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Histology and fine structure of epidermal papillomas in the Alpine newt *Ichthyosaura alpestris* (Urodela: Salamandridae)

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Abstract

Epidermal papillomas of alpine newts (*Ichthyosaura alpestris*) collected in the field (Germany, Austria) were studied by histology (LM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Papillomas were found on the head, the trunk and the tail, with the most and largest on the head of males. They protruded beyond the body surface exhibiting an appearance like a cauliflower. The head of one specimen studied by SEM had a large papilloma and was densely populated with bacteria, fungi and sessile ciliates. The surface of papillomas was covered either by stratum corneum cells, or by deeper cell layers that may be exposed by injuries. Histology revealed that papillomas consisted of compact bulbous extensions that were deeply embedded into the dermis and separated from each other by small septa (papillae) of connective tissue. Bulbs were distinctly demarcated by a thin basal lamella that was continuous with the basal lamella of the adjacent non-altered epidermis. An invasion of papilloma-cells through the basal lamella in the underlying connective tissue could not be unequivocally demonstrated; only once we found an area by TEM, which could be interpreted in this way. Bulbs may have two types of cavities or cysts. One type contained masses of keratinized cell layers, the other appeared either largely empty, or contained cellular debris and/or PAS-positive substances discharged by secretory cells lining the cyst. Tumor cells within a bulb are offen arranged in clusters or nests. Generally, cells appeared relatively undifferentiated having large euchromatic or heterochromatic nuclei, prominent nucleoli, and a moderate amount of cell organelles. Also the amount of tonofilamets and number and size desmosomes (macuale adhaerentes) seemed to be reduced. Virus-like particles were found neither in the cytoplasm nor in the nucleus. Compared to the unaltered epidermis, in which no mitoses were seen, mitotic cells occurred in all papillomas examined. In addition, the neoplastic tissue always contained macrophages and further 'leucocytes', but necrotic areas were rare. Dermal papillae separating the bulbs from each other and the dermal tissue immediately beneath the basal lamina of papillomas contained a high number of cells (*e.g.*, fibroblasts and cells of the immune system).

Kurzfassung

Epidermale Papillome bei Bergmolchen (*Ichthyosaura alpestris*) aus Deutschland und Österreich, die im Freiland gesammelt worden waren, wurden histologisch (LM) und feinstrukturell (SEM, TEM) charakterisiert. Die z. T. stark aufgewölbten und wie ein Blumenkohl gefurchten Papillome, fanden sich am Kopf, am Rumpf und am Schwanz, die größten und meisten jedoch vor allem am Kopf der Männchen. Dieser war bei einem Männchen relativ dicht von Bakterien und Pilzhyphen sowie von einigen sessilen Ciliaten besiedelt. Die Oberfläche der Papillome war entweder mit Stratum corneum-Zellen oder einer weniger stark keratinisierten Zelllage bedeckt oder war so verletzt, dass noch tiefer gelegene Zellschichten sichtbar waren. Das histologische Bild zeigte kompakte epidermale Verdickungen, überwiegend aus Keratinocyten, die weit in die Dermis ragten und hier durch bindegewebige Papillen voneinander getrennt waren. Die Verdickungen waren deutlich von der darunter liegenden Dermis durch eine intakte Basallamelle abgegrenzt, die kontinuierlich in die Basallamelle der benachbarten, offenbar nicht veränderten Epidermis-Bereiche überging. Eine Invasion neoplastischer Keratinocyten durch die Basallamelle in das Bindegewebe konnte nicht zweifelsfrei belegt werden; nur einmal fanden wir in Ultradünnschnitten einen kleinen Bereich, der in diesem Sinne interpretiert werden könnte. In den Papillomen kamen zwei Typen von Cysten oder Höhlen vor. Ein Typ enthielt große Mengen keratinisierter Zelllagen, der andere Typ erschien entweder weitgehend leer oder enthielt Zellreste und/oder PAS-positive Substanzen von sekretorischen Zellen der Cystenbegrenzung. Innerhalb der Papillome schienen die Keratinozyten oft in Gruppen angeordnet, zu sein. Die Keratinozyten waren relativ undifferenziert, besaßen große eu- und heterochromatische Kerne, auffällig gestaltete Nucleoli sowie einer mäßige Anzahl von Organellen; zudem schienen auch die Menge an Tonofilamenten sowie Anzahl und Größe der Desmosomen (maculae

