Functional morphology of mammalian rhinariumn skin

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

The naked skin surrounding the nostrils in most mammals is called a rhinarium. Rhinarium skin exhibits several unique characteristics, including an ultrastructure of pits or furrows on surface corneocytes, formed in a unique epidermal differentiation process (Paper I). Rhinarium skin is often assigned a mechanosensory function because the rhynoglyphic pattern of epidermal domes, ridges or polygons resemble the digital skin in higher primates. This is corroborated by the presence of mechanosensory Eimer's organs in the rhinarium skin of various insectivores, indicating that it is indeed a tactile surface in these species. Interestingly, the rhinarium skin of a distantly related prosimian primate, the ring-tailed lemur also contains Eimer's-like mechanosensory organs (Paper II). Although rhinarium skin structure in all studied mammals exhibits comparable features, differences in innervation pattern suggest that its sensory function varies amongst species (Paper III). The rhinarium function becomes even more curious if we consider its peculiar temperature dynamics in carnivorous mammals. In alert dogs, the rhinarium skin surface is normally kept a few degrees below ambient temperature. The rich vascularization and the arrangement of blood vessels in the canine rhinarium (Paper IV) suggests that it may be cooled actively and that the low tissue temperature is of functional importance. Due to the risk of tissue damage, this relative reduction in skin temperature cannot be sustained in freezing climates. Nevertheless, the rhinarium surface temperature in cold-acclimatized dogs can decrease below the cold pain threshold previously measured in mammals without eliciting any behavioural signs of distress (Paper V). In addition, the cold-transducing channels TRPM8 and TRPA1 in dog behave similarly to their human orthologs, suggesting that the cold tolerance observed in naked rhinarium skin does not rely on the innate characteristics of these channels (Paper V). Considered together, the findings presented in this thesis lay the groundwork for future studies into this enigmatic structure.

Key words: nasolabial, skin, epidermis, cutaneous innervation, canine, vascularization, cold acclimatization

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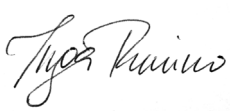
Signature  Date 2020-08-31

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II. Elofsson, R., Tuminaite I., Kröger, R.H.H. (2015) A complex sensory organ in the nose skin of the prosimian primate *Lemur catta. Journal of Morphology,* 276(6), 649-656.

III. Tuminaite, I., Kröger, R.H.H. Rhinarium skin morphology and innervation in selected mammals. Under second revision in Journal of Morphology.

IV. Tuminaite, I., Kröger, R.H.H. Anatomical arrangement of blood vessels in the dog rhinarium and their role in maintaining low tissue temperature. *Manuscript*.

V. Tuminaite, I., Derouiche, S., Kröger, R.H.H., Tominaga M. Naïve TRPM8 and TRPA1 in dog are unable to detect temperatures observed in naked skin of cold-exposed dogs. *Manuscript.*

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Author contributions

I. RE and IT conceived the idea, IT collected the samples, RE and IT performed the experiments and analysis, RE wrote the manuscript with input from IT and RHHK.

II. RE conceived the idea, IT collected the samples, RE performed the experiments with some assistance from IT, RE wrote the manuscript with input from IT and RHHK

III. IT conceived the idea, collected the samples, performed the experiments and analysis, and wrote the manuscript with input from RHHK

IV. RHHK and IT conceived the idea, IT performed the experiments and analysis, and wrote the manuscript with input from RHHK

V. All the authors contributed to conceiving the idea, IT performed the experiments and analysis with help from SD, IT wrote the manuscript with input from SD, RHHK, and MT.

Popular Science Abstract

The naked and pronounced nose tip is a characteristic feature in the face of most mammals. It is normally referred to as simply “the nose”, which implies a connection to the sense of smell. In reality, this small naked skin patch, also called the rhinarium, can have different sensory specializations but our current understanding of these are limited. This thesis comprises five studies that aim at achieving a better understanding of the rhinarium structure and function in mammals.

The rhinarium surface pattern, formed by small domes and ridges visible to the naked eye, is unique in each species and individual, similar to a fingerprint. In addition to this pattern, the rhinarium skin surface is also sculptured on the cellular level. A pattern of small pits or furrows can be seen on the superficial skin cells under high magnification. This pattern, and the process through which it is formed, makes the rhinarium skin unique compared to skin on other parts of the body (Paper I). Although there are similarities in overall rhinarium skin structure amongst mammals, the major differences emerge when one looks at its sensory innervation. In a number of species, including the ring-tailed lemur, rhinarium skin is specialized to detect pressure and touch (Paper II). In other mammals, the rhinarium skin is likely most sensitive to chemical substances in contact with the skin surface or temperature (Paper III).

Most dog owners have likely noticed that the dog rhinarium feels cold to the touch. Indeed, the rhinarium surface temperature is normally a few degrees lower than ambient temperature in dogs and other carnivores. Achieving this is not a trivial task and would require specific arrangements of blood vessels both close to the skin surface and deeper in the tissue. The peculiar arrangement of venous blood vessels in the dog rhinarium might provide several ways to cool it (Paper IV).

In freezing winter climates, the rhinarium faces other challenges. While we rely on warm clothing to be comfortable in sub-zero temperatures, the naked and wet rhinarium is directly exposed to the freezing ambient temperature. This does not seem to bother the dogs as much as one would expect - their rhinarium surface temperature can be several degrees lower than what we would consider painful. Nevertheless, the cold receptors in skin, that function as molecular thermometers, are tuned to similar temperatures in both dogs and humans making their cold tolerance even more surprising (Paper V).

In summary, the work presented in this thesis stress the uniqueness of the mammalian rhinarium and lays the groundwork for future studies on this exciting organ.

Populärvetenskapligt dikt

Hej, jag är en hund
Precis som andra kära hundar
Näsvis, men oförstående
På vilket vis näsan funkar

Ja, den har gropar och gupp
Och under lupp mer än vad som tros
En labyrint av nerver i rhinariet
Som jag kallar för min nos

Det finns hos nästan alla djur,
Men ändå unik som ett fingeravtryck
Och till skillnad från en ringsvanslemur
Känner den inte av tryck

Oavsett temperatur,
Är den anpassningsbar
Så min nos kan vara coolare
Än ett kallt klimat

En outforskad sak
Som kan testas mer
En doft av kunskap
Om nosens komplexitet

J. Landenfelt

Scope of this thesis

The survival of any living organism is dependent on its ability to correctly interpret its immediate environment. Information about the surroundings is available in a variety of forms and relevant stimuli are detected with more or less advanced sensory systems. Since temperature can affect all biological processes, it is critical for an organism to keep track of its thermal environment – to seek out preferable temperatures and to avoid dangerous extremes. To function properly, body tissues typically require a stable thermal environment. For animals that are unable to regulate their temperature, being exposed to temperature extremes or rapid changes in temperature can therefore be fatal. To avoid this, more advanced animals such as mammals have evolved a variety of physiological adaptations that keep their inner body core temperature stable (Schmidt-Nielsen, 1997). In combination with behavioral adaptations, such as seeking shelter or shade, this allows mammalian species to survive in a broad range of ambient temperatures.

In polar and subarctic climates, where winter temperatures can drop to below -50 °C, the difference between the mammalian body core and ambient temperature can reach more than 80 °C. Species that are adapted to these conditions boast effective structural and physiological adaptations that include brown adipose tissue, increased metabolic rates, hibernation, and, of course, a thick fur cover (Blix, 2016). Even though these adaptations allow arctic mammals to successfully cope with freezing temperatures, not all of their body surfaces are covered by thick fur. Hairless (glabrous) skin patches on the paw pads and the nose tip (also called rhinarium or planum nasale) are exposed to the cold environment. Greatly reduced skin temperatures induce a pain response, warning the animal of potential tissue damage and inducing appropriate behavioural responses. For example, humans have multimodally distributed cold pain thresholds of 23.7 °C, 13.2 °C, and 1.5 °C (Lötsch et al., 2015). To survive harsh winter climates, we must rely on clothing and shelter. Arctic mammals must supply warm blood to the glabrous skin patches to keep the surface temperature at a level that is tolerable. Keeping the naked skin at lower temperatures would reduce heat loss, but might result in pain and frostbite. So how do cold-adapted mammal species solve this dilemma? To date, nothing is known about the cold pain threshold of arctic and subarctic mammals and the molecular mechanisms behind it. It remains unclear how these animals perceive low temperatures and at which point they are experienced as painful. My initial objective

was to focus on this knowledge gap, which inevitably generated a broad range of interesting research questions regarding the mammalian rhinarium.

Since avoiding heat loss is a priority in cold climates, any naked and uninsulated skin patch should be of great functional importance. The paw pads have to be cold and naked for better traction on icy surfaces, but the reasons for keeping the nasolabial skin naked remain somewhat enigmatic. The 'pebbled' skin surface texture of the mammalian rhinarium has previously received some scientific interest (Osman Hill, 1948), but many questions regarding its skin morphology, innervation, and blood supply remained unanswered. I therefore chose to address some of these knowledge gaps in this thesis.

Since a prominent glabrous rhinarium is a characteristic of most mammals, I could compare this trait among a broad range of species exhibiting different ecologies and from different thermal habitats. The choice of model animals was, however, limited by the availability of tissue samples. The projects described in this thesis therefore often used easily accessible domesticated species as dog (*Canis familiaris*) and cow (*Bos taurus*), or species abundant in local zoos, such as the ring-tailed lemur (*Lemur catta*).

I began my exploration into the functional morphology of mammalian rhinarium skin with a detailed examination of the rhinarium skin surface and epidermal differentiation in a variety of placental mammals (Paper I). The following two studies address the sensory innervation in rhinarium skin in ring-tailed lemur, cow, brown bear, and dog, which was crucial for attaining a better understanding of the function of rhinaria in these species (Papers II and III). I then studied the rhinarium blood supply in dogs, which display peculiar rhinarium surface temperature dynamics (Paper IV). Finally, I addressed the cold detection mechanisms and tolerance in the glabrous skin of a species physiologically equipped to live in arctic and subarctic climates (where I used dog as a model species, Paper V). Each of these studies was aimed at addressing important gaps in our understanding of the structure and function of the mammalian rhinarium and required utilizing a variety of morphological and molecular biology techniques.

Mammalian skin

The mammalian skin is a multipurpose organ that constitutes a protective barrier and has prominent roles in thermoregulation and the perception of the immediate environment. It consists of two primary layers, the epidermis and the dermis. The overlying epidermal layer constantly regenerates and is primarily composed of specialized cells called keratinocytes that are tightly connected by a multitude of desmosomes to ensure tissue integrity. The underlying dermal layer is a connective tissue containing collagen, elastin, and glycosaminoglycans. The deepest layer of the skin is the hypodermis, which additionally contains adipocytes (fat cells) (McGarth & Uitto, 2016). In the following sections, I introduce the general organization and sensory structures in mammalian skin before discussing the specific features of rhinarium skin.

Epidermal layer

The epidermal layer in mammals is typically subdivided into (starting from the deepest layer of the epidermis): stratum basale, stratum spinosum, stratum granulosum, and stratum corneum (Fig. 1). All of these layers undergo a constant renewal process where a keratinocyte formed in the stratum basale will end up as an anuclear corneocyte in the stratum corneum and finally be shed from the skin surface. This keratinocyte differentiation, called cornification, is a controlled form of cell death that ensures a protective layer of cells devoid of organelles on the skin surface (Eckhart et al., 2013).

