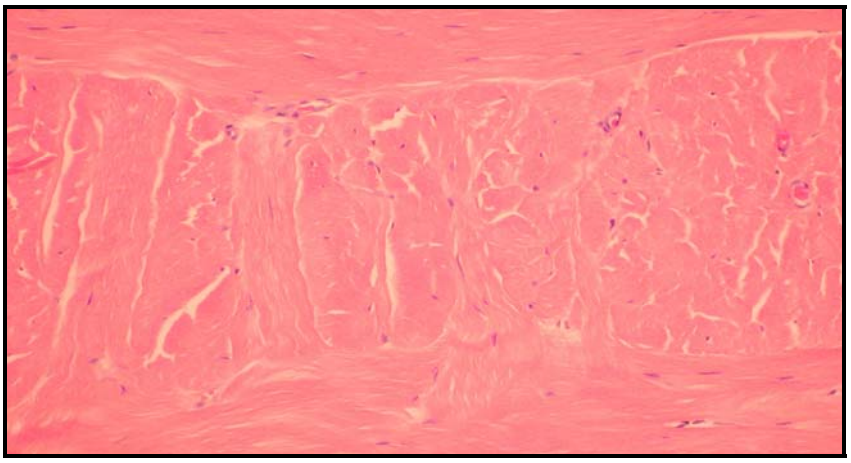

Figure 3-47. MSW03170, male calf, site 14, H&E, 100X, dermis.

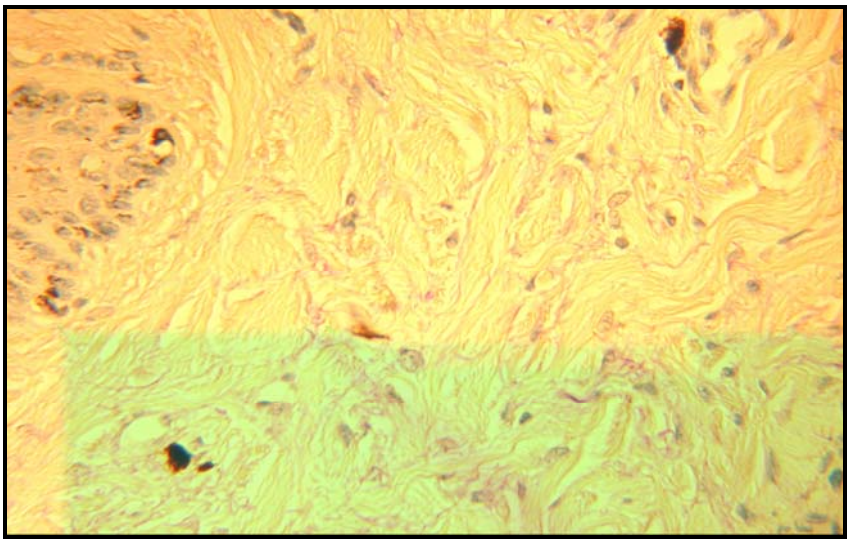

Figure 3-48. MSW03170, male calf, site 12, Luna's stain for mast cells, 250X, elastin fibers (violet) are fine and numerous in this area.

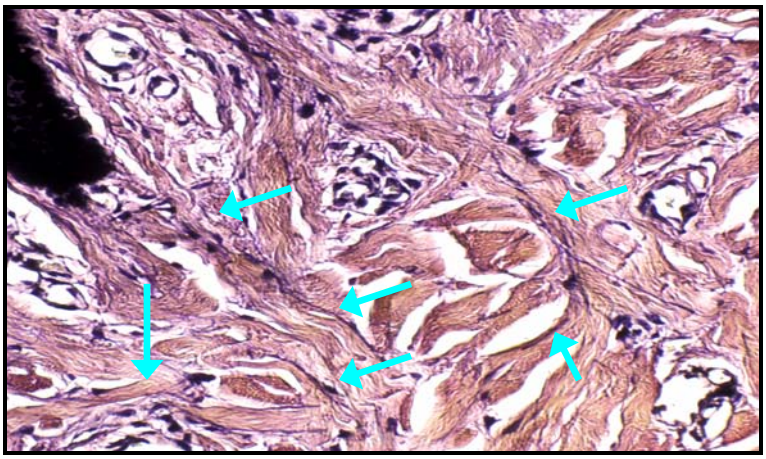

Figure 3-49. MEC0348, adult male, site 11, Verhoff-Van Geison, 200X, very fine elastin fibers, indicated by arrows, in the upper dermis.

Ventral Flipper Skin (Sites 16, 17, 18, and 19)

The ventral surface of the flipper is exceptionally rough compared to the dorsal surface of the flipper. The skin of the flipper, like all areas of the manatee skin, is rigid, exhibits a thick stratum spinosum and stratum corneum, and the epidermis is composed of three layers; the stratum basale, stratum spinosum, and stratum corneum. The ventral flipper skin epidermis, like the ventral fluke skin epidermis, exhibits fairly uniform thickness around the perimeter.

The epidermis in the ventral center of the flipper is the thinnest out of all the ventral flipper samples. The thinnest epidermis was measured at 0.2 mm in a medium male calf, and thickest at 6.7 mm in a medium adult male. The stratum corneum has a wide range of cell layers in the ventral flipper. There are as few as nine cell layers in a medium male calf and as numerous as 115 in a medium adult female. In some areas the stratum corneum was too compact to clearly see the individual cell layers (Figure 3-53). The undulating ridges present in the ventral flipper range from 3–7 per linear 275µm. The epidermal pegs are abundant in the calf, from 14–18 per linear 275µm, and fewer in the

adult, 8–15 per linear 275µm. The epidermal pegs in the ventral flipper vary in depth and thickness as do the undulating ridges. There are melanocytes present in the stratum basale and in the papillary dermis. The dermis of the ventral flipper is thin compared to the rest of the body. In a small male calf the dermal thickness was 1.8– 4.1 mm at the edges, with the thinnest dermis on the ventral tip of the flipper. The adult male dermis ranges from 2.9–9.6 mm at the edges with the thinnest dermis at the ventral tip of the flipper. The epidermis of the ventral center skin of the flipper is thicker than at the edges ranging from 1.8 mm (calf) to 6.7 mm (adult). The flipper, like the fluke, is very vascular with the largest arteries and vessels in the deep reticular dermis. There is no hypodermis in the ventral flipper. There are some sparse fat cells in the deep reticular dermis. In the papillary dermis there are a few pacinian corpuscle present (Figure 3-56). The dermis of the flipper is attached to skeletal muscle in all parts (Figure 3-62), except the ventral tip, where it is attached by connective tissue to the phalange. The elastin fibers of the flipper skin exist as very thin fibers ascending throughout the dermis and ending at the dermal papillae and epidermal pegs (Figures 3-57 – 3-61). They are more numerous here than in the dorsal and ventral body samples and fluke samples. Melanocytes are seen in the ventral flipper skin. As in other areas of the manatee skin the melanocytes are found in the stratum basale and in the papillary dermis. Special mechanoreceptors, called pacinian corpuscles, can be seen in the ventral flipper (Figure 3-56).

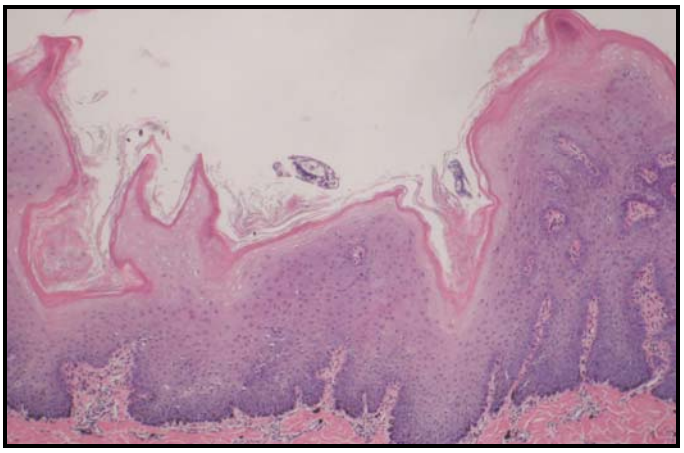

Figure 3-50. MSW03170, male calf, site 16, H&E, 40X, epidermis.

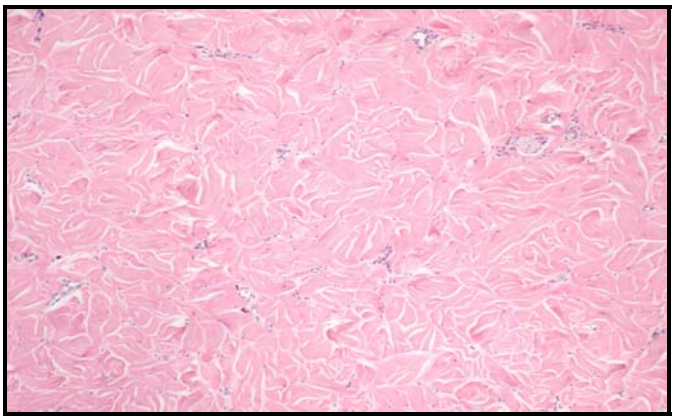

Figure 3-51. MSW03170, male calf, site 16, H&E, 40X, dermis.

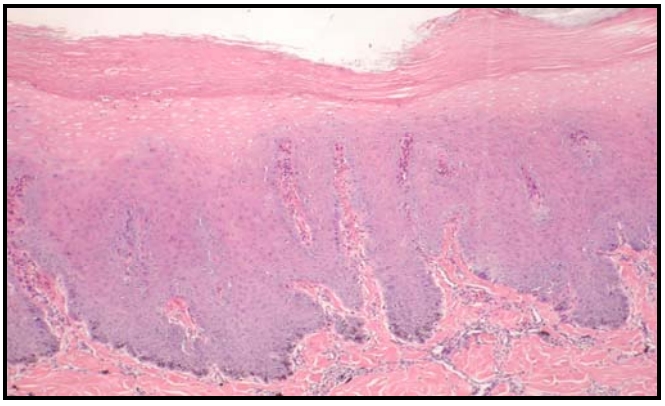

Figure 3-52. MSW03170, male calf, site 18, H&E, 40X, epidermis.

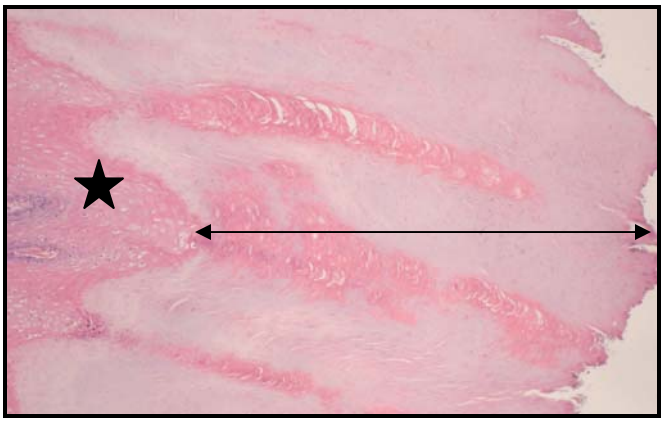

Figure 3-53. MSW03170, male calf, site 18, H&E, 40X, ventral flipper tip. Stratum corneum (arrow) with a little stratum spinosum (star) present.

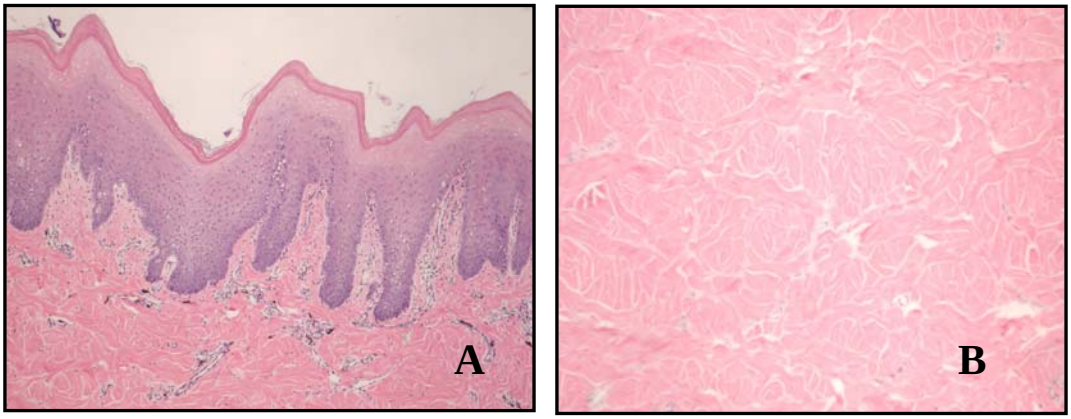

Figure 3-54. MSW03170, male calf, site 19, H&E, 40X, A)epidermis and B)dermis.

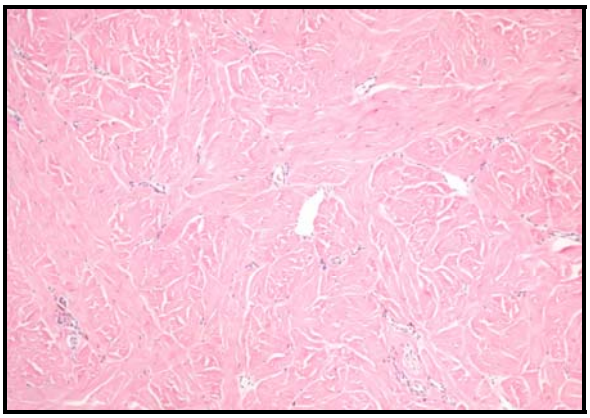

Figure 3-55. MSW03170, male calf, site 17, H&E, 40X, dermis.

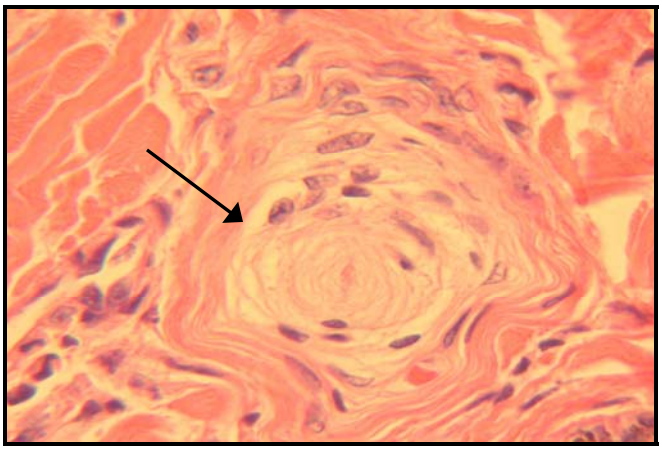

Figure 3-56. MNW0347, male calf, site 16, H&E, 400X, pacinian corpuscle (arrow).

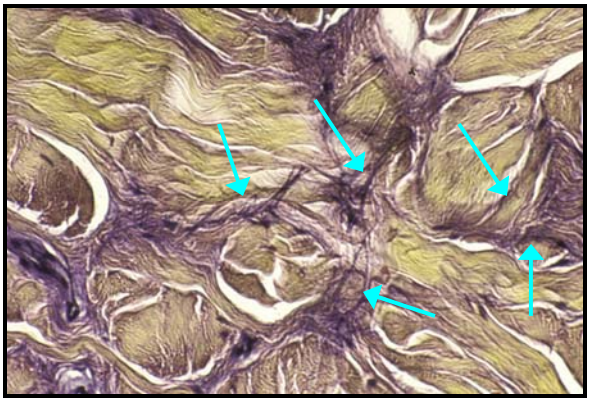

Figure 3-57. MEC0348, adult male, site 19, Verhoff-Van Geison, 200X, dermis. Elastin fibers are very fine and black (arrows).

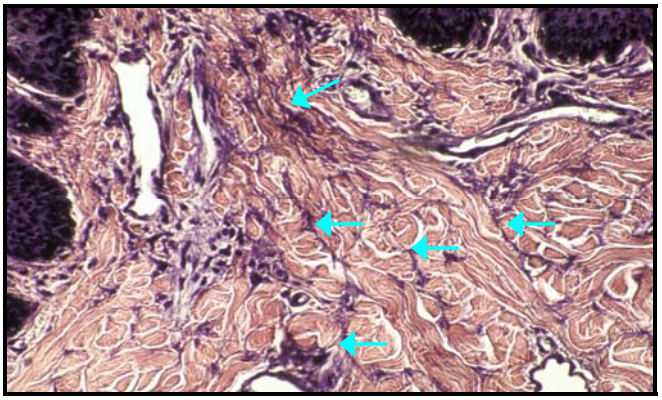

Figure 3-58. MEC0348, adult male, site 18, Verhoff-Van Geison, 200X, elastin fibers in the papillary dermis.

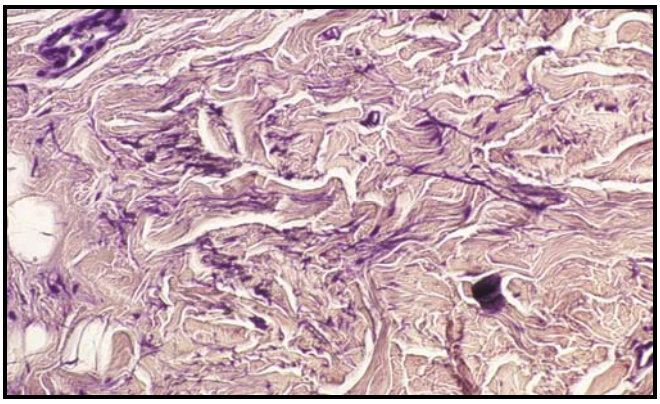

Figure 3-59. MEC0348, adult male, site 18, Verhoff-Van Geison, 200X. Elastin fibers (black) in the reticular dermis.

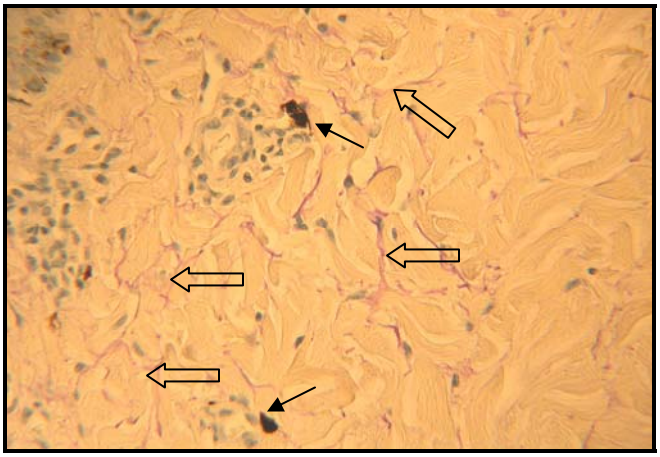

Figure 3-60. MSW03170, male calf, site 16, Luna's stain for mast cells, 250X, elastin fibers (stained violet, block arrows). Notice the melanocytes (arrows) present in the dermis.

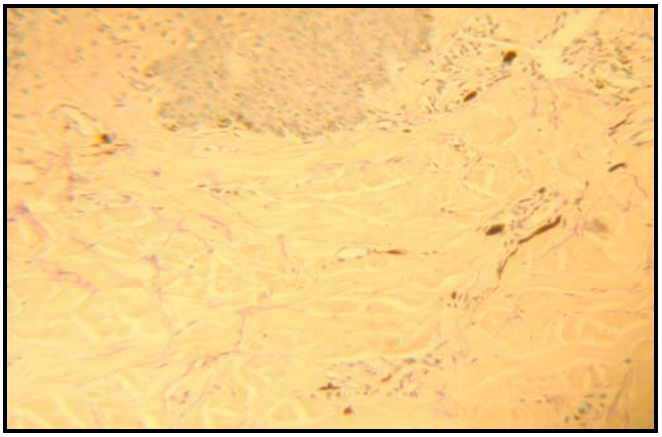

Figure 3-61. MSW03170, male calf, site 19, Luna's stain for mast cells, 100X, elastin fibers (violet) in the dermis.

Figure 3-62. LPZ101820, male calf, site 19, Masson's Trichrome, 100X, dermis transitioning directly to skeletal muscle.

Urogenital Skin (Sample Site 15)

The urogenital skin of the manatee does not differ in structure between the male and female. This area of the skin is very thick and consists of many folds. The epidermis has three layers, the stratum basale, stratum spinosum, and stratum corneum. The urogenital skin is hyperkeratotic and hyperplastic when compared to other mammalian skin, but is normal for the skin of the manatee (Figure 3-63, 3-64, and 3-65). The epidermis in a male calf was 0.4 mm thick. In an adult male the epidermis ranged from 0.4 mm up to 3.4 mm, compared to an adult female which was between 0.8 mm- 2.6 mm. The undulating ridges of the urogenital skin are irregular, and vary in number from manatee to manatee, in adults there were as few as 2 per linear 275µm, and as many as 5 per linear 275µm which was also the same for the calf. The epidermal pegs vary in depth and thickness. In a linear 275µm length there can be as many as 15, and as few as 8 in two adult males. The stratum corneum in some of the samples is extremely thick and too compact to count the individual cell layers, and very thin in some samples. The range of cell layers is between 22–86 with the thinnest being measured in a male calf, and the thickest in an adult male. The female urogenital skin can be too compact in areas to count

the individual layers. There are melanocytes present in the urogenital skin, but are not as numerous compared to the other sampled areas, 1–10 melanocytes per epidermal peg. They are seen in the stratum basale and also in the papillary dermis. The dermis is thick, being comparable in thickness to dermis measurements of sample site 9. Unlike the other ventral body dermis samples, the urogenital dermis does not have an organized collagen network, lacking a distinct pattern. In a male calf the dermis was as thin as 2.5 mm. In the adult male the dermis measures between 13–16.3 mm in thickness, and the female dermis is 15–16.3 mm thick. The urogenital skin is very vascular, with several small vessels and arteries present in the upper dermis, and larger vessels in the reticular dermis. At a depth between 0.6 mm–2 mm in the dermis, depending on the size of the manatee, there are several bundles of smooth muscle present (Figure 3-66, 3-69, and 3-72). These bundles of smooth muscle are surrounded by thick elastin fibers, which are seen throughout the dermis up to the junction of the epidermis (Figure 3-68, 3-70, and 3-71).

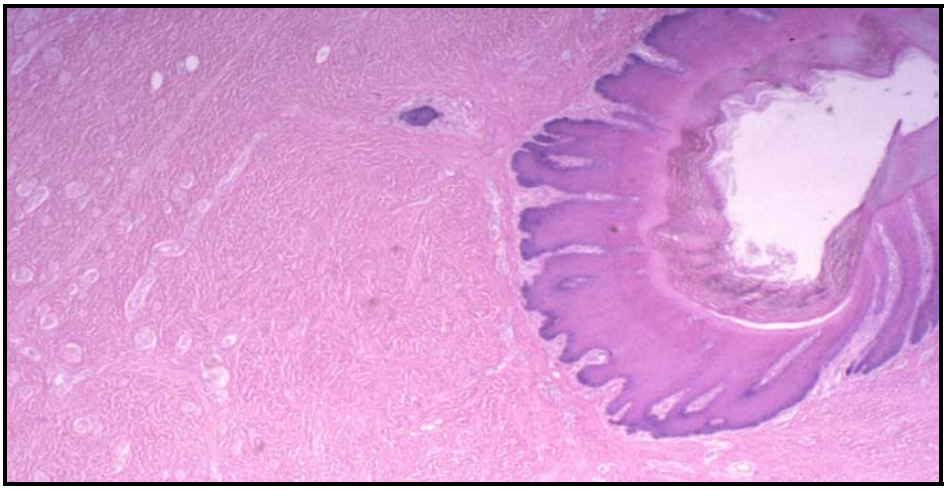

Figure 3-63. MNW0342, adult female, site 15, H&E, 20X, epidermis and dermis. Notice the fold in the skin and the smooth muscle bundles.

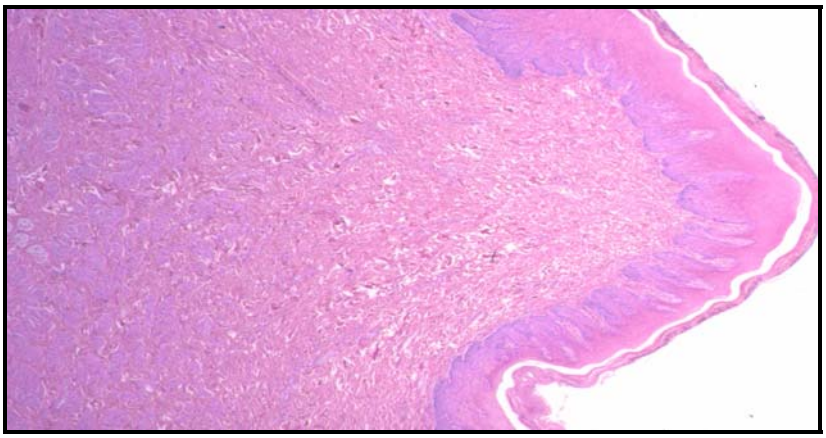

Figure 3-64. MEC0348, adult male, site 15, H&E, 20X, epidermis and dermis.The space between the stratum corneum and stratum spinosum is an artifact of processing.

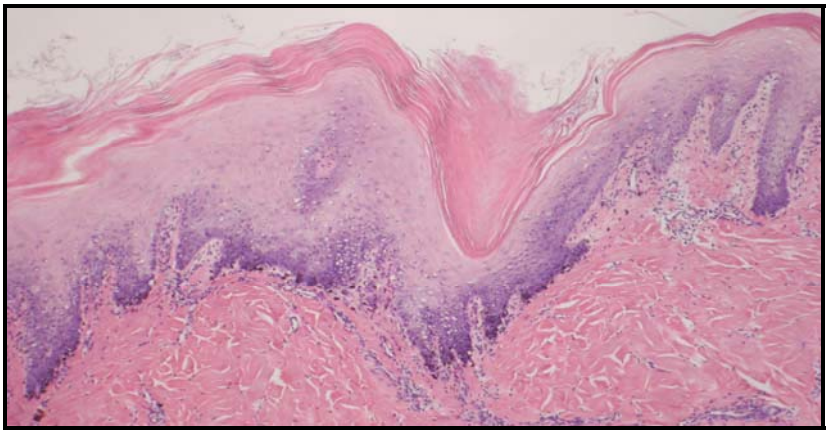

Figure 3-65. MSW03170, male calf, site 15, H&E, 40X, epidermis and papillary dermis.

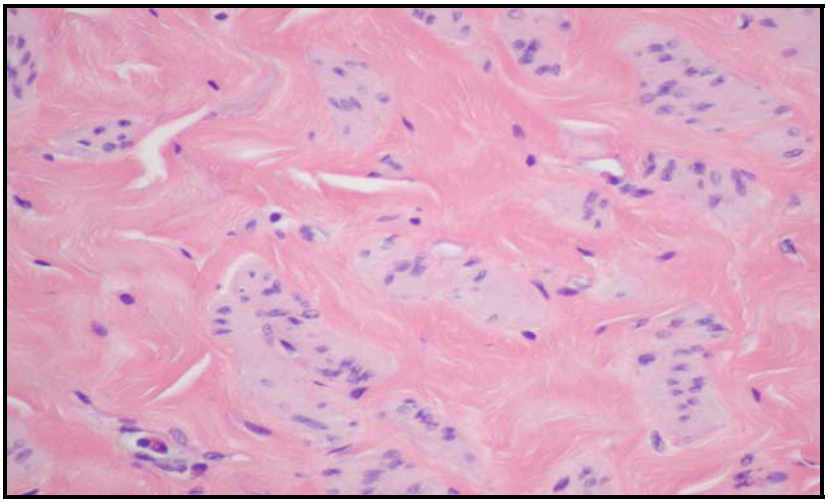

Figure 3-66. MSW03170, male calf, site 15, H&E, 200X, smooth muscle bundles within the dermis.

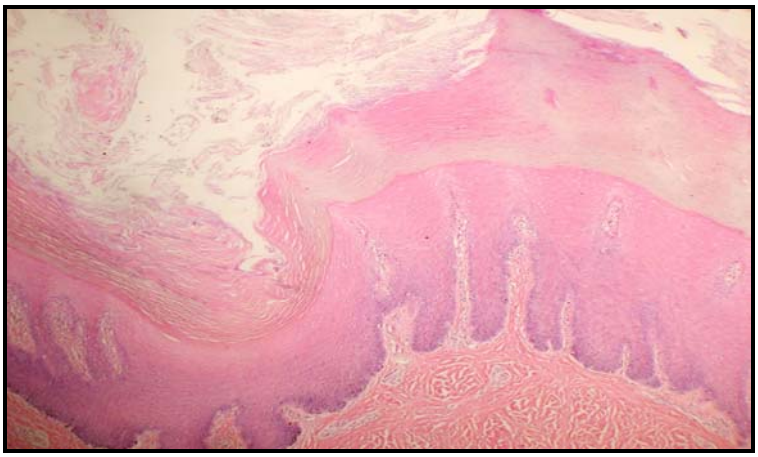

Figure 3-67. MNW0342, adult female, site 15, H&E, 40X, epidermis.

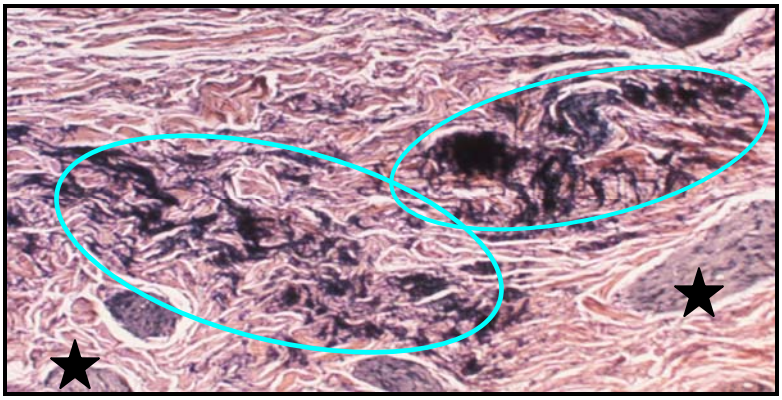

Figure 3-68. MEC0348, adult male, site 15, Verhoff-Van Geison, 100X, elastin fibers within the dermis. Elastin fibers are marked by circles, and smooth muscle bundles are marked by stars.

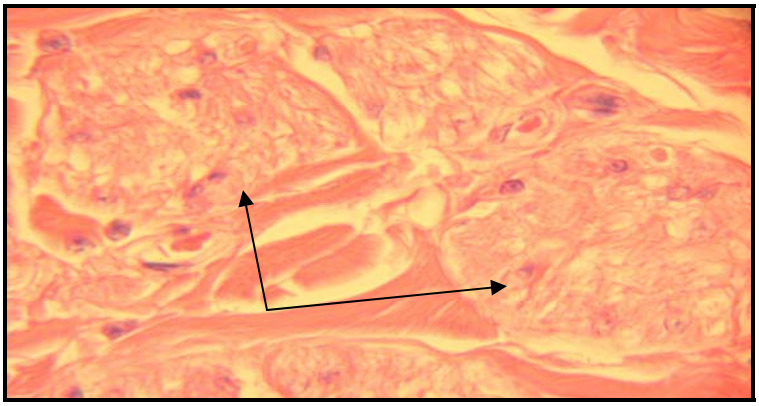

Figure 3-69. MNW0347, male calf, site 15, H&E, 400X, smooth muscle bundles (arrows).