adhaerentes) reduziert zu sein. Virusähnliche Partikeln wurden weder im Kern noch im Cytoplasma gefunden. Verglichen mit der benachbarten Epidermis war die Mitoserate im Tumor erhöht. Im Tumorgewebe verstreut waren zudem Makrophagen und andere ‚Leukozyten'; nekrotische Bereiche waren aber selten. Darüber hinaus enthielten die bindegewebigen Papillen, welche die einzelnen Verdickungen voneinander trennten sowie das Bindegewebe basal der Papillome eine auffallend hohe Anzahl von Zellen (Fibroblasten, Zellen des Immunsystems).

Key words

Urodela, spontaneous neoplasia, skin tumor, neoplastic keratinocytes.

Introduction

Neoplasias (synonymously used with the terms neoplasms and tumors; Stacey & Parker, 2004), i.e., diseases in which genetically altered cells escape from the normal cell-cycle regulation and monitoring of the immune system, have been reported in various organs of Anura and Urodela (= Caudata), whereas no such reports are known from Gymnophiona (reviewed in Schlumberger & Lucke, 1948; Ashashima *et al*., 1987; Green & Harshbarger, 2001; Stacy & Parker, 2004; Densmore & Green, 2007). Generally, however, neoplasias are relatively rare in Amphibia, due to the fact that especially anurans possess effective antitumor responses (Robert, 2010), and that urodeles have a remarkable regenerative capacity (e.g. Laurens, 1997; Stacy & Parker, 2004; Ruggiero & Bustuoabad, 2006).

In Urodela the most frequently described neoplasias are spontaneous (i.e. without previous exposition to a known carcinogen) epidermal papillomas (also named squamous papillomas, warts, epitheliomas, see Stacy & Parker, 2004). These epithelium-derived tumors are usually benign, but occasionally may turn malignant. They have been reported and in part histologically examined in several species (e.g. *Triturus cristatus*: Seilern-Aspang *et al*., 1966; *Ichthyosaura* (formerly *Triturus*) *alpestris*: Champy & Champy 1935; Darquenne & Matz 1971 fide Ashashima *et al*., 1987; Hachtel & Greven, 2014; *Ambystoma mavortium*: Rose, 1981; Harshbarger *et al*., 1989; *Cynops pyrrhogaster*: Bryant 1973; Pfeiffer *et al.* 1979, 1989; Ashashima *et al*., 1982, 1985, 1986; *Hynobius lichenatus*: Asashima & Meyer-Rochow, 1988; *Andrias japonicus*: Frye *et al*., 1989 fide Trauth *et al*., 2002; *Cryptobranchus alleganiensis*: Trauth *et al*., 2002; Harshbarger & Trauth, 2002).

The most detailed information of epidermal papillomas in Urodela currently available is from the salamandrid *C. pyrrhogaster.* Studies on this species include field and laboratory investigations about the geographical variation of epidermal papillomas, seasonality, prevalence, temperature dependence, growth, spontaneous regression (Pfeiffer *et al*., 1979; Asashima, & Komazaki, 1980; Ashashima *et al*., 1982, 1985; Ashahima & Koyama, 1986; Ashahima *et al*., 1986; Oka *et al*., 1992). Ultrastructural studies on such papillomas have been published by Pfeiffer *et al*. (1979, 1989), and a few TEM images are found in Trauth et al. (2002), in which some slight alterations of the papilloma-tissue were described compared to the unaffected epidermis. In some papillomas of *C. pyrrhogasters* virus-like particles were demonstrated by TEM (Pfeiffer *et al*., 1979, 1989).

Recently we reported on epidermal papillomas in free living alpine newts *Ichthyosaura alpestris* (Salamandridae) collected 2013 in Germany (Hachtel & Greven, 2014). A more recent collection of a larger number of newts from Austria showing the same symptoms prompted us to study these papillomas in more detail and discuss our findings in a broader context.