The stratum basale is separated from the dermal layer by a basement membrane and composed of a single layer of epidermal stem cells, melanocytes, and Merkel cell-neurite complexes (mechanoreceptive structures sensitive to touch). From the basal layer, the daughter cells generated through asymmetrical division migrate up to the stratum spinosum, which is the thickest of the epidermal sublayers. It is characterized by large intercellular spaces (Falck et al., 1981) and pronounced desmosomes, that give the layer its characteristic prickly appearance. Langerhans cells, which function as microphages, are found between the keratinocytes in this layer (Menon, 2015). The keratinocytes gradually flatten and elongate as they approach the upper part of the layer and the transition to the granular layer. The expression of the epidermal differentiation complex leads to keratinocytes losing

their nuclei and organelles. The intracellular space shrinks, the keratinocytes flatten further and become filled with keratohyalin granules containing profillaggrin and loricrin (Eckhart et al., 2013). The skin cells continue upwards to become corneocytes, anuclear keratinocytes filled with cross-linked proteins. The corneocytes form the stratum corneum that retains the basic organizational structure as the deeper layers of the epidermis with corneodesmosomes holding the cells together. Corneodesmosomes are formed primarily from corneodesmosin that is expressed in the granular layer and binds to the extracellular parts of desmosomes to form desmosomal plaques. The corneodesmosomes slowly degrade and corneocytes gradually shed from the skin surface to be replaced with new cells (Ishida-Yamamoto & Igawa, 2015).

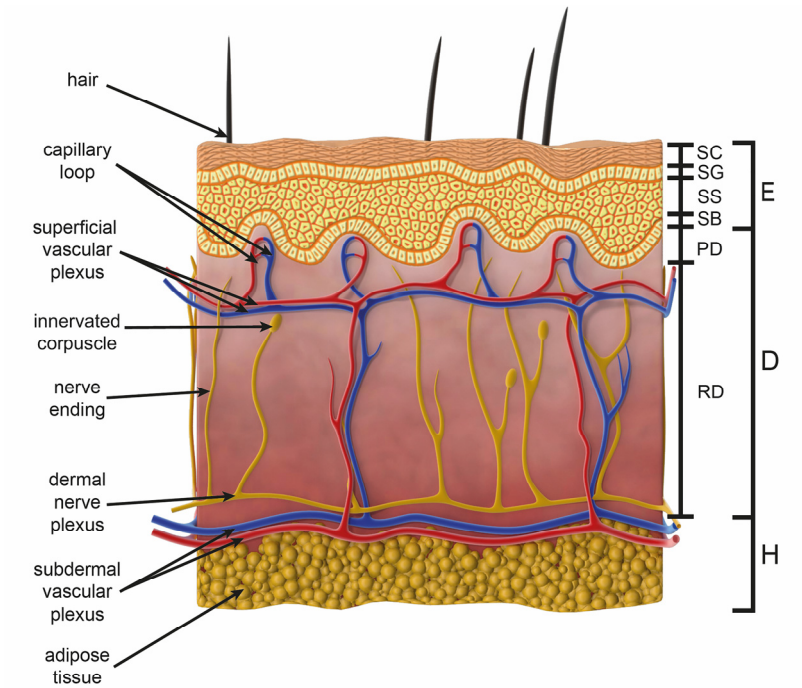


Figure 1. Schematic representation of the structures in hairy skin. The layers of the epidermis *(E)*, dermis *(D)*, and the underlying hypodermis *(H)* are indicated on the right side of the image, *SC* - stratum corneum, *SG* - stratum granulosum, *SS* - stratum spinosum, *SB* - stratum basale, *PD* - papillary dermis, *RD* - reticular dermis.

Dermal layer

Collagen, elastin, and glycosoaminoglycans, which constitute the dermal extracellular matrix in the dermis, are produced by fibroblasts. Other cell types in the dermis include phagocytes and mast cells that are part of the innate immune

system. The dermal layer is typically subdivided into papillary and reticular (Fig. 1). The papillary layer is most prominent in body parts exposed to abrasion. The dermal papillae extend into the epidermis and increase the contact area between the dermal and epidermal layers, this way strengthening their linkage. The papillary layer contains innervated mechanosensitive corpuscles and blood capillaries that nourish the avascular epidermis. The underlying reticular dermis is denser and richly vascularized. It houses innervated mechanosensitive corpuscles, but also sweat glands, hair follicles and associated sebaceous glands. It furthermore contains a nerve plexus from which free nerve endings enter the upper layers of the dermis and the epidermis (McGarth & Uitto, 2016). Hypodermis lies under the dermal layer and is primarily composed of adipocytes (Fig. 1).

Sensory structures in the skin

Sensory stimuli in the skin are transmitted by neurons in the peripheral nervous system. Cutaneous sensory nerves enter the upper skin layers from the subepidermal nerve plexus in the dermis. The nerve fiber density decreases when moving away from the body trunk to distal parts of the limbs, with the exception of high innervation densities in tactile areas, such as fingertips, facial, and genital areas (Lauria et al., 1999). In broad terms, cutaneous sensory fibers are classified as Aβ-fibers (thickly myelinated), A∂-fibers (lightly myelinated), and C-fibers (unmyelinated). These fibers can be further classified based on the stimuli they detect and transmit. In mammalian skin, temperature information is usually transmitted by C- and A∂-fibers, while mechanical stimuli are encoded mainly by Aβ-fibers. Painful temperature and mechanical stimuli are encoded by subpopulations of the above-mentioned fiber types (McGlone & Reilly, 2010).

Receptor proteins and receptor ion channels in cell membranes of cutaneous nerve fibers determine what stimuli the fibers can detect. In addition to nerve fibers, sensory stimuli can also be detected by keratinocytes that in their turn activate adjacent nerve fibers (Denda, 2012).

Skin mechanosensitive organs are innervated multicellular structures specialized to detect touch, vibrations, and stretch. These include Merkel cell-neurite complexes, Ruffini endings, Meissner corpuscles, and Pacinian corpuscles that are all associated with or innervated by Aβ-fibers. Merkel cell-neurite complexes are unique among the described mechanoreceptors since they are not encapsulated. Merkel cells are derived from the epidermis and were previously mentioned when describing the stratum basale layer, where they are situated. They are innervated by slowly adapting fibers, meaning that they are best suited to detect constant pressure and

low frequency vibrations (Morrison et al., 2009). A capsule of flattened Schwan cells, fibroblasts, and collagen fibers are otherwise characteristic for all dermal mechanoreceptors. Ruffini endings, which also adapt slowly, are situated in the reticular dermal layer. They are elongated and aligned parallel to the skin surface, making them especially sensitive to cutaneous stretch (Chambers et al., 1972). Meissner corpuscles are situated in the top of dermal papillae of glabrous skin and encapsulated in a layer of fibroblasts. The capsule contains stacked lamellar Schwann cells and can be innervated by several neurons. They are rapidly adapting and detect small force changes in the skin and are especially important for grip control (Paré et al., 2001). Pacinian corpuscles, positioned deep in the dermis, are large and have onion-like capsules in which the lamellae are separated by fluid filled spaces. They adapt rapidly and are therefore suited to detect high frequency vibrations and fine surface structures (Johnson, 2001). In addition to the above described mechanosensory skin organs, mechanical stimuli are also detected by innervated hair follicles and free nerve endings (reviewed in e.g. Djouhri, 2016).

Rhinarium skin

The naked nasolabial skin surrounding the nostrils seen on most mammalian muzzles exhibits a rhinoglyphic pattern of macroscopic epidermal domes, ridges and polygons that is both species and individual specific, and often compared to human finger tips (see Fig. 2 for an overview of rhinarium skin structure). The following sections introduce the findings from previous work on rhinarium skin and present the findings of studies on rhinarium skin morphology conducted as part of this thesis (Paper I and Paper III).

Epidermal domes and basic skin morphology

The rhinoglyphic pattern of epidermal domes is recurrent over several mammalian orders, monotremes, and marsupials (Osman Hill, 1948). The basic morphological features of nasolabial skin have been described in a variety of mammals (e.g. cat: Abrahams et al., 1987; camel: Eshrah, 2017; pig: Jacobi et al., 2005; tree shrew: Loo & Kanagasuntheram, 1972; rat: Macintosh, 1975), but there are few comparative studies on rhinarium skin. In order to get some overview of the dome size and compare basic skin morphology, I examined the morphological features of rhinarium skin in cow (*Bos taurus)*, ring-tailed lemur (*Lemur catta*), brown bear (*Ursus arctos)*, and dog (*Canis familiaris*) (Paper III). In the same study I also describe the innervation pattern in the epidermal domes, which will be detailed in a later section. Interestingly, the epidermal dome diameter varies. The dome diameter is largest in cow and smallest in lemur, indicating a possible correlation with body size. The corneous layer thickness increases with increasing dome size in all examined animals except for dog. Epidermal domes in all species share similar traits such as a thick epidermis and elongated dermal papillae, but the positioning and number of dermal papillae varies. The border between the dermal and epidermal layers, from which the dermal papillae extend, is linear in lemur, brown bear, and dog, but rounded in cow. The rounded border between epidermal and dermal layers in cow domes might provide additional anchoring of the skin, enabling it to withstand the substantial mechanical forces the rhinarium is exposed to during grazing. In lemur, the dermal papillae are normally positioned at junctions between the separate domes, while cow, bear, and dog epidermal domes contain several dermal papillae across each dome. A single, centrally positioned papilla and an

invagination on the skin surface just above it was observed in a population of lemur epidermal domes. Pronounced central dermal papillae were found in bear and dog epidermal domes. In dog, the central papilla extends almost to the surface of the skin, and terminates in a rounded bulge that protrudes into the corneous layer. The bear central dermal papilla is instead wider at the base and divides into several smaller dermal protrusions when approaching the skin surface. In contrast to the central papilla in dog, it does not extend markedly higher towards the skin surface than surrounding papillae. In cow epidermal domes, the central part is instead occupied by a large gland duct that is connected to glandular tissue in the dermis, that was only found in cow, despite extensive searching in the other animals. Eccrine nasolabial glands and their secretions have been described in several domesticated and wild grazers. The nasolabial glands are of salivary nature and hypothesized to have a functional role in food intake. The high concentrations of complex carbohydrates in the gland secretions also provide protection from microorganisms and help retain moisture on the skin surface (Fukui et al., 2012; Meyer & Tsukise, 1989; Yasui et al., 2005; Yasui et al., 2003). To my knowledge, the North American raccoon is the only carnivoran reported to possess nasolabial skin glands. The glands are less numerous than in ruminants and the composition of their secretions hints to a protective function, less connected with food intake (Yasui et al., 2006).

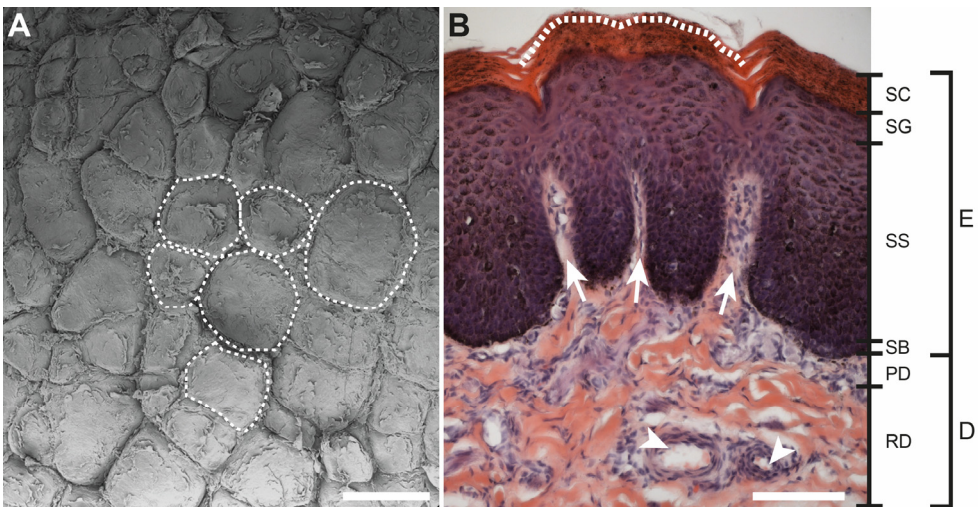


Figure 2. The surface and structure of rhinarium skin. (A) Scanning electron micrograph of rhinarium skin surface in brown bear. Epidermal domes of varying size are encircled (white dashed line). Scale bar 500 µm. (B) Cross-section of rhinarium skin in ring-tailed lemur. The surface of a single epidermal dome is outlined (white dashed line). The layers of the epidermis (*E*) and dermis (*D*) are indicated on the right side of the image, *SC* - stratum corneum, *SG* - stratum granulosum, *SS* - stratum spinosum, *SB* - stratum basale, *PD* - papillary dermis, *RD* - reticular dermis. The dermal papillae protruding into the epidermis are indicated by white arrows. Blood vessels in the reticular dermis are indicated by white arrowheads. Scale bar 100 µm. Image B modified from Paper III.