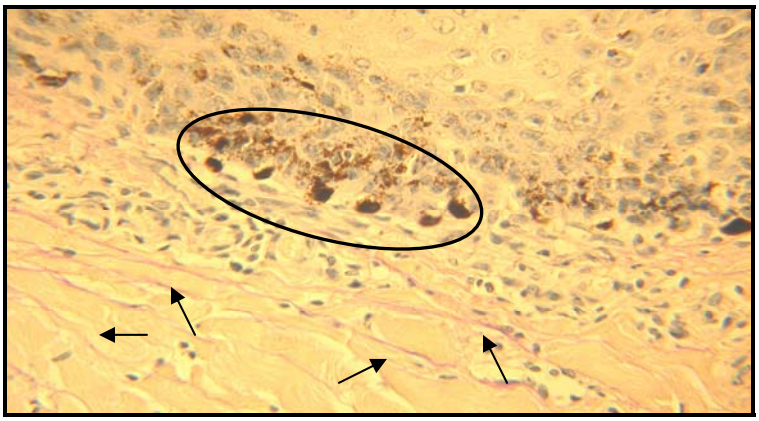

Figure 3-70. MSW01370, male calf, site 15, Luna's stain for mast cells. 250X, epidermis and papillary dermis. This stain not only stains for mast cells but also stains elastin fibers violet (arrows), also notice the numerous melanocytes (dark brown, circled) in this one portion of this epidermal peg.

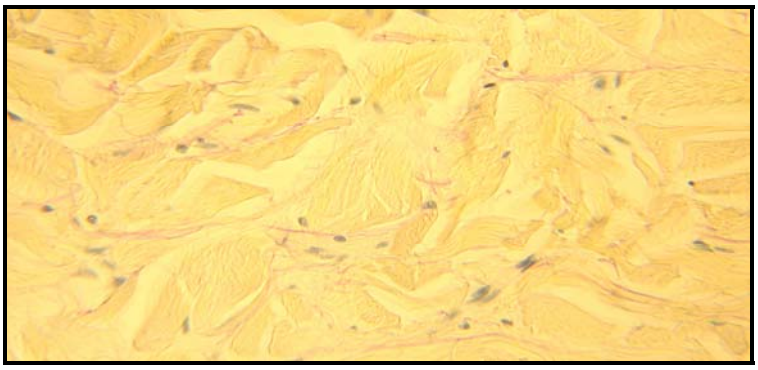

Figure 3-71. MSW03170, male calf, site 15, Luna's stain for mast cells, 250X, elastin fibers in the dermis.

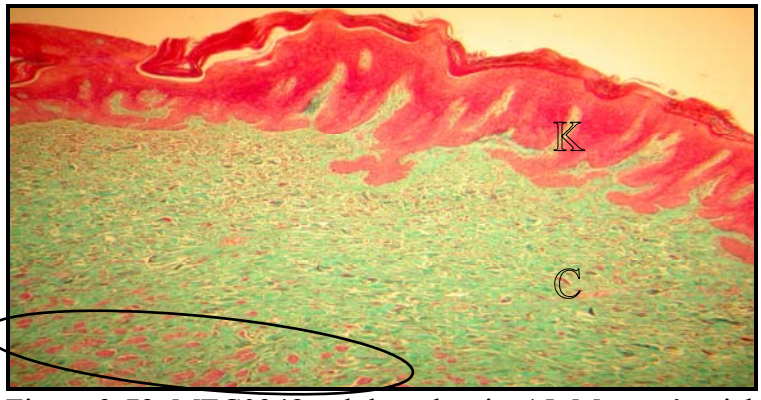

Figure 3-72. MEC0348, adult male, site 15, Masson's trichrome, 20X, epidermis and dermis. Here you can see that the smooth muscle bundles within the dermis are red (circled), the keratin (K) that composes the epidermis stains red, and the collagen (C) stains green.

Eyelid Skin (Sample Site 24)

The skin of the eyelid exhibits a thickened stratum spinosum, a slightly thickened stratum corneum, and has three layers in the epidermis, like all other areas of the manatee skin. The epidermis in this area has the thinnest epidermis and dermis of the entire body. The epidermis can measure 0.2 mm in a male calf and be as thick as 2.2 mm in an adult male. The surface of the eyelid is smooth, and the undulating ridges are more rounded at the apex, and shorter than most other areas of the manatee skin. In a neonate there are 12 undulating ridges per linear 275µm, 5 undulating ridges per 40X view in a male calf, and 3 per view in the adult male. The stratum corneum is very thin, with 5–12 individual cell layers in a neonate, 6–60 in the calves, and 7–75 in the adults.

The epidermal pegs vary in depth and thickness, but for the most part are uniform in depth. The calf has the most pegs per 40X view (16) and the adult has the least (7). There are melanocytes present in the stratum basale and the papillary dermis, and melanocytes are numerous in the eyelid, with anywhere between 2–12 melanocytes per epidermal peg. There are nerves associated with vasculature present as well as pacinian corpuscles. The dermis is not very thick, ranging from 1.5 mm (male calf)–5.4 mm (adult female). The collagen of the dermis has no defined pattern like many other areas of the manatee skin. It is very vascular and has most of the dermis infiltrated with skeletal muscle. There are no lacrimal or mybobian glands present, but as you near the conjunctiva there is an exceptionally large accessory gland present that is mucous secreting. Elastin fibers are present in the dermis of the eyelid, they are thin and mostly present in the papillary dermis.

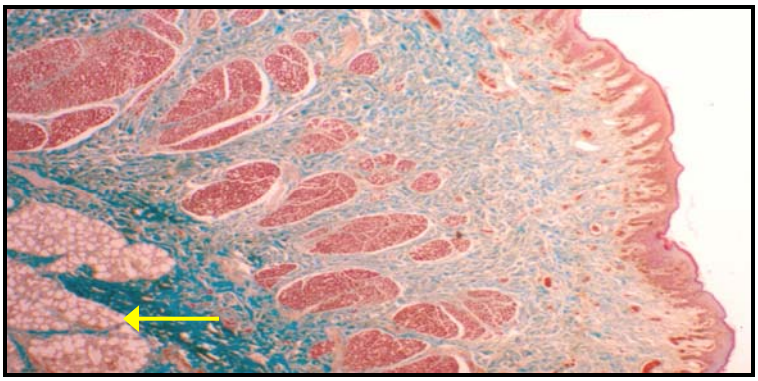

Figure 3-73. MEC0348, adult male, site 24, Masson's trichrome, 20X, muscle and keratin stain red, collagen of the dermis is stained green, and the accessory glands are white (arrow).

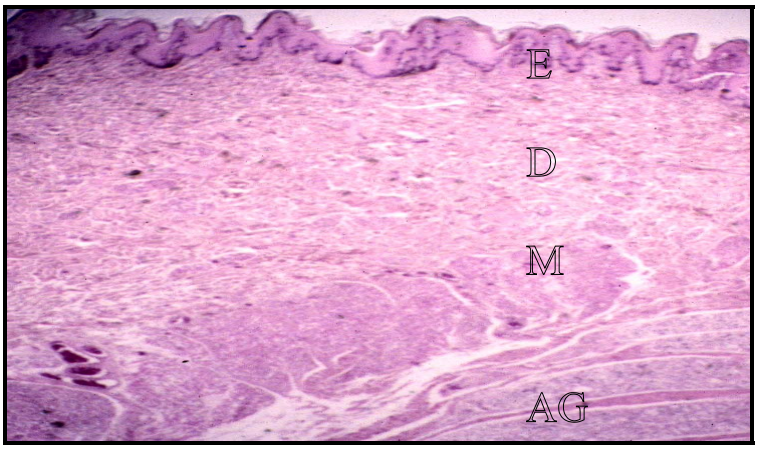

Figure 3-74. TM0311, neonate, site 24, H&E, 20X. Eyelid: epidermis (E), dermis (D), muscle (M), and accessory glands (AG).

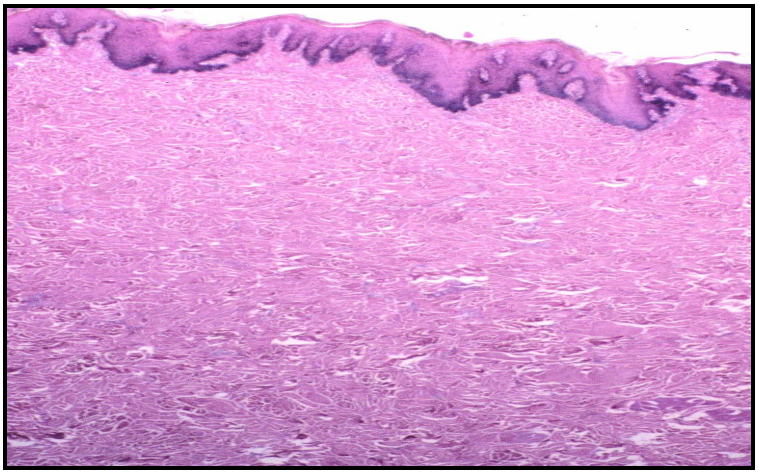

Figure 3-75. MEC0348, adult male, site 24, H&E, 20X, epidermis and dermis.

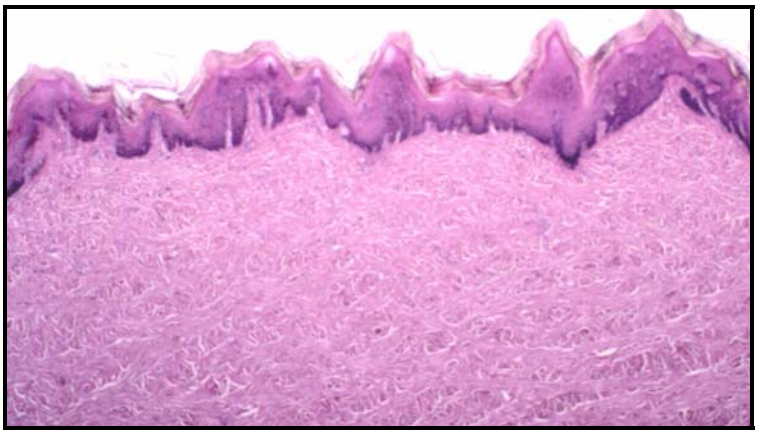

Figure 3-76. LPZ101820, male calf, site 24, H&E, 20X, epidermis and dermis.

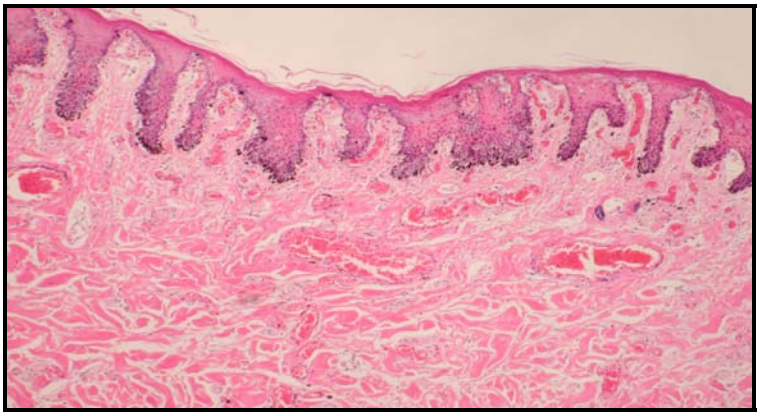

Figure 3-77. MNW0342, adult female, site 24, H&E, 40X, epidermis and dermis.

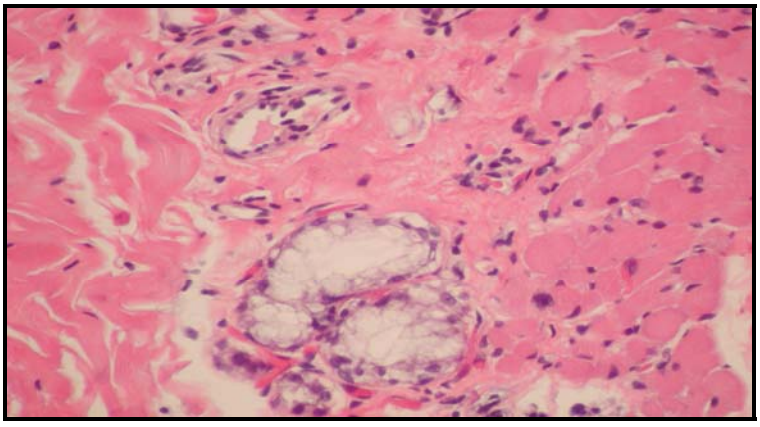

Figure 3-78. MSW03170, male calf, site 24, H&E, 200X, the accessory gland is surrounded in this picture by collagen, small vessels, and skeletal muscle on the right.

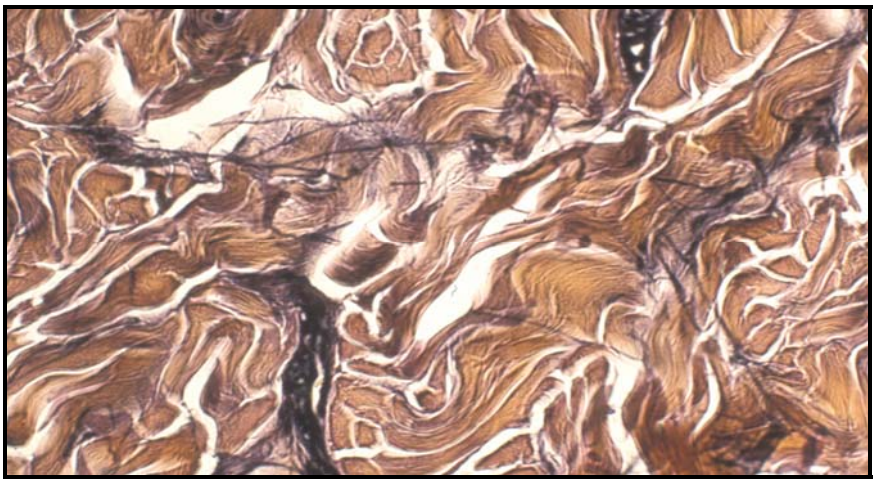

Figure 3-79. MEC0348, adult male, site 24, Verhoff-Van Geison, 200X, elastin fibers within the dermis are fine and stained black.

Nostril Skin (Sample Site 25)

The nostril of the manatee is unique compared to most mammals because it has the capability to seal off the opening to prevent water from entering. The exterior epidermis of the nostril is thick and consists of three layers, lacking a stratum lucidum and stratum granulosum. The inner epidermis, lining the airway, is similar in structure to the exterior epidermis except that it is not as thick, does not have distinct undulating ridges, and has few cell layers that make up the stratum corneum (Figure 3-80 – 3-82).

The skin lining the airway is completely keratinized. The epidermis lining the airway is as thin as 0.2 mm, and the exterior epidermis is as thick as 2.3 mm. The stratum corneum of the interior of the nostril is between 5–17 cell layers, whereas the exterior stratum corneum has between 20–101 cell layers depending on the age of the manatee. In a neonate there are 14 undulating ridges present per linear 275µm. There are 3–4 present per linear 275µm in the calf and 2–4 undulating ridges present per linear 275µm in the adult. The nostril skin has several epidermal pegs; 14 per linear 275µm in the neonate, 16 per linear 275µm in the calf, and anywhere between 8–16 in the adult. Melanocytes are present not only in the stratum basale but are also seen in the papillary dermis (Figure 3-

81). They range from 1–9 melanocytes per epidermal peg. The dermis of the nostril is 1.8 mm a male calf and up to 10.1 mm in an adult female. Although it is dense, there is no distinct organization present in the dermis. There are nerves associated with vasculature, and pacinian corpuscles present (Figure 3-83). The nostril has the most pacinian corpuscles of all the skin samples. There are blood sinus hairs present in the exterior nostril skin as well as the interior skin lining the airway. The dermis of the nostril, like the eyelid, contains a lot of skeletal muscle. The nostril, out of all areas of the skin sampled, has the most amount of elastin fibers. The fibers are thin in the papillary dermis, and become thicker in the reticular dermis (Figure 3-84 – 3-87).

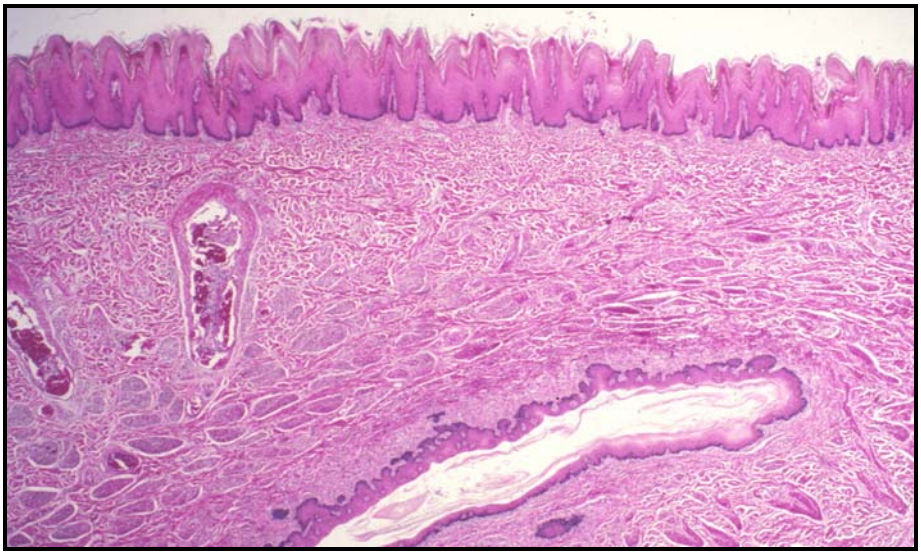

Figure 3-80. TM0311, neonate, site 25, H&E, 20X, exterior nostril epidermis, interior nostril epidermis, dermis, and blood sinus hair follicles.

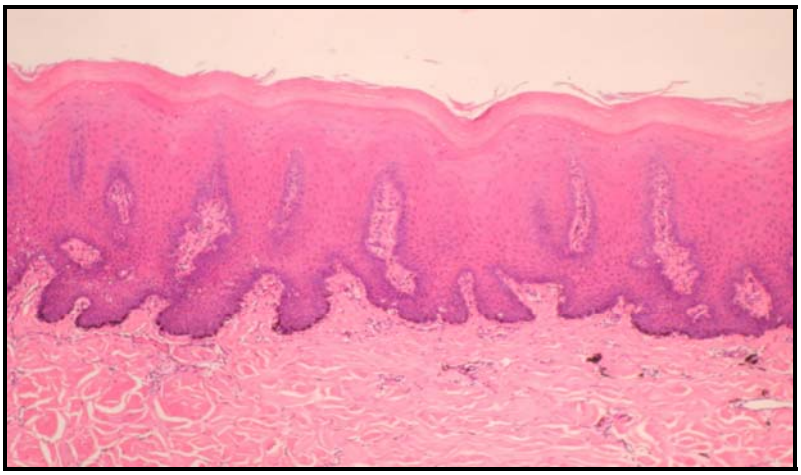

Figure 3-81. MSW03170, male calf, site 25, H&E, 40X, exterior nostril epidermis and dermis.

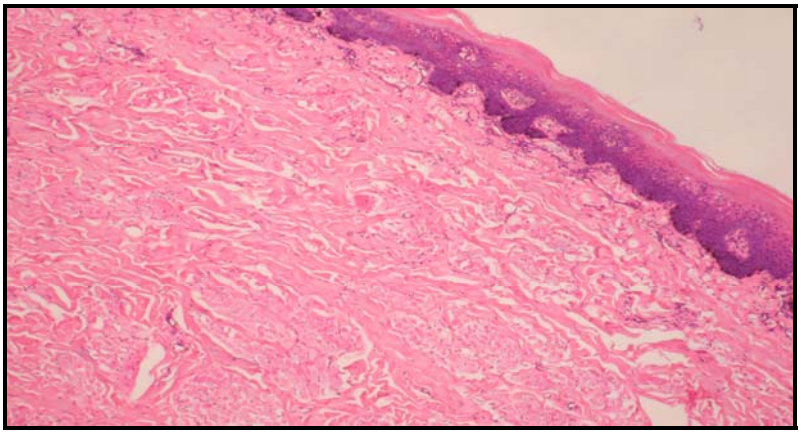

Figure 3-82. MSW03170, male calf, site 25, H&E, 40X, interior nostril epidermis and dermis.

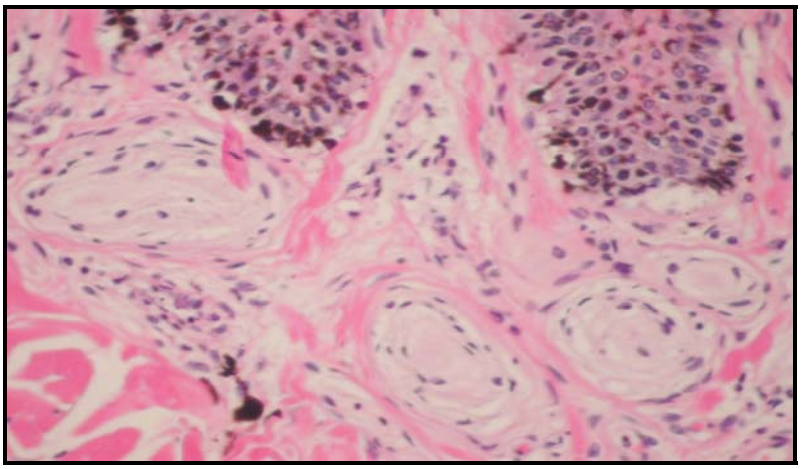

Figure 3-83. MNW0342, adult female, site 25, H&E, 100X, interior nostril. Just below the epidermis there are several pacinian corpuscles present.

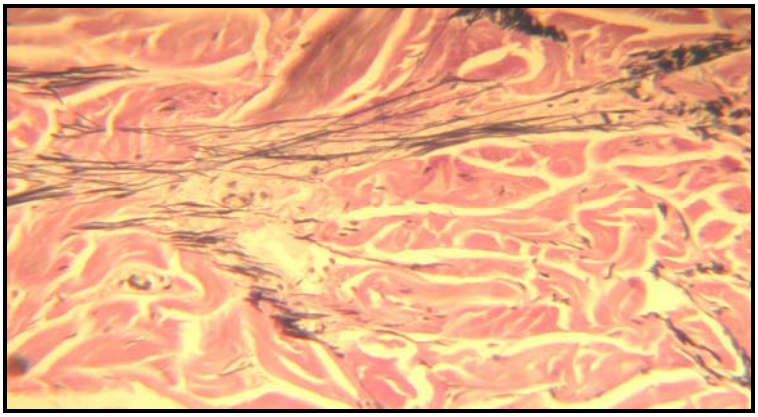

Figure 3-84. MEC0348, adult male, site 25, Verhoff-Van Geison, 100X, networks of long thin elastin fibers (black) in the dermis of the nostril.

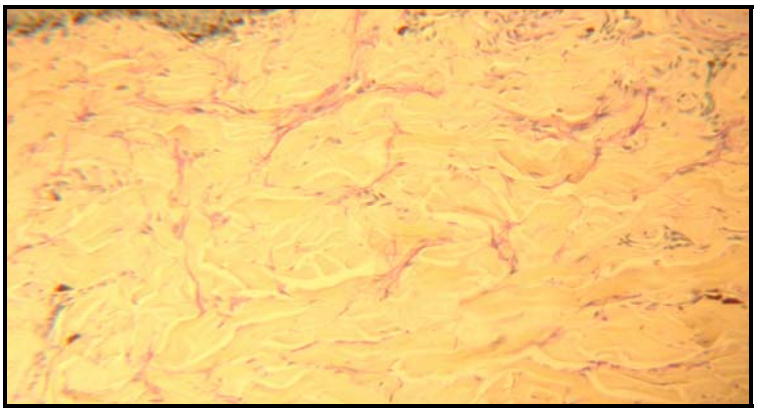

Figure 3-85. MSW03170, male calf, site 25, Luna's stain for mast cells, 100X, several elastin fibers (violet) are present just below the epidermis of the nostril.

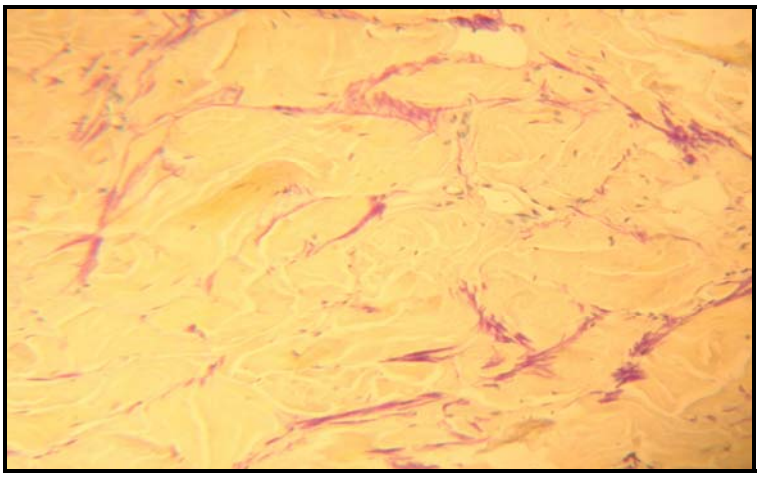

Figure 3-86. MSW03170, male calf, site 25, Luna's stain for mast cells, 100X, deeper into the dermis of the nostril, there are thicker elastin fibers (violet) present.

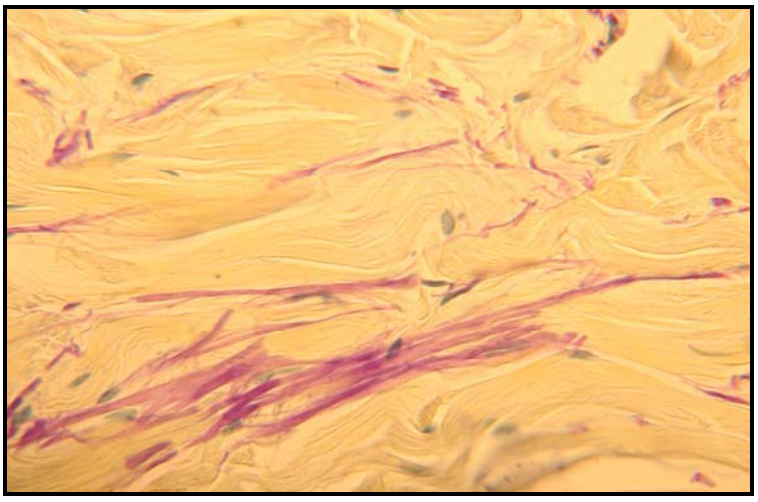

Figure 3-87. MSW03170, male calf, site 25, Luna's stain for mast cells, 250X, higher magnification of Fig. 3-86, elastin fibers (violet).

Normal Ectoparasites and Organisms

Many skin sections of the manatee contained a variety of ectoparasites and organisms. None of these organisms were identified because skin scrapings were not taken in this study. In previous studies several ectoparasites have been identified. Humes (1964) has documented a species of copepod, *Harpacticus pulex*, which can inhabit the stratum corneum of the manatee. These copepods have also been identified by Husar (1977), and Hartman (1979). The copepods seen in this examination of the normal manatee integument seemed to pose no health threat to the manatees due to the fact that there was no associated injury to the skin or inflammation (Figure 3-88 and 3-89). Zeiller (1981) noted two cases of captive manatees at the Miami Seaquarium where copepods were associated with skin lesions. It was not determined whether the copepods were responsible for the lesions or secondarily invaded them. The situation was remedied by changing the tank water to freshwater and adding copper sulfate (Zeiller, 1981). Other organisms previously found in the skin of the manatee are barnacles, remoras, blue-green algae, red algae, diatoms, protozoans, ostracods, amphipods, isopods, dipteran larvae,

gastropods, and small leeches (Forrester, 1992). Algae were present in some skin samples encountered during this study. Their identity is unknown since microbiological analysis was not performed in this study (Figure 3-90 and 3-91).

Several types of bacteria have been cultured from the skin of the manatee. These include: *Bacillus* sp., *Corynebacterium* sp., *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudonmonas aeruginosa, Pseudomonas florescens, and Pseudomonas putrefaciens* (Forrester, 1992). Forrester also notes that *Citrobacter* sp., *Proteus* sp., and *Pseudomonas* sp. were cultured from skin wounds in the manatee. In the present study bacteria were found in both the normal skin and wounded skin of the manatee. Classification of these bacteria was not obtained because cultures were not taken from these samples. One ectoparasite found on the skin of the manatee that is not mentioned in manatee literature is a species of nematode (Figure 3-92 and 3-93).

In a study conducted by Dr. Kendal Harr (2003) consisting of skin sampling with fungal cutures, grown out for species identification, several species were documented to inhabit the stratum corneum of the manatee. These species consisted of *Penicillium sp.*, *Nectria* sp., *Mucor circinelloides*, *Mucor Ramosissimus, Pestalotiopsis* sp., *Fusarium semitectum*, *Nigrospora* sp., *Trichoderma* sp., and *Aspergillus niger*. Some of these fungi were found to be present in animals with skin lesions and some were found in normal skin from manatees. In the present study culturing the skin for microorganisms and fungus was not implemented in the sampling protocol. Fungus was seen histologically in 4 out of the 10 manatees sampled in this study (Figure 3-94). In three of these cases, the fungus was present on the dorsal and ventral fluke, and the dorsal flipper with no signs of inflammation, and in one case fungus was detected in multiple regions of the body, both

dorsal and ventral sites, that was associated with inflammation. The findings of Dr. Harr, and those of the present study, suggests that some fungal growth in the stratum corneum of the manatee skin may be normal. The manatee skin is unlike other marine mammals due to its thick stratum corneum and reduced rate of skin sloughing. Many cetaceans have a constant sloughing of their stratum corneum layers which would inhibit fungal growth unless there were an injury to the skin or the animal's health was compromised. More research is required to determine if the fungal growth seen in the skin of the manatee is normal for these bottom resting marine mammals, or whether those manatees that do have fungal growth without inflammation are compromised in some other aspect of their health.