Material and methods

Origin of the specimens

Five specimens of *Ichthyosaura alpestris* with epidermal papillomas were found on May 3 and June 2013 in the Königsdorfer Forst bei Kerpen (Rhein-Erft-Kreis, NRW, Germany) (see Hachtel & Greven, 2014).

In April 2016 more than 100 specimens of *I. alpestris* and *Lissotriton vulgaris* captured in a eutrophic pond of the school in Rankweil (Austria) were inspected. From these only *I. alpestris* (approx. 25 – 30%) showed epidermal papillomas (K. Zimmermann, in litt). Concerning number and location of papillomas a total of 27 affected specimens were more carefully examined.

Macrophotography

The Austrian animals were photographed with a Nikon D4 and macro- lenses (AF Micro Nikkor 60 mm) without flash. Details of heads were photographed using a SMZ 745T Nikon Binocular with camera head (DS-5M).

Histology (LM), scanning (SEM) and transmission electron microscopy (TEM)

For histology and ultrastructure 7 specimens (2 from Germany, 5 from Austria) were anesthetized with MS 222

Tab. 1. Number, and site of papillomas in 27 specimens of *Ichthyosaura alpestris* from Austria. Se = sex; He = head; TB = trunk and belly, Ta = tail.

Nr	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Se	♀	♀	♀	♀	♀	♀	♀	♀	♀	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂
He	4	3	2	2	1	3	1	0	1	1	4	3	1	5	7	4	2	1	3	1	1	6	9	1	1	1	0
TB	3	0	1	1	3	0	0	1	3	0	1	3	0	2	2	0	0	1	1	2	2	2	3	1	0	0	1
Ta	0	0	2	8	0	0	0	0	2	0	0	0	4	1	4	1	0	2	0	2	0	1	0	0	0	0	0
Total	**7**	**3**	**5**	**11**	**4**	**3**	**1**	**1**	**6**	**1**	**5**	**6**	**5**	**8**	**13**	**5**	**2**	**4**	**4**	**5**	**3**	**9**	**12**	**2**	**1**	**1**	**1**

Tab. 2. Relation between the total numbers (no) of papillomas in the different body regions of females and males (F, M) of *Ichthyosaura alpestris*. The tail was taken as reference point (= 1).

				Relations		
	♀	♂	Σ	♀	♂	Σ
No	9	18	27			
Head	17	50	67	1.2	4.2	2.6
Trunk/Belly	12	21	33	0,9	1.8	1.3
Tail	14	12	26	1	1	1

(Sigma) and skin samples were excised from the papillomas and from unaffected body areas.

LM: Samples of the Austrian newts were either fixed in buffered 4% formalin for several hours, routinely embedded in paraffin, sectioned at 7–10 µm, and subjected to the PAS-reaction; nuclei were counterstained (Mulisch & Welsch, 2019) or processed for TEM (see below).

Further, 1 µm sections from resin embedded material (see below) from the German and the Austrian sample were stained with Azur B/Toluidin blue according to Richardson *et al.* (1960). Histological sections were examined and photographed using the NDP.view procedure (Hamamatsu) and the Olympus, Vanox-T AH-2-microscope with a digital camera (Olympus C-3030-Z)

SEM: One Austrian specimen with a single papilloma on its head was euthanized (see above) and decapitated. The head was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, 2, washed in buffer, dehydrated, critical point dried, sputtered with gold and examined under the SEM (Leo 1430 (Fa. Zeiss). Thereafter the head was cut lengthwise to expose the interior of the papilloma, and sputtered and viewed again

TEM: Small pieces of the skin of the samples from Germany were excised from the back and the flanks, were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4, washed in the same buffer and postfixed with 1% osmiumtetroxide + 1.5% potassiumferrocyanide. The osmium-ferrocyanide method enhances the electron density of membranes, and structures known to contain acidic mucopolysaccharides including intercellular material. However, staining is somewhat unreliable resulting in variable filling of extracellular space and uneven staining of membranes (see Karnovsky, 1971; Schnepf *et al*., 1982; Aguas, 1982). After postfixation specimens were washed with the same buffer, dehydrated in 70%, 90% and 100% ethanol 15 min each, and embedded in Araldite. Ultrathin sections were made with a diamond knife, stained with uranyl-acetate and examined with a Zeiss transmission electron microscope EM 109.