Epidermal differentiation

To begin to understand the function of the rhinarium epidermis, we compared its epidermal layer morphology with that of the interfollicular abdominal skin across different mammalian species. Using scanning electron and transmission electron microscopy, we described the epidermis morphology of cow (*Bos taurus*), pig (*Sus scrofa domesticus*), sheep (*Ovis aries*), rat (*Rattus norvegicus*), ring-tailed lemur (*Lemur catta*), brown bear (*Ursus arctos*), dog (*Canis familiaris*), cat (*Felis catus*), and ferret (*Mustela putorius*) (Paper I). In all species examined, we found that the rhinarium epidermis is considerably thicker than the abdominal skin due to additional keratinocyte rows in the spinous, granular and corneal epidermal layers. The border between the dermal and epidermal layers in abdominal skin is fairly smooth, in contrast to the finger-like dermal protrusions observed in rhinarium skin. The epidermis differentiation is similar between rhinarium and abdominal interfollicular skin, except for a few, but important differences. In the stratum granulosum of the rhinarium, the keratinocytes lack keratohyalin granules, normally characteristic for cells in this layer, and are instead filled with thick fiber bundles. This may be the reason why the nasolabial epidermis of dog, also included in our study, has previously been described as lacking a granular layer (Evans & de Lahunta, 2013). The corneocytes in rhinarium skin are also considerably thicker than in abdominal skin. An additional difference in the cornification process of the two types of skin is the formation of corneodesmosomes. In the granular layer of abdominal skin, the cellular protrusions with desmosomes retract to later form corneodesmosomes that are seen as electron-dense patches on the cell membrane. In rhinarium skin, these protrusions do not retract entirely and instead project into neighbouring cells. This, in turn, creates invaginations in adjacent corneocytes of the corneal layer. When surface corneocyte layers are shed off, a structured cell surface is revealed (Fig. 3). This ultrastructure is independent from the macroscopic rhinoglyphic pattern of epidermal domes seen on mammalian rhinaria.

Two types of ultrastructural pattern can be identified on the rhinarium corneocyte cell surface. All of the species examined possess a pattern of pits, except for the pig, that instead has a pattern formed of closely apposed furrows. The shape of the pits varies and can be cylindrical (cattle and sheep), embedded (ring-tailed lemur, dog, ferret, and bear, see Fig. 3A-B) or compressed (cat and rat). The mean diameter of the pits is approximately 370 nm, they can be up to 1000 nm deep and conceal an intricate interior. Capillary forces have been shown to be sufficient to spread moisture on artificial surfaces with similar sized pores (Bico et al., 2002), which suggests that the ultrastructural pit pattern promotes wetting of the rhinarium surface. Pigs, that use their snout for digging and rooting, normally have a dry nasolabial skin surface. Unlike the other examined species, their nasolabial skin lacks the pit pattern and instead exhibits a pattern of furrows. This observation

further corroborates the proposed functional role of the pit pattern in wet-nosed species.

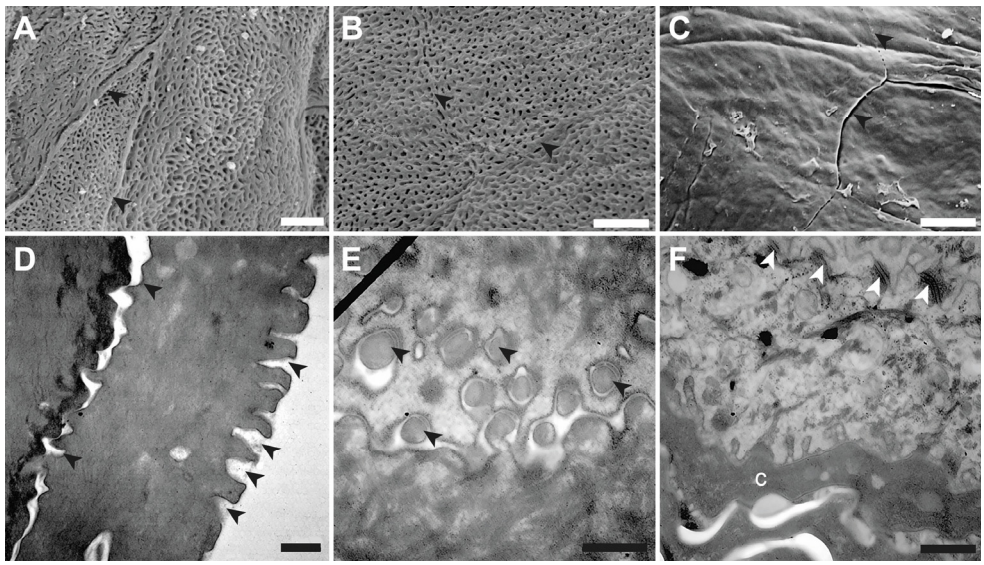


Figure 3. Scanning electron and transmission electron micrographs of rhinarium and abdominal skin. (A) Ultrastructure on rhinarium surface corneocytes in brown bear. The borders of corneocytes are indicated by black arrowheads. Scale bar 3 µm. (B) Ultrastructure on rhinarium surface corneocytes in ring-tailed lemur. The borders of corneocytes are indicated by black arrowheads. Scale bar 3 µm. (C) Smooth corneocyte surface in ring-tailed lemur abdominal skin. The borders of corneocytes are indicated by black arrowheads. Scale bar 3 µm. (D) Transmission electron micrograph of a cross-sectioned corneocyte in ring-tailed lemur rhinarium skin. The invaginations in the corneocyte surface are indicated by black arrowheads. Scale bar 0.5 µm. (E) Transmission electron micrograph of a corneocyte sectioned parallel to the skin surface in ring-tailed lemur rhinarium. The perturbances from the underlying corneocyte are indicated by black arrowheads. Scale bar 0.5 µm. (F) Transmission electron micrograph of the upper granular layer in ring-tailed lemur rhinarium. The cell membrane of the granular keratinocyte and the desmosomes that later form the perturbances are indicated by white arrowheads. *c* - neighboring corneocyte. Scale bar 0.5 µm. Images D-F courtesy of R. Elofsson.

Following this study, it was reported that dog and dingo glabrous skin, including the rhinarium skin, exhibits a unique pattern of pigmentation (Elofsson & Kröger, 2018). Rather than being restricted to the basal epidermal layer as normal for mammalian skin (Schartl et al. 2016), melanocytes in rhinarium skin are found in the basal and spinous sublayers of the epidermis and across the dermal layer. New melanocytes and melanosomes are continuously formed in the epidermal layers. The reason for having high amounts of migrating melanocytes is unknown. It is nevertheless of interest since most wild mammalian species possess highly pigmented rhinaria. This feature, in addition to the unique epidermal differentiation process described in this section, distinguishes rhinarium skin from interfollicular skin in mammals.

Sensory innervation in rhinarium skin

The innervation of glabrous rhinarium skin has been studied in a variety of mammals in the latter half of 1900, but has since received less attention. In the following sections, I introduce the results from many of these studies as well as results from Papers II and III, without dwelling on what the sensory structures in rhinarium skin might imply about its function (which will be discussed in the following chapter).

Innervated corpuscles

The rhinarium skin innervation has been studied in some artiodactyls, carnivorans, rodents, insectivores, monotremes, and basal primates. Although the innervation patterns are highly variable, there are some common characteristics. Merkel cell-neurite complexes are numerous in rhinarium skin and they are positioned at the base of epidermal intrusions, in between dermal papillae. Ruffini-endings as well as Meissner corpuscles have not been reported (Montagna et al., 1975).

A variety of encapsulated nerve endings and simple Pacinan-like innervated corpuscles have also been described in rhinarium skin. The position and minor details in morphological appearance of these corpuscles vary among species, which has complicated any generalization. Encapsulated nerve endings at the top of dermal papillae were described in the nasolabial skin in the pig (Tachibana et al., 1989), rat (Macintosh, 1975; Silverman et al., 1986), and mole rat (Klauer et al., 1997). These innervated corpuscles bear similarities to Meissner corpuscles, which otherwise are lacking from rhinarium skin. In rat, each such corpuscle may be innervated by several nerve fibers that form a loose bundle enclosed by lamellar cells and connective tissue (Macintosh, 1975). Lamellated corpuscles, resembling simple Pacinian corpuscles or the Krause corpuscle and positioned at the base or sides of epidermal intrusions, have been described in rhinarium skin of tree shrew (Loo & Kanagasuntheram, 1972), pig (Nafstad, 1971), cat (Abrahams et al., 1987), rat (Macintosh, 1975; Silverman et al., 1986), and opossum (Loo & Halata, 1985). In cat rhinaria, encapsulated nerve endings are often found in clusters. Each corpuscle formed of several lamellae houses a single nerve terminal. The same exhaustive study performed on cat rhinarium skin also revealed that the innervation pattern in

individual epidermal domes is relatively consistent, indicating that each dome could be seen as a functional unit (Abrahams et al., 1987).

Eimer's organ

The rhinarium epidermal domes of some species possess a complex mechanosensory organ found exclusively in rhinaria, called Eimer's organ. It was first described in moles (*Talpidae*) (Eimer, 1871), but has since also been discovered in monotremes (Andres et al., 1991; Manger et al., 1997; Manger & Hughes, 1992; Manger & Pettigrew, 1996). This complex sensory skin organ is located in the center of rhinarium epidermal domes, with its base under the epidermal intrusions, and stretches from the papillary dermis to the surface of the skin. It is comprised of several lamellated Pacinian corpuscles in the dermis, Merkel cell-neurite complexes in the stratum basale, specialized keratinocytes, and free nerve endings in the epidermis. All of the components share innervation from a common nerve plexus in the dermis. The epidermal nerve fibers are arranged in a circle, with one central fiber positioned in the middle. An associated column of keratinocytes reaches the corneal layer where it is covered by a corneal cap (Catania, 2000; Marasco et al., 2007). In moles, the Eimer's organ was demonstrated to create small receptive fields and high sensitivity to mechanical stimulation (Sachdev & Catania, 2002), which makes it ideal for gathering tactile information in the animal's dark habitat.

Eimer's-like organ

Structures similar to Eimer's organ, also exlusively present in the epidermal intrusions of rhinarium epidermal domes, have been described in other mammalian species, including several marsupials (Loo & Halata, 1985; Munger, 1965) and the European hedgehog (Malinovsky, 1981). In Paper II, we report that rhinarium epidermal domes in the ring-tailed lemur (*Lemur catta*) contain Eimer's-like organs. We found that, as in other species that possess an Eimer's-like organ, the organ in lemurs lacks the specialized keratinocyte column and the central fiber. The epidermal nerve fibers are arranged in a wide ring (Fig. 4A). In the ring-tailed lemur rhinarium skin, the epidermal nerves form small spines, filled with mitochondria, which penetrate into keratinocytes (Fig. 4A), similar to what has been described in the coast mole, *Scapanus orarius* (Marasco et al., 2007). These epidermal nerve fibers thus constitute a distinct population of unmyelinated nerve endings designed for mechanoreception. In accordance to other authors, we suggest that the three main components (lamellated corpuscles in the dermis, Merkel cell-neurite complexes in the stratum basale, and unmyelinated nerve endings in the epidermis) positioned in

the base of epidermal protrusions, make the organ equivalent to Eimer's organ described in moles and monotremes.