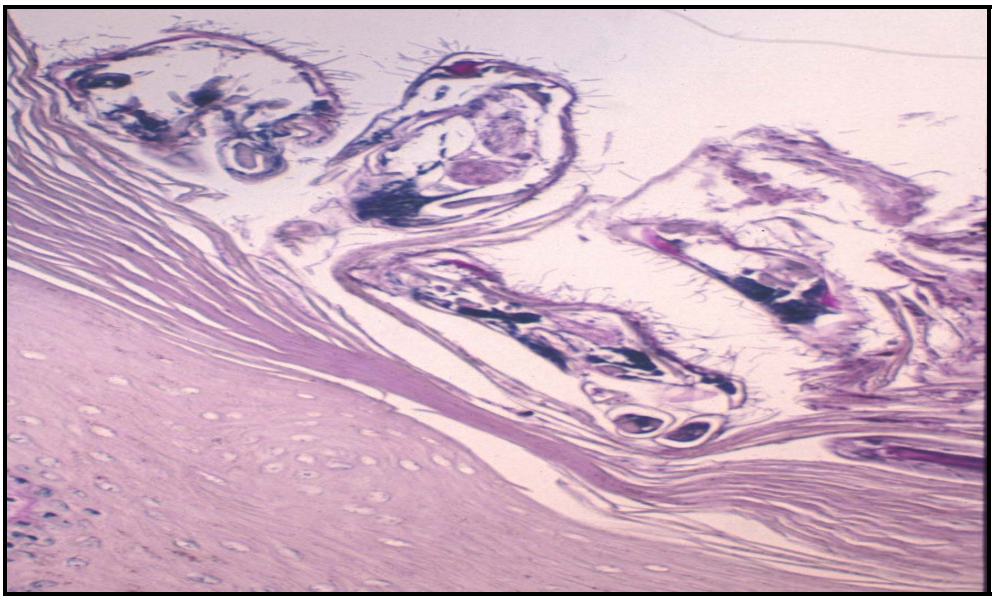

Figure 3-88**.** MNW0342, adult female, site 17, PAS, 200X, arthropods/copepods in the stratum corneum

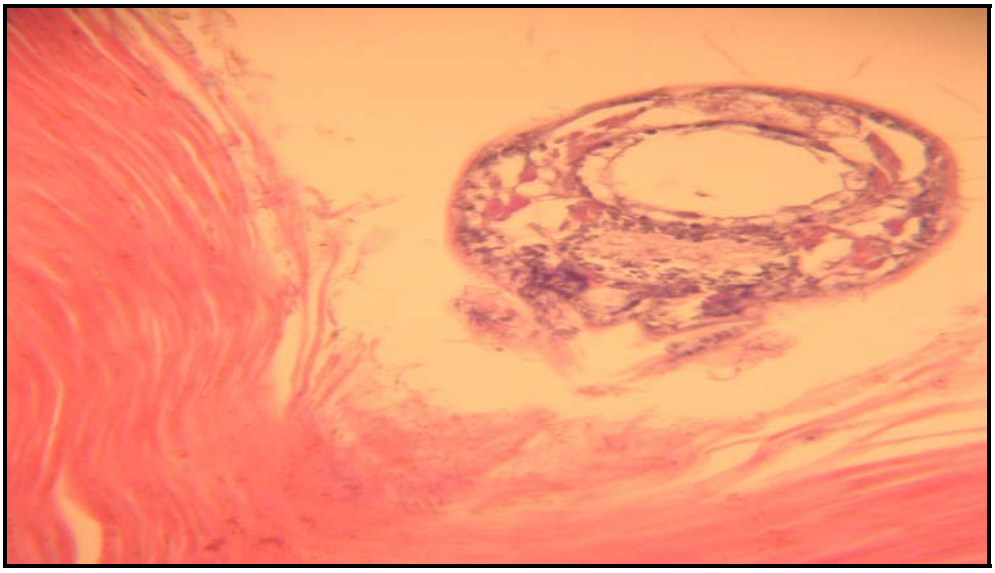

Figure 3-89 MSW03170, male calf, site 3, H&E, 400X, arthropod/copepod in the skin of the manatee.

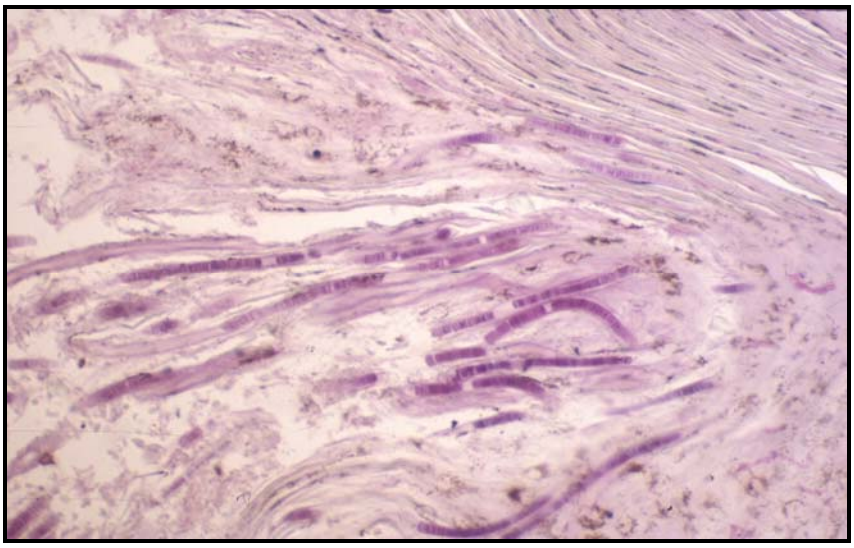

Figure 3-90. MEC0348, adult male, site 6, PAS, 200X, algae present in the stratum corneum.

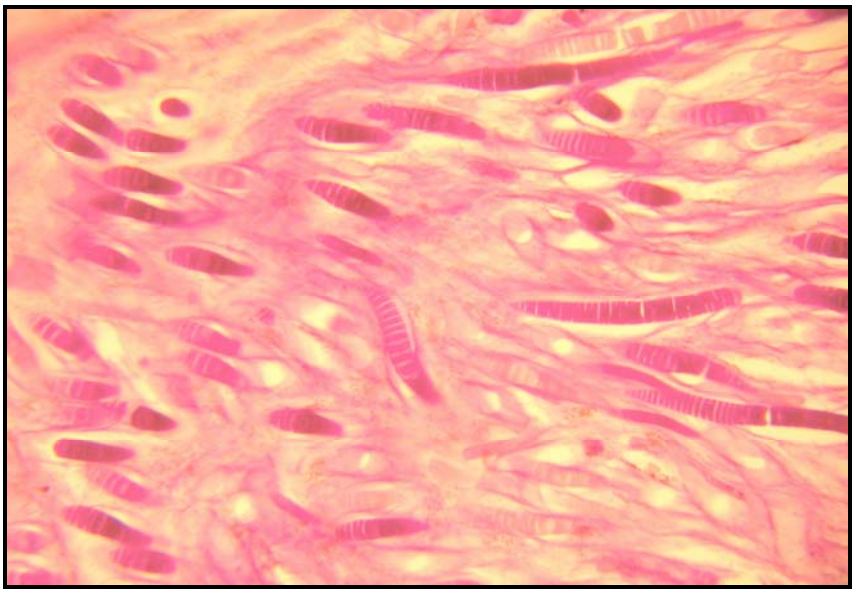

Figure 3-91.TM0406, adult male, site 9, H&E, 400X, algae in the stratum corneum.

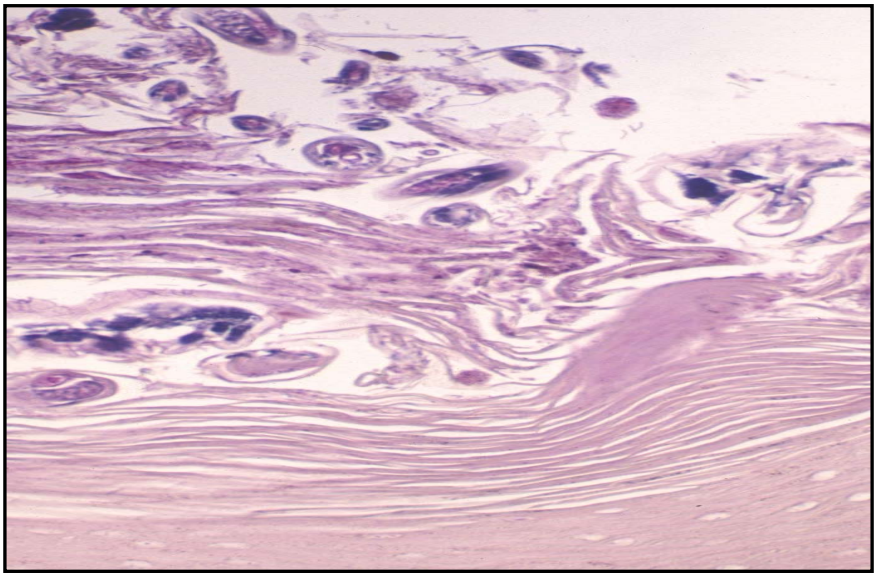

Figure 3-92. MNW0342, adult female, site 17, PAS, 100X, nematodes present in the stratum corneum.

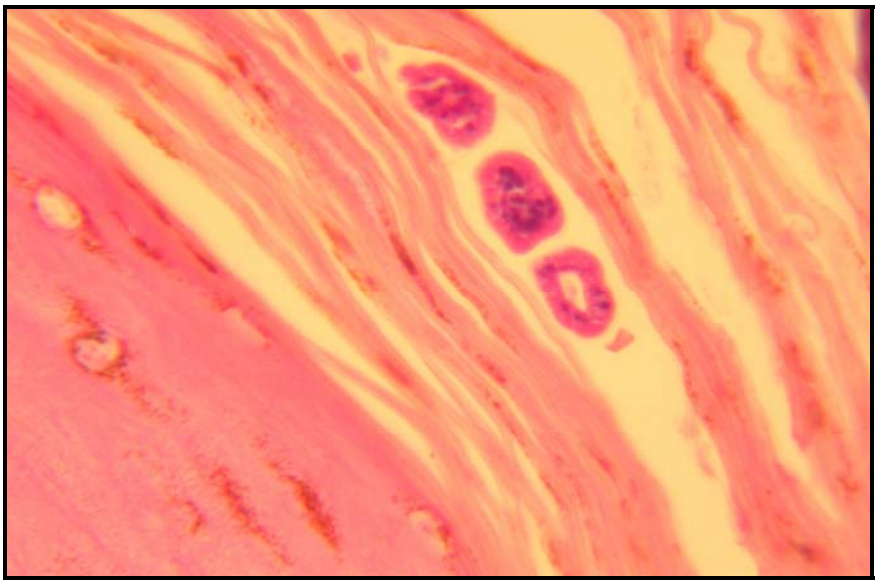

Figure 3-93. MSW03170, male calf, site 10, H&E, 400X, nematode present in the stratum corneum.

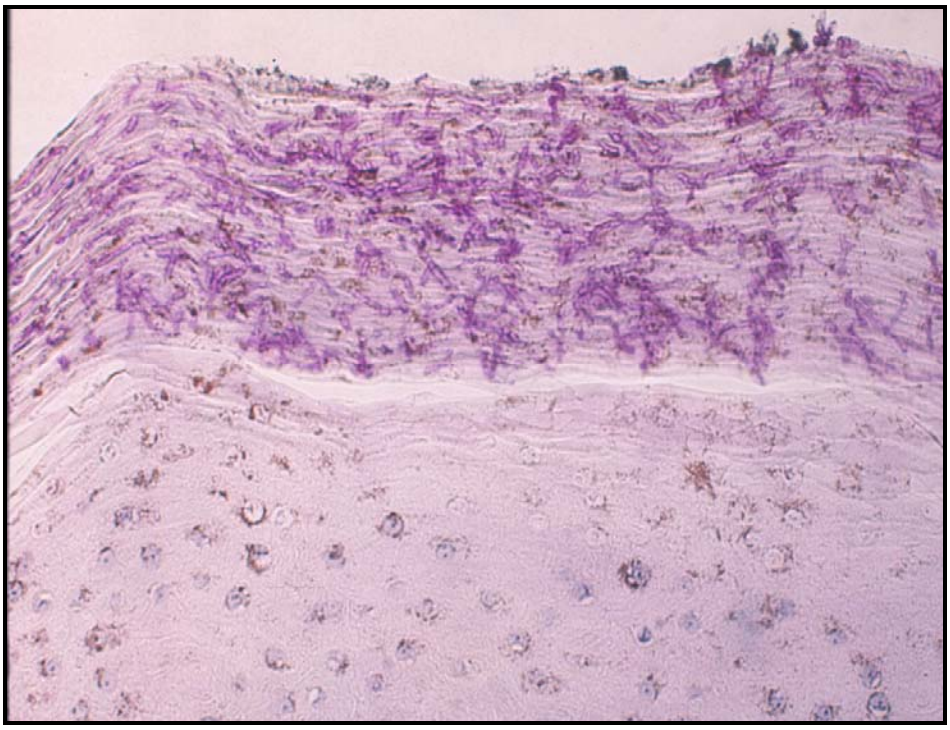

Figure 3-94. MNW0342, adult female, site 5, PAS, 200X, fungus present in the stratum corneum with no signs of inflammation present.

CHAPTER 4
DISCUSSION OF NORMAL MANATEE INTEGUMENT

The skin of the manatee, like many anatomical and physiological aspects of the species, is distinctive and at times unique in comparison to most other marine mammals. The integument of the manatee has many features that are normal for them but would be characterized as abnormal in other mammals. Normal manatee is hyperplastic and hyperkeratotic, both conditions that in many domestic mammals are abnormal except for specialized locations (Yager, 1994). Cetaceans display this hyperplastic characteristic as well, and is also a normal feature of their integument (Ling, 1974). The hyperplastic and hyperkeratotic epidermis aids as a protective device from constant abrasion from water current, rocks, seafloor, and other debris below the water. This protective function is comparable to that of many other mammals where hyperkeratotic skin is present, such as the palms of our hands, soles of our feet, and the pads on the paws of most land mammals. Another function may be to protect against ectoparasites. The extremely thick stratum corneum provides a niche for several microorganisms to live in. None of these organisms are seen to penetrate the stratum spinosum. One potential reason for the presence of algae, copepods, bacteria, and other organisms to live on the skin of the manatee could be the slow sloughing cycle. This could allow these organisms to live on the manatee for prolonged periods of time and flourish.

Regardless of age, the epidermis of the manatee is thinnest in two specialized areas: the eyelid and the nostril (Table 4-1 and 4-2). The thickest epidermis is found at the mid-dorsal point (site 2) of the back. There is a similarity in dermal thicknesses between the

dorsal and ventral body of the manatee. Both the adult and juvenile manatees show a trend where the dermis is thickest at the mid-point of either the dorsal or ventral aspect of the body and towards the fluke and head the dermis is thinner than at the mid-point. The thickest epidermis occurs at sample site 18 in both adult and juvenile manatees. The edges of the flipper are practically the same in epidermal and dermal thickness, with the flipper tip being thinner (sites 18 and 21), as well as the central sample sites of the flipper (sites 19 and 23).

The organization of collagen throughout the manatee skin changes based on the region of the body. The dorsal and ventral body of the manatee, at all ages, has a collagen network that is a very dense, criss-cross, diagonal weave. It is a very distinct pattern that is not seen in most mammals. In the fluke, both dorsal and ventral, the organization changes to a dense, criss-cross, 90º weave that gives the appearance of a checkerboard. The flipper has a similar organization to the fluke but it is not always a 90º criss-cross. There are three areas of the manatee skin that do not contain a distinct pattern of organization. These sample sites are the urogenital, eyelid, and nostril skin. There were no differences in collagen organization based on age or sex. Although the collagen is very dense, there is no obvious direction as to the orientation of the collagen bundles. The thick dermis of the skin provides structure and support, to give the manatee its shape. The pressure of an aquatic environment requires a thick integument to give marine mammals the support needed. The unique and intricate collagen network in different regions of the manatee integument probably serves to aid in the function of movement. The dense collagen bundles combined with the complex weaving, found in the flippers and fluke, makes these areas extremely rigid to better propel the manatee through water and aid in

pushing off from the bottom after resting. The areas of the eyelid, nostril and urogenital skin do not have any type of organization among the collagen bundles. These areas are sensory areas that require the skin to stretch (breathing, opening and closing eyelid, giving birth, mating) and for this reason most likely lack an intricate collagen network similar to the rest of the body.

In most mammals the presence of pigment observed below the stratum basale, in the papillary dermis, is considered a pathological feature (Yager, 1994). In all manatee integument samples examined, macrophages that ingest melanin, also known as melanophages, are present in the dermis, strongly suggesting that in the manatee this feature is normal. The function of the melanophages in the dermis is not clear. Pigmentary incontinence is usually due to immune-mediated damage to the epidermal-melanin unit, but can also reflect a "spillover" from epidermal hyperpigmentation from a variety of causes (Yager, 1994). This characteristic has not been reported in other marine mammals.

The numerous rete ridges, that give the skin its texture, and epidermal pegs provide the external structure to shape the manatee's body, as well as to increase the surface area of attachment between the dermis and epidermis thereby giving the skin more strength and support. In cetaceans, undulating ridges are not very prominent if present at all. They do, however, have many long invaginating epidermal pegs similar to the manatee, with some being as deep as half the dermis (Geraci et al., 1986). Terrestrial mammals that have epidermal pegs are quite different from those of the manatee and cetaceans. They are normally very uniform in depth and are very shallow, as well as broad. In the manatee these epidermal pegs vary considerably in depth, shape, and width,

depending on the area of the body. The thinnest epidermis and dermis occurs in the eyelid. The thickest epidemis is found in the fluke, with both the dorsal and ventral samples having similar measurements. The thickest dermis is the centermost dorsal point on the manatee's back.

The manatee epidermis consists of three layers: stratum basale, stratum spinosum, and stratum corneum. This result is in agreement with the earlier work done by Sokolov (1982). In order to establish the normal integument of the manatee it is necessary to have a good baseline normal from as many manatees as possible. Previous research (Dosch, 1915; Sokolov, 1982) was based on comparatively small number of samples from a few manatees. Dosch's research involved embryos, and a calf, with random sampling from embryo to embryo. Sokolov's research has the same drawbacks, only a few manatees were sampled and from very few, select areas of the body. In fact, Sokolov's description of the manatee skin was based on single samples, from a single individual, and were not comparable to other manatees because the same areas were not sampled on each manatee. Therefore these results were questionable.

The lack of a stratum granulosum is a normal trait for not only the manatee, but also cetaceans (Sokolov, 1982; Ling, 1974). Some researchers in the past have not wanted to commit to the fact that cetacean skin is completely keratinized because of this fact. The manatee skin is completely cornified, as shown by the present finding that manatees have an extremel thick stratum corneum, that in most mammals would be considered hyperkeratotic, in which the cells of this layer lack a nuclei. It is possible for the skin to completely keratinize without the stratum granulosum. The keratohyalin granules in the stratum granulosum do contribute to the process of keratinization, but it

has been found that they are not essential. The process of keratinization without a stratum granulosum is called trichelemmal keratinization (Kimura, 1983; Poblet et al., 1996). Trichelemmal keratinization has been observed in hair, various tumors, and cysts (Kimura, 1983). Although trichelemmal keratinization has not been seen or described to occur in the skin of any mammals, it is a hypothesis of how the manatee integument, and possibly cetacean skin, keratinizes without the presence of the stratum granulosum.

The only type of hairs present on the postcranial body of the manatee were blood sinus hair follicles, which are in accordance with Reep et al. (2002). Nerves can be seen throughout the skin, mainly associated with vessels and seen in the dermal papillae. To see the network and identify the different types of nerves present in the skin, further research must be done. Pacinian corpuscles are located in the skin of the nail and nostril, with the latter of the two locations having several pacinian corpuscles. Pacinian corpuscles act as mechanoreceptors that in the manatee most likely aid in detecting objects in the water and more importantly, above the surface of the water, with regard to the nostrils.

Several stains were applied in this study to observe mast cells specifically in the integument of the manatee. Unfortunately, none of the stains revealed their presence in the manatee skin. As a result, the question of whether manatee skin contains mast cells remains unanswered. There may be some biochemical component to the mast cells of the manatee that does not allow the usual stains for mast cells to react with their granules. This area of study needs further pursuit in order to understand the involvement that the mast cell has with not only the wound healing process but the immune system as a whole.

All skin specimens for this study had to be stained separately according to area of the body. Different areas of the manatee integument that underwent the same staining procedure would turn out lighter or darker than one another, including specimens from the same animal just different sample sites of the body. It is possible that the skin of the manatee has different components within the extracellular matrix based on the area of the body that the sample is from. This difference in staining was seen throughout all stains applied in this study.

Table 4-1. Ranges, averages, and characteristics of adult manatees.

Site	Range Epidermis (mm)	Range Dermis (mm)	Average Epidermis (mm)	Average Dermis (mm)	Pigment	Collagen Organization	Elastin	Hypodermis	Vascularity
1	0.3-1.9	16.8-21.3	0.6-1.4	18.9-20.5	+++	Dense, criss-cross diagonal weave	+	Present	+
2	0.65-2.3	16.1-22.1	0.8-1.7	19.3-20.7	+++	Dense, criss-cross diagonal weave	+	Present	+
3	0.4-1.6	13.6-18.9	0.6-1.3	15.2-16.8	+++	Dense, criss-cross diagonal weave	+	Present	+
4	0.4-3.6	3.0-9.8	0.9-2.3	5.4-8.2	++	Dense, cross-cross 90º weave	++	Absent	+++
5	0.5-3.1	1.8-5.0	0.82-2.3	2.9-4.7	++	Dense, cross-cross 90º weave	++	Absent	+++
6	0.4-1.7	7.1-10.8	0.7-1.4	9.2-9.6	++	Dense, criss-cross, diagonal weave	++	Absent	+++
7	0.3-3.4	3.0-6.6	0.88-2.5	4.1-6.2	++	Dense, cross-cross 90º weave	++	Absent	+++
8	0.3-1.8	8.6-16.5	0.6-1.3	13.8-14.3	++	Dense, criss-cross, diagonal weave	+	Present	+

Table 4-1. Continued.

Site	Range Epidermis (mm)	Range Dermis (mm)	Average Epidermis (mm)	Average Dermis (mm)	Pigment	Collagen Organization	Elastin	Hypodermis	Vascularity
9	0.63-2.0	12.3-19.6	0.8-1.5	14.9-16.4	++	Dense, criss-cross diagonal weave	+	Present	+
10	0.3-3.8	8.5-17.0	0.7-2.1	11.2-14.3	++	Dense, criss-cross diagonal weave	+	Present	+
11	0.7-2.6	6.25-15.2	0.76-2.1	9.5-10.5	++	Dense, criss-cross 90º weave	++	Absent	+++
12	0.75-5.1	2.5-4.4	1.1-3.4	3.0-4.2	++	Dense, cross-cross 90º weave	++	Absent	+++
13	0.5-2.9	4.1-10.5	0.85-2.5	5.4-8.4	++	Dense, cross-cross 90º weave	++	Absent	+++
14	0.7-1.9	8.1-10.3	0.9-1.7	8.6-9.8	++	Dense, criss-cross, diagonal weave	++	Absent	+++
15	0.4-3.4	9.4-16.3	0.7-2.4	11.5-12.8	+	Dense, undefined	+++	Present	+++
16	0.8-2.9	3.7-7.8	0.89-2.1	5.5-6.1	++	Dense, 90°criss-cross	+++	Absent	++
17	0.5-2.0	3.4-9.6	0.7-1.7	4.8-6.2	++	Dense, 90° criss-cross	+++	Absent	++
18	1.0-6.7	2.0-6.0	1.6-3.9	3.4-4.7	++	Dense, 90°criss-cross	+++	Absent	++
19	0.5-1.8	3.6-6.6	0.62-1.5	4.4-5.8	++	Dense, 90°criss-cross	+++	Absent	++
20	0.5-2.7	3.8-7.1	0.93-2.0	4.5-5.9	++	Dense, criss-cross	+++	Absent	++
21	0.5-2.2	2.0-5.2	0.96-1.45 nail 3.3	2.8-4.6	++	Dense, 90° criss-cross	+++	Absent	+++
22	0.4-2.8	4.0-8.3	0.87-2.1	2.8-4.6	++	Dense, criss-cross	+++	Absent	++
23	0.3-2.6	3.8-7.5	0.65-1.5	4.6-5.5	++	Dense, 90°criss-cross	+++	Absent	++
24	0.2-2.2	1.5-4.3	0.3-1.4	2.2-3.9	+++	Dense, undefined	++	Absent	+++
25	0.2-2.3	2.5-8.0	0.5-1.8	4.8-6.5	+++	Dense, undefined	+++	Absent	+++

Table 4-2. Ranges, averages, and characteristics of juvenile manatees.

Site	Range Epidermis	Range Dermis	Average Epidermis	Average Dermis	Pigment	Collagen Organization	Elastin	Hypodermis	Vascularity
1	0.05-2.5	5.4-17.8	0.33-1.33	12.7-14.2	++	Dense, criss-cross diagonal weave	+	Present	+
2	0.3-1.0	14.3-15.0	0.3-1.0	14.3-15.0	++	Dense, criss-cross diagonal weave	+	Present	+
3	0.3-2.3	8.3-16.6	0.5-1.4	11.47-13.3	++	Dense, criss-cross diagonal weave	+	Present	+
4	0.4-1.8	3.3-5.2	0.4-1.7	3.3-4.7	+++	Dense, cross-cross 90º weave	++	Absent	+++
5	0.3-2.7	1.7-4.1	0.7-1.7	2.5-3.5	+++	Dense, cross-cross 90º weave	++	Absent	+++
6	0.3-1.4	6.4-8.4	0.4-1.1	6.9-8.0	+++	Dense, criss-cross, diagonal weave	++	Absent	+++
7	0.3-2.3	3.6-6.0	0.5-2.0	3.7-5.8	+++	Dense, cross-cross 90º weave	++	Absent	+++
8	0.2-2.2	6.5-10.1	0.4-1.3	8.4-9.0	++	Dense, criss-cross, diagonal weave	+	Present	+
9	0.3-2.3	7.0-12.1	0.56-1.5	9.3-10.7	++	Dense,criss-cross diagonal	+	Present	+
10	0.3-2.2	6.1-11.8	0.5-1.57	8.7-10.0	++	Dense, criss-cross diagonal weave	+	Present	+
11	0.5-3.0	3.3-9.0	0.8-2.2	4.4-6.8	++	Dense, criss-cross 90º weave	++	Absent	++
12	0.3-2.8	2.0-4.7	0.7-2.0	2.5-4.0	++	Dense, criss-cross 90º weave	++	Absent	++
13	0.3-3.3	2.7-6.3	1.1-2.5	3.3-5.1	++	Dense, criss-cross 90º weave	++	Absent	++
14	0.4-2.4	4.1-7.5	0.7-1.5	5.7-6.6	++	Dense, criss-cross, diagonal weave	++	Absent	++

Table 4-2. Continued.

Site	Range Epidermis (mm)	Range Dermis (mm)	Average Epidermis (mm)	Average Dermis (mm)	Pigment	Collagen Organization	Elastin	Hypodermis	Vascularity
15	0.4-3.5	2.5-10.0	0.5-2.6	5.5-9.7	++	Dense, undefined	+++	Present	+++
16	0.2-2.3	2.2-7.3	0.37-1.7	3.6-6.5	++	Dense, 90°criss-cross	+++	Absent	++
17	0.3-2.4	2.1-9.0	0.6-1.6	3.2-6.2	++	Dense, 90°criss-cross	+++	Absent	++
18	0.6-3.8	1.4-3.7	0.9-3.2	1.8-2.8	++	Dense, 90°criss-cross	+++	Absent	++
19	0.3-2.4	1.8-6.3	0.5-1.6	3.2-5.7	++	Dense, 90°criss-cross	+++	Absent	++
20	0.4-2.3	3.4-7.8	0.7-1.6	4.6-6.2	++	Dense, 90°criss-cross	+++	Absent	++
21	0.4-3.5 nsil-3.7	2.2-5.0	0.8-2.3 nail-2.6	2.2-4.8	++	Dense, 90°criss-cross	+++	Absent	+++
22	0.2-2.5	3.5-8.3	0.5-1.6	3.2-5.7	++	Dense, 90°criss-cross	+++	Absent	++
23	0.3-2.1	1.9-4.8	0.4-1.4	2.7-4.3	++	Dense, 90°criss-cross	+++	Absent	++
24	0.1-2.0	1.5-5.1	0.2-1.3	2.0-4.6	+++	Dense, undefined	+++	Absent	+++
25	0.3-2.2	1.8-7.5	0.5-1.3	4.3-5.2	+++	Dense, undefined	+++	Absent	+++

CHAPTER 5
COMPARISON OF THE MANATEE AND ELEPHANT INTEGUMENT

The manatee's closest living terrestrial relative is the elephant. These two mammals have different external appearances and live in completely different habitats. The manatee is completely aquatic, and the elephant is completely terrestrial. Elephants have been known to be attracted to the water; even to depths well over their heads. Fossil evidence and molecular data have provided the association between these seemingly unrelated orders of mammals. The specialized, unique morphology of the wrist bones organized serially, their horizontal tooth replacement, and experiments with amino acid sequencing of proteins all have proven the manatee and elephant are related (Shoshani, 1992)

The manatee and elephant share similarities in their integuments, including epidermal organization and overall thickness. It is interesting to note that the stratum granulosum of the epidermis is mostly underdeveloped in the elephant and non-existent in the manatee. Though similar in some ways the manatee skin is more irregular with respect to the extremely thick stratum spinosum and stratum corneum. The dermis of the manatee and elephant vary in anatomical architecture and density as well, being more organized and pronounced in development in the manatee, especially in specific regions such as the fluke.

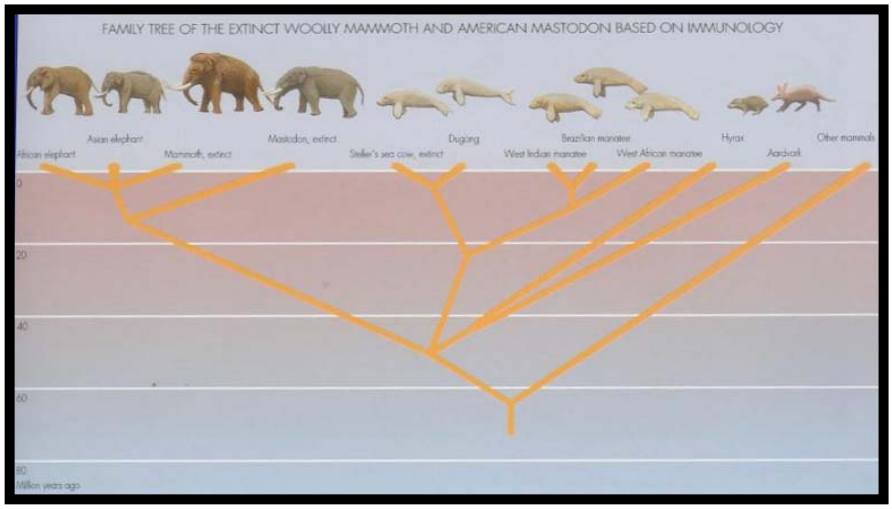

Figure 5-1. Family tree linking elephants and manatees (Shoshani, 1992).

Samples were collected from 25 sites of the manatee body (Figure 2-1), including both dorsal and ventral regions where applicable. Analogous sites of the elephant were used for this histological comparison. Samples were fixed upon collection in 10% buffered formalin. Samples went through routine processing and were embedded in paraffin and cut at 6 microns. These samples were analyzed morphologically through paraffin embedding, and routine H & E. staining.