Skin pieces of the samples from Austria were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, washed in the same buffer and postfixed with 1% osmiumtetroxide in the same buffer, dehydrated and also embedded in Araldite or Epon. Ultrathin sections were stained as above and examined mainly with a Zeiss transmission electron microscope EM 109 (Düsseldorf).

Results

Outer inspection and external appearance

A closer inspection of 27 Austrian specimens (9 females, 18 males) with skin papillomas allowed some statements on the number, location and appearance of papillomas. Regarding the macroscopically visible tumors, their numbers ranged from 1 to 12 (in some cases counting was somewhat arbitrary, especially when papillomas were very close to each other or seemed to be fused.). Their size (longest diameter) varied between 1 mm and approx. 1 cm (Tab. 1, 2; Fig. 1 A–D).

Generally, papillomas were distributed over the entire body. However, independent of their external appearance, most were seen on the head and males had more papillomas on the head and the trunk than females. Females had a similar number of papillomas on the trunk and the tail (see Table 1, 2). In between the lesions the integument appeared unaltered.

Concerning coloration and surface, papillomas were somewhat different in appearance. Some were relatively small and, depending on the location, black (dorsum) or silvery (flanks) (Fig. 1 E). Larger ones show a pink surface convoluted or folded like a cauliflower with a few pigment cells delimitating single bulbs (Fig. 1 F, G). In some cases papillomas appeared seriously injured showing some haemorrhages (Fig. 1 H).

The single specimen, whose head was studied with SEM, revealed some lesions on the surface of the papil-