The ring-tailed-lemur rhinarium skin was further examined in Paper III, where I determined the innervation density and pattern across whole rhinarium epidermal domes. It became evident that in some epidermal domes, the central dermal papilla and the slight invagination seen in the skin surface above it in effect separate the epidermal dome in to two functional units, each served by an Eimer's-like organ. This is an additional difference between the Eimer's organ in *Talpidae* species and the Eimer's- like organ in lemur rhinarium skin.

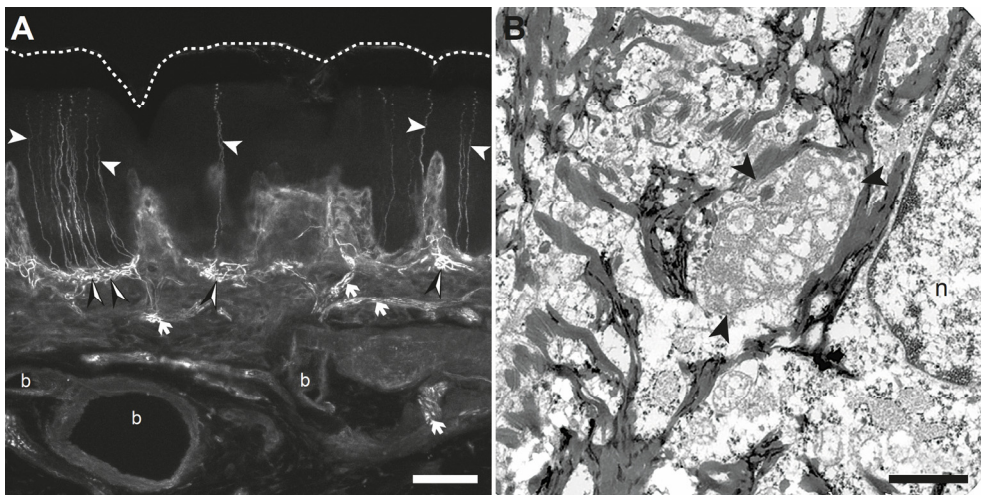


Figure 4. The Eimer's-like organ in the rhinarium skin of ring-tailed lemur. (A) Neural tissue in a cross-section of rhinarium skin in the ring-tailed lemur. The skin surface is indicated by a white dashed line. Epidermal nerves belonging to the Eimer's- like organ (white arrowheads) ascend in clusters from the lamellated bodies (partially filled arrowheads) in the papillary dermis. Thick nerve bundles in the reticular dermis are indicated by white arrows. *b* - blood vessels. Scale bar - 100 µm. (B) Transmission electron micrograph of lemur rhinarium skin (section parallel to the skin surface). A mitochondria-filled nerve terminal (black arrowheads) in a keratinocyte. *n* - keratinocyte nucleus. Scale bar 1 µm. Image B courtesy of R. Elofsson.

A new morphological type of skin sensory organs

In Paper III, I briefly describe an innervated structure in the dog rhinarium epidermal domes that deviates from the above described corpuscles. It is found in the top of a pronounced centrally positioned dermal papilla and is supplied by multiple nerve fibers that ascend from the underlying nerve plexus (Fig. 5A). The nerves terminate in a bundle structure that is separated from the skin surface only by a few layers of keratinocytes (Fig. 5B). In contrast to the Meissner-like corpuscles in the top of dermal papillae described in some non-canine rhinaria, it is more heavily innervated and positioned much closer to the skin surface. A recent electron microscopy study

has classified it as a new morphological type of somatosensory skin organ (Elofsson & Kröger, 2020). In that more in-depth study, it was determined that the multiple myelinated nerve fibers entering the dermal papilla are enclosed by a tube formed of fibrocytes. The tube ends distally in the papilla and the nerve fibers then lose their myelin sheet before they fan out towards the epidermis. Most importantly, unlike other dermal mechanosensory skin organs and the Meissner-like corpuscles described in other species, the organ lacks a capsule and surrounding collagen fibers. The nerve fibers are instead surrounded by supporting cells that together with the axon branches form a loose bundle, merely 25 µm from the skin surface.

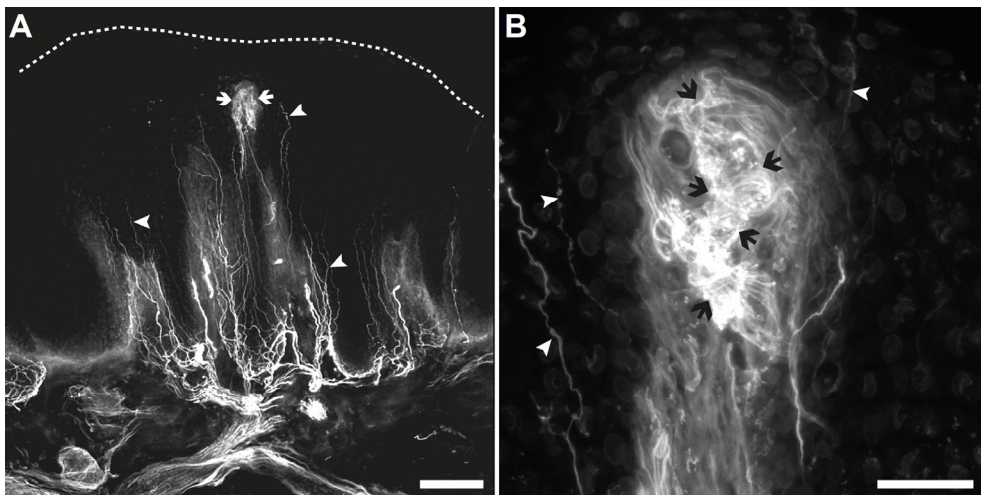


Figure 5. Neural tissue in dog rhinarium skin. (A) Cross-section of a rhinarium epidermal dome in dog. The skin surface is marked by a white dashed line. Epidermal nerve fibers are indicated by white arrowheads. A heavily innervated structure (white arrows) is visible in the top of a centrally positioned dermal papilla. Scale bar 100 µm. (B) Higher magnification image of the superficial dermal somatosensory organ in dog rhinarium skin. A network of intertwined nerves (black arrows) occupy the top of the central dermal papilla. Single nerve fibers entering the epidermis from the dermal papilla are indicated by white arrowheads. Scale bar 30 µm. Figure modified from Paper III.

Intraepidermal nerve fibers

In addition to the skin sensory organs described above, rhinarium epidermis is richly innervated by free nerve endings. In all examined species, the free nerve endings, arising from the dermal nerve plexus, reach the granular epidermal layer (Macintosh, 1975) and often terminate just below the corneous layer of the epidermis (Abe, 1954; Abrahams et al., 1987; Ono, 1956). However, previous studies, where traditional staining methods were used on relatively thin tissue sections, often provide an incomplete picture of the morphology and fail to consider rhinarium epidermal domes as functional units. Although all authors state that the rhinarium skin is richly innervated, estimations of intraepidermal nerve fiber

densities are lacking, which further hinders comparisons of rhinarium skin specialization in different mammalian species.

In Paper III, my goal was to provide a direct comparison of the rhinarium epidermal dome innervation in four mammalian species in order to recognize any similarities that would indicate a common function. The presence of eccrine glands and the salivary nature of their secretions in rhinarium skin in some grazer species has led researchers to hypothesize that the rhinarium has a role in food intake. To test this, the species for this study were chosen based on different feeding behaviours. These included: cow (*Bos taurus,* herbivore, grazer), brown bear (*Ursus arctos,* omnivore), and dog (*Canis familiaris,* carnivore). The other and more widely accepted hypothesis regarding the rhinarium function in mammals is that it constitutes a tactile surface. The ring-tailed lemur (*Lemur catta,* herbivore, browser), that possess Eimer's-like organs in rhinarium skin, was included in the study to provide a reference of how the innervation in rhinarium skin can be optimized for tactile sensitivity. Cutaneous nerve fibers in thick consecutive tissue sections were labelled with antibodies against a neuronal marker and analysed using confocal microscopy. I mapped the intraepidermal nerve fiber density (IENFD) and the innervation pattern across whole rhinarium epidermal domes in lemur, bear, and dog. Due to its large size (2-3 mm in diameter), it was only possible to examine the widest part of the cow epidermal dome (six consecutive cross-sections). From my analysis, I could establish that the density of epidermal fibers is not homogenous across the lemur, bear, and dog epidermal domes, but is instead higher in the center, with few or no fibers in the peripheral sections. The mean IENFDs determined across the width of the sections are similar in cow, lemur, and dog, but lower in bear (Fig. 6).

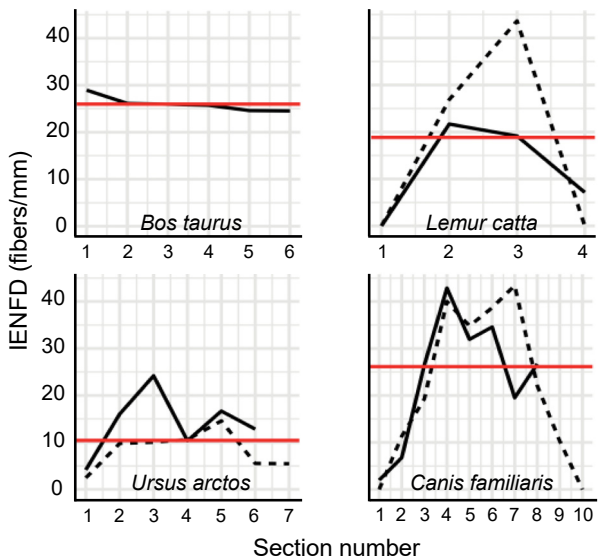
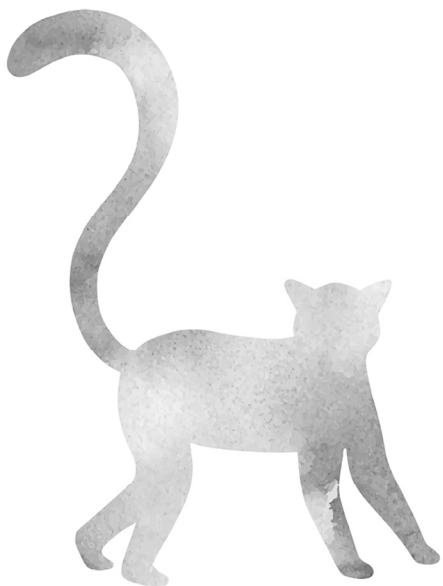


Figure 6. Graphic representation of intraepidermal nerve fiber density (IENFD) in rhinarium epidermal domes in four mammals. Due to the large epidermal dome size in Bos taurus, only the central part of the dome was examined. When more than one was analyzed, data from both domes are presented (solid and dashed lines). The mean IENFD determined across the length of the sections is indicated by a red line. Figure modified from Paper III.

The pattern of intraepidermal nerve fibers in cow, bear, and dog rhinarium epidermal domes differ substantially from the ordered nerve fiber clusters in the ring-tailed lemur. As described in Paper II, the lemur epidermal domes contain Eimer's-like organs of which the majority of nerve fibers entering the epidermis are a part. Nerve fibers enter the cow, bear and dog epidermis at various depths, both from the elongated dermal papillae and from the baseline of the epidermis-dermis border. The centrally positioned gland duct in cow epidermal domes lacks innervation. The sensory organ in dog epidermal domes (described in a previous section) is positioned within the dermis. As previously mentioned, the IENFD is unevenly distributed across the domes (Fig. 6). Higher nerve fiber densities in the central part of the dome indicate increased sensitivity in this part and further support the view of rhinarium epidermal domes as functional units. Interestingly, in all four species, the most superficial epidermal nerve fibers terminate slightly thickened, just below the corneous layer.