Urogenital Skin

The urogenital skin of the manatee has a thicker epidermis than the elephant, yet both have a similar organization of the epidermis (Figure 5-1A and 5-1B). The epidermis of the adult female manatee measured 0.8–2.6 mm, where as the epidermis of the adult female elephant was 0.1–0.75 mm. The manatee epidermis, as described in chapter 3, has a thick stratum spinosum and stratum corneum. The elephant epidermis in this area does not display a thick stratum spinosum but it does exhibit a thick stratum corneum. The manatee has at least 79 layers in the stratum corneum, the maximum number of layers is not able to be determined because it is too compact at points to count. In the elephant there are a minimum of 21 layers, and as many as 42. The number of undulating ridges

per linear 275µm is greater in the female elephant than in the manatee. The manatee has two undulating ridges compared to the elephant's seven. Like the number of undulating ridges, the number of epidermal pegs is more numerous in the elephant as well. The female urogenital skin of the elephant has approximately 20 epidermal pegs per linear 275µm while the manatee has 12. A similarity between the two species in this area occurs with regard to the irregular depth and width of the epidermal pegs. The manatee has three layers in the epidermis where the elephant has four layers. The elephant exhibits the stratum basale, stratum spinosum, stratum cornuem and a thin stratum granulosum. The stratum granulosum is present in the crevasses of the undulating ridges, but is not visible at the apexes of the undulating ridges. Melanocytes are present in both species in this area, but are more prevalent in the manatee. The collagen network in the dermis is similar in both animals, and there are no glands present. The manatee urogenital skin has the presence of numerous smooth muscle bundles in the dermis whereas the elephant does not (Figure 5-4 and 5-5).

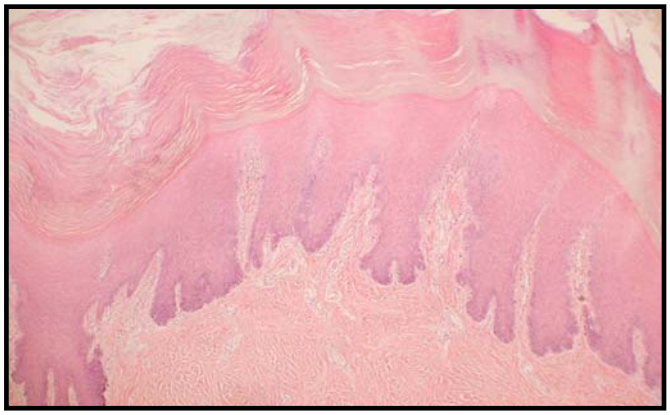

Figure 5-2 Manatee urogenital skin, MNW0342, female, H&E, 40X

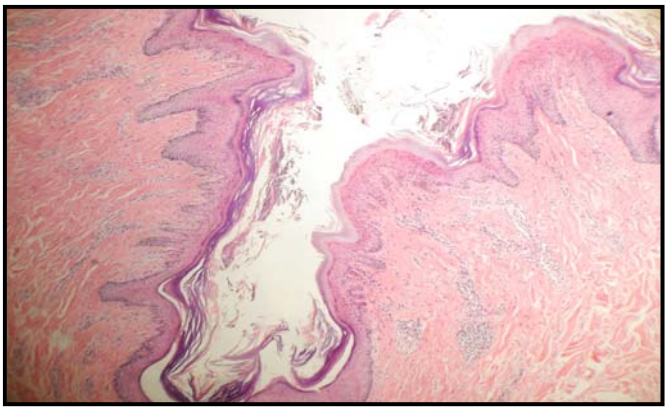

Figure 5-3 Elephant urogenital skin, female, H&E, 40X.

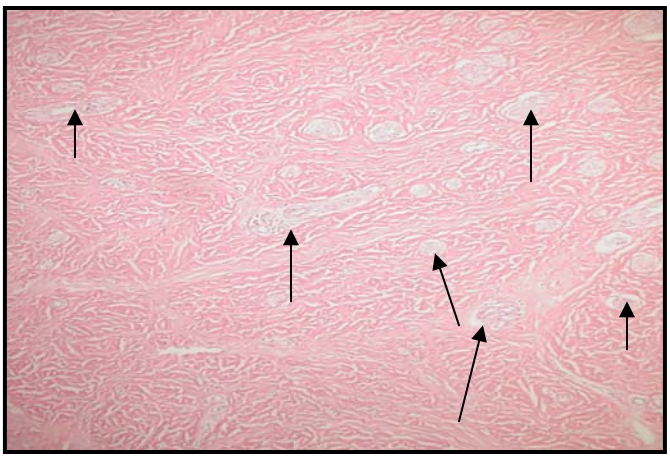

Figure 5-4 Dermis of 5-2, H&E, 40X, arrow point to bundles of smooth muscle.

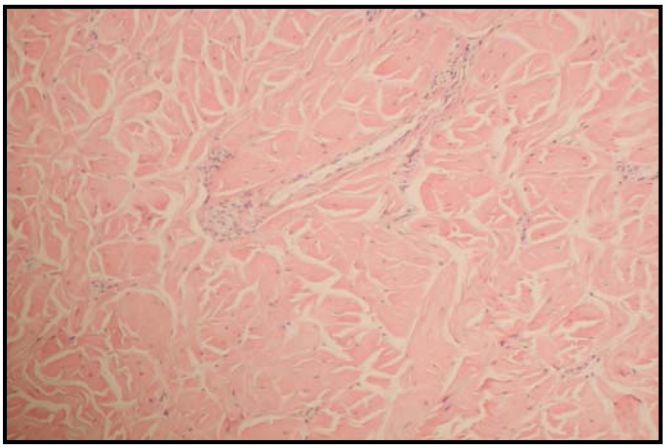

Figure 5-5 Dermis of 5-3, H&E, 40X.

The juvenile male urogenital skin in both species is similar to that of the female, yet there are some differences. The same layers of the epidermis are present for each species, the epidermis is hyperkeratotic (Figure 5-6 and 5-8), but in the elephant the

undulating ridges are fairly uniform in distance from one to the next, and the epidermal pegs are even in depth (Figure 5-6). The epidermis of the male manatee sub-adult is 0.4–1.70 mm, whereas the juvenile male elephant measured 0.1–1.5 mm. The number of undulating ridges in the elephant is seven and the manatee is four per linear 275µm. The epidermal pegs of the male elephant urogenital skin were counted at 15 pegs per linear 275µm, and the manatee has 16. The organization of the dermis is the same as in the female urogenital skin. The thickness varies in the elephant from 4.1–6.6 mm and 8.6–10.0 mm in the manatee. As in the female urogenital skin there are no glands present in either species, but there are several smooth muscle bundles present in the manatee skin that are not seen in the male elephant skin (Figure-5-7 and 5-9).

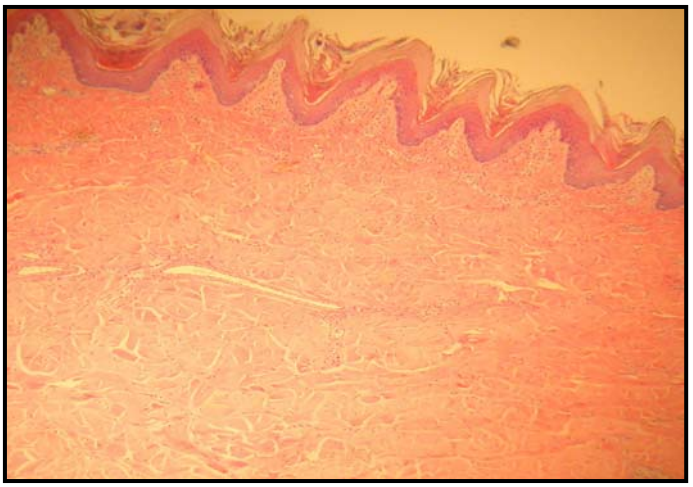

Figure 5-6 Juvenile male elephant, urogenital skin, 20X, H&E, epidermis and dermis.

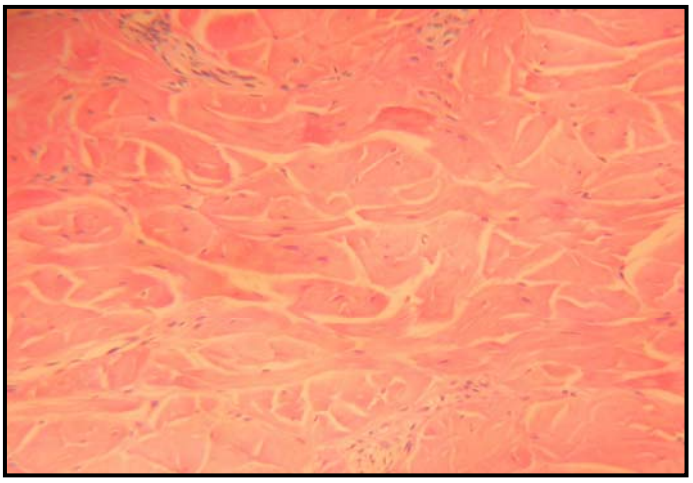

Figure 5-7 Juvenile male elephant, urogenital skin, 100X, H&E, dermis.

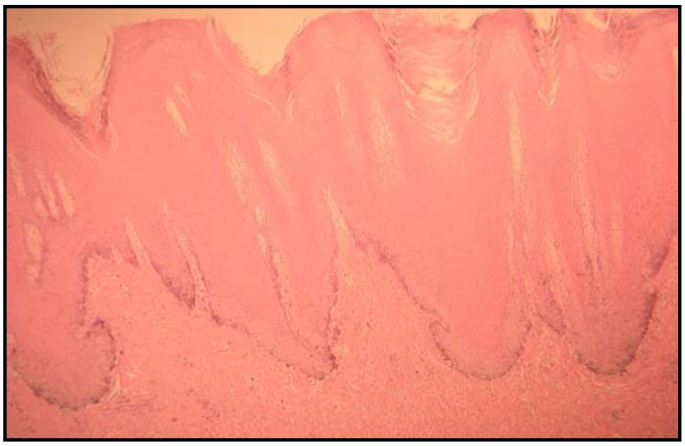

Figure 5-8 Male manatee calf, urogenital skin, 20X, H&E, epidermis and dermis.

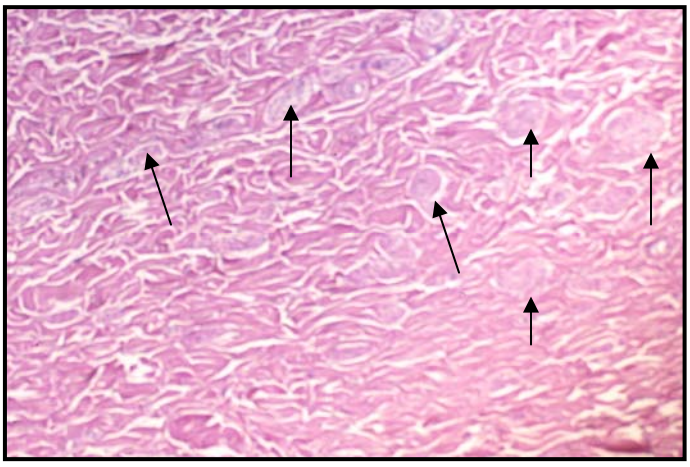

Figure 5-9 Male manatee calf, urogenital skin, 100X, H&E, dermis (arrows point to smooth muscle bundles).

Nail

The organization of the nail and skin associated with the nail of both species is nearly identical (Figure 5-10 and 5-11). The epidermis of the manatee consists of three layers; the stratum corneum, stratum spinosum, and the stratum basale. In the epidermis of the elephant there are the same three layers that the manatee has in addition to a very faint stratum granulosum; that when visible is only one to two layers. The epidermis in both species in this area has an exceptionally thick stratum spinosum and stratum corneum. The epidermis in the elephant is thicker than the manatee. The minimum measurement of the elephant epidermis is 3.5 mm with the thickest measurement being 5.5 mm at the actual nail. The epidermis of the manatee measures 1.3–3.3 mm with the nail measuring 5.5 mm. Both the elephant and the manatee skin near the nail are flat and do not exhibit any prominent undulating ridges. In the adult elephant there are approximately 7 epidermal pegs present per linear 275µm, while the adult manatee has 12. In the juvenile elephant there are 14 epidermal pegs per linear 275µm and 17 in the manatee calf. Melanocytes are present in both species, with the manatee having more melanocytes seen per linear 275µm. The dermis in both species is similar, but the organization of collagen bundles in the manatee is more intricate and density of collagen is greater. The adult elephant dermis is 5.8–11.3 mm, while the adult manatee dermal thickness is 3.5–5.8 mm. Both species have several pacinian corpuscles present in this area of skin and are very vascular (Figure 5-12 and 5-14). The major difference in this area of skin between these two species is the presence of interdigital glands in the elephant skin (Figure 5-13). The glands are arranged into lobules composed of secretory glandular units and ducts and are of the eccrine type.

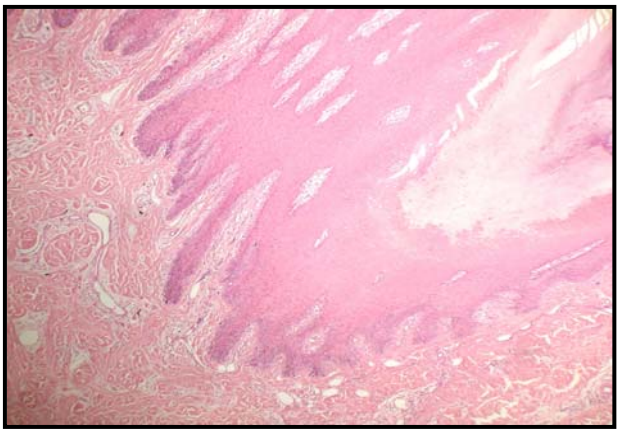

Figure 5-10 Nail of the manatee, MNW0342, female, H&E, 40X.

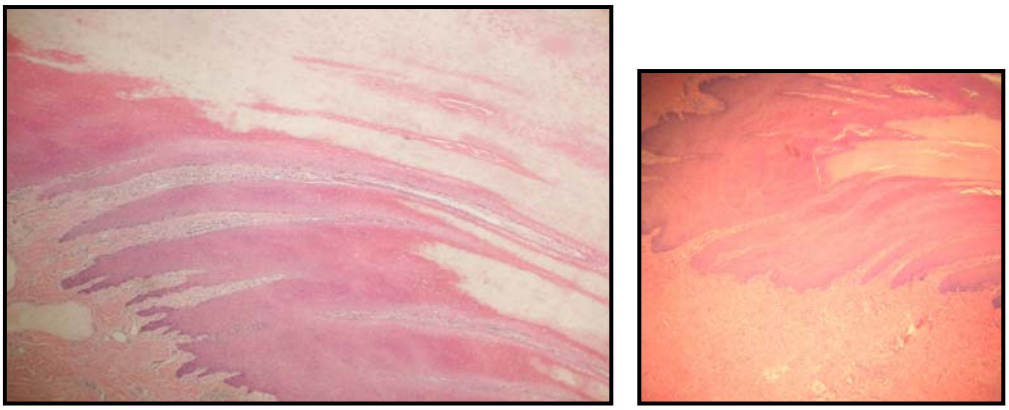

Figure 5-11 Nail of the elephant, female, H&E, 40X.

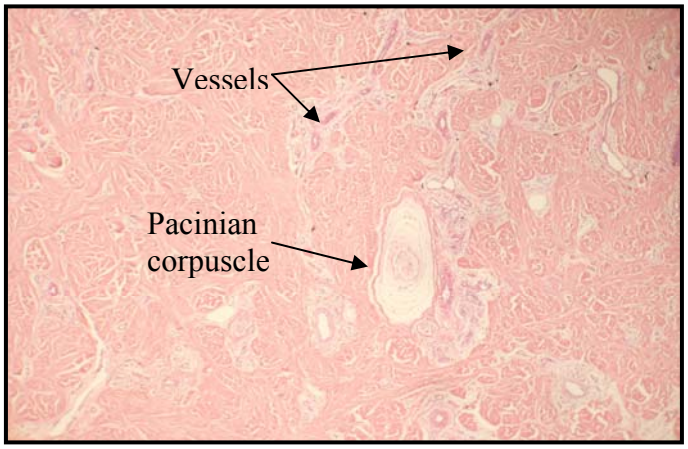

Figure 5-12 Dermis of 5-10, H&E, 40X.

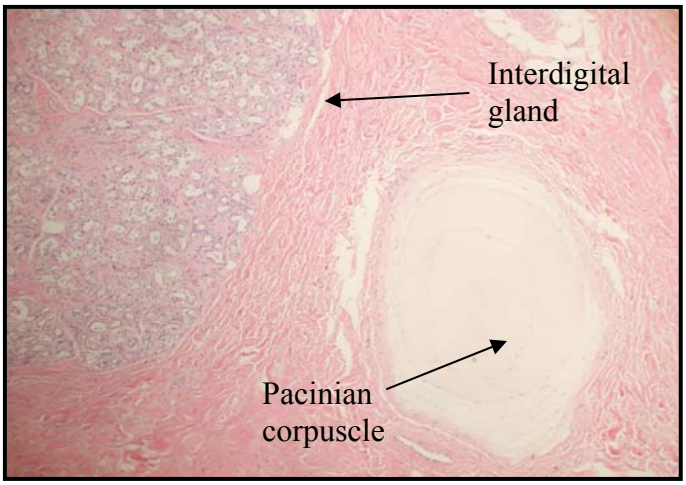

Figure5-13 Dermis of 5-11, H&E, 40X.

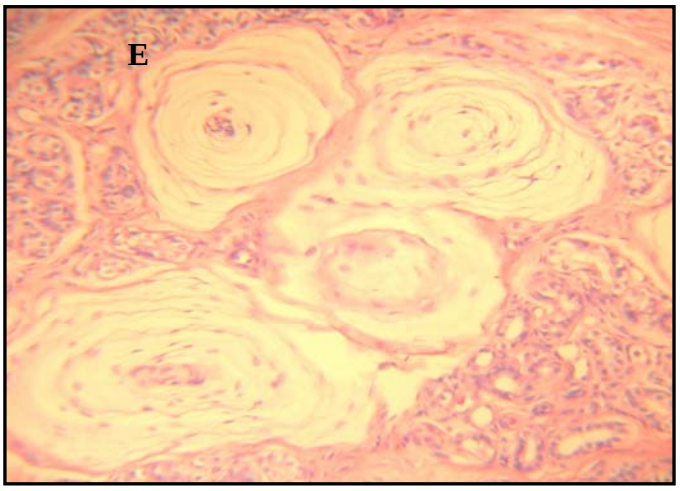

Figure 5-14 Interdigital gland of the elephant, juvenile, male, H&E, 100X. Notice the pacinian corpuscles are surrounded by the interdigital gland.

Back

The manatee epidermis is thicker than that of the elephant in this region. In the manatee the stratum spinosum and stratum corneum are very thick, in the elephant only the stratum corneum is exceptionally thick. The slight presence of a stratum granulosum in the elephant separates it from the manatee's three layers of the epidermis, excluding the stratum granulosum (Figure 5-15 and 5-16). The undulating ridges present in the elephant are more numerous and evenly distanced from one to the next than those of the manatee. There are approximately nine undulating ridges per linear 275µm in the

elephant, and three undulating ridges in the manatee. The number of epidermal pegs is similar in both the manatee and the elephant, both having 14 pegs per linear 275µm. The epidermal pegs of the manatee differ more in depth, shape, and broadness than they do in the elephant. Melanocytes are observed in both species, yet are much more numerous in the manatee. Melanin granules can be seen in the manatee well into the stratum corneum. The dermis of the manatee has a denser weave of collagen fibers than that of the elephant, with the pattern of the collagen bundles being oriented in two directions in the elephant versus three directions in the manatee (Figure 5-17 and 5-18).

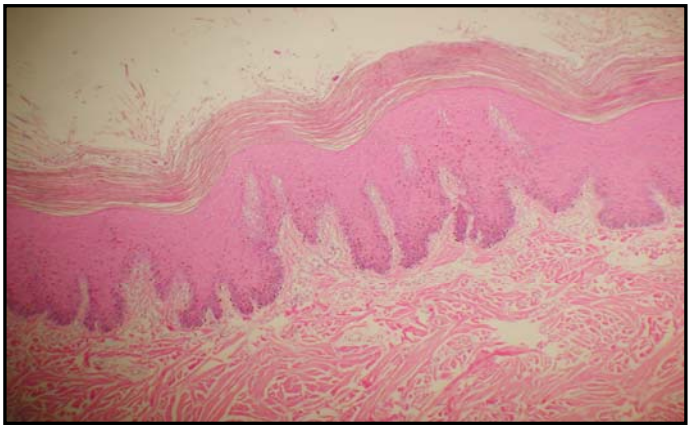

Figure 5-15 Back skin from the manatee, female, H&E, 40X.

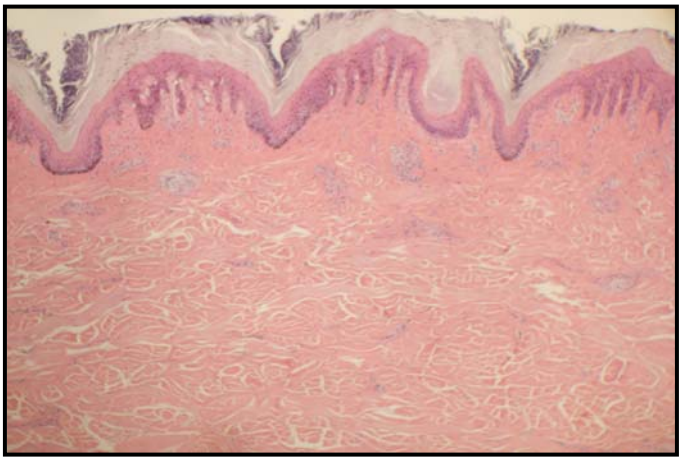

Figure 5-16 Back skin from the elephant, female, H&E, 40X.

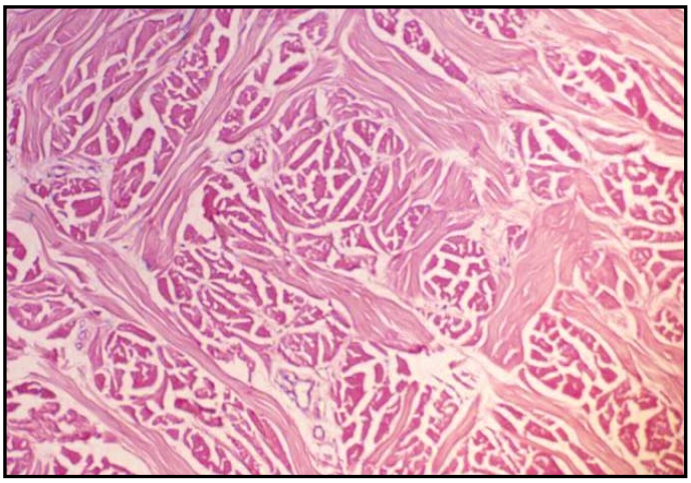

Figure 5-17 Dermis of the manatee back skin from 5-15, H&E, 40X.

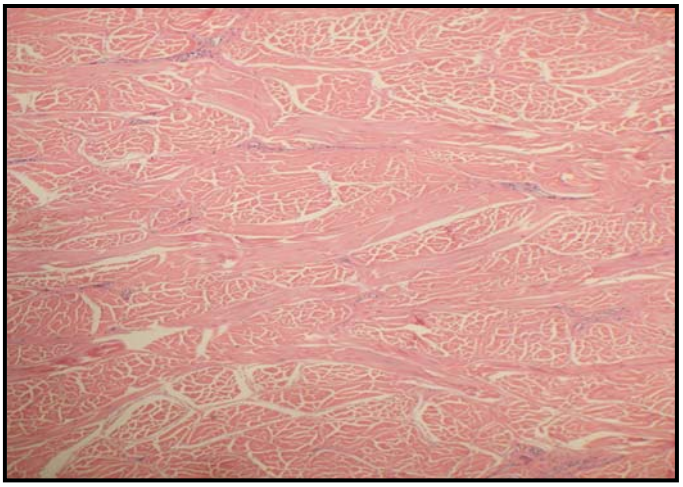

Figure 5-18 Dermis of the elephant back skin from 5-16, H&E, 40X.

Ventral Body

In this region of the body, the manatee epidermis is thicker than that of the elephant. Like other areas, the manatee skin displays a very thick stratum spinosum and stratum corneum, while the elephant skin only exhibits a very thick stratum corneum (Figure 5-19 and 5-20). The manatee epidermis measures 0.4–1.6 mm and the elephant epidermis is 0.2–0.6 mm thick. There are more undulating ridges and epidermal pegs present in the elephant per linear 275µm than in the manatee. The elephant has seven undulating ridges that are uniform in distance from one to the next. By comparison, the undulating ridges of the manatee are irregular in distance from one another and are seen

three per linear 275µm. The epidermal pegs of the elephant are seen at two levels; deep and shallow. The adult elephant contained approximately 21 epidermal pegs per linear 275µm. The epidermal pegs in the manatee are seen at all different levels, consisting of 10 per 275µm. The dermis of the manatee has a dense, intricate weave, whereas the elephant has a collagen framework that consists mainly of the collagen bundles that run parallel with the longitudinal line of the body (Figure 5-21 and 5-22). The dermis of the manatee ranges from 11.8 mm up to 13.0 mm. The minimal dermal measurement of the elephant is 11.4 mm, with the maximum dermal measurement at 12.5 mm.

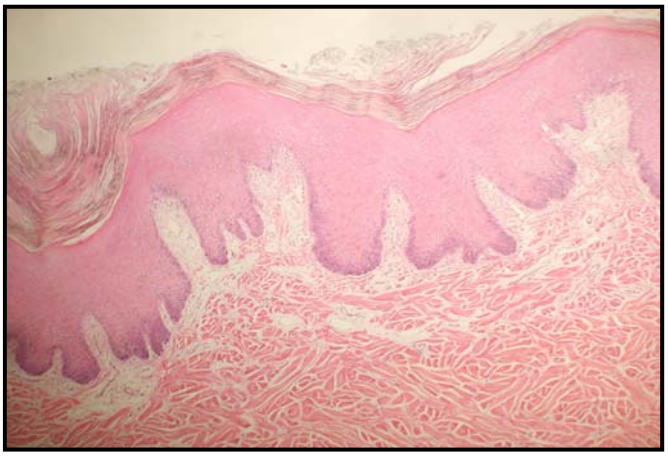

Figure 5-19 Manatee skin, female, H&E, 40X, epidermis.

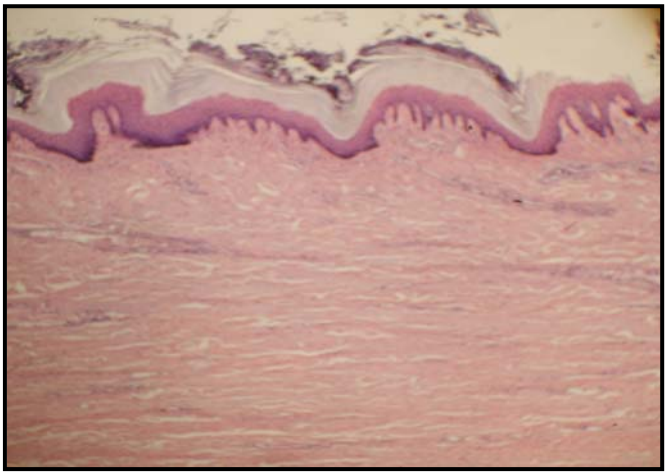

Figure 5-20 Elephant skin, female, H&E, 40X, epidermis.

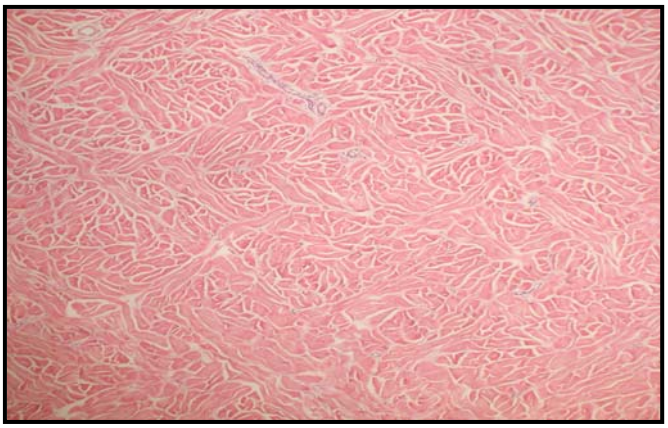

Figure 5-21 Dermis of 5-19, H&E, 40X.

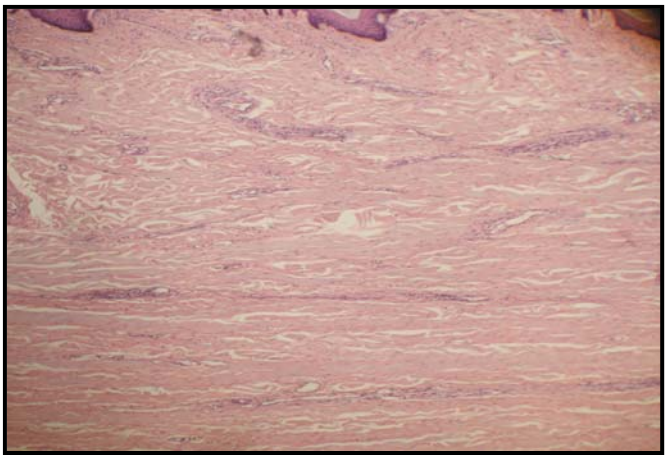

Figure 5-22 Dermis of 5-20, H&E, 40X.

Nostril Skin

The nostril skin of these two species is quite different. The manatee epidermis of this area has three layers; the stratum basale, stratum spinosum, and stratum corneum (Figure 5-23). The elephant skin has four layers; stratum basale, stratum spinosum, stratum granulosum, and stratum corneum. Here the stratum granulosum is easily observed compared to most areas of the elephant skin (Figure 5-28 and 5-29). The epidermis in this area of both species exhibits an exceptionally thick stratum spinosum and stratum corneum (Figure 5-23 and Figure 5-30). The undulating ridges of the

manatee are broad and not well defined. In the elephant they are comprised of sharp peaks and appear well defined.

There are approximately four undulating ridges per linear 275µm in the manatee, and four in the elephant as well. In each animal, the epidermal pegs are both highly irregular in depth and shape. There are 20 epidermal pegs per linear 275µm in the manatee and 14 epidermal pegs present per linear 275µm in the elephant. In both species the inner epidermis lining the nasal passage is thinner than that of the external skin. The undulating ridges are broad and flat, and the stratum corneum is not hyperkeratotic and only exists in as many as 31 cell layers thick. Externally, the manatee stratum corneum is anywhere from 14–108 cell layers thick, while the elephant can be as few as 32, but is mainly very thick, being too compact to count the individual cell layers. Melanocytes are present in this area in both animals, but there are more in the manatee skin. The dermis of these two species is made up of a dense collagen network, that in the manatee is infiltrated with nerves, vessels, and pacinian corpuscles, and bundles of muscles (Figure 5-25 and 5-26). The elephant dermis is also very vascular, pacinian corpuscles are present and are more numerous than in the manatee (Figure 5-30, 5-33, and 5-34). Since the nostril of the elephant is part of the trunk, the dermis is not very thick before it transitions to an enormous weave of muscle (Figure 5-32 and 5-35). Blood sinus hair follicles are found in the nostril of the manatee, these hairs also occur in the nostril of the elephant (Figure 5-26 and Figure 5-31).