Rhinarium – a sensory organ

The rhinarium is innervated by a sensory branch of the trigeminal nerve (Ashton & Oxnard, 1958) and its frontal position is ideal for exploring the immediate environment. The trigeminal somatosensory system can pick up stimuli such as touch, temperature and chemical irritants (Darian-Smith, 1973; Nguyen et al., 2017). The rhinarium is described as wet in most species but eccrine glands in rhinarium skin have been described only in a handful of species. In other species, the moisture could be coming from serous glands draining into the vestibule of the nasal passages (Blatt et al., 1972) or licking the rhinarium skin surface but this remains to be confirmed.

A trait of particular interest is the surface temperature of the rhinarium, which differs considerably between mammalian taxa. In the species relevant for this thesis, the rhinarium surface temperature is correlated with ambient temperature, albeit in different ways: herbivorous even- and odd-toed ungulates normally warm their rhinaria to temperatures higher than ambient, prosimians keep their rhinaria at a temperature close to ambient (in the limited range of subtropical temperatures they are naturally exposed to), and in carnivorans, rhinarium skin surface temperature is often below ambient (Gläser & Kröger, 2017). The surface temperature of the carnivoran rhinarium will be discussed in greater detail in the following chapter. In the following sections, I discuss the possible primary and secondary sensory specializations of the rhinarium in different mammals based on literature and on my own observations (Papers II and III).

A tactile fovea

Due to the ridges and papillae on the rhinarium skin surface (Fig. 2A), rhinarium skin is often compared to primate digital skin, which is specialized for tactile sensitivity. Across the species in which the rhinarium skin has been examined, the basic morphology suggests that the rhinarium surface is well equipped to withstand mechanical stress. Similar to the skin on primate hand and foot soles, the epidermis is thick in rhinarium skin and dermal papillae protruding high into the epidermal layer provide additional rigidity (Maiolino et al., 2016).

Mechanosensory Eimer's and Eimer's-like organs are present in a number of taxa – in monotremes and insectivores that are considered close to the mammalian stem forms and in prosimian primates such as the ring-tailed lemur (Paper II), which is more derived. This indicates that these organs are either plesiomorphic structures that have been lost in most mammalian taxa, or a derived feature due to convergent evolution and therefore present only in certain species. A complex mechanosensitive organ in rhinaria of ring-tailed lemurs, which have both well-developed eyes and can manipulate objects with their front paws, suggests that the rhinarium surface may be important for determining fine structural details. Barker and Welker (1969) found no significant differences in receptive field sizes of first order neurons in the nasolabial skin in raccoons (*Procyon lotor*), that use their paws to manipulate objects, and coatis (*Nasua nasua*), that primarily use their snout for exploring the environment. However, the density of rhinarial receptors was higher in coatis, which could provide the central nervous system with more sensory information.

Interestingly, the innervation of rhinarium skin in raccoons is higly similar to their digital skin. As with rhinarium skin, raccoon digital skin has a high density of Merkel cells and simple Pacini-like lamellated corpuscles. True Pacinian corpuscles are few, Ruffini-endings and Meissner corpuscles are lacking (Munger & Pubols, 1972). Neurophysiological studies performed on rhinarium skin in raccoon, and cat revealed that the majority of receptor fibers are rapidly adapting, which correlates well with the high densities of simple lamellated Pacini-like corpuscles in the upper dermal layer (Barker & Welker, 1969; Donevan & Abrahams, 1993). Merkel cell-neurite complexes, innervated by slowly adapting fibers and plentiful in rhinarium skin, are thought to detect textural details, shapes, and edges. As mentioned previously, a variety of encapsulated nerve endings and simple corpuscles can also be found in rhinarium skin. To establish whether they are sensitive to mechanical stimuli, their components and response characteristics should be explored further, also in other species. Despite the diversity observed, and remaining open questions regarding these unclassified innervated corpuscles, the authors of the above-mentioned studies unanimously conclude that the mammalian rhinarium is a tactile surface. However, cutaneous mechanoreceptors like Meissner corpuscles and Merkel cells-neurite complexes are now known to detect more than only mechanical stimuli (Bouvier et al., 2018; Pare et al., 2001) and the high density of intraepidermal free nerve endings is rarely considered when discussing the sensory specializations of rhinaria. Furthermore, when discussing the mammalian rhinarium as a "tactile fovea", one must consider the skin surface temperature, since it affects tactile sensitivity. Improved sensory function is often actively obtained by warming a sensory organ (Mauck et al., 2000), and increasing skin tactile sensitivity by warming the nasolabial skin is suggested to be advantageous for animals grazing in cold climates (Ince et al., 2012). A cold rhinarium surface, as observed in most carnivorans, may have a negative effect on the mechanosensory function of the rhinarium, since low temperatures decrease tactile sensitivity (Gescheider et al., 1997). This feature raises questions

regarding the generalization of rhinaria as a surface primarily for tactile discrimination.

Detection of chemical signals

Many irritants and poisonous compounds can be detected by receptors in cutaneous nerve fibers encoding temperature and pain, thus warning the animal of these unsafe substances. Trigeminal and cutaneous chemoreception differs from both taste and smell since it involves direct activation of free nerve endings through ion channels rather than initiating a transduction cascade in receptor cells (Viana, 2010). In Paper III, I concluded that cow, ring-tailed lemur, brown bear and dog epidermal domes all have high densities of superficially terminating intraepidermal nerve fibers (Fig. 7).

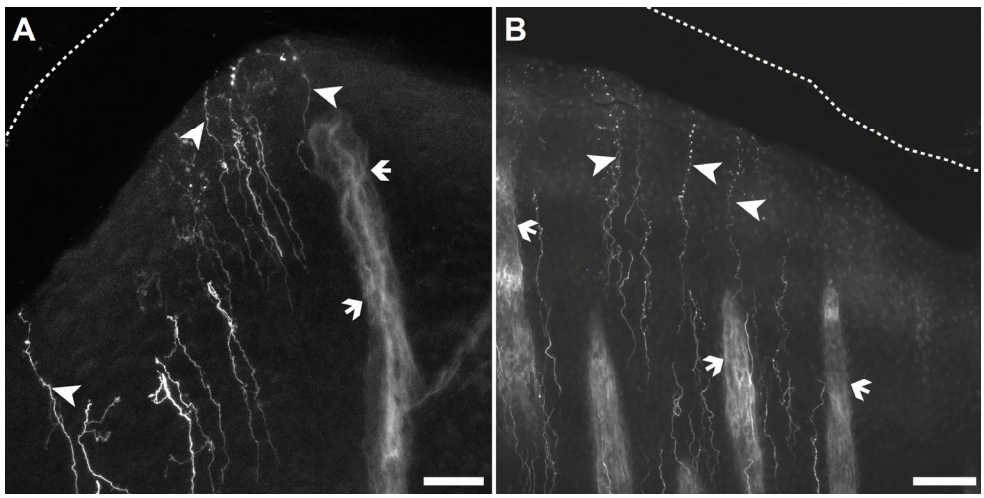


Figure 7. Superficially terminating epidermal nerve fibers in rhinarium skin: in dog (A) and cow (B). The cutanous nerve fibers are indicated by white arrowheads and the dermal papillae are indicated by white arrows. The skin surface is marked with a white dashed line. Scale bars: A - 30 µm, B - 100 µm.

Superficially terminating cutaneous nerve fibers are exposed to chemical signals and irritants that come in to contact with the skin, making it an ideal surface for chemoreception. A moist rhinarium surface could further facilitate the collection of substrate-bound chemical signals. Although it is well known that rhinarium skin has a high density of intraepidermal nerve fibers, a chemosensory function of the rhinarium has rarely been discussed, and if so, exclusively in species possessing eccrine glands in rhinarium skin. Cow, one of the focus species in Paper III, was previously reported to have glands in rhinarium skin (Meyer & Tsukise, 1989). In cow epidermal domes, nerve fibers enter the epidermis in a seemingly unorganized

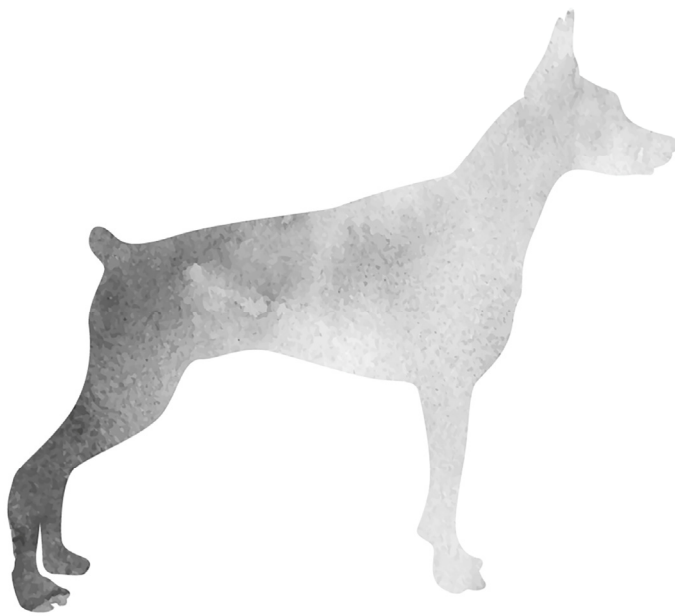
manner and the dermis lacks heavily innervated skin sensory organs. These findings are indicative of a primarily chemosensory role of cow rhinarium skin. Grazers, such as cow, forage for food in low vegetation and would benefit from detecting irritants in contact with the rhinarium skin in order to avoid inedible vegetation. Interestingly, a similarly unorganized innervation pattern was observed in brown bear rhinarium epidermal domes. This suggests that the rhinarium could be utilized as a chemosensory surface in other mammalian species, such as brown bear, that often digs and browses amongst low vegetation to collect food items.

Temperature detection

Cutaneous thermoreceptors are crucial for discriminating the temperature of objects or surfaces in contact or within close proximity to the skin. A recent study has examined the sensitivity of dog rhinaria to radiating heat using both behavioural methods and brain imaging (Bálint et al., 2020). It was found that dogs could be successfully trained to detect a warm object from a distance of approximately 1.6 m based on heat radiation in a two-way discrimination task. In fMRI scans, the brain of awake dogs showed significantly increased activity in the left somatosensory association cortex when they were presented with a stimulus approximately 10.7 °C warmer than ambient temperature at a distance of 240 mm. The authors argue that dogs could detect infrared radiation from distant prey using the cold rhinarium and the same function is likely in other cold-nosed species. At this time, the limitations of this sense and possible transduction mechanisms are unknown. Interestingly, in Paper III, dog rhinarium skin was found to have a disproportionally thin stratum corneum layer. A thin stratum corneum and, consequently, a smaller distance to the nerve receptors in the tissue would be advantageous for detecting stimuli not in direct contact with the skin surface, such as temperature. Detection of temperature was suggested as one of the possible functions for the newly discovered somatosensory organ in dog rhinaria (Elofsson & Kröger, 2020; Fig. 5). Its response to thermal stimuli remains to be tested, but the claim is supported by morphological features, such as proximity to the skin surface as well as lack of a capsule and collagen fibers normally present in mechanosensory skin organs.