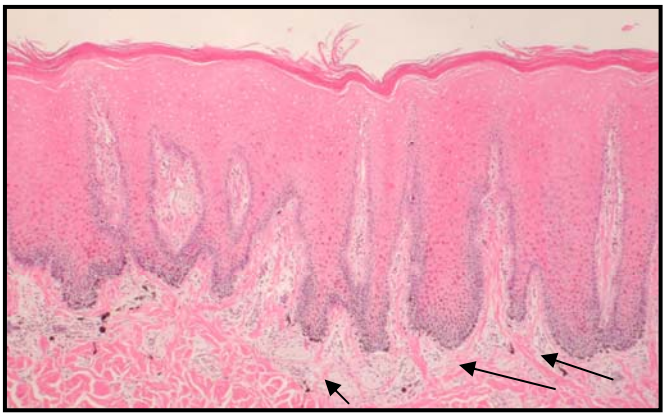

Figure 5-23 Adult manatee, nostril skin, female, H&E, 40X, epidermis.

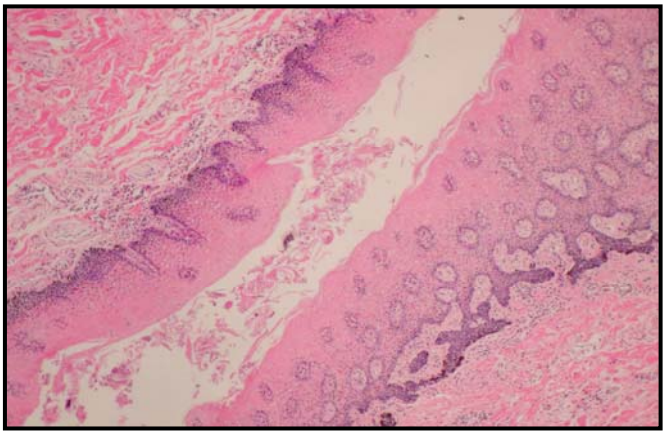

Figure 5-24 Adult manatee, skin lining the nasal passage, female, H&E, 40X.

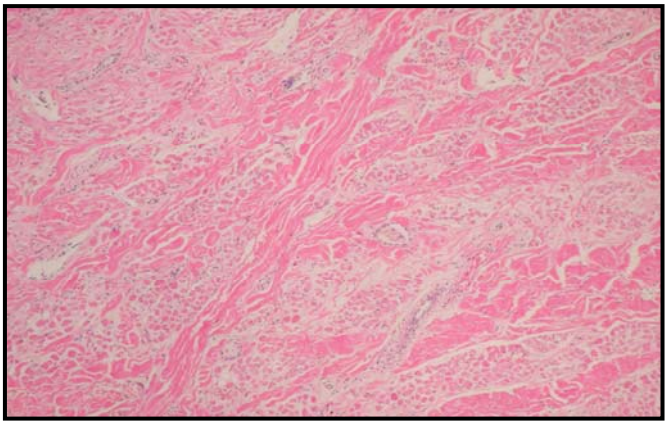

Figure 5-25 Adult manatee, nostril skin, female, H&E, 40X, dermis.

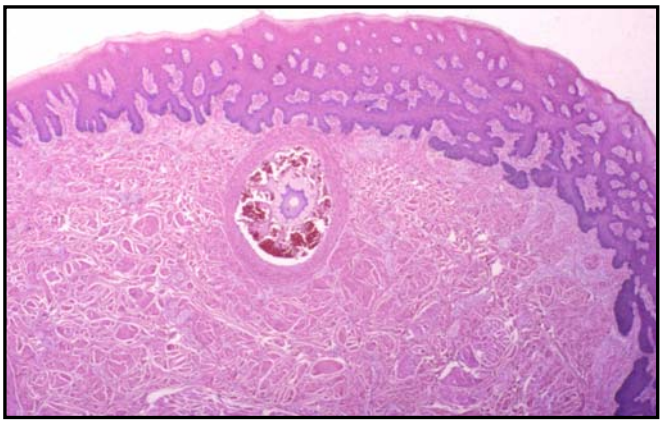

Figure 5-26 Manatee calf, nostril skin, male, H&E, 40X, epidermis and dermis.

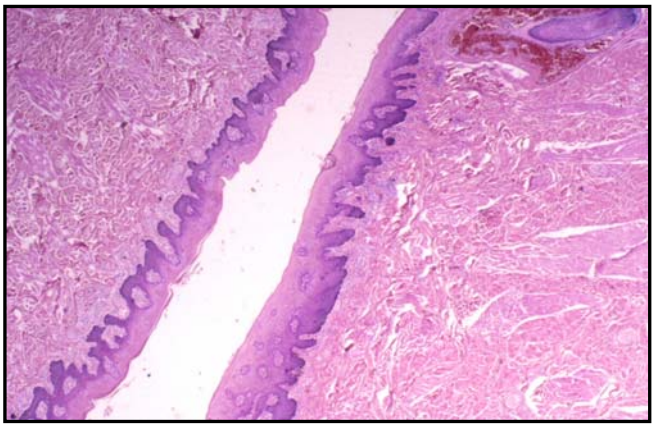

Figure 5-27 Manatee calf, skin lining the nasal passage, male, H&E, 40X, epidermis and dermis.

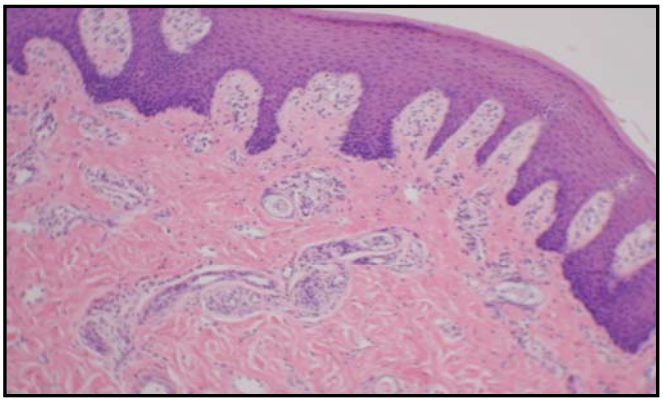

Figure 5-28 Adult interior nostril skin, female, H&E, 40X.

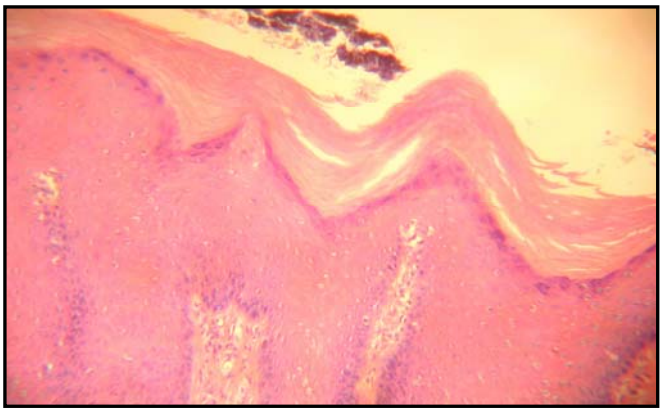

Figure 5-29 Juvenile elephant, external nostril skin, male, H&E, 100X, epidermis with stratum granulosum.

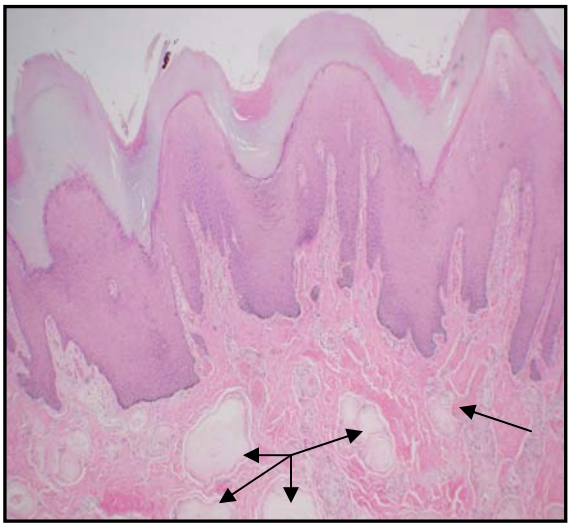

Figure 5-30 Adult elephant, external nostril skin, female, H&E, 40X (arrows point to Pacinian corpuscles).

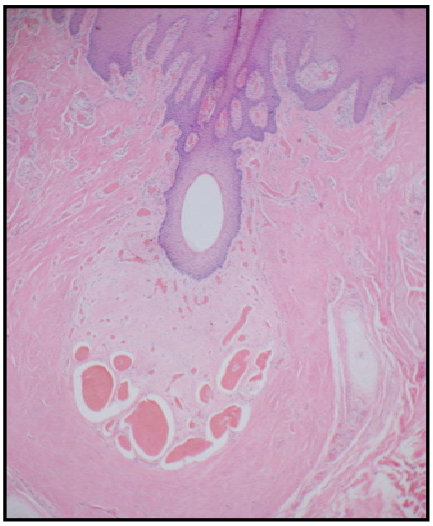

Figure 5-31 Adult elephant, external nostril skin, female, H&E, 40X, blood sinus hair follicle.

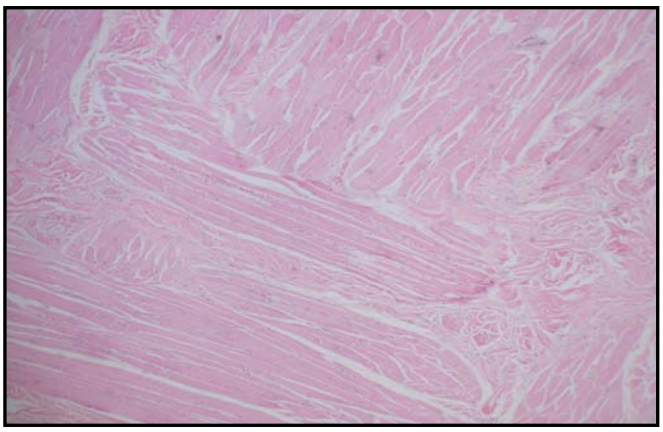

Figure 5-32 Adult elephant, nostril skin, female, H&E, 40X, muscle.

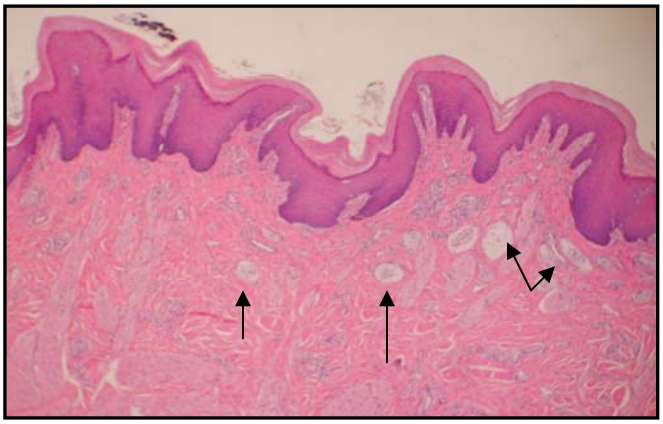

Figure 5-33 Juvenile elephant, external nostril skin, male, H&E, 40X, epidermis and dermis (arrows point to Pacinian corpuscles).

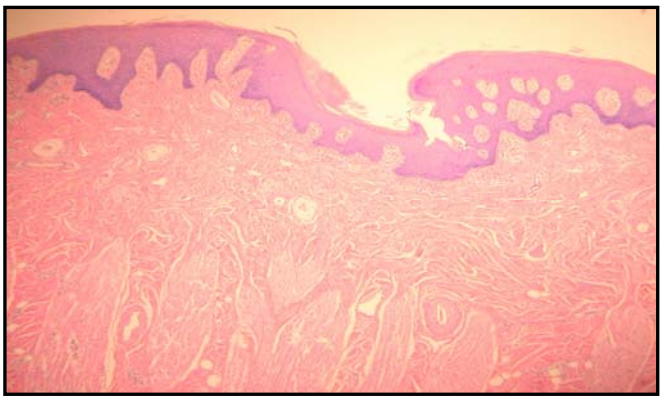

Figure 5-34 Juvenile elephant, skin lining nasal passage, male, H&E, 40X, epidermis and dermis.

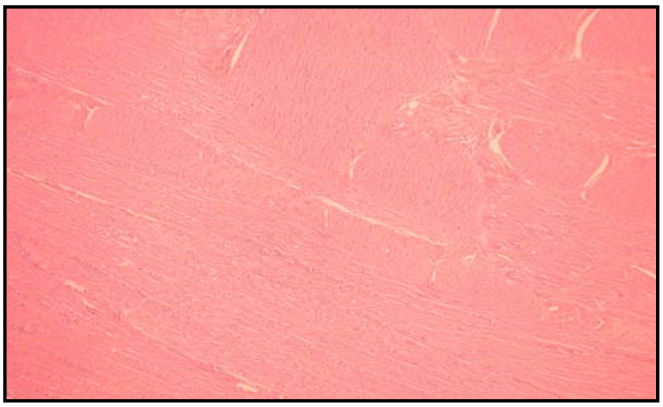

Figure 5-35 Juvenile elephant, nostril skin, male, H&E, 40X, muscle.

Eyelid

The eyelids of the manatee and the elephant are quite different. The manatee eyelid has three layers comprising its epidermis, where the elephant has four. The elephant has several long stiff hairs (eyelashes) that extend from the eyelid and the manatee does not have any hairs associated with the eyelid (Figure 5-36 and 5-39). The manatee eyelid epidermis is thicker than the elephant's eyelid epidermis (Figure 5-36 and 5-39), but the dermis of the elephant eyelid is thicker than the manatee.

The manatee does not have any glands present in the upper dermis near the epidermis, the only glands present are accessory glands deep in the dermis near the conjunctiva (Figure 5-37 and 5-38). The elephant not only has accessory glands located near the conjunctiva but also has well developed sebaceous glands that are associated with the hair follicles in the eyelid (Figure 5-39). Both species do not have a lacrimal gland present in the orbit and upper eyelid. The epidermis of the manatee has a thick stratum spinosum in the eyelid, but the elephant does not. The epidermis of the elephant eyelid measures between 0.13–0.55 mm, and the manatee's eyelid epidermis measures 0.1–1.2 mm in thickness. The irregular organization of the epidermal pegs is similar in both animals. In the elephant there are 19 epidermal pegs per linear 275μm whereas the

manatee has 16 epidermal pegs. The undulating ridges of the manatee eyelid skin are fairly flat and broad, while the elephant undulating ridges are more prominent peaks. The manatee has approximately three undulating ridges present per linear 275µm, and the elephant has six per linear 275µm, which is twice as many as the manatee. Melanocytes are present in both species, but are observed more frequently in the manatee. The dermis of the manatee and elephant differ in development of collagen, being more dense in the manatee dermis than the elephant dermis (Figure 5-37 and 5-39), but both are similar in that neither have any pattern to the network of collagen.

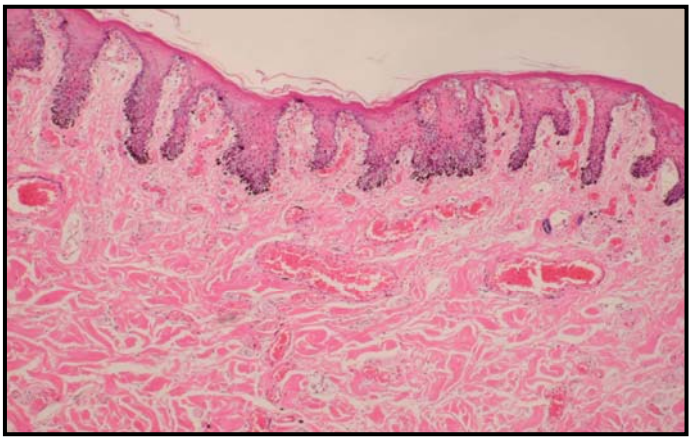

Figure 5-36 Adult manatee, eyelid, female, H&E, 40X, epidermis and dermis.

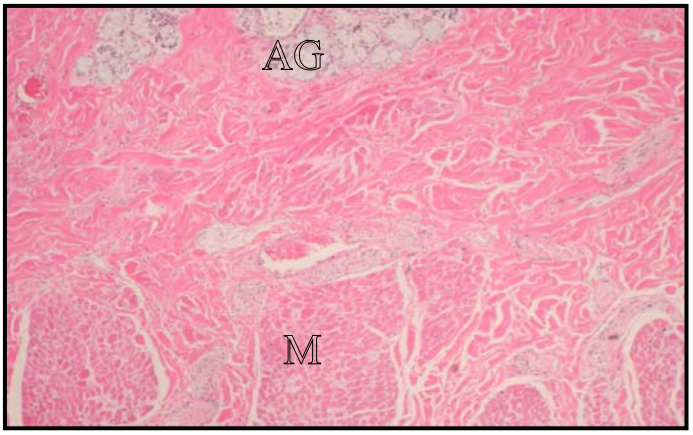

Figure 5-37 Adult manatee, eyelid, female, H&E, 40X, dermis with muscle (M) and accessory glands (AG).

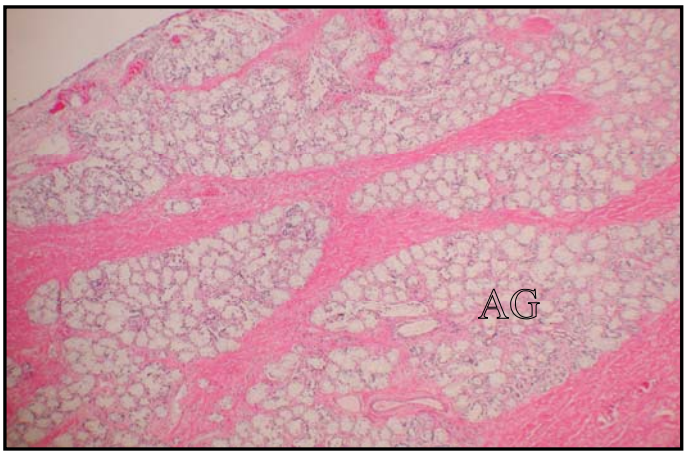

Figure 5-38 Adult manatee, eyelid, female, H&E, 40X, accessory glands (AG) present near the conjunctiva.

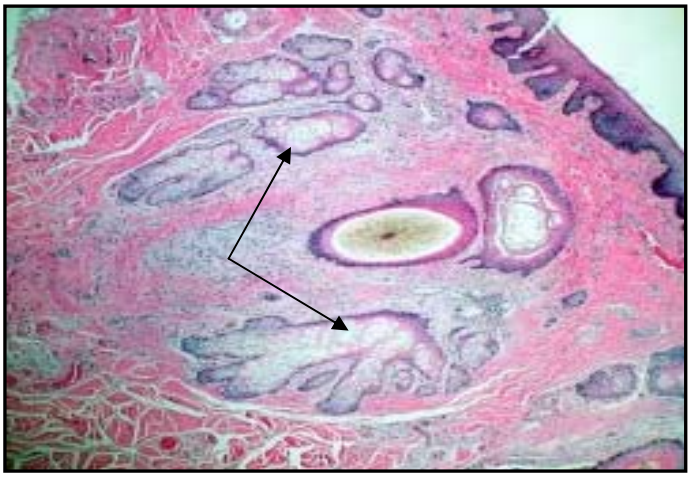

Figure 5-39 Adult elephant, eyelid, female, H&E, 40X, epidermis and dermis with associated sebaceous glands (arrows).

Discussion

Many of the findings in this research on the elephant skin are in agreement with other authors (Shoshani, 1992; Spearman, 1970; Luck and Wright, 1964; Rasmussen, 1996; and Sokolov, 1982). Both the manatee and elephant have glabrous skin. The manatee has many blood sinus hair follicles throughout its body (Reep et al., 2002), but the hair follicles seen throughout the elephant lacks a blood sinus. The only area where the blood sinus hair follicle occurs is on the trunk. Since the blood sinus hair follicle is thought to aid in touch, this occurrence is not unexpected as the elephant's trunk

possesses many functions, such as feeding, sensing and feeling objects, and greet conspecifics. Comparable tactile function in the elephant reinforces the theory of the function of blood sinus hair follicles in the manatee (Reep et al., 2002).

The presence of a stratum granulosum is typical for terrestrial mammals and those marine mammals with a pelage. The lack of a stratum granulosum in the manatee is similar to other marine mammals without a pelage. It was suggested by Spearman (1970), that the elephant stratum corneum contains structural and chemical components which in humans are associated with consistency of corneal cells, and thus thickening of the horny layer occurs. Thickening of the horny layer in conjunction with extensive epidermal proliferation can greatly reduce water loss (Grice and Bettley, 1967). Both animals have an unusually thick epidermis, but the manatee's stratum corneum is thicker than the elephant's stratum corneum. Also, the manatee epidermis exhibits an exceptionally thick stratum spinosum throughout, and the elephant does not. Both of these factors contribute to the thickness of the epidermis. While the elephant is a much larger mammal than the manatee, the manatee epidermis is thicker.

The dermis of the elephant and manatee are very similar in thickness. The manatee's dermis forms different patterns of intricate networks of collagen bundles, whereas the elephant's dermis is simple in organization, being comprised of collagen bundles that are directed in one or two directions. This dense and highly organized dermis of the manatee is most likely due to the aquatic environment that it lives in. The structure of the skin gives the manatee's body its contour. The number of Pacinian corpuscle present in the elephant skin is at least twice that seen in the manatee. An interesting finding is that the mechanoreceptive, Pacinian corpuscles are found in the

same areas in both species, principally the nail and nostril skin. Their presence suggests that these areas may be important in touch sensation.

The undulating ridge- epidermal peg organization is similar in both animals, although it is more irregular in the manatee. This organization increases the surface area of the mitotic stratum basale, and increases the contact between the epidermis and dermis, therefore strengthening the skin (Wright and Luck, 1984). The numerous dermal papillae each have capillary loops within them. The vascularization close to the skin surface increases the circulation, and therefore increases thermoregulation and hydration. According to Wright and Luck (1984), the dermal papillae with large surface area of the basal layer and its proximity to the capillary circulation will ensure a fluid driving force to maintain hydration.

Glands exist in the manatee only in the eyelid, and the elephant has glands in the eyelid as well as a gland associated with the foot. This gland is called the interdigital gland, and is thought to function as a sweat gland, producing perspiration that accumulates onto the cuticle (Lamps et al., 2001). The manatee and elephant have very similar skin coloration, yet the manatee skin possesses more pigmentation than the skin of the elephant. There are several factors that influence the amount of pigment, such as hormones, ultraviolet light, inflammation, and friction, all being able to increase pigmentation. Some factors that inhibit pigmentation are an excess/deficiency of chemicals in the body, genetics, hormones, physical destruction through injury, and immune mediated destruction of melanocytes. Which of these factors accounts for the difference in amount of melanocytes between these two species remains to be known.

CHAPTER 6
ELECTRON MICROSCOPY RESULTS AND DISSCUSION

A dorsal skin sample (sample site 1) was processed and embedded in plastic for transmission electron microscopy evaluation. In Figure 6-1, two portions of the epidermis are seen, the stratum basale and stratum spinosum. It is the cells of the stratum spinosum that will continue on and become keratinized, eventually forming the stratum corneum (Figure 6-2). As the cells move upward, they accumulate more keratin until it replaces all metabolically active cytoplasm. The keratinocytes in the stratum spinosum are attached to one another by the cell junctions, desmosomes (Figure 6-3) and contain several melanin granules. The melanin granules aggregate above the nucleus forming "nuclear caps" (Figure 6-4 and 6-5). As the keratinocytes progress towards the stratum corneum they become flattened in shape and tonofilaments become more numerous and prominent forming a well-developed meshwork (Figure 6-6). These closely stacked, well-developed intercellular bridges outline the periphery of the cells of the stratum spinosum (Figure 6-6). There was no stratum granulosum detected in any of the samples evaluated by light microscopy; this was confirmed through electron microscopy (Figure 6-7 and 6-8). There were a few cells with keratohyalin-like granules at the transmission electron microscopy level with concomitant degeneration of the nucleus (Figure 6-9). But there is no presence of a stratum granulosum layer. The stratum corneum is extremely compact, having a complex end-to-end interdigitation of adjacent cornified cells, with sparse lipid droplets throughout (Figure 6-5 and 6-6), and, in this sample, the presence of fungi (Figure 6-10 and 6-11). Although melanocytes were not observed by ultrastructure, in

one micron sections these cells were visible, and some were even seen at the stage where the melanin granules had been dispersed (Figure 6-12).

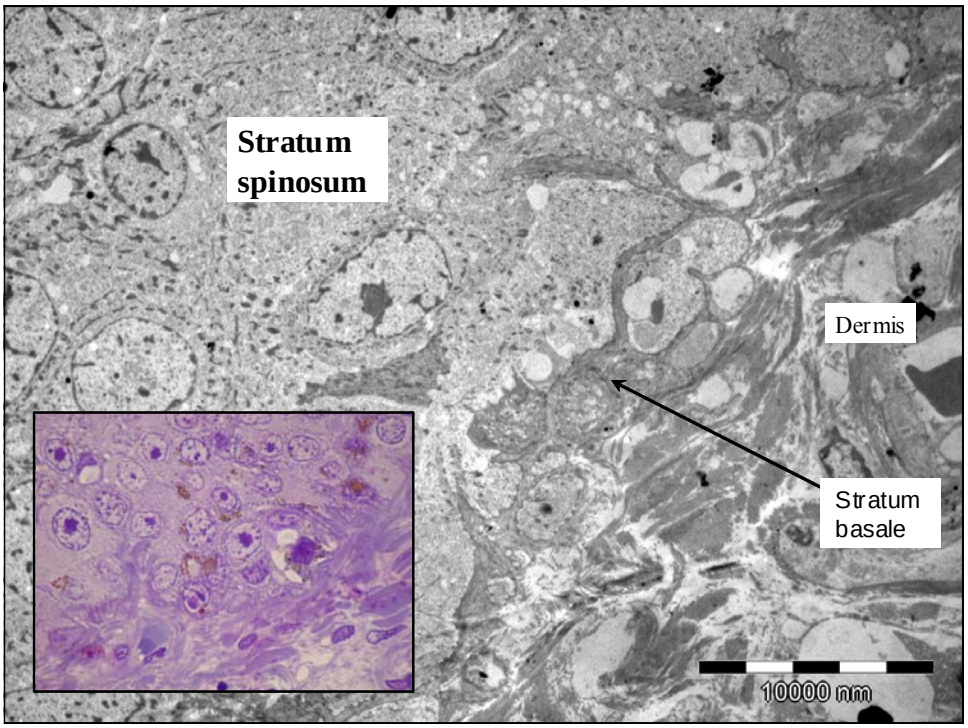

Figure 6-1. Electron micrograph, MSW03169, junction of the epidermis and dermis with the stratum basale and stratum spinosum present. Insert: Photomicrograph, 1µ plastic section, Methylene Blue, 1000X, junction of epidermis and dermis.

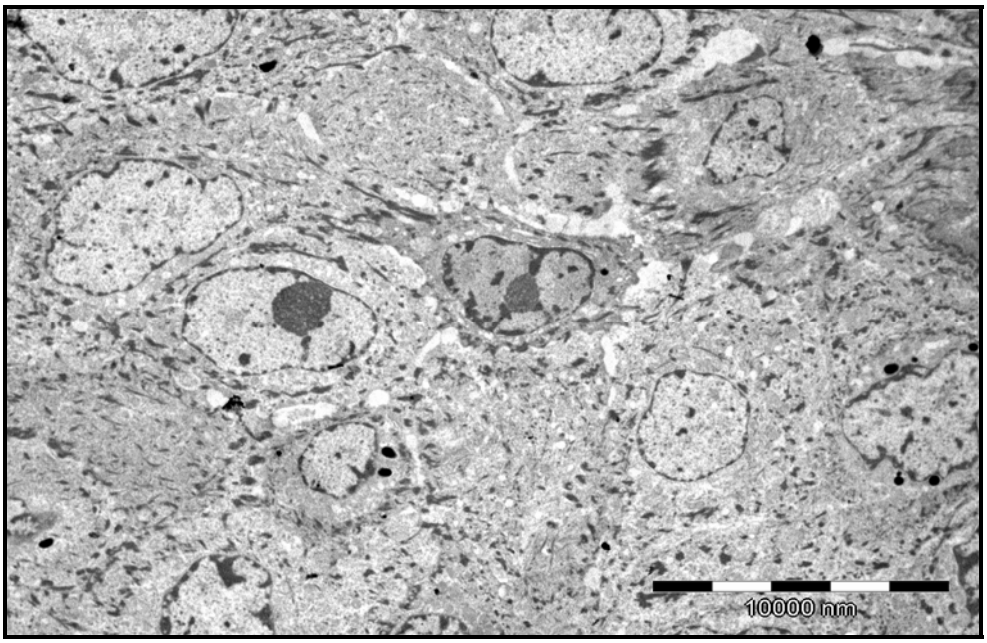

Figure 6-2. Low power electron micrograph. MSW03169, keratinocytes within the stratum spinosum.

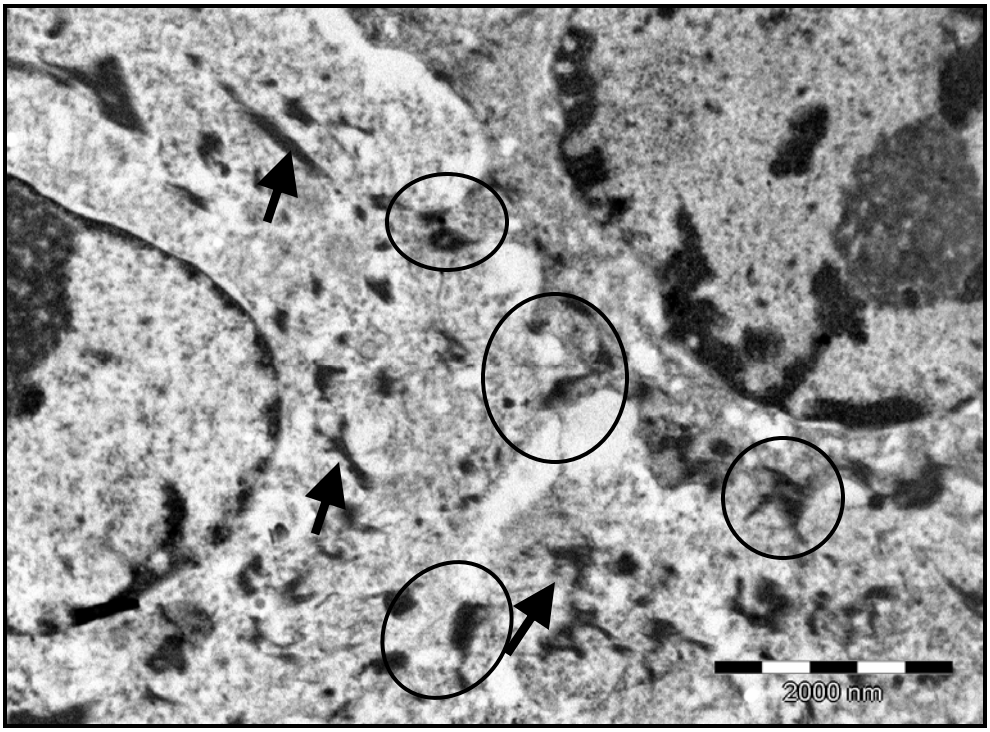

Figure 6-3. High power electron micrograph, MSW03169, close up of Figure 6-2. Notice the distinct cell junction, marked by the numerous tonofilaments (arrows) and desmosomes (circles).

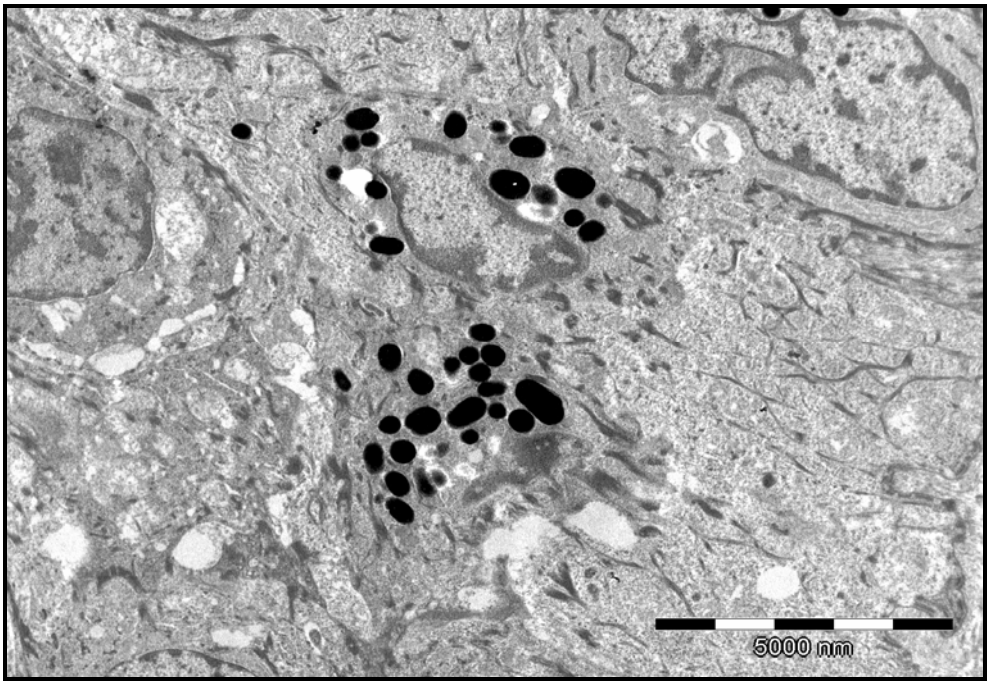

Figure 6-4. Low power electron micrograph, MSW03169, aggregates of melanin forming nuclear caps in keratinocytes.

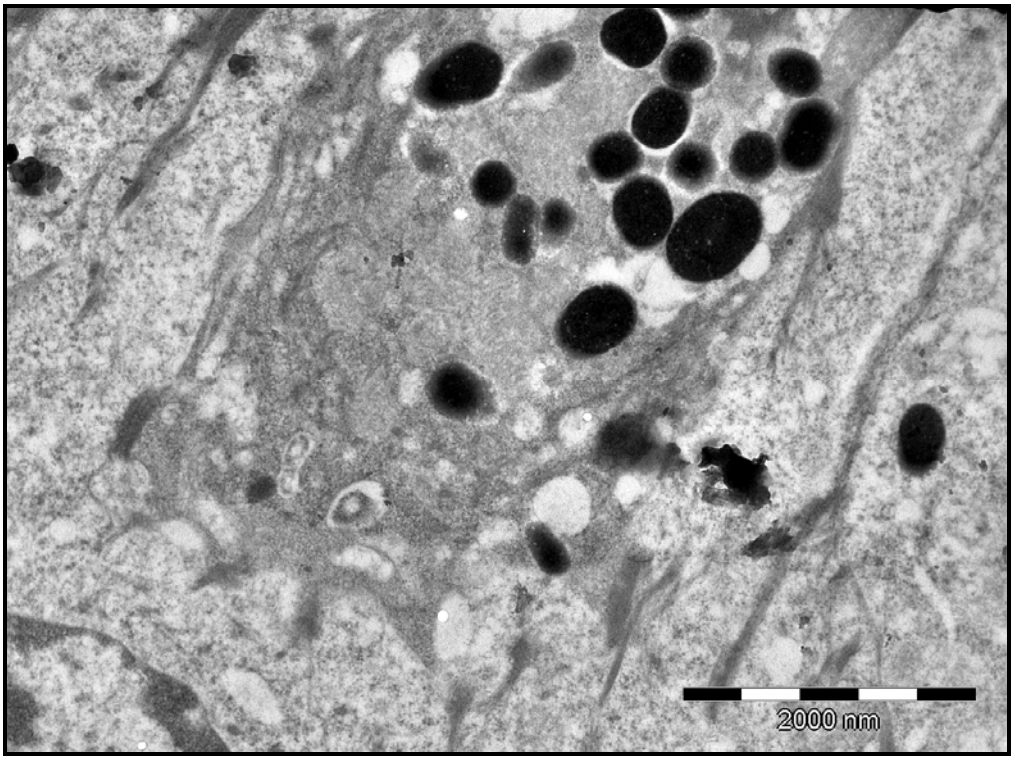

Figure 6-5. High power electron micrograph, MSW03169, higher magnification of a keratinocyte with nuclear cap of melanin.

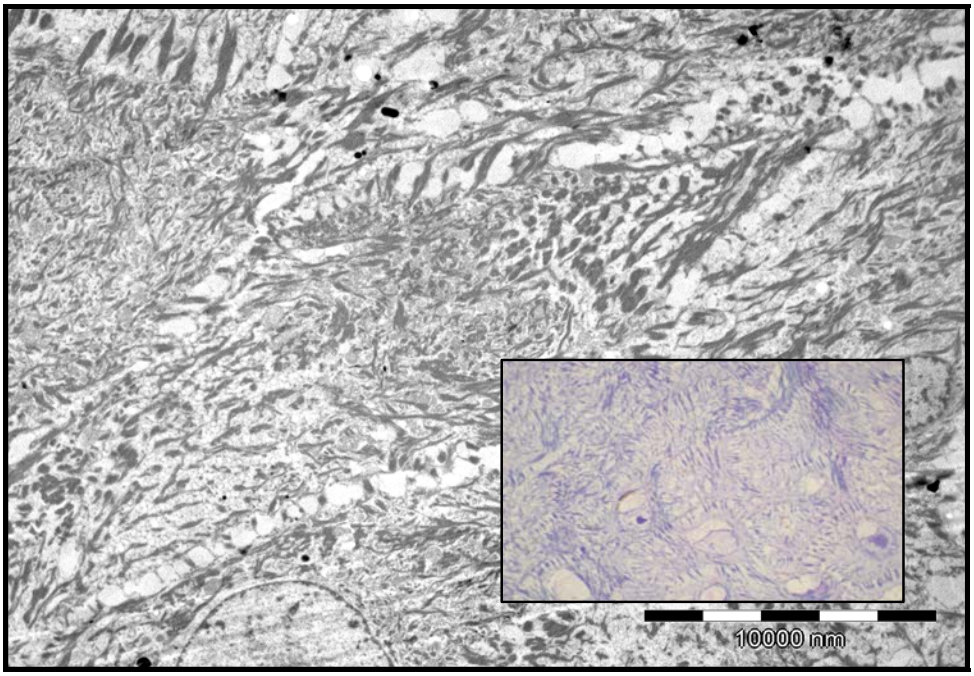

Figure 6-6. Low power electron micrograph, MSW03169, keratinocyte in the upper stratum spinosum. Insert: 1µ plastic section, Methylene blue, 1000X, upper stratum spinosum.

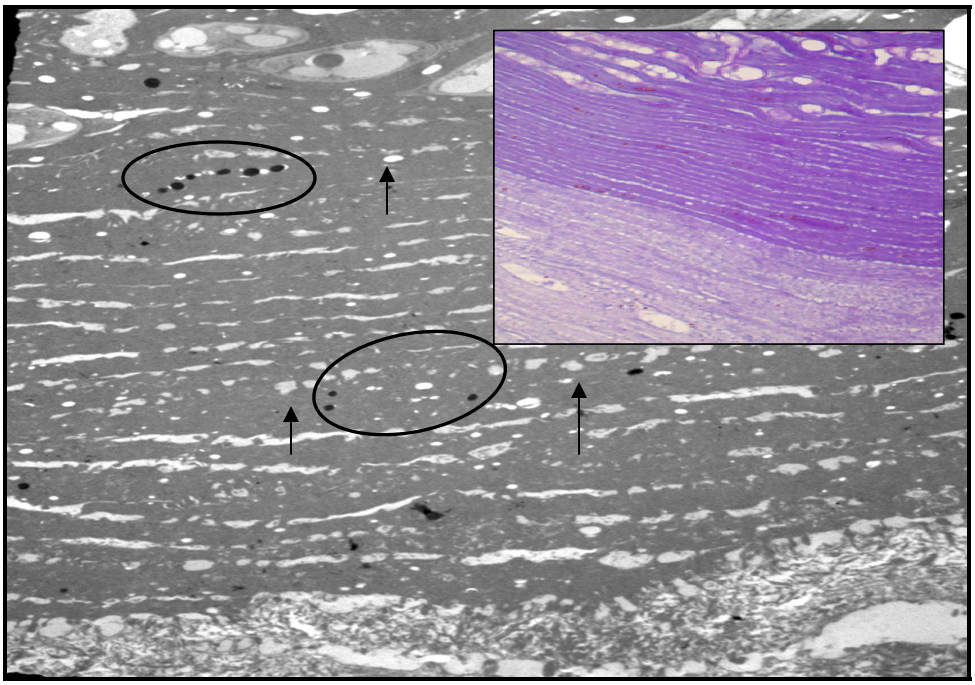

Figure 6-7. Low power electron micrograph, MSW03169, junction of the stratum spinosum and stratum corneum. Notice the lipid droplets (arrows) and melanin (circle) present in the stratum corneum. Insert: 1µ plastic section, Methylene Blue, 1000X, junction of stratum spinosum and stratum corneum.

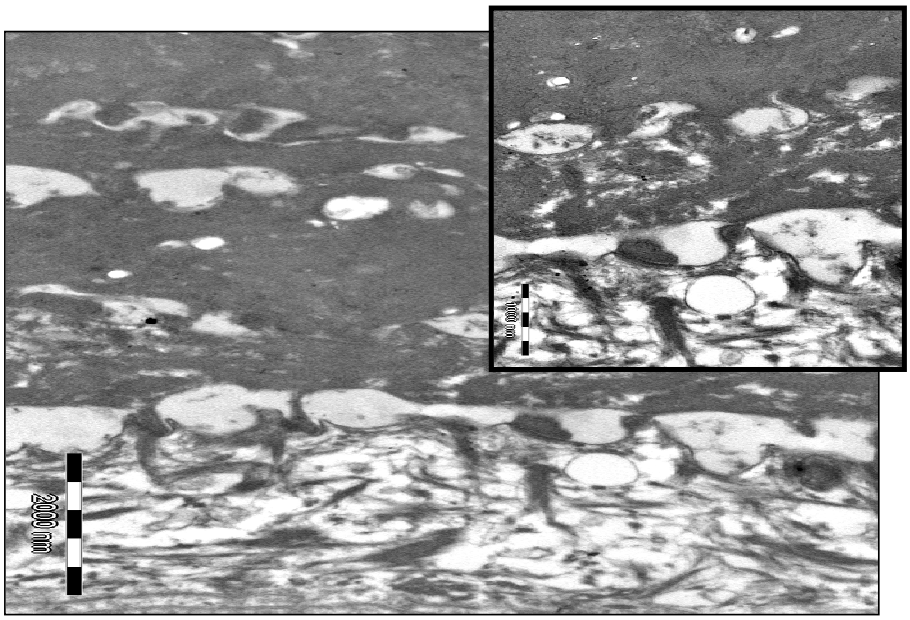

Figure 6-8. Higher power electron micrograph of figure 6-7. Notice there is no presence of a stratum granulosum at the junction. Insert: Higher magnification.

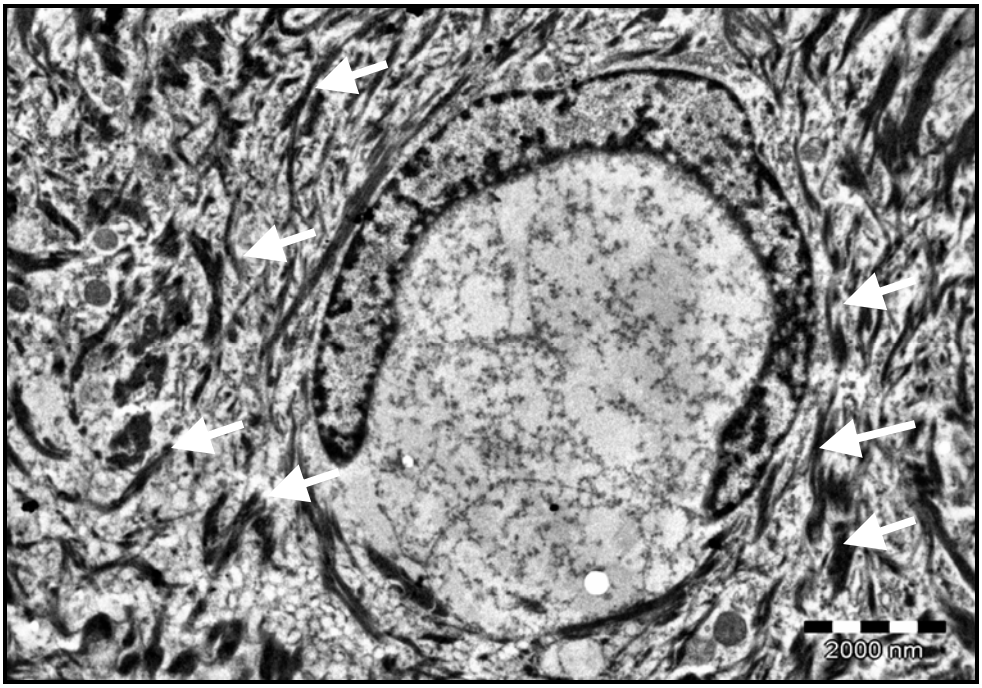

Figure 6-9. High power electron micrograph, MSW03169, cell present in upper stratum spinosum with keratohylain-like granules. Notice the numerous tonofibrils (arrows).

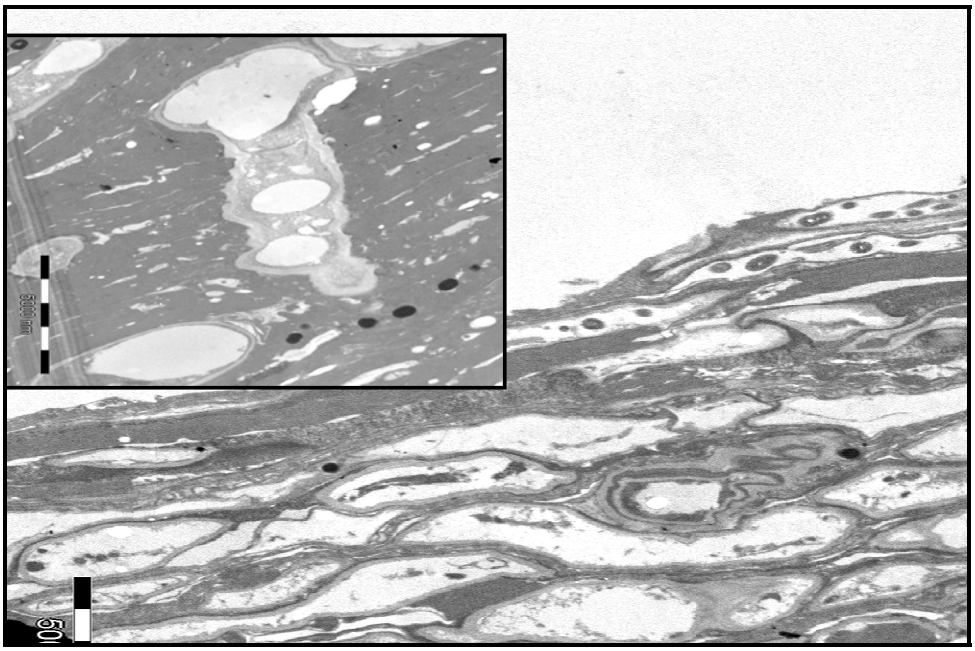

Figure 6-10. High power electron micrograph, MSW03169, outermost layers of the stratum corneum invaded by fungi. Insert: Higher magnification of fungi penetrating the layers of the stratum corneum.

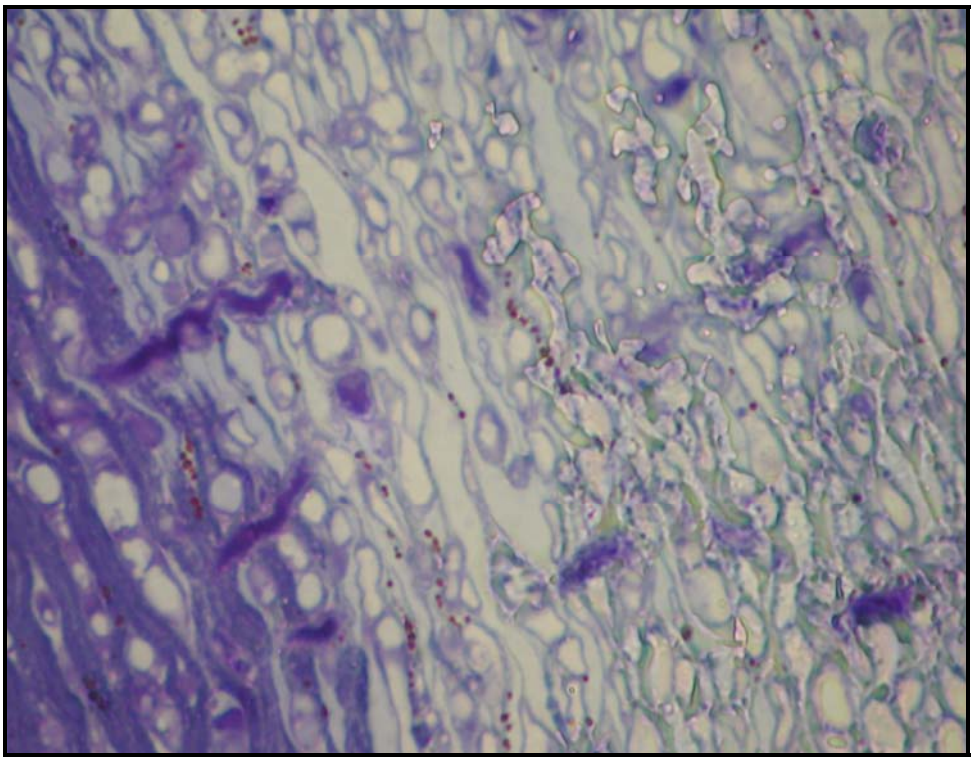

Figure 6-11. Photomicrograph, MSW03169, 1µ plastic section, Methylene Blue, 1000X, stratum corneum invaded by fungi.

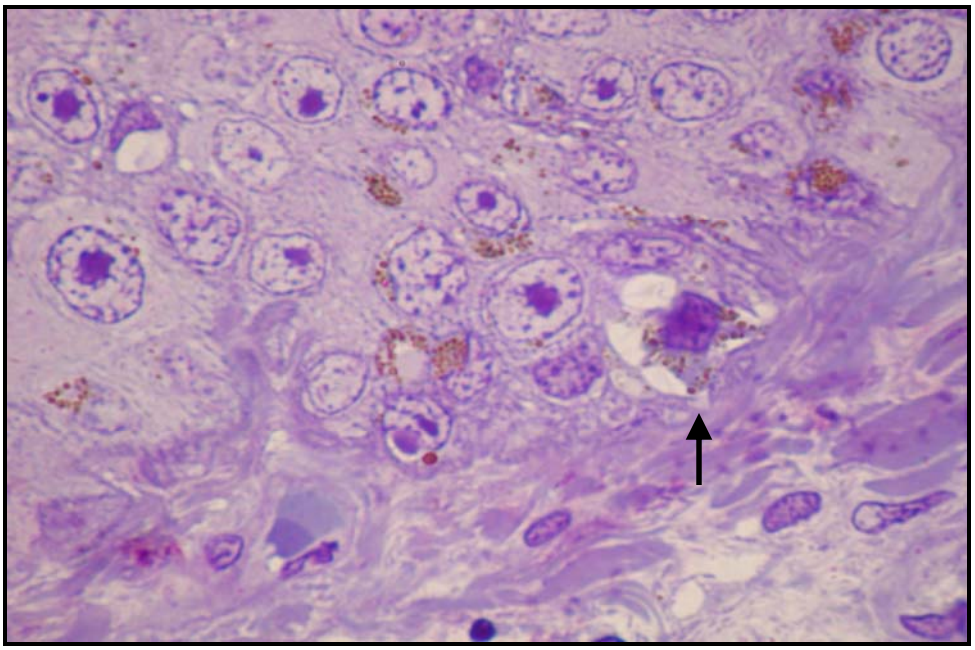

Figure 6-12 Photomicrograph, MSW03169, 1µ plastic section, 1000X, junction of the stratum basale and the dermis with some stratum spinosum present. The melanocyte indicated by the arrow has most of the melanin within dispersed.

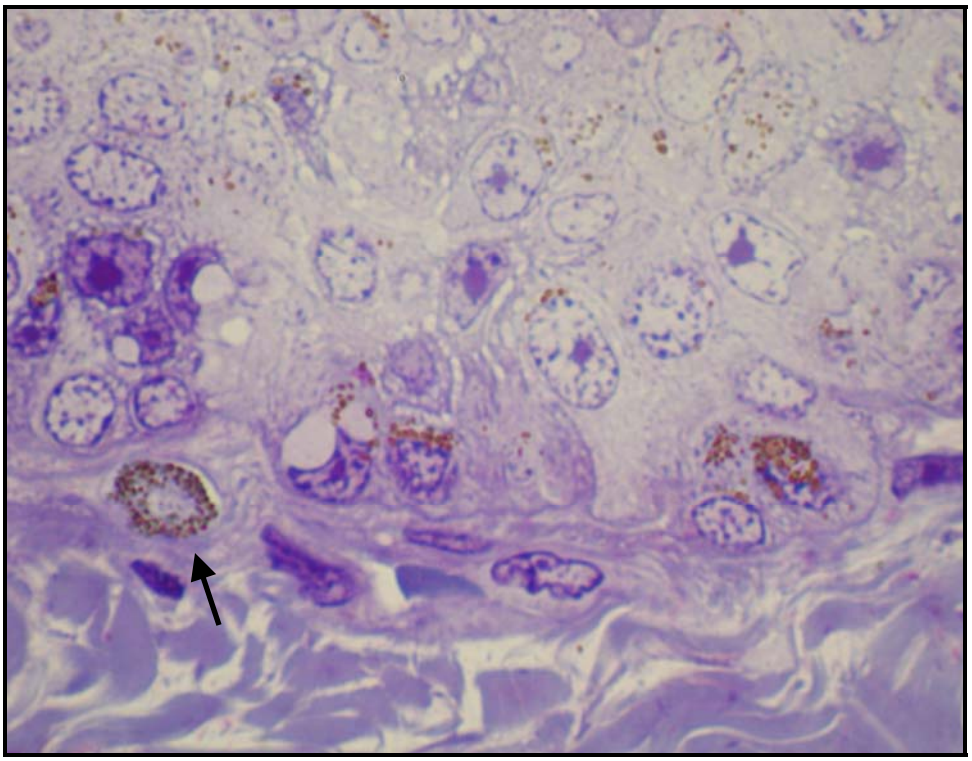

Figure 6-13. Photomicrograph, MSW03169, 1µ plastic section, 1000X, junction of the stratum basale and the dermis with some stratum spinosum present. The melanocyte indicated by the arrow, the melanin has not yet been dispersed.

The purpose of performing electron microscopy was to examine stages of keratinization in the absence of a stratum granulosum in the manatee skin. Even though the stratum granulosum was not detectable through routine light microscopy staining, it was important to obtain certainty that this was not due to chemical properties or thickness of sectioning. Research on cetacean skin, by Geraci (1986), which included transmission electron microscopy, revealed that there was no stratum granulosum seen in *Tursiops truncatus.* As in the manatee, there were a few sparse cells that contained keratohyalin-like granules.

One main difference in the electron microscopy between these two species was that the dolphin stratum corneum contained far more lipid droplets than in the manatee. The function of the numerous lipid droplets in the stratum corneum of the dolphin is not fully understood, but this epidermal layer does serve as a permeability barrier, which is thought to prevent the dolphin from transcutaneous salt-loading (Geraci et al., 1986). The intracellular spaces of the stratum corneum appear electron-lucent and empty, but these spaces actually contain material which corresponds to the surfaces of apposing membranes, known as glycocalyx (Holbrook, 1987 and Fritsch, 1975). The glycocalyx is visible at the light microscopy level by staining samples with Periodic Acid Schiff's stain (Fritsch, 1975) and was visible in the manatee skin samples. Electron microscopy of mouse, guinea pig and human skin showed similar cellular organization and characteristics that were found in the manatee (Odland and Reed, 1967). The major ultrastructural difference was the presence of a stratum granulosum and keratohyalin granules. Further electron microscopy would need to be performed to determine whether the keratohyalin-like granules seen in the manatee are truly

keratohyalin granules.

Lamellar bodies are commonly found in the keratinocytes of the epidermis and function to produce an enzyme that triggers the stratum corneum to be shed. The lamellar bodies were not seen in the manatee skin sampled, further electron microscopy on more samples at higher magnification would be necessary to determine if lamellar bodies are present in the epidermis of the manatee. If they are not present, then the manatee skin does not have the proper enzymes to shed its skin. This information would help to explain why the manatee retains its stratum corneum.

CHAPTER 7
IMMUNOHISTOCHEMISTRY OF NORMAL AND WOUNDED MANATEE SKIN

Ageing of wounds by histology and immunohistochemical methods has been done for many years. Unfortunately, there is much biological variability which introduces uncertainty. While probabilities can be suggested, a definitive timeline that can be applied universally is at this time, unobtainable. The smaller the animal, the more rapid the wound healing process, and more primitive animals have been known to demonstrate far greater powers of regeneration than that of man (Knight, 1991).

The major phases of the wound healing process in mammals consists of the inflammatory stage, proliferative stage, and remodeling stage. In the inflammatory stage neutrophils and monocytes are the first cells to migrate to the wound site after the blood clot is formed. This migration is induced by several cytokines and growth factors, including platelet-derived growth factor and interleukin-1 (IL-1). Interleukin-1 is stored in the epidermis of the intact skin and released post-wounding (Moulin, 1995). During this stage the neutrophils and monocyte-derived macrophages eliminate bacteria and cell and matrix debris by phagocytosis and secretion of proteases (Kiritsy, 1993). Neutrophils have been shown to be a source of pro-inflammatory cytokines that serve as some of the earliest signals to activate local fibroblasts and keratinocytes (Martin, 1997).

The proliferative stage of wound healing includes re-epithelialization, angiogenesis, and fibroplasias, which results in the development of granulation tissue. Fibroblasts have been noted to enter the wound between 48 and 72 hours (Clark, 1988). The fibroblast serves several functions and concominantly undergoes several associated

phenotypic changes during the course of healing (Kirsner, 1993). Fibroblasts are responsible for wound contraction, but must undergo a phenotypic change for this to occur. The fibroblasts become activated and transform into myofibroblasts. The main feature of the myofibroblast is its contractile apparatus which is similar to that of smooth muscle (Gabbiani et al., 1971), involving in particular the expression of smooth muscle α- actin (Hinz, 2001). Wound contraction occurs as early as three days post-injury (Bertone, 1989) but can occur as late as nine days (Swaim, 1990). Once scar tissue has formed, myofibroblasts expressing smooth muscle α- actin disappear, most likely as a result of apoptosis (Desmouliere, 1995).

Various members of the matrix metalloproteinase (MMP) family are upregulated during the wound healing process. MMPs are produced by several different types of cells in skin including fibroblasts, keratinocytes, macrophages, endothelial cells, mast cells, and eosinophils (Lobmann et al., 2002). MMPs are not constitutively expressed in skin but are induced temporarily in response to signals such as cytokines and growth factors. MMPs participate in the removal of devitalized tissue, angiogenesis, contraction of wound matrix, and migration of fibroblasts, and synthesis of new connective tissue (Lobmann, 2002). MMPs that are prominent in injured skin are not present in normal skin (Parks, 1999). In a study involving wound healing using a rat model, the peak of matrix metalloproteinase-9 was observed at early timepoints (within 48 hours after injury) and then decreased rapidly thereafter, having been consistent with an acute inflammatory response (Paul et al., 1997). In contrast, matrix metalloproteinase-2 was seen later (during day 3, after wounding) and then decreased gradually, having been consistent with its role as one of the predominant enzymes involved in the remodeling process (Paul et al, 1997).

Based on the properties and functions of the matrixmetalloproteinases and smooth muscle actin reported in the wound healing processes in other mammals, and the timeline associated with them, it was decided to try to localize for MMP-2, MMP-9, and smooth muscle actin in the wounds of the manatee. A timeline of the wound healing process of the manatee would provide crucial information to be able to age wounds for forensic purposes, and to understand the process to try to help those manatees in rehabilitation..

Results

Wounds used for immunohistochemistry in this study were aged by a pathologist. It was determined, based on histology, that there were three different ages of wounds present in two different manatees. One of the wounded manatees had an acute wound (12–24 hours old) on its dorsum, and also had suffered from an old boat strike on its dorsum, that had resolved and formed scars. The other wounded manatee had sub-acute (24–48 hours old) wounds present on its dorsum. Based on previous studies, normal skin should not localize for MMP-2, MMP-9, or smooth muscle α- actin localization (except in the vasculature) and therefore normal skin from the dorsum of the manatee was included.

Matrix metalloproteinase-9 was localized in only one section of an acute wound (Figure 7-1). This reaction was faint but positive. No localization was observed in the sub-acute wounds, scars (Figure 7-2), or normal skin (Figure 7-3). Matrix metalloproteinase-2 localized in the same acute wound that localized for MMP-9 (Figure 7-4), but the reaction was stronger than that of MMP-9. MMP-2 also localized in the sub-acute wounds (Figure 7-5), but did not localize in the scarred or normal skin samples (Figure 7-6).

The presence of smooth muscle α-actin was detected in the acute (Figure 7-7) and

subacute wounds (Figure 7-8). The localization was present in the fibroblasts, which with the expression of smooth muscle α-actin, have most likely become myofibroblasts. In the scarred skin sample and normal skin samples, the only localization was in the vasculature (Figure 7-9 and 7-10).

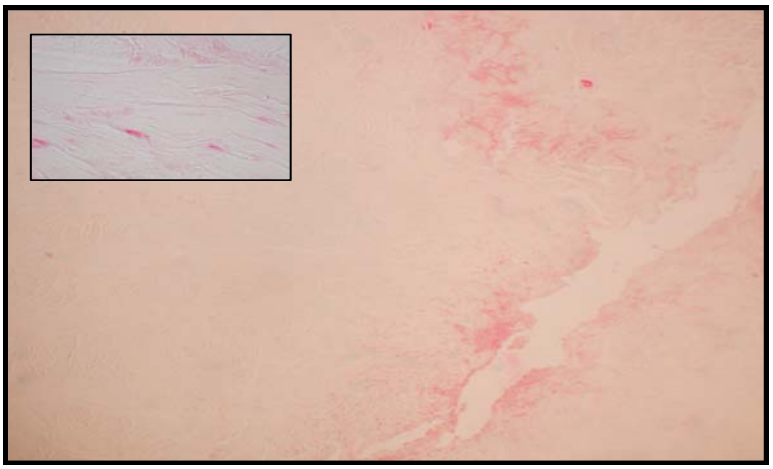

Figure 7-1. MEC0348, adult male, acute wound, MMP-9 localization, 40X. Insert: 1000X magnification showing cellular localization.