In addition to detecting temperature of objects and surfaces, temperature-sensitive cutaneous nerve fibers in mammalian skin detect ambient temperature, which is important for regulating physiological and behavioural responses that prevent any large fluctuations in body core temperature. The body core temperature is kept within a narrow interval using feedback and feedforward mechanisms. The feedback responses are triggered when the inner core temperature strays from its normal interval. These temperature changes are detected by specialized thermoreceptors in the brain, spinal cord, and internal organs in the abdomen (Morrison & Nakamura, 2019). Detection of ambient temperature changes by thermosensitive afferents in

the skin is thought to constitute a feedforward mechanism. Hairy skin covers most of the body surface in mammals and due to its large surface area is the primary source for feedforward signals. However, temperature detection by glabrous skin on the face plays a disproportionally large role in thermoregulation despite its much smaller surface area (Nadel et al., 1973). The rhinarium skin that is not covered with hair and has a high density of cutaneous nerve fibers most likely sensitive to thermal stimuli could be utilized as a surface for providing precise feedforward signals regarding ambient temperature. The presence of polymodal and exclusively temperature sensitive nerve fibers was confirmed in neurophysiological studies on sensory afferents in rhinarium skin (Barker & Welker, 1969; Duclaux et al., 1980; Heinz et al., 1990), but it remains to be explored whether their contribution to body thermoregulation is particularly important.



The cold rhinarium

The cold carnivoran rhinarium is puzzling with regard to the maintenance of the low tissue temperature. Vasoconstriction is an effective way to reduce tissue temperature, but it cannot be maintained over prolonged periods of time. In arctic and subarctic mammalian species, keeping glabrous skin patches cold helps minimizing heat loss that would occur from the skin if it was kept at temperatures much higher than ambient. This is achieved through the use of vascular counter-current heat exchangers that can effectively recycle heat in animal limbs and other protruding body parts (Blix, 2016). However, the carnivoran rhinarium was consistently observed to have a lower than ambient surface temperature (Gläser & Kröger, 2017), indicating that an additional or a completely different cooling mechanism is in place. The rhinarium temperature dynamics are best described in the domestic dog (Kröger & Goiricelaya, 2017), which prompted me to use dog as a model species to explore possible rhinarium cooling mechanisms in carnivores. In this section, I give a short introduction to the rhinarium surface temperature dynamics in canids, explain the concept of vascular heat exchangers, and describe the structural features of blood vessels in dog rhinaria (Paper IV).

Rhinarium surface temperature dynamics in canids

The odd temperature profile of the carnivoran rhinarium has previously been described in foxes (*Vulpes*) and dogs (*Canis*) (Klir & Heath, 1992; Kröger & Goiricelaya, 2017). In foxes, the rhinarium surface is lower than ambient at ambient temperatures between 20 °C and 30 °C. The hairless and wet rhinarium surface in foxes has been suggested to be important for dissipating excessive body heat at high ambient temperatures. In alert dogs, the nasolabial skin is kept a few °C under ambient temperature down to ambient temperatures of about 15 °C. However, the skin is warm when the animal is asleep or after strenuous exercise. This behaviour-dependent thermal profile is evident already in newborn pups. The authors of the latter study claim that the small surface area of the rhinarium would be negligible for body cooling and instead suggest that a low rhinarium temperature is important for its sensory function. Significance of the cold rhinarium surface for detection of heat signals has since been demonstrated in behavioural experiments by Balint et al. (2020).

Vascular heat exchangers

Counter-current heat exchangers that prevent heat loss have been well studied and their elegant heat conservation principle is broadly applied in engineering. The counter-current exchangers in animal extremities transfer heat from warm arterial blood coming from the body core to cold venous blood returning from an extremity. This mechanism minimizes both the loss of body heat by supplying the extremity with cooled arterial blood as well as lessens the harm the returning cold blood would cause the heart and body core. This system is made possible by the close proximity of arteries and veins with opposite directions of blood flow. It is used for saving body heat and, in a few cases, for dissipating excessive thermal energy by increasing blood flow and instead redirecting venous blood to more superficial veins. The heat exchanger is then bypassed and excessive heat in both arterial and venous blood is lost to the surroundings (Blix, 2016; Schmidt-Nielsen, 1997). In the dog foot, triads formed of two veins and an artery run in parallel, and the close proximity of the vessels allows for effective heat exchange. The foot pad contains numerous veins that anastomose and form a well-developed plexus, which can be used as a reservoir for warm blood when the tissue temperature becomes too low (Ninomiya et al., 2011). In addition to reducing heat loss from the body, keeping the glabrous foot pad skin temperature close to 0 °C ensures minimal melting of snow or ice that comes in contact with the skin, which would otherwise substantially reduce friction.

Brain cooling system

An additional blood cooling system can be found at the base of the brain in many mammalian species. Its function is to cool arterial blood entering the brain to keep it at its optimal operating temperature when in extreme thermal environments or during strenuous physical exercise. In the brain cooling system, also known as the carotid rete or *rete mirabile*, the arterial blood heading to the brain is cooled by the cold venous blood coming back from the nasal mucosa, where it has been cooled by evaporation of water (Baker, 1979). The carotid rete is poorly developed in dogs (Daniel et al., 1953) but their brain cooling system is nevertheless very effective (Baker & Chapman, 1977). Dogs have a highly convoluted mucous membrane in the maxilloturbinate part of the nasal cavity with sufficient area to cool the blood flowing through the respiratory epithelia while breathing or panting (Craven et al., 2007). Kröger and Goiricelaya (2017) suggest that the cooled venous blood returning from the nasal cavities could also be used to cool the rhinarium skin when brain cooling is not needed. This is based on their observation that the nasolabial

skin remains cold in animals experiencing moderate heat stress, but warms up in overheated animals that might need to cool their brains.

Blood vessels in the dog rhinarium and snout

In Paper IV, I explore whether a vascular heat exchanger could be used to maintain low rhinarium tissue temperature. To visualize the intricate network of blood vessels in the dog rhinarium, I performed tomography scans on vascular corrosion casts of the dog snout. In order to resolve the smaller details in regions of interest, I also analysed parts of the cast using scanning electron microscopy (SEM). I searched for structural features that are typical for vascular heat exchangers such as a close proximity between arteries and veins and extensive branching of these structures. I also explored whether there are any indications that the cooling of the rhinarium is connected to the brain cooling system, by mapping the large veins in the snout.

The primary arterial blood supply to the rhinarium comes from the lateral nasal arteries. These superficial arteries follow the lateral sides of the snout prior to entering the rhinarium from the ventral side. Thick, freely anastomosing venous vessels form an intricate venous plexus in the rhinarium. This plexus is drained by branches of the dorsal nasal veins on the dorsal and ventral sides. The lateral nasal arteries and branches of the dorsal nasal vein do not come in contact with each other outside of the rhinarium, making it impossible for them to participate in effective heat exchange along the length of the snout (Fig. 8).

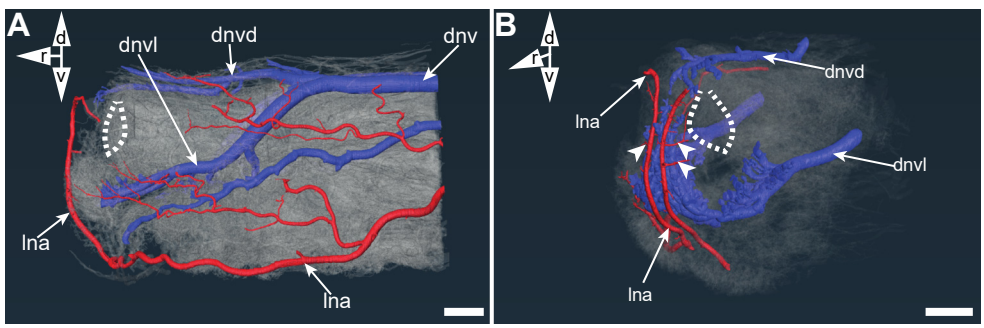
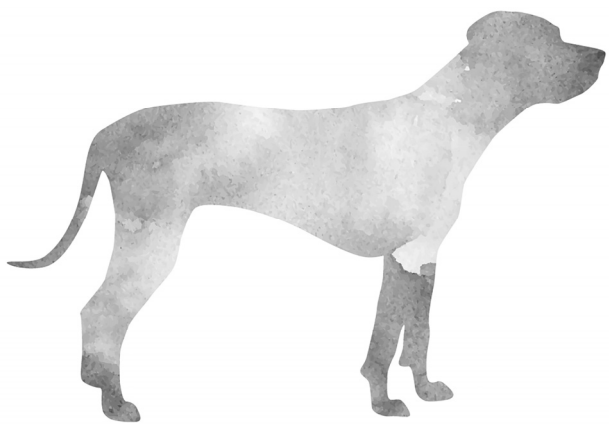


Figure 8. Blood vessels in the canine snout and rhinarium. Surfaces of the arteries (red) and veins (blue) recreated from tomography scans of dog snout corrosion casts. The remaining vessels of the corrosion cast are imaged as a partially transparent surface (grey). The lateral nasal artery (lna) and branches of the dorsal nasal vein (dnv, dorsal branch - dnvd, lateral branch - dnvl) are indicated by arrows. The position of the left nostril is outlined by a white dashed line. The dorsal (d), ventral (v), and rostral (r) directions are indicated in the left upper corner. (A) Lateral view of the dog snout and its major vessels. The rhinarium vessels in this cast were partially damaged and were therefore not segmented. Scale bar 500 µm. (B) Frontal-lateral view of the rhinarium and its major vessels. The interconnections between the lateral nasal arteries (lna) and the rhinarium venous plexus are indicated by white arrowheads. Scale bar 500 µm. Figure modified from Paper IV.

Superficial damages to the fragile corrosion casts did not allow any further analysis of the microcirculation in rhinarium skin. In dog paw skin, the microcirculation is arranged in a manner that promotes heat loss from the capillaries in order to ensure a close to ambient temperature of returning venous blood. In regard to basic skin morphology, paw skin exhibits features similar to rhinarium skin, including pronounced dermal papillae. In paw dermal papillae, the blood in its superficial capillaries is cooled when in contact with a cold surface, and the cold blood is then collected in centrally positioned venules (Ninomiya et al., 2011). If the capillaries and venules in rhinarium dermal papillae are arranged in a similar way, the blood in superficial capillaries could be brought close to ambient temperature and additional cooling could be achieved by water evaporation from the wet rhinarium surface. The cooled blood then pooled in the larger veins and the rhinarium venous plexus might be sufficient to maintain a slightly lower than ambient temperature in the rhinarium. The microcirculation in rhinarium dermal papillae should be investigated in more detail to explore this possibility.

The rhinarium venous plexus could also function as a local heat exchanger. The intricate network of venous vessels results in a large surface area that could be used to cool arterial blood. In my SEM preparations I observed a small artery meandering through the posterior side of the rhinarium venous plexus. Given a supply of cold venous blood, the plexus would be able to cool the blood passing through this artery. Apart from the branches of the dorsal nasal vein, I did not observe any other venous vessels interconnecting with the plexus therefore limiting the possible supply of cold venous blood from the nasal cavities, as suggested by Kröger and Goiricelaya (2017). Nevertheless, corrosion casts are fragile and their smaller vessels are easily damaged. Functional studies that map the directions of blood flow in alert dogs and other cold-nosed animals are therefore necessary to determine whether the rhinarium cooling mechanism is interconnected with the brain cooling system.



The very cold rhinarium

Regardless of how the low rhinarium surface temperature in dogs is sustained, it leads to further questions regarding the cold tolerance of skin. Domestic dogs have accompanied humans for thousands of years and are therefore widespread around the world, including at high latitudes. Their ability to withstand cold temperatures comes as no surprise considering their evolutionary history. Fossil remains of an ancestral canid, a common ancestor of both wolves and dogs, were discovered in Siberia, the far north of Russia. To a varying extent, its ancestral DNA is present in modern dog breeds (Lee et al., 2015) and these animals still exhibit physical adaptations necessary for living in cold climates, including counter-current heat exchangers that reduce heat loss from their glabrous paw pads (Ninomiya et al., 2011). Little is known about the cold pain response threshold in mammals other than humans and rodents. In humans, cold pain sensations may occur already at temperatures above 20 °C (Lötsch et al., 2015). Freely behaving laboratory rats show nociceptive behaviors when placed on a cold plate of 5 °C -9 °C (Allchorne et al., 2005). In winter, pet dogs living in northern Sweden are daily exposed to freezing temperatures, making them an attractive resource for studying acclimatization to cold temperatures and the limits of skin cold tolerance. The frontal position of the rhinarium also allows for an easy determination of naked skin surface temperature without interfering with the animals' normal behaviour. In the following sections, I describe the basics of cold detection and transmission. I focus on the ion channels that are considered the main candidates for detection (TRPM8 and TRPA1) and propagation ($Na_v1.8$ and $Na_v1.9$) of cold and cold pain signals in nerve fibers. I also introduce what is known from acclimatization studies, as well as results from my own study that explores the cold receptor membrane channels in dog (Paper V).