Figure 7-2. MEC0348, adult male, scar, MMP-9 localization, 40X. Notice that there is no localization present and the brown seen is pigment.

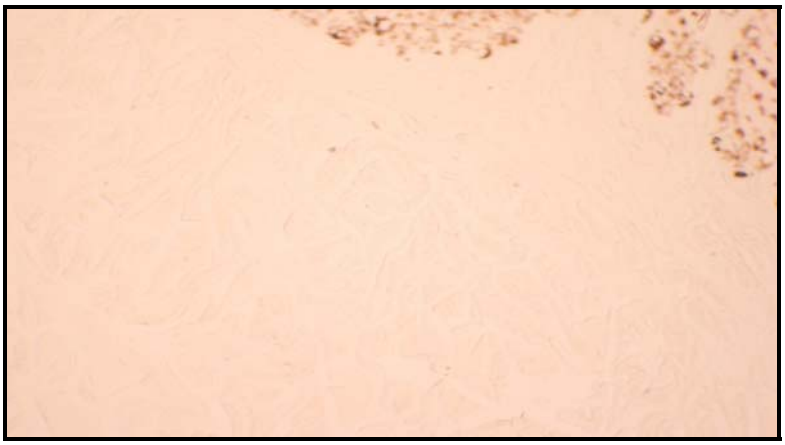

Figure 7-3. MEC0348, adult male, normal skin, MMP-9, 200X, no localization present. The brown color seen is melanin granules in the epidermis.

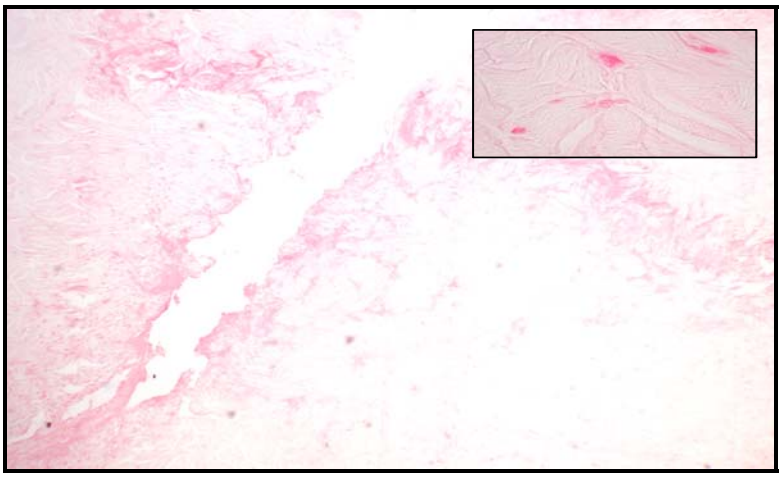

Figure 7-4. MEC0348, adult male, acute wound, MMP-2 localization, 40X Insert:1000X, cellular localization.

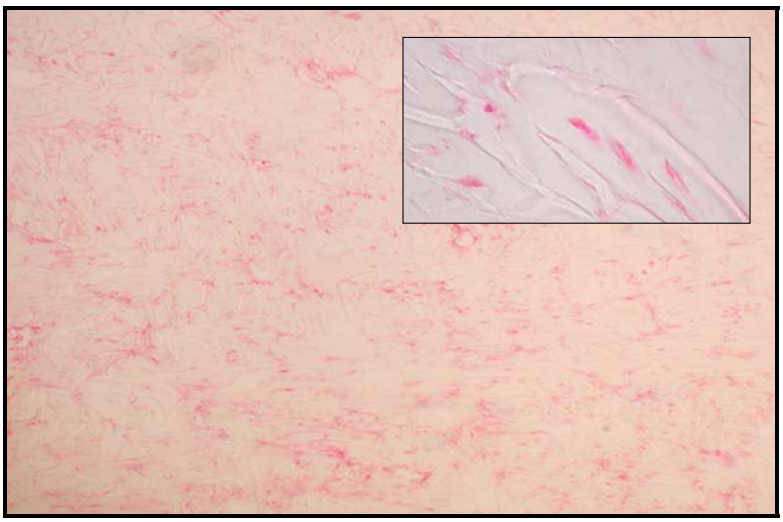

Figure 7-5. LPZ101763, adult female, sub-acute wound, MMP-2 localization, 40X. Insert: 1000X cellular localization.

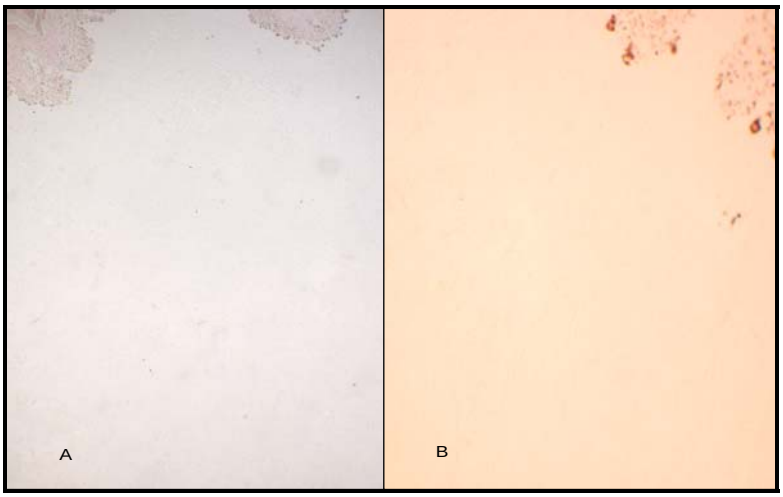

Figure 7-6. MEC0348, adult male, A-normal skin, B-scar, MMP-2 localization, 40X, notice there is no localization observed. Pigmentation present from epidermis.

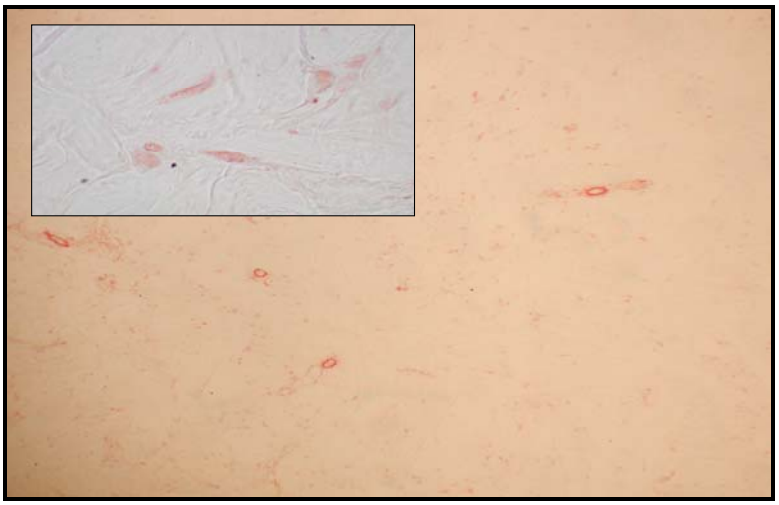

Figure 7-7. MEC0348, adult male, acute wound, α-SMA localization, 40X. Insert: 1000X, notice that the strongest localization is within the vasculature present, but there are also several positively localized cells as well.

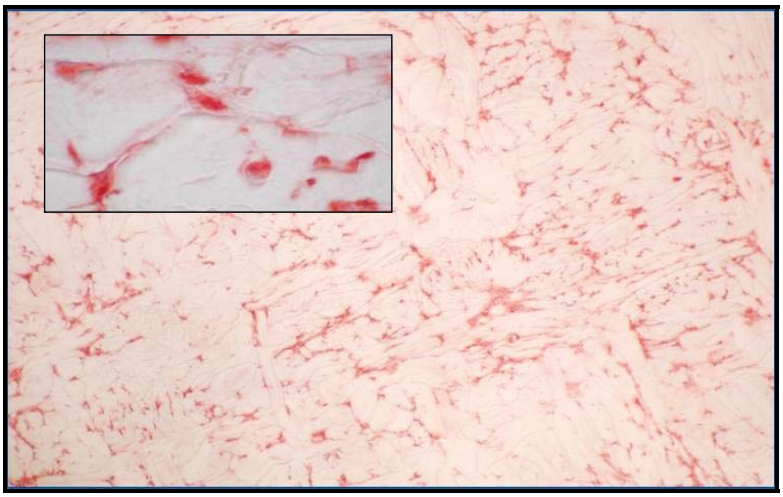

Figure 7-8. LPZ101763, adult female, sub-acute wound, α-SMA localization, 40X. Insert: 1000X, showing cellular expression.

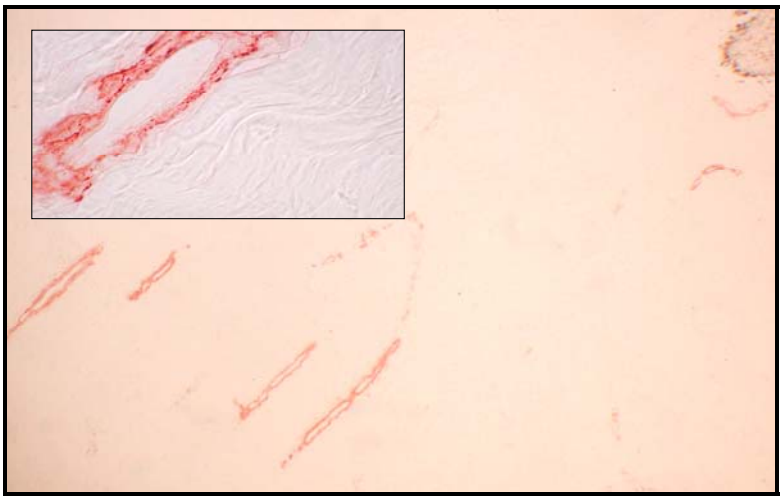

Figure 7-9. MEC0348, adult male, scar, α-SMA localization, 40X. Notice that the only localization is in the vessels. Also the vessels seen here are perpendicular to the epidermis, typical morphology of healed wound vasculature. Insert:1000X.

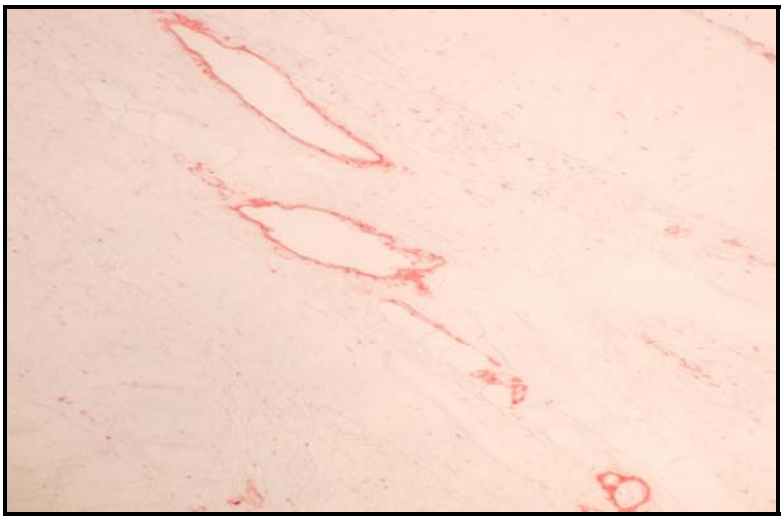

Figure 7-10. MEC0348, adult male, normal skin (site 1), α-SMA localization, 40X, α-SMA is only found in the vasculature in the normal skin.

Discussion

The immunohistochemistry performed in this study is preliminary. More research needs to be performed to determine whether the use of immunohistochemistry would be a way to determine age of wounds in the manatee. The pathological aging of the acute and sub-acute wounds does not completely coincide with the immunohistochemical results. The acute wound with its weak presence of MMP-9, along with the localization of MMP-2 appears to be slightly older than the 12–24 hours old time frame given based on pathological assessment. Previous studies suggest MMP-9 localization peaks around 48 hours (Parks et al., 1997). In the present wound the presence of MMP-9 appears to be decreasing based on the stronger localization of MMP-2. The presence of smooth muscle α-actin was detected in this wound, which also suggests that the wound is older than 12–24 hours.

In the sub-acute histologically aged wound, only the presence of MMP-2 and smooth muscle α-actin were detected, which collectively suggests that the wound is slightly older than 24–48 hours. In the scarred and normal skin samples there was no

detection of MMP-9, MMP-2, and the only localization of smooth muscle α-actin was in the vasculature. These findings were consistent not only with histological aging but also previous studies in other animal models.

Based on the preliminary results of this research, more work needs to be completed to determine proper aging of manatee wounds. Based on standard mammalian criteria, the morphology of the tissue and character of the associated inflammation the wounds were aged histologically. It is not known how closely these criteria correlate with the manatee wound healing process. It is possible that the manatee wound healing process varies slightly from other mammals due to the difference in pathological aging and immunohistochemical localization. Further research that includes more samples from a wider range of wound healing stages is required to arrive at an understanding of the aging of manatee wounds by histological and immuohistochemical methods.

Conclusions

Manatee skin is different from other marine mammals mainly with regard to the excessive thickness of the stratum corneum. The skin of the manatee is very different from terrestrial mammals. When comparing the two, the manatee skin would be considered to exhibit hyperplasia and hyperkeratosis. After examining the manatee skin it is clear that these characteristics are normal for the manatee. The integument of the manatee varies by region of the body in epidermal and dermal thickness, collagen organization, and amount of elastin fibers and vasculature present. There are no major differences between males and females, but there are slight variations between age groups which consist of dermal/epidermal thickness, and numbers of epidermal pegs and rete ridges per linear 275µm. In the adult the thickness of the epidermis and dermis is

thicker, and there are more rete ridges and epidermal pegs present in the juvenile. Except for the fluke and the pectoral flippers a hypodermis is present throughout the integument of the manatee. The manatee skin is an opportunistic environment for algae, bacteria, and other microorganisms to flourish on. One probable cause for this might be that the manatee has an extremely slow skin sloughing cycle which allows these organisms to live on the manatee for quite some time. Further research on the skin sloughing cycle of the manatee would be of interest and help to determine its length. Although fungi were seen in normal skin samples with no inflammation, further research is needed to determine whether it is normal for fungi to inhabit the stratum corneum of the manatee or whether there may be an underlying issue involving compromised health (i.e., cold stress). Further investigation on specific dermatitis of the skin of the manatee, especially those in captivity, need to be performed in the future to determine how prevalent they are in the manatee.

The morphology of the manatee skin appears to provide proper protection for the potentially abrasive environment that it inhabits. The thick dermis of the skin provides appropriate structure and support for its body shape. The pressure of an aquatic environment requires a thick integument to give marine mammals the support needed. The unique and intricate collagen network in different regions of the manatee integument probably serves to aid in the function of movement. The dense collagen bundles combined with the complex weaving, found in the flippers and fluke, makes these areas more rigid so as to propel the manatee more effectively through water and aid in pushing off from the bottom after resting. The areas of the eyelid, nostril and urogenital skin do not have any comparable type of organization among the collagen bundles. These areas

are sensory areas that require the skin to stretch (breathing, opening and closing eyelid, giving birth, mating) and for this reason most likely lack an intricate collagen network similar to the rest of the body.

Wounds analyzed in this research provided preliminary results. Further efforts in researching the wound healing process of the manatee is needed for a timeline of this process to be determined, for wounds to be properly aged. Histological and immunohistochemical evaluation of the wounds examined showed a difference in the potential age of the wound. Histological evaluation determined the ages of the wounds at an earlier estimation than the immunohistochemical results, which suggested the ages of the wounds were a few days later in the wound healing process. Additional research using many stages of the wound healing process need to be collected and analyzed by histological and immunohistochemical methods to try to determine a timeline to further understand the manatee's wound healing process.

APPENDIX A
PROCESSING AND STAINING PROCEDURES

TRANSMISSION ELECTRON MICROSCOPY PROCESSING SCHEDULE MANUAL

Working phosphate buffer, 3 changes .....15 minutes each
Osmium tetroxide, 1.0% phosphate buffered ..... 1 hour
Distilled water, 4 changes……15 minutes each
Uranyl acetate, 1% aqueous..... 1 hour
50% ethyl alcohol .....15 minutes
75% ethyl alcohol .....15 minutes
95% ethyl alcohol .....15 minutes
100% (absolute) ethyl alcohol, 4 changes.....15 minutes each
Equal parts 100% ethyl alcohol and propylene oxide....15 minutes
Propylene oxide, 4 changes.....15 minutes each
Equal parts propylene oxide and epoxy resin ..... 1 hour
Epoxy resin, 3 changes ……..1 hour each
Epoxy resin .....2 hours
Embed

PARAFFIN EMBEDDED TISSUE PROCESSING SCHEDULE AUTO

80% alcohol. . . . . .1 hour
95% alcohol, 3 changes. . . . . .1 hour each
100% alcohol, 3 changes. . . . . .1 hour each
Xylene, 3 changes. . . . . .1 hour each
Paraffin, 3 changes. . . . . ..1 hour each
Paraffin, under vacuum. . . . . .1 hour
Embed.

Masson's Trichrome (modified)

Deparaffinize and hydrate to water		
Bouin's solution (In oven at 56°C)	1 change	60 minutes
Running tap water 1 change until yellow disappears		
Weigert's iron hematoxylin	1 change	20 minutes
Tap water rinse		
Biebrich's Scarlet-Acid fuschin	1 change	1 minute
Rinse in water		
5% phosphotungstic acid	1 change	4 minutes
Light green solution	1 change	2 minutes
Rinse in water		
0.5% glacial acetic acid water	1 change	2 minutes
Distilled water rinse		
95% ethanol	2 changes	2 minutes
100% ethanol	2 changes	2 minutes
Xylene	3 changes	2 minutes
Mount with Fisher Scientific Mounting Media		

H&E

Deparaffinize and hydrate to water		
Harris' hematoxylin	1 change	6–9 minutes
Tap water rinse	1 change	10 minutes
Acid alcohol differentiation		
Tap water rinse	1 change	5 minutes
Ammonia water rinse		
Running tap water	1 change	10 minutes
Dip in 95% alcohol	1 change	30 seconds
Eosin	1 change	30 seconds–2 minutes
95% ethanol	2 changes	2 minutes
100% ethanol	2 changes	2 minutes
Xylene	3 changes	5 minutes
Mount with Fisher Scientific Mounting Media		

PAS

Deparaffinize and hydrate to water		
.5% Periodic Acid	1 change	5 minutes
Running tap water	1 change	5 minutes
Schiff Reagent	1 change	15 minutes
Running tap water	1 change	10 minutes
Harris Hematoxylin	1 change	3 minutes

Running tap water	1 change	5 minutes
Differentiate in:		
1 % acid alcohol	1 change	1 dip
Tap water rinse		
Blue in ammonia water		
Running tap water 1 change		
95% ethanol	2 changes	
100% ethanol	2 changes	
Xylene	3 changes	
Mount with Fisher Scientific Mounting Media		

Luna's Method for Mast Cells

Deparaffinize and hydrate to 95% alcohol		
Aldehyde Fuschin solution	1 change	30 minutes
Rinse 95% alcohol		
Weigert's iron hematoxylin working solution	1 change	1 minute
Running water	1 change	10 minutes
Rinse 95% alcohol		
Counterstain Methyl Orange	1 change	5 minutes
95% ethyl alcohol	2 changes	2 minutes
100% ethyl alcohol	2 changes	2 minutes
Xylene	2 changes	2 minutes
Mount with Fisher Scientific Mounting Media		

Gaffney's One-Hour Giemsa

Deparaffinize and hydrate to water.		
Geimsa working solution	1 change	60 minutes
100% alcohol	3 changes	2 minutes
Xylene	3 changes	2 minutes
Mount with Fisher Scientific Mounting Media		

Toluidine Blue for Mast Cells

Deparaffinize and hydrate to water		
Toluidine Blue	1 change	10 minutes
Rinse in water		
Quickly dehydrate through 95% and 100% alcohols.		
Xylene	3 changes	2 minutes
Mount with Fisher Scientific Mounting Media		

Wolbach's Geimsa

Deparaffinize and hydrate to distilled water.
Remove mercuric chloride crystals with iodine and clear with sodium thiosulfate.
Wash in running water for 15 minutes.

Working Wolbach's Giemsa solution	1 change	overnight

Differentiate in working rosin alcohol solution until sections assume a purplish pink color.

Dehydrate in 100% alcohol	2 changes	2 minutes
Xylene	2 changes	2 minutes

Mount with Fisher Scientific Mounting Media

Verhoff's-Van Gieson

Deparaffinize and hydrate to water.

Verhoff's working solution	1 change	15 minutes
Rinse in running water	1 change	20 minutes
Differentiate in 2% Ferric Chloride	1 change	until black fibers can be seen Microscopically
5% Sodium thiosulfate	1 change	1 minute
Rinse in water	1 change	5 minutes
Counterstain in Van Geison solution	1 change	1 minute
Dehydrate rapidly through:		
95% ethyl alcohol	2 changes	
100% ethyl alcohol	2 changes	
Clear in xylene	2 changes	2 minutes

Mount with Fisher Scientific Mounting Media

Brown and Brenn Gram Stain

Deparaffinize and hydrate to water.
Pour 1 mL crystal violet and 5 drops sodium bicarbonate onto slides for 1 minute.
Rinse with water
Flood slides with Gram's Iodine for 1 minute.
Rinse with water.
Decolorize with acetone.
Flood slides with Basic fuschin working solution for 1 minute.
Dip individually into acetone, and then immediately into picric acid-acetone solution until background is yellow.
Rinse quickly with acetone.
Dip in acetone-xylene solution.

Clear in xylene	2 changes	2 minutes

Mount with Fisher Scientific Mounting Media

APPENDIX B
MANATEE SKIN MEASUREMENTS

Table B-1. Manatee skin measurements, MNW0346.

MANATEE #	**# SC layers**	**Epi. Thickness**	**Dermis Thickness**
MNW0346 Adult Male		(in mm)	(in mm)
1	27-72	.55-1.9	18.3-19.5
2	28-82	.65-1.25	20.4-22.1
3	19-49	.53-1.35	14.0-15.3
4	32-102	1.2-2.6	4.9-6.8
5	36-Tc	.83-3.1	1.8-4.9
6	21-49	.58-1.6	9.7-10.4
7	19-69	.83-2.3	3.6-6.6
8	12-35	.8-1.5	15.6-16.4
9	21-64	.63-1.5	19.0-19.6
10	24-36	.48-1.7	14.4-15.9
11	22-Tc	.8-2.6	8.3-10.1
12	34-66	1.3-2.8	2.5-4.4
13	29-65	.9-2.9	5.0-7.3
14	24-61	.78-1.6	9.5-10.3
15	22-56	.7-2.4	9.4-10.9
16	28-62	.95-2.3	5.0-7.5
17	25-47	.78-1.98	3.7-5.9
18	55-.2mm	1.5-4.8	3.3-6.0
19	21-62	.58-1.8	3.6-6.6
20	34-87	1.4-2.7	5.0-7.1
21	24-79	1.0-4.5	2.4-5.0
22	16-36	.65-2.0	6.6-7.2
23	21-35	1.1-2.6	4.0-6.1
24	7-51	.23-.73	1.8-4.3
25	18-101	1.4-2.1	2.5-6.0
Scar	20-42	1.1-2.8	14.1-16.0
Tc= too compact			

Table B-1. Continued.

MANATEE#	Cassette #	Peg Depth Max. (mm)	Peg Min. (mm)	# Peaks	Dist.peaks (mm)	# pegs/view
MNW0346	1	1.9	.55	2	.75-2.0 a-1.5	14
Adult Male	2	1.25	.65	3	1.3-2.1 a-1.8	16
	3	1.35	.53	3	1.0-1.8 a-1.4	17
	4	2.6	1.2	3	1.0-2.5 a-1.5	12
	5	3.1	.83	3	1.1-2.3 a1.6	16
	6	1.6	.58	4	.5-1.5 a-.9	14
	7	2.3	.83	3	1.0-2.5 a-1.6	13
	8	1.5	.8	4	.75-2.0 a-1.1	12
	9	1.5	.63	4	.75-1.6 a-1.1	16
	10	1.7	.48	3	1.0-2.0 a-1.6	16
	11	2.6	.8	4	.75-1.8 a-1.6	14
	12	2.8	1.3	4	.75-2.3 a-1.0	13
	13	2.9	.9	4	1.0-2.1 a-1.4	16
	14	1.6	.78	4	1.0-1.6 a-1.3	14
	15	2.4	.7	3	1.0-3.0 a-1.5	13
	16	2.3	.95	3	.75-2.1 a-1.3	12
	17	2.0	.78	3	.9-1.8 a-1.1	15
	18	4.8	1.5	4	1.1-2.0 a-1.4	12
	19	1.8	.58	4	.75-1.9 a-1.0	13
	20	2.7	1.4	4	.75-1.5 a-1.0	13
	21	4.5	1.0	5	.63-1.5 a-.9	16
	22	2.0	.65	4	.75-2.0 a-1.0	15
	23	2.6	1.1	4	.75-1.5 a-1.0	15
	24	.73	.23	3	.63-1.3 a-1.1	16
	25	2.1	1.4	3		13
	Scar	2.8	1.1	flat	N/A	10

Table B-2. Manatee skin measurements, TM0406.

MANATEE #	**# SC layers**	**Epi. Thickness**	**Dermis Thickness**
TM0406 Adult Male		(in mm)	(in mm)
1	31-69	.75-1.8	20.4-21.3
2	41-125	1.1-2.3	21.3
3	64-102	.9-1.6	13.6-16.2
4	N/A	1.1-3.6	7.7-9.8
5	55-124	1.2-2.7	4.8
6	80-93	.9-1.7	10.8
7	30-124	1.4-3.4	3.8-6.0
8	53-87	1.0-1.8	15.8-16.5
9	74-109	1.1-2.0	13.3-14.8
10	48-71	1.4-3.8	8.5-12.8
11	N/A	N/A	N/A
12	30-51	1.2-5.1	2.7-4.4
13	63-95	1.4-2.8	4.1-7.8
14	56-107	1.2-1.9	8.1-8.9
15	Tc	1.0-3.4	13.1-16.3
16	26-64	.8-2.9	6.1-7.8
17	25-72	.6-1.8	7.7-9.6
18	Tc	3.2-6.7	2.9-3.7
19	N/A	N/A	N/A
20	Tc	1.5-2.5	3.8-5.0
21	Tc	1.2-2.2nail4.6	2.6-3.9
22	Tc	.8-2.1	6.3-8.3
23	47-92	.6-1.3	3.8-4.2
24	11-75	.4-2.2	1.5-2.8
25	32-108	.3-2.3	5.2-6.1

Table B-2. Continued.

MANATEE#	Cassette #	Peg Depth Max. (mm)	Peg Min. (mm)	# Peaks	Dist.peaks (mm)	# pegs/view
TM0406	1	1.3	.75	4	.5-1.8 a-1.0	11
Adult Male	2	2.3	1.1	5	.5-1.6 a-.9	9
	3	1.5	.98	3	1.3-1.6 a-1.5	9
	4	3.6	1.1	3	1.1-2.0 a-1.8	12
	5	2.7	1.2	7	.4-1.4 a-.9	11
	6	1.7	.9	4	.4-1.9 a-1.0	9
	7	3.4	1.4	5	.3-2.4 a-.9	11
	8	1.8	1.0	4	.8-1.8 a-1.1	10
	9	2.0	1.1	4	.6-2.5 a-1.0	7
	10	3.8	1.4	4	.3-1.3 a-1.1	12
	11	N/A	N/A	N/A	N/A	N/A
	12	5.1	1.2	5	.3-1.3 a-.9	12
	13	2.8	1.4	4	.6-1.6 a-.9	10
	14	1.9	1.2	3	.8-2.2 a-1.7	7
	15	3.4	1.0	5	.6-2.1 a-1.0	17
	16	2.9	.8	5	.5-1.6 a-1.0	10
	17	1.8	.6	4	.3-2.2 a-1.0	8
	18	6.7	3.2	3	.9-1.6 a-1.3	12
	19	N/A	N/A	N/A	N/A	N/A
	20	2.5	1.5	3	.9-1.6 a-1.4	13
	21	2.2	1.2	7	.4-1.5 a-.5	10
	22	2.1	.8	5	.3-2.6 a-1.1	10
	23	1.3	.6	5	.7-1.9 a-1.0	10
	24	2.2	.4	3	.1-2.9 a-1.4	7
	25	2.3	.3	3	.1-2.6 a-1.4	8

Table B-3. Manatee skin measurements, MEC0348.

MANATEE #	**# SC layers**	**Epi. Thickness**	**Dermis Thickness**
MEC0348 Adult Male		(in mm)	(in mm)
1	57-85	.3-.8	20.2-20.7
2	N/A	N/A	N/A
3	26-57	.4-.9	17.6-18.9
4	27*	.4-.7	6.0-6.6
5	26*	.5-.9	3.0-5.0
6	33-54*	.4-.8	7.1-8.0
7	15-31*	.3-1.9	5.9-6.6
8	15-23	.3-.8	8.6-9.2
9	27-43	.7-1.3	12.3-12.8
10	Fungi*	.3-1.2	10.6-11.3
11	17*	.7-1.3	13.9-15.2
12	N/A	N/A	N/A
13	30-43	.6-1.8	6.4-7.9
14	26-38	.7-1.6	8.1-10.3
15	31-41	.4-1.1	10.5-11.0
16	11-1.0mm	.8-2.0	4.0-5.9
17	35-69	.5-1.1	4.6-5.0
18	75-90	1.1-1.8	3.3-4.0
19	Tc*	.7-1.6	5.1-5.7
20	14-58	.5-1.8	5.3-6.3
21	25-36	.5-1.2 nail 1.4	4.7-5.2
22	31-58	.4-1.2	5.0-5.5
23	18-39	.3-.9	4.2-4.6
24	14-26	.2-.9	1.8-3.3
25	2-39	.2-1.1	6.0

Table B-3. Continued.

MANATEE#	Cassette #	Peg Depth Max. (mm)	Peg Min. (mm)	# Peaks	Dist.peaks (mm)	# pegs/view
MEC0348	1	.8	.3	4	.9-1.8 a-.9	12
Adult Male	2	N/A	N/A	N/A	N/A	N/A
	3	.9	.4	4	.9-1.5 a-1.4	14
	4	.7	.4	4	.9-1.1 a-.9	13
	5	.9	.5	5	.5-1.5 a-1.1	12
	6	.8	.4	4	.5-1.6 a-1.0	16
	7	1.9	.3	4	.8-1.9 a-1.1	11
	8	.8	.3	4	.9-1.4 a-1.0	14
	9	1.3	.7	4	.9-1.5 a-1.3	11
	10	1.2	.3	4	.9-1.5 a-1.3	10
	11	1.3	.7	4	.5-1.8 a-1.4	11
	12	N/A	N/A	N/A	N/A	N/A
	13	1.8	.6	3	1.0-2.0 a-1.4	12
	14	1.6	.7	4	.5-2.0 a-1.0	12
	15	1.1	.4	2	2.5-5.5 a-	8
	16	2.0	.8	6	.5-2.0 a-1.0	9
	17	1.1	.5	3	.9-2.4 a-2.0	10
	18	1.8	1.1	5	.8-2.1 a-1.4	10
	19	1.6	.7	3	.8-2.0 a-1.3	10
	20	1.8	.5	2	1.0-2.5 a-2.0	12
	21	1.2 nail 1.4	.5	5	.6-1.5 a-.9	12
	22	1.2	.4	3	1.1-2.3 a-1.8	12
	23	.9	.3	3	.9-2.0 a-1.5	15
	24	.9	.2	3	1.0-2.3 a-1.0	14
	25	1.1	.2	4	.5-1.9 a-1.0	12

Table B-4. Manatee skin measurements, LPZ101820.

MANATEE #	**# SC layers**	**Epi. Thickness**	**Dermis Thickness**
LPZ101820 Male Calf		(in mm)	(in mm)
1	63-85	.4-1.2	15.8-17.1
2	61-102	.3-1.0	14.3-15.0
3	70-124	.4-1.0	12.9-14.0
4	49-118	.4-1.8	3.3-4.2
5	57-Tc	.5-1.4	3.2-4.1
6	48-83	.5-1.4	7.4-8.4
7	63-85Tc	.7-2.3	3.8-6.0
8	31-90	.4-.8	9.0-9.6
9	34-104	.4-1.0	10.0-11.4
10	42-98	.4-1.4	8.9-10.6
11	63-Tc	.7-2.1	6.0-9.0
12	*	.7-1.8	3.0-4.7
13	Tc	1.7-3.3	2.7-3.5
14	56-tc	.4-1.1	7.1-7.5
15	N/A	N/A	N/A
16	32-50	.4-1.3	4.5-7.3
17	25-97	.4-1.1	3.8-5.1
18	90-Tc	.6-2.4	1.8-2.3
19	32-84	.4-1.2	4.5-6.3
20	29-54Tc	.4-1.2	5.3-5.8
21	N/A	N/A	N/A
22	36-72*	.5-1.2	5.4-8.3
23	52-93	.5-1.0	3.6-4.3
24	9-50	.1-1.2	2.3-5.1
25	N/A	N/A	N/A

Table B-4. Continued.

MANATEE#	Cassette #	Peg Depth Max. (mm)	Peg Min. (mm)	# Peaks	Dist.peaks (mm)	# pegs/view
LPZ101820	1	1.2	.4	4	.4-1.8 a-1.1	14
Male Calf	2	1.0	.3	4	.5-1.5 a-1.3	12
	3	1.0	.4	4	.4-1.8 a-1.0	11
	4	1.8	.4	5	.4-2.0 a-1.0	14
	5	1.4	.5	5	.5-1.5 a-.9	12
	6	1.4	.5	4	.5-1.4 a-1.0	12
	7	2.3	.7	5	.5-1.5 a-.8	11
	8	.8	.4	5	.5-1.4 a-.9	11
	9	1.0	.4	5	.5-1.3 a-.8	15
	10	1.4	.4	5	.5-1.0 a-.8	13
	11	2.1	.7	5	.5-1.6 a-1.0	13
	12	1.8	.7	5	.5-1.8 a-1.0	11
	13	3.3	1.7	5	.5-1.4 a-.9	11
	14	1.1	.4	5	.5-1.3 a-.9	13
	15	N/A	N/A	N/A	N/A	N/A
	16	1.3	.4	5	.5-1.5 a-.8	11
	17	1.1	.4	5	.5-1.6 a-1.0	12
	18	2.4	.6	5	.4-1.8 a-1.1	13
	19	1.2	.4	5	.5-1.6 a-1.0	12
	20	1.2	.4	5	.8-1.6 a-1.0	11
	21	N/A	N/A	N/A	N/A	N/A
	22	1.2	.5	5	.6-1.5 a-1.0	11
	23	1.0	.5	4	.5-1.3 a-.9	12
	24	1.2	.1	4	.8-1.9 a-1.3	10
	25	N/A	N/A	N/A	N/A	N/A

Table B-5. Manatee skin measurements, TM0311, MNW0323, MSW0353, and TM0339.

MANATEE #	# SC layers	Epi. Thickness	Dermis Thickness
TM0311cb Neonate		(in mm)	(in mm)
Back (1)	2-11	50-.8	5.4-6.0
24	5-12	.3-.8	1.9-3.3
25	29-61	.3-.8	4.3
MNW0323 Adult			
16	21-43	1.0-1.6	4.1-4.8
17	33-64	.9-2.0	3.4-5.1
18	Tc	1.1-3.5	2.0-4.7
19	24-43	.5-1.1	4.6-6.5
20	28-45	.7-1.6	5.0-5.6
21	Tc	3.0-3.9	1.1-1.8
22	34-51	1.1-2.3	4.0-4.6
23	20-26	.5-1.4	6.5-7.5
MSW0353 Adult Female			
Fluke scar	Tc	3.0-4.3	3.3-4.3
Scar site 1	Tc	3.8-6.1	15.5-17.8
Lynnie- CS Lesion	23-52	1.0-3.1	Biopsy punch
Chiquita-CS Lesion	22-Tc	1.3-2.6	Biopsy punch
TM0339 Adult Male			
1	*	1.9-3.8	13.6-17.0

Table B-5. Continued.

MANATEE#	Cassette #	Peg Depth Max. (mm)	Peg Min. (mm)	# Peaks	Dist.peaks (mm)	# pegs/view
TM0311cb	1	.8mm	50 µm	11	.4-.9 a-.4	14
Neonate	24	.8	.3	12	.3-.8 a-.4	15
	25	.8	.3	14	.3-.8 a-.3	14
MNW0323	16	1.6	1.0	5	.6-1.5 a-.9	15
Adult	17	2.0	.9	4	.8-1.8 a-1.0	15
	18	3.5	1.1	3	.9-2.1 a-1.8	15
	19	1.1	.5	4	.5-1.6 a-1.0	15
	20	1.6	.7	4	.6-1.4 a-1.0	17
	21	3.9	3.0	Flat	Flat	12
	22	2.3	1.1	5	.8-1.8 a-1.0	14
	23	1.4	.5	4	.8-2.1 a-.9	15
MSW0353	Fluke scar	4.3	3.0	2	1.2-1.6 a-1.5	11
Adult Female	Scar 1	6.1	3.8	3	1.4-2.2 a-1.5	9
Lynnie Adult Fem.	CS lesion	3.1	1.0	3	1.0-2.5 a-1.5	8
Chiquita Adult Fem.	CS lesion	2.6	1.3	3	.6-2.1 a-1.5	10
TM0339 Adult Male	1	3.8	1.9	3	.9-1.5 a-1.4	15

Table B-6. Manatee skin measurements, MSW03170.

MANATEE #	**# SC layers**	**Epi. Thickness**	**Dermis Thickness**
MSW03170 Male calf		(in mm)	(in mm)
1	48-106	.3-.8	17.0-17.8
2	N/A	N/A	N/A
3	34-107	.3-.8	15.2-16.6
4	35-110	.4-1.5	3.3-5.2
5	16-25	.3-1.1	2.6-3.9
6	40-112	.3-.8	6.4-7.6
7	39-129	.3-1.6	3.6-5.5
8	28-89	.2-.9	9.7-10.1
9	29-95	.3-1.3	11.0-12.1
10	34-113	.3-1.1	11.3-11.8
11	24-67	.5-1.5	3.3-5.3
12	18-77	.3-1.4	2.4-3.6
13	26-64	.3-1.4	3.3-6.3
14	16-62	.4-1.1	5.9-7.0
15	N/A	.4-1.7	8.6-10.0
16	9-26	.2-1.4	4.0-10.0
17	12-67	.3-1.5	3.6-9.1
18	37-1.2mm	.6-3.8	1.4-2.3
19	14-42	.3-1.2	3.4-5.1
20	16-51	.5-1.2	5.1-7.8
21	25-58	.4-1.1 nail-1.6mm	2.2-4.5
22	9-46	.2-1.1	5.3-7.4
23	18-47	.3-1.2	2.5-4.8
24	6-20	.2-1.2	2.3-4.5
25	11-34	..5-1.0	6.8-7.5

Table B-6. Continued.

MANATEE#	Cassette #	Peg Depth Max. (mm)	Peg Min. (mm)	# Peaks	Dist.peaks (mm)	# pegs/view
MSW03170	1	.8	.3	4	.5-1.3 a-1.0	17
Male calf	2	N/A	N/A	N/A	N/A	N/A
	3	.8	.3	5	.4-1.3 a-1.0	20
	4	1.5	.4	5	.4-1.6 a-1.0	16
	5	1.1	.3	4	.5-1.8 a-1.0	17
	6	.8	.3	5	.4-1.1 a-.9	17
	7	1.6	.3	5	.5-1.6 a-1.0	15
	8	.9	.2	6	.4-1.1 a-.8	16
	9	1.3	.3	5	.4-1.3 a-.8	15
	10	1.1	.3	6	.4-1.1 a-.8	16
	11	1.5	.5	6	.4-1.4 a-.8	16
	12	1.4	.3	5	.4-1.3 a-.8	16
	13	1.4	.3	5	.5-1.3 a-.9	17
	14	1.1	.4	6	.4-1.1 a-.9	16
	15	1.7	.4	flat	N/A	16
	16	1.4	.2	6	.4-1.5 a-.8	18
	17	1.5	.3	5	.5-1.6 a-.8	15
	18	3.8	.6	flat	N/A	14
	19	1.2	.3	4	.5-1.9 a-.8	18
	20	1.2	.5	4	.6-1.5 a-1.0	15
	21	1.1	.4	6	.4-1.1 a-.5	14
	22	1.1	.2	5	.4-1.4 a-.9	16
	23	1.2	.3	6	.5-1.1 a-.8	16
	24	1.2	.2	4	.8-1.6 a-1.0	16
	25	1.0	.5	4	.8-1.6 a-1.0	15

Table B-7. Manatee skin measurements, MSW03169.

MANATEE #	**# SC layers**	**Epi. Thickness**	**Dermis Thickness**
MSW03169 Adult Male		(in mm)	(in mm)
1	47-59	.75-1.25	16.75
2	39	.75-1.48	16.1-18.73
3	74	.63-1.3	15.75
4	*	.88-2.25	3.0-9.5
5	32-58	.75-2.4	2.0-4.0
6	35-61	.88-1.5	9.25
7	60-94	.98-2.45	3.0-5.5 tip 1.75
8	*	.48-1.2	15.1
9	*	.7-1.35	18.3
10	*	.6-1.5	17.0
11	*	.88-2.33	6.25
12	67-140	.75-2.15	3.88
13	*	.5-2.6	6.0-10.5
14	N/A	N/A	N/A13.0-18.1
15	65	.8-2.1	4.63-5.5
16	N/A	N/A	N/A
17	40-53	.73-1.5	4.4-5.3
18	*	1.0-2.75	4.1-6.1
19	24-43	.73-1.45	4.5-6.1
20	*	.55-1.6	4.3-5.3
21	30-54 nail 1.3mm	.78-3.1	3.0-4.3
22	*	1.4-2.8	4.5-6.1
23	*	.73-1.5	4.3-5.3
24	7-50	.3-1.9	3.75-5.1
25	22-56	.95-1.5	5.4-8.0

Table B-7. Continued.

MANATEE#	Cassette #	Peg Depth Max. (mm)	Peg Min. (mm)	# Peaks	Dist.peaks (mm)	# pegs/view
MSW03169	1	1.25	.75	4	.6-1.8 a-	9
Adult Male	2	1.48	.75	3	.8-2.3 a-	11
	3	1.30	.63	5	.6-2.1 a-	12
	4	2.25	.88	4	.5-2.0 a-1.0	12
	5	2.40	.75	5	.6-1.5 a-1.0	12
	6	1.50	.88	5	.4-1.1 a-	11
	7	2.45	.98	5	.4-1.5 a-1.1	11
	8	1.18	.48	5	.4-1.5 a-.9	12
	9	1.35	.70	5	.8-1.5 a-1.1	13
	10	1.50	.60	4	.8-1.8 a-1.3	12
	11	2.33	.88	5	.4-1.5 a-.8	13
	12	2.15	.75	5	.3-1.8 a-.6	13
	13	2.60	.50	4	.5-1.9 a-1.1	10
	14	N/A	N/A	N/A	N/A	N/A
	15	2.10	.80	4	.8-2.5 a-1.0	13
	16	N/A	N/A	N/A	N/A	N/A
	17	1.50	.80	4	.8-2.0 a-1.5	11
	18	2.75	.73	N/A flat	N/A flat	11
	19	1.45	1.00	3	.8-2.5 a-1.5	11
	20	1.60	.73	3	.3-2.6 a-1.0	12
	21	2.80	.55	4	.8-1.5 a-1.1	13
	22	3.1	1.40	2	1.5-2.8 a-	12
	23	1.50	.73	3	.8-1.6 a-1.0	12
	24	1.90	.30	3	.6-1.8 a-	14
	25	1.50	.95	2	.8-3.3 a-	13

Table B-8. Manatee skin measurements, MNW0347.

MANATEE #	**# SC layers**	**Epi. Thickness**	**Dermis Thickness**
MNW0347 Male calf		(in mm)	(in mm)
1	19-63	.73-2.4	9.3-10.2
2	N/A	N/A	N/A
3	*	.78-2.6	8.3-9.3
4	N/A	N/A	N/A
5	17-65	1.3-2.7	1.7-2.6
6	N/A	N/A	N/A
7	N/A	N/A	N/A
8	21-52	.7-2.2	6.5-7.4
9	38-61	1.0-2.3	7.0-8.7
10	28-83	.78-2.2	6.1-7.6
11	38-*	1.2-3.0	3.8-6.2
12	30-102	1.2-2.8	2.0-3.6
13	24-96	1.3-2.8	4.0-5.5
14	17-62	1.4-2.4	4.1-5.2
15	36-86	.6-3.6	2.5-9.4
16	19-63	.5-2.3	2.2-4.1
17	24-55	1.1-2.4	2.1-4.3
18	23-62	1.7-3.4	2.1-3.7
19	25-46	.9-2.5	1.8-5.6
20	29-45	1.1-2.3	3.4-5.0
21	31-*	1.3-3.5	2.1-5.0
22	23-44	.8-2.5	3.5-4.5
23	*	1.0-2.1	1.9-3.7
24	14-60	.3-2.0	1.5-5.3
25	17-49	.6-2.2	1.8-3.7

Table B-8. Continued.

MANATEE#	Cassette #	Peg Depth Max. (mm)	Peg Min. (mm)	# Peaks	Dist.peaks (mm)	# pegs/view
MNW0347 Male Calf	1	2.4	.73	5	.6-1.3 a-1.0	11
	2	N/A	N/A	N/A	N/A	N/A
	3	2.6	.78	5	.6-1.5 a-.6	12
	4	N/A	N/A	N/A	N/A	N/A
	5	2.7	1.3	6	.4-1.9 a-.6	12
	6	N/A	N/A	N/A	N/A	N/A
	7	N/A	N/A	N/A	N/A	N/A
	8	2.2	.7	5	.5-1.0 a-.6	14
	9	2.3	1.0	6	.5-1.3 a-.8	12
	10	2.2	.78	5	.5-1.3 a-.6	12
	11	3.0	1.2	5	.5-1.6 a-.8	12
	12	2.8	1.2	5	.5-1.5 a-.8	12
	13	2.8	1.3	6	.5-1.5 a-.6	12
	14	2.4	1.4	4	.5-1.5 a-.8	14
	15	3.5	.6	5	.5-1.0 a-.8	12
	16	2.3	.5	4	.5-1.5 a-1.0	14
	17	2.4	1.1	7	.3-1.0 a-.5	14
	18	3.4	1.7	4	.3-1.4 a-.5	14
	19	2.5	.9	5	.4-1.3 a-1.0	14
	20	2.3	1.1	4	.5-1.5 a-1.0	18
	21	3.5	1.3	5	.3-.9 a-.3	17
	22	2.5	.8	4	.8-1.5 a-1.0	15
	23	2.1	1.0	6	.3-1.1 a-.6	18
	24	2.0	.3	5	.5-1.9 a-.8	16
	25	2.2	.6	3	.8-1.8 a-1.4	16

Table B-9. Manatee skin measurements, MNW0342.

MANATEE #	**# SC layers**	**Epi. Thickness**	**Dermis Thickness**
MNW0342 Adult Female		(in mm)	(in mm)
1	70-162	.5-1.2	20.2-20.6
2	75-149	.8-1.3	21.1-21.5
3	66-89	.7-1.6	12.9-16.0
4	74-135	1.1-3.9	2.8-5.4
5	*	.9-2.6	5.2-5.9
6	66-110	.7-1.4	9.8-10.9
7	91-170	1.1-2.8	4.5-7.2
8	29-50	.6-2.0	10.7-12.7
9	59-99	.4-1.6	11.8-13.0
10	59-86	.9-1.4	12.1-13.4
11	66-95*	1.3-3.8	7.2-8.4
12	51-156	.9-2.1	3.7-4.9
13	72-112	1.1-4.6	3.6-4.9
14	58-143	.9-1.8	10.8-11.8
15	79*	.8-2.6	15.0-16.3
16	21-83	.6-2.5	4.7-6.0
17	26-73	.6-3.4	2.9-5.0
18	71-115	1.1-2.6	4.2-5.3
19	30-54	.6-1.7	5.6-6.7
20	34-62	.7-2.4	5.3-6.8
21	67*	1.3-3.3 nail 5.5	3.5-5.1
22	39-109	.7-1.9	5.3-6.3
23	20-52	.5-1.6	7.1-7.5
24	16-37	.1-1.2	3.1-5.4
25	14-31	.6-2.3	3.0-10.0
Wound #2	N/A	N/A	19.7

Table B-9. Continued.

MANATEE#	Cassette #	Peg Depth Max. (mm)	Peg Min. (mm)	# Peaks	Dist.peaks (mm)	# pegs/view
MNW0342	1	1.2	.5	3	1.4-2.1 a-1.6	14
Adult Female	2	1.3	.8	3	.6-2.5 a-1.3	13
	3	1.6	.7	3	1.0-1.8 a-1.5	10
	4	3.9	1.1	3	1.0-2.0 a-1.6	12
	5	2.6	.9	3	1.0-2.0 a-1.5	11
	6	1.4	..6	3	1.0-1.6 a-1.3	11
	7	2.8	1.1	3	.8-2.0 a-1.3	13
	8	2.0	.6	3	.5-2.3 a-1.5	12
	9	1.6	.4	3	1.0-2.1 a-1.6	10
	10	1.4	.9	3	1.3-2.1 a-1.5	11
	11	3.8	1.3	3	1.3-2.5 a-1.5	12
	12	2.1	.9	4	.6-2.1 a-1.4	11
	13	4.6	1.1	4	.9-1.6 a-1.3	12
	14	1.8	.9	3	.8-1.8 a-1.4	12
	15	2.6	.8	2	N/A	12
	16	2.5	.6	4	.5-2.3 a-1.6	12
	17	3.4	.6	3	1.0-2.3 a-1.5	11
	18	2.6	1.1	4	1.0-1.6 a-1.3	12
	19	1.7	.6	3	.5-2.1 a-1.0	11
	20	2.4	.7	3	.9-1.8 a-1.4	12
	21	3.3	1.3	Flat	N/A	12
	22	1.9	.7	3	1.0-2.0 a-1.5	12
	23	1.6	.5	4	.8-2.5 a-1.3	11
	24	1.2	.1	3	.8-2.0 a-1.1	21
	25	2.3	.6	4	.5-2.5 a-1.1	20

APPENDIX C
MAXIMUM AND MINIMUM MEASUREMENTS

Table C-1. Maximum and minimum measurements.

Site	**Epidermis (mm) Manatee# /Age/Gender**	**Dermis (mm) Manatee #/ Age/ Gender**	**# of layers in stratum corneum**	**# rete ridges per 40X field**	**#epidermal papillae per 40X field**
1 Thickest	1.9 MNW0342 Adult F	21.3 TM0406 Adult M	162 MNW0342 Adult F	11 TM0311 Neonate	17 MSW03170 Med. Calf M
Thinnest	.05 TM0311 Neonate	5.4 TM0311 Neonate	2 TM0311 Neonate	3 MNW0342 Adult F	9 MSW03169 Adult M
2 Thickest	2.3 TM0406 Adult M	22.1 MNW0346 Sub Adult M	149 MNW0342 Adult F	16 MNW0346 Sub Adult M	17 MSW03170 Med. Calf M
Thinnest	.3 LPZ101820 Med. Calf M	14.3 LPZ101820 Med. Calf M	28 MNW0346 Adult M	3 MNW0342 Adult F	11 MNW0342 Adult F
3 Thickest	1.7 TM0406 Adult M	10.9 MNW0346 Sub Adult F	112 MSW03170 Med. Calf M	5 MSW03170 Med. Calf M	17 MSW03170 Med. Calf M
Thinnest	.3 MSW03170 Med. Calf M	6.4 MSW03170 Med. Calf M	21 MNW0346 Sub Adult M	3 MNW0342 Adult F	9 TM0406 Adult M
4 Thickest	3.4 MNW0342 Adult F	9.8 TM0406 Adult M	135 MNW0342 Adult F	5 MSW03170 Med. Calf M	16 MSW03170 Med. Calf M
Thinnest	.4 MSW03170 Med. Calf M	2.8 MNW0342 Adult F	27 MEC0348 Adult M	3 MNW0342 Adult F	12 MNW0342 Adult F
5 Thickest	3.1 MNW0346 Sub Adult M	5.9 MNW0342 Adult F	124 TM0406 Adult M	6 MNW0347 Sm. Calf M	17 MSW03170 Med. Calf M
Thinnest	.3 MSW03170 Med. Calf M	1.7 MNW0347 Sm. Calf M	16 MSW03170 Med. Calf M	3 MNW0342 Adult F	11 MNW0342 Adult F

Table C – 1 Continued

Site	Epidermis (mm) Manatee# /Age/Gender	Dermis (mm) Manatee #/ Age/ Gender	# of layers in stratum corneum	# rete ridges per 40X field	#epidermal papillae per 40X field
6 Thickest	1.7 TM0406 Adult M	10.9 MNW0346 Sub Adult M	112 MSW03170 Med. Calf M	5 MSW03170 Med. Calf M	17 MSW03170 Med. Calf M
Thinnest	.3 MSW03170 Med. Calf M	6.4 MSW03170 Med. Calf M	21 MNW0346 Sub Adult M	3 MNW0342 Adult F	9 TM0406 Adult M
7 Thickest	3.4 TM0406 Adult M	7.2 MNW0342 Adult F	170 MNW0342 Adult F	5 MSW03170 Med. Calf M	15 MSW03170 Med. Calf M
Thinnest	.3 MSW03170 Med. Calf M	3.0 MSW03169 Sm. Adult M	15 MEC0348 Sm. Adult M	3 MNW0346 Sub Adult M	11 LPZ101820 Med. Calf M
8 Thickest	2.0 MNW0342 Adult F	16.5 TM0406 Adult M	90 LPZ101820 Med. Calf M	6 MSW03170 Med. Calf M	16 MSW03170 Med. Calf M
Thinnest	.2 MSW03170 Med. Calf M	6.5 MNW0347 Sm. Calf M	12 MNW0346 Sub Adult M	3 MNW0342 Adult F	10 TM0406 Adult M
9 Thickest	2.0 TM0406 Adult M	19.6 MNW0346 Sub Adult M	109 TM0406 Adult M	6 MNW0347 Sm. Calf M	16 MNW0346 Sub Adult M
Thinnest	.3 MSW03170 Med. Calf M	7.0 MNW0347 Sm. Calf M	15 MEC0348 Adult M	3 MNW0342 Adult F	9 TM0406 Adult M
10 Thickest	3.8 TM0406 Adult M	17.0 MSW03169 Adult M	113 MSW03170 Med. Calf M	6 MSW03170 Med. Calf M	16 MNW0346 Sub Adult M
Thinnest	.3 MSW03170 Med. Calf M	6.4 MSW03170 Med. Calf M	24 MNW0346 Sub Adult M	3 MNW0342 Adult F	10 MEC0348 Adult M
11 Thickest	3.8 MNW0342 Adult F	17 MEC0348 Adult M	102 MEC0348 Adult M	6 MSW03170 Med. Calf M	16 MSW03170 Med. Calf M
Thinnest	.5 MSW03170 Med. Calf M	3.3 MSW03170 Med. Calf M	17 MEC0348 Adult M	3 MNW0342 Adult F	11 MEC0348 Adult M

Table C – 1 Continued

Site	Epidermis (mm) Manatee# /Age/Gender	Dermis (mm) Manatee #/ Age/ Gender	# of layers in stratum corneum	# rete ridges per 40X field	#epidermal papillae per 40X field
12 Thickest	5.1 TM0406 Adult M	4.9 MNW0342 Adult F	156 MNW0342 Adult F	5 MNW0347 Sm. Calf M	16 MSW03170 Med. Calf M
Thinnest	.3 MSW03170 Med. Calf M	2.0 MNW0347 Sm. Calf M	18 MSW03170 Med. Calf M	4 MNW0342 Adult F	11 MNW0342 Adult F
13 Thickest	4.6 MNW0342 Adult F	10.5 MSW03169 Adult M	112 MNW0342 Adult F	6 MNW0347 Sm. Calf M	17 MSW03170 Med. Calf M
Thinnest	.3 MSW03170 Med. Calf M	2.7 LPZ101820 Med. Calf M	24 MNW0347 Sm. Calf M	3 MEC0348 Adult M	10 TM0406 Adult M
14 Thickest	1.9 TM0406 Adult M	11.8 MNW0342 Adult F	143 MNW0342 Adult F	6 MSW03170 Med. Calf M	16 MSW03170 Med. Calf M
Thinnest	.4 MSW03170 Med. Calf M	4.1 MNW0347 Sm. Calf M	16 MSW03170 Med. Calf M	3 TM0406 Adult M	7 TM0406 Adult M
15 Thickest	3.4 TM0406 Adult M	16.3 TM0406 Adult M	86 MNW0347 Med. Calf M	5 MNW0347 Sm. Calf M	17 TM0406 Adult M
Thinnest	0.4 MSW03170 Med. Calf M	2.5 MNW0347 Sm. Calf M	22 MNW0346 Sub Adult M	2 MNW0342 Adult F	8 MEC0348 Adult M
16 Thickest	2.9 TM0406 Adult M	7.8 TM0406 Adult M	83 MNW0342 Adult F	6 MSW03170 Med. Calf M	18 MSW03170 Med. Calf M
Thinnest	.2 MSW03170 Med. Calf M	2.2 MNW0347 Sm. Calf M	9 MSW03170 Med. Calf M	3 MNW0346 Sub Adult M	9 MEC0348 Adult M
17 Thickest	3.9 MNW0342 Adult F	9.6 TM0406 Adult M	97 LPZ101820 Med. Calf M	7 MNW0347 Sm. Calf M	15 MSW03170 Med. Calf M
Thinnest	.3 MSW03170 Med. Calf M	2.1 MNW0347 Sm. Calf M	12 MSW03170 Med. Calf M	3 MNW0342 Adult F	8 TM0406 Adult M

Table C-1 Continued

Site	Epidermis (mm) Manatee# /Age/Gender	Dermis (mm) Manatee #/ Age/ Gender	# of layers in stratum corneum	# rete ridges per 40X field	#epidermal papillae per 40X field
18 Thickest	6.7 TM0406 Adult M	6.0 MNW0346 Sub Adult M	115 MNW0342 Adult F	5 MEC0348 Adult M	15 MNW0323 Adult M
Thinnest	.6 MSW03170 Med. Calf M	1.4 MSW03170 Med. Calf M	23 MNW0347 Sm. Calf M	3 MNW0323 Adult M	10 MEC0348 Adult M
19 Thickest	1.8 MSW03169 Adult M	6.7 MNW0342 Adult F	84 LPZ101820 Med. Calf M	5 MNW0347 Sm. Calf M	18 MSW03170 Med. Calf M
Thinnest	.3 MSW03170 Med. Calf M	1.8 MNW0347 Sm. Calf M	14 MSW03170 Med. Calf M	3 MNW0342 Adult F	10 MEC0348 Adult M
20 Thickest	2.7 MNW0346 Sub Adult M	7.1 MNW0346 Sub Adult M	81 MNW0346 Sub Adult M	5 LPZ101820 Med. Calf M	18 MSW0347 Sm. Calf M
Thinnest	.4 LPZ101820 Med. Calf M	3.4 MNW0347 Sm. Calf M	14 MEC0348 Adult M	2 MEC0348 Adult M	12 MNW0342 Adult F
21 Thickest	4.6 TM0406 Adult M	5.2 MEC0348 Adult M	N/A	5 MNW0347 Sm. Calf M	17 MNW0347 Sm. Calf M
Thinnest	.5 MEC0348 Adult M	2.0 MNW0323 Adult M	24 MNW0346 Sub Adult M	All are mostly flat near nail	10 MSW03169 Adult M
22 Thickest	2.8 MSW03169 Adult M	8.3 TM0406 Adult M	109 MNW0342 Adult F	5 MSW03170 Med. Calf M	17 MSW03170 Med. Calf M
Thinnest	.2 MSW03170 Med. Calf M	3.5 MNW0347 Sm. Calf M	9 MSW03170 Med. Calf M	2 MSW03169 Adult M	10 TM0406 Adult M
23 Thickest	2.6 MNW0346 Sub Adult M	8.2 MNW0342 Adult F	93 LPZ101820 Med. Calf M	6 MSW03170 Med. Calf M	18 MNW0347 Sm. Calf M
Thinnest	.3 MSW03170 Med. Calf M	1.9 MNW0347 Sm. Calf M	18 MSW03170 Med. Calf M	3 MSW03169 Adult M	10 TM0406 Adult M

Table C-1 Continued

Site	Epidermis (mm) Manatee# /Age/Gender	Dermis (mm) Manatee #/ Age/ Gender	# of layers in stratum corneum	# rete ridges per 40X field	#epidermal papillae per 40X field
24 Thickest	2.2 TM0406 Adult M	5.35 MNW0342 Adult F	75 TM0406 Adult M	12 TM0311 Neonate	16 MNW0347 Sm. Calf M
Thinnest	.2 MSW03170 Med. Calf M	1.5 MNW0347 Sm. Calf M	5 TM0311 Neonate	3 TM0406 Adult M	7 TM0406 Adult M
25 Thickest	2.3 TM0406 Adult M	10.1 MNW0342 Adult F	108 TM0406 Adult M	14 TM0311 Neonate	17 MNW0347 Sm. Calf M
Thinnest	.2 MEC0348 Adult M	1.8 MNW0347 Sm. Calf M	2 MEC0348 Adult M	2 MSW03169 Adult M	10 MSW03169 Adult M

APPENDIX D
LOCATIONS OF THE MANATEES USED

Figure D-1. Salvage and recovery locations of the manatees used in this research.

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