Molecular basis of cold detection

In mammals, detection of temperature is thought to mainly rely on a group of temperature-gated transient receptor potential (TRP) channels. These calcium permeable cation channels are incorporated in the membranes of cutaneous nerve fibers. There are in total seven known TRP gene subfamilies, of which six are found in mammals: ankyrin (TRPA), classical (TRPC), melastatin (TRPM), mucolipin

(TRPML), polycistin (TRPP), and vaniloid (TRPV) (Nilius & Flockerzi, 2014). The vaniloid receptor TRPV1, activated by noxious heat and capsaicin (the molecule responsible for the "hot" taste of chili), was the first one to be identified as a thermal TRP channel (Caterina et al., 1997). This discovery led to further investigations into the thermosensitivity of TRP channels.

TRPM8 was simultaneously identified as a cold sensor by two independent research groups (McKemy et al., 2002; Peier et al., 2002). The channel was originally discovered in the prostate (Tsavaler et al., 2001), but its expression was since confirmed in other tissues, including the trigeminal and dorsal root ganglions (Dhaka et al., 2008). When tested in a heterologous expression system, TRPM8 is activated by ≈21 °C to 26 °C (McKemy et al., 2002; Peier et al., 2002). The channel can, however, be activated by both higher and lower temperatures, because it adapts to ambient temperature through phospholipase C mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), a lipid component of the cell membrane. This mechanism is claimed to be crucial for adaptation to fast temperature changes, and possibly acclimatization to seasonal differences (Fujita et al., 2013; Rohács et al., 2005). TRPM8 has been proposed to be the primary detector of both noxious and innocuous cold via its modulation by potassium currents (Madrid et al., 2006) but this is contradicted by lack of co-expression of TRPM8 and known nociceptive markers (Dhaka et al., 2008). TRPM8 deficient mice exhibit reduced sensitivity to cold but respond to noxious cold similarly as wild-type mice (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007; Winter et al., 2017).

Since its discovery, TRPA1, the only member of the ankyrin subfamily in mammals, has been recognized as the main candidate for a noxious cold receptor. TRPA1 is expressed in dorsal root and trigeminal ganglions as well as visceral primary sensory neurons (Lapointe & Altier, 2011; Story et al., 2003). The channel is a polymodal sensor for tissue damage and plays a prominent role in chemo-nociception. Despite TRPA1 being established as a nocicepetor, its role in transduction of noxious thermal stimuli remains somewhat controversial (Saito & Tominaga, 2017). TRPA1 of invertebrates, basal vertebrates (lizards and snakes), and birds is activated by heat (Laursen et al., 2015). In a comparative study on mammalian TRPA1 orthologs it was reported that rat and mouse TRPA1 can be activated by cold (18 °C - 16 °C), while human and rhesus monkey orthologs do not react to cold in whole-cell or single channel recordings (Chen et al., 2013). Purified human TRPA1 inserted in a planar lipid bilayer was reported to be both heat and cold sensitive. The channel was gated by cold only when in a redox state (after being treated by H_2O_2) (Miyake et al., 2016; Moparthi et al., 2016). Studies on the cold sensitivity in *trpa1* knock-out mice have delivered contradicting results (e.g. Karashima et al., 2009; Knowlton et al., 2010). In some studies, however, lack of TRPA1 seemed to lead to higher heat tolerance (Hoffmann et al., 2013; Vandewauw et al., 2018).

Transmission of cold temperature by cutaneous fibers

Cutaneous nerve fibers reacting exclusively to innocuous cold have been described in several mammals and frogs. They have constant action potential activity and exhibit a bell-shaped stimulus response function. The responses of these fibers are transient within the innocuous temperature range, meaning that they return to steady state activity after discharging due to a cooling stimulus. Results from studies on transmission speed and blocking of these fibers indicate that innocuous cold is signalled by thinly myelinated A-fibers (A∂) in mammalian skin (Schepers & Ringkamp, 2010). Some C-fibers are also receptive of innocuous cold, but are unlikely to have any greater role in encoding it (Mackenzie et al.,1975).

In contrast to specialized fibers transducing innocuous cold, afferents reacting to noxious cold are often polymodal A∂ and C-fibers (which are also sensitive to mechanical stimulation and heat). The fiber response increases linearly with decreasing temperature in both A∂ and C-fibers (Schepers & Ringkamp, 2010). Deeper peri-vascular sensory fibers are also involved in encoding cold pain and are responsible for the persistent pain sensation that remains even after more superficial afferents are numbed (Arndt & Klement, 1991). Curiously, an electrophysiological study on cats has revealed populations of cold activated fibers in the cat rhinarium skin that show static responses at temperatures below 10 °C and therefore could potentially signal lower temperatures than the assumed mammalian cold pain threshold. As expected of cold activated fibers, most of the cold sensitive afferents in the study showed dynamic responses as the fibers were gradually cooled down to 10 °C. One group of fibers did however exhibit static discharges at 10 °C and could be activated by temperatures down to -5 °C (Duclaux et al., 1980). A population of cold sensitive fibers in the rhinarium skin with static discharges at 10 °C has also been described in the rat (Heinz et al., 1990). Since the identification of thermosensitive ion channels in sensory afferent membranes, the research focus in this field has shifted to the characterization of temperature activated protein channels and their expression in cold sensitive fibers.

Acclimatization to cold

Investigations that address long term cold adaptation effects on cold sensitive afferents are sparse and it was long assumed that peripheral cold receptors do not undergo long term adaptation. In rats adapted to cold (5 °C for six weeks), there was a significant decrease in cold activated fiber sensitivity (Kozyreva, 2006a). However, the study only considers afferents that are activated by innocuous cold. Hensel and Schäfer (1982) performed similar electrophysiological measurements on cats (two groups, held in 30 °C or 5 °C for 4.7 years). They tested the response

of cutaneous nerve fibers in the rhinarium to static and dynamic (in cooling steps of 5 °C) cold stimuli. There was little difference in the response to static cold stimuli between the two groups, but the average peak frequencies in response to dynamic cold stimuli were considerably lower in the group kept at 5 °C. These results indicate that prolonged exposure to a certain ambient temperature changes the response profile in cutaneous cold receptors. In another study, the number of cold spots in forearm skin was compared between humans that spend substantial time working outside in freezing temperatures and a control group (Kozyreva, 2006b). Each cold spot in the skin has a diameter of around 1 mm and is innervated by at least one cold sensitive afferent. Cold adapted subjects had a significantly lower number of cold spots (tested with a 4 °C probe), indicating lower cold sensitivity and therefore higher sensory cold tolerance in the skin.

It is reasonable to assume that mammals thriving in cold climates can tolerate lower skin temperatures than humans, but since such tolerance involves slow-acting acclimatization (Scholander et al., 1950), results from studies on laboratory populations may not reflect the full temperature tolerance range of a species. In Paper V, using thermography, I measured the rhinarium skin surface temperature in pet dogs that are repeatedly exposed to the freezing outside temperatures in northern Sweden. This was done in order to determine what naked skin surface temperatures they normally experience and tolerate. In controlled cold-acclimatization studies on rats, the peripheral skin thermoreceptors undergo long-term cold adaptation while the mean skin temperature remains just below 37 °C (Kozyreva, 2006a). We were surprised to find that more than half of the dogs (12 of 22 individuals) in the thermographic experiments had a rhinarium surface temperature lower than 5 °C (some as low as 1.06 °C), while showing no behavioral signs of distress (Fig. 9). Temperatures below 5 °C in dog rhinarium skin raise several questions about cold tolerance and pain in these mammals, which should be addressed on both receptor and neural levels.

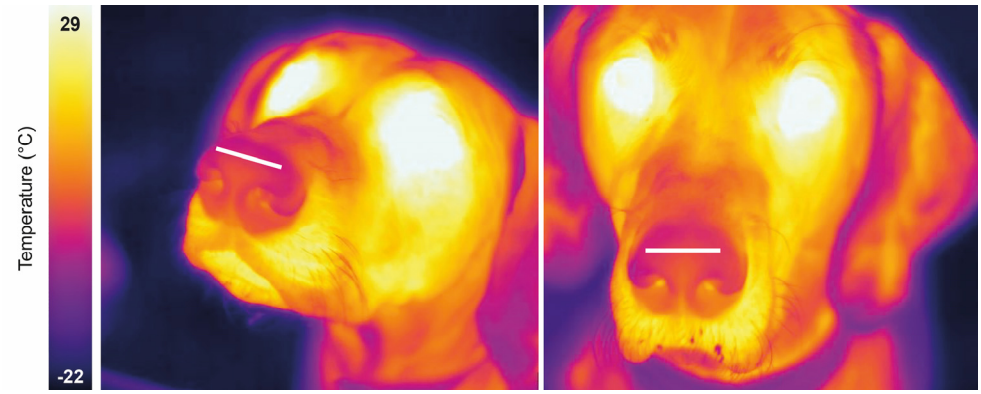


Figure 9. Thermography images of dogs in an ambient temperature of -21 °C. The temperature scale for both images is provided on the left side. The rhinarium surface temperature was determined by averaging pixel values across the frontal rhinarium (white line). The rhinarium surface temperature in the two dogs was 4.2 °C (left image) and 5.3 °C (right image).

TRPM8 and TRPA1 in dog

Surviving harsh winter climates would require some adaptation of cold transduction mechanisms to avoid cold-induced pain at non-critical temperatures and the development of protective behavioural responses when the skin temperature is low enough to risk tissue damage. The low surface temperatures recorded on dog rhinaria in northern Sweden indicate that transduction mechanisms in dog might be intrinsically tuned to lower temperature. Dog TRPM8 was reported to have a slightly lower activation threshold temperature than the human and rodent orthologs (Liu et al., 2006), but it remained untested whether and to which extent the channel can adapt to ambient temperature. Since mammalian TRPA1 orthologs exhibit discrepancy regarding their thermal sensitivities (Chen et al., 2013), the response to both cold and heat needed to be addressed in the previously thermally uncharacterised dog TRPA1 in order to determine whether it might function as a noxious cold transducer in dogs.

In paper V, I report the results of a thermal characterization study of dog TRPM8 and TRPA1. The main goal of this study was to determine whether TRPM8 and TRPA1 in dog have lower activation threshold temperatures than their orthologs in humans. The characterization was performed on the channels expressed in human embryonic kidney cells (HEK293T) using calcium-imaging and electrophysiological methods. I found that the activation threshold temperature of dog TRPM8 is only slightly lower than in human TRPM8 and also changes depending on the ambient temperature the channel is exposed to prior to the application of a cold stimulus (Fig. 10A-B). The adaptability of dog TRPM8 is similar to what has been reported for the human TRPM8 channel (Fujita et al.,

2013). This does not exclude TRPM8 as a noxious cold sensor but it is evident that the channel in its naïve form cannot account for the observed cold tolerance in dogs. In my experiments, dog TRPA1 showed no response to cold (also in its reduced state) but was instead activated by moderate heat. Since TRPA1-deficient mice exhibit reduced sensitivity to heat (Hoffmann et al., 2013; Vandewauw et al., 2018), it is likely that TRPA1 is also involved in heat transduction in dogs. Together, these results suggest that the cold tolerance of mammals living in arctic and subarctic climates does not depend on the characteristics of unmodified TRPM8 and TRPA1 and that one should instead focus on investigating different channel modification pathways, expression and even other candidates for molecular cold receptors.

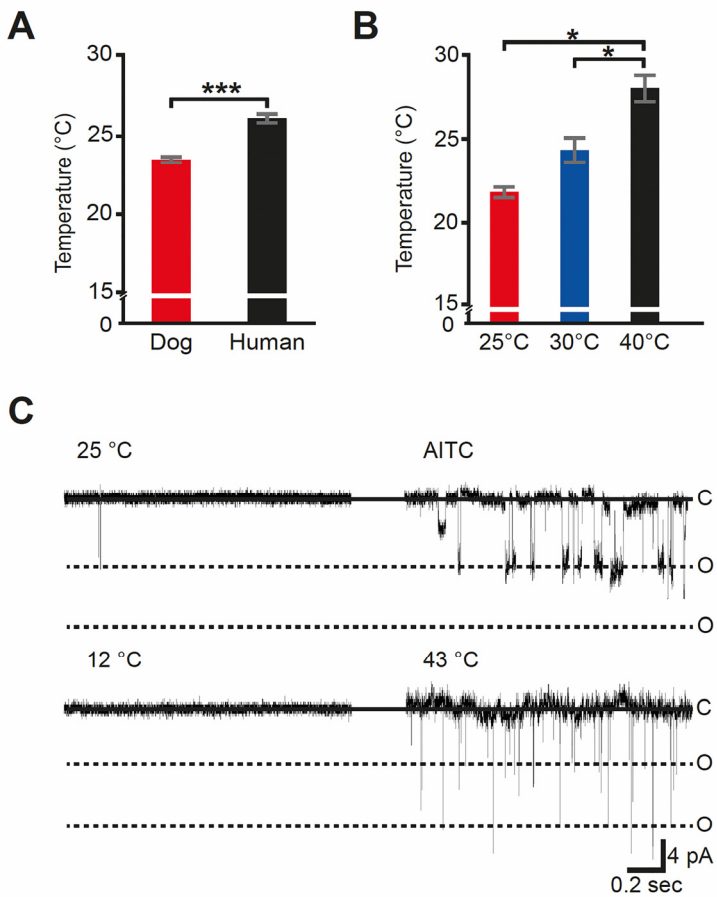


Figure 10. Characteristics of dog TRPM8 and TRPA1 channels tested in HEK293T cells. (A) Comparison of activation threshold temperatures in dog and human TRPM8 determined in whole-cell patch-clamp recordings (ambient temperature 30 °C). Student's t-test, S.E.M. indicated by error bar, ***P ≤ 0.001. (B) Comparison of activation threshold temperatures in dog TRPM8 adapted to different ambient temperatures (*25 °C, 30 °C, 40 °C*) as determined in whole-cell patch-clamp recordings. ANOVA, followed by a two tailed multiple t-test with Bonferroni correction. S.E.M. indicated by error bars, *P ≤ 0.05. (C) Single-channel events of dog TRPA1 in HEK293T cells at Vp= -60 mV, cell-attached mode. The channels remain at closed levels (*c*, solid line) when at 25 °C and 12 °C. Channel openings (*o*, dashed lines) are observed when the cell is exposed to AITC (50 µm, channel agonist) and 43 °C. Scale bar in right lower corner. Figure modified from Paper V.

Transmission of painful stimuli and cold-induced pain

Transmission of any nervous signal relies on voltage-gated sodium channels (VGSCs) that belong to the Na_v protein family. These ion channels play a crucial role in generating action potentials in the membranes of nerves and other excitable cells. The pharmacology of these channels and their contribution to pain signalling have been studied extensively (de Lera Ruiz & Kraus, 2015; Kwong & Carr, 2015). In sensory neurons $Na_v1.8$ and $Na_v1.9$, channel activity is less affected by cooling compared to other sodium channels. $Na_v1.8$ is essential for retaining sensitivity to noxious stimuli when the skin is cooled (Zimmermann et al., 2007), while $Na_v1.9$ is a sub-threshold amplifier for cold transducer signals (Lolignier et al., 2015).

The low skin surface temperatures recorded on the rhinaria of cold-acclimatized dogs prompted me to explore the expression of $Na_v1.8$ and $Na_v1.9$ in rhinarium cutaneous nerve fibers. A full study on this was limited due to a lack of tissue samples from cold-acclimatized dogs. Nevertheless, the available dog tissue samples provided some indications of the channel expression pattern in dog rhinaria. I observed that both $Na_v1.8$ and $Na_v1.9$ are expressed in keratinocytes throughout the epidermis, but not in epidermal free nerve endings. However, a significant and consistent expression of $Na_v1.8$ was observed in single nerve fibers on the border between the dermis and epidermis, along the dermal papillae (Fig. 11A). Expression of Nav1.9 could not be confirmed in any nerve fibers in the analysed sections (Fig. 11B).

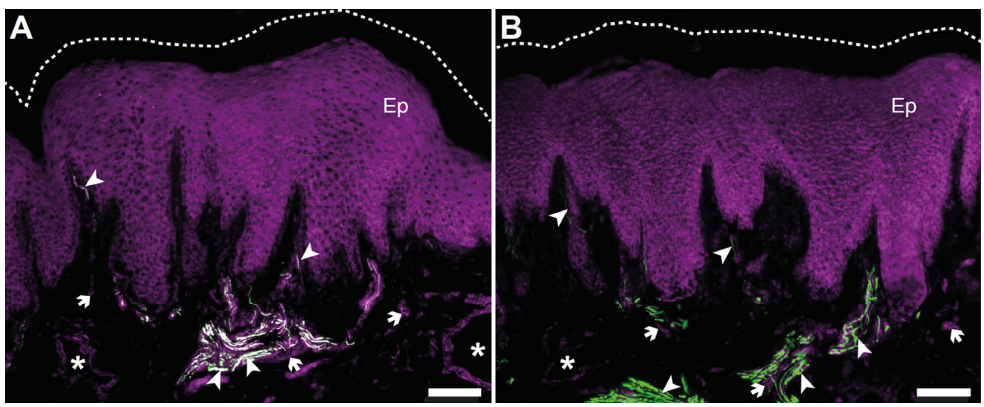


Figure 11. Expression of $Na_v1.8$ and $Na_v1.9$ in canine rhinarium skin. Expression of $Na_v1.8$ (A) and $Na_v1.9$ (B) is shown in magenta, neural tissue is labelled with NF-200 and shown in green. The skin surface is marked by a white dashed line. (A) $Na_v1.8$ signal was present in keratinocytes across the epidermis (*Ep*) and colocalized with NF-200 in a population of nerve fibers (colocalization shown in white, white arrowheads). $Na_v1.8$ is additionally expressed in fibroblasts (in the demis and within dermal nerve bundles (white arrows)) and endothelial cells of blood vessels (marked *). (B) $Na_v1.9$ is expressed in the keratinocytes across the epidermis (*Ep*), fibroblasts (in the dermis and surrounding nerve bundles (white arrows)) and endothelial cells of blood vessels (marked *). No expression was observed in nerve fibers (white arrowheads). Scale bars 100 µm.

In the dermis, both $Na_v1.8$ and $Na_v1.9$ are expressed in fibroblast cells. In dermal nerve bundles, $Na_v1.8$ is expressed in neural fibers and adjacent fibroblast cells. The expression of $Na_v1.9$ within the nerve bundles appears to be limited to fibroblast cells. Both $Na_v1.8$ and $Na_v1.9$ are expressed in endothelial cells of blood vessels in the dermis (Fig. 11). Expression of $Na_v1.8$ in a distinct population of nerve fibers in the upper layer of the dermis in dog rhinarium skin could be ensuring that sensitivity to harmful stimuli is maintained at low skin temperatures. Absence of $Na_v1.9$, which is an amplifier of cold transducer signals in nerves, suggests that the nerve fibers in rhinarium skin are less sensitive to cold-induced pain. The function of VGSCs in non-excitable cells as keratinocytes, fibroblasts, and endothelial cells is poorly understood and deserves further attention.

Conclusions and perspectives

The work on mammalian rhinaria presented in this thesis provides the first few building blocks towards a more complete understanding of this fascinating structure. Firstly, we observed clear morphological differences in the epidermal layers of rhinarium and interfollicular skin (Paper I). The thick corneocytes and their surface ultrastructure in rhinarium skin constitute a unique surface morphology not found on other parts of the body. Morphology of the rhinarium epidermal layers suggests that the keratinocytes in rhinarium skin undergo a unique differentiation process. The observed morphological differences between interfollicular and rhinarium epidermis hint that the gene expression pattern must differ in rhinarium keratinocytes but whether it does and, in that case, how, remains an open question.

The discovery of an Eimer's-like organ in a prosimian primate (Paper II) has disturbed the common belief that the organ is restricted to the rhinarium of insectivores and marsupials. This in turn raises the question of whether Eimer's or Eimer's-like organs exist in additional mammalian species and whether it is more correlated to phylogeny or ecology. Despite Eimer's organs previously described in rhinaria of certain species, the innervation pattern in rhinarium epidermal domes has received little attention and was generally described as poorly organized. Rhinarium skin contains few mechanoreceptors otherwise found in mammalian glabrous skin, which urges us to consider other options than it being primarily a surface for tactile discrimination.

The comparison of rhinarium epidermal dome innervation in four mammalian species (Paper III) has resulted in several new insights. The inhomogeneous distribution of intraepidermal nerve fibers within an epidermal dome suggests that each dome constitutes a functional unit. Amongst the four examined species, a mechanosensory function of rhinarium was obvious only in the ring-tailed lemur, that possess Eimer's-like organs in rhinarium skin. The observed differences in sensory innervation and skin morphology suggest that there will likely be no universal answer regarding the function of mammalian rhinaria. A more functional approach, including electrophysiological, behavioural, and observational studies in the animals' natural environments, is necessary to determine the sensory specializations of rhinaria.

The peculiar surface temperature dynamics in dogs has received attention both in scientific and layman circles. The cold rhinarium surface in active dogs is puzzling

since keeping the tissue at lower than ambient temperatures for prolonged periods of time would require a cooling mechanism. A closer look at the blood vessels in the canine snout and rhinarium (Paper IV) has provided some clues regarding the possible rhinarium cooling mechanisms. However, further studies that address the rhinarium skin microcirculation and examine the blood flow in rhinarium vessels are necessary to reach any further conclusions.

Pet dogs repeatedly exposed to winter temperatures in northern Sweden provided an opportunity to explore the cold tolerance limits of the naked rhinarium skin (Paper V). I discovered that the rhinarium surface temperature in these animals can be much lower than what is considered to be the cold pain threshold in most mammals. The observed cold tolerance appears to be independent of the intrinsic characteristics of cold sensitive channels TRPM8 and TRPA1, which both are similar to their human orthologs. In fact, in my experiments, dog and human TRPA1 appeared to instead be gated by moderate heat. Despite these interesting new results, it remains unclear how dogs tolerate and detect cold temperatures. Further efforts are needed to elucidate the cold transduction and transmission mechanisms in these animals and these should not be limited to ion channels. Nevertheless, dog TRPM8 and TRPA1 should be investigated in their native cells, rather than a heterologous expression system. This would provide more insight into whether cofactors or other proteins are necessary for their function. Also, the expression of these and other temperature sensitive channels should be mapped in the rhinaria of dogs acclimatized to cold climates, in order to determine whether other ion channels could be acting as transducers of cold. If performed, such a study would likely provide answers to many of the remaining open questions regarding skin cold tolerance and cold transduction in dogs.

Dictum factum.

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Tack

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