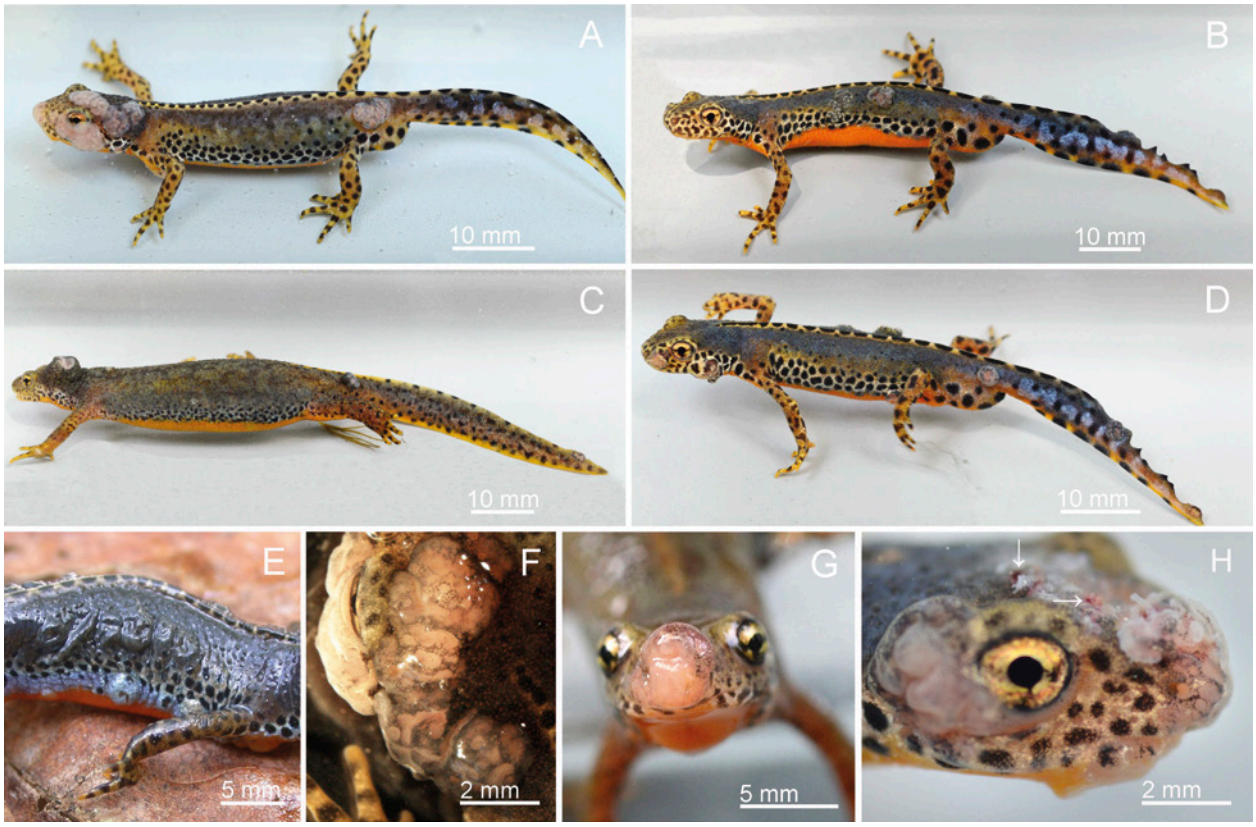


Fig. 1. Multiple papillomas on the skin of *Ichthyosaura alpestris*. **A**, **B**, **D** – Males. **B**, **D**: The same male from both sides. **C**: Female. Note location, site and different aspects of papillomas. **E**–**H**: Details showing papillomas of different age and different developmental stages. **E** : Early stages covered with an intact surface and coloured according to the dermal pigment cells (from Hachtel & Greven, 2014). **F** : Largely uncoloured papilloma with circular arrays of pigment cells delineating individual papilloma bulbs. **G** : Unpigmented large papilloma on the tip of the snout. **H**: Papillomas behind the eye and on the tip of the snout. Note haemorrhages on the head (arrows).

loma (Fig. 2 A). Cells of the exposed surface had small microridges (Fig. 2 B) similar to the surface of the unaffected skin, and both surfaces merged continuously with each other on the tumor edges that were characterised often by a step caused by the elevation of the papilloma above the skin surface. In other probably unaffected areas microridges appeared thicker and swollen (Fig. 2 C). Noteworthy are the numerous fungal hyphae and bacteria scattered over the entire head surface (Fig. 2 D, E) and the presence of some peritrichous ciliates (Fig. 2 E, F); their relatively long stalk, coiled like a spring, suggests specimens of *Vorticella* sp. Longitudinal fractures of papillomas show the arrangement of partly elongated cells in clusters (Fig. 2 G, see below).

Histology

Compared to the adjacent, non-affected epidermis that consisted of 3 to 5 cell layers depending on the body region (see Fig. 4 A), the epithelial component of the papilloma tumor was considerably thickened bulging upwards beyond the surface, downwards into the dermis and laterally (Fig. 3 A, 4 B). In the environment of some papillomas thickness of the epidermis increased gradually. Papillomas mostly form multiple bulges separated from one another by thin strands of connective tissue (dermal papillae; Fig. 3 A, 4 H–K).

The outermost layers of cells in the tumor epidermis were either keratinized (stratum corneum) as in normal skin or keratinized layer(s) were missing exposing the underlying non-keratinized cell layer, i.e. either the transitional or replacement layer (stratum granulosum) or, depending on the severity of the disruption, more deeper cells of the stratum intermedium (= stratum spinosum) (Fig. 3 B, 4 B, E). Generally, keratinocytes appear to have intercellular spaces more widened apically than basally (Fig. 4 B, E). The basal cells (stratum basale) and the basal lamina of papillomas were continuous with the adjacent seemingly non-affected epidermis and basal lamina clearly separated epithelia from the underlying dermis (Fig. 3 C, 4 H) No clear evidence for breaks in the basal lamina was found at the light microscopical level (Fig. 4 H). The bulk of papilloma cells consisted of more or less tightly packed keratinocytes that often appeared to be arranged in clusters or nests not specifically separated from adjacent clusters (Fig. 4 C, D). The cell population of the neoplastic tissue appeared relatively uniform, especially in the lower third of a papilloma, less differentiated; they contained large, often lobate euchromatic and heterochromatic nuclei in varying proportions. Some of the cells appear to undergo apoptosis (Fig. 4 E, I, K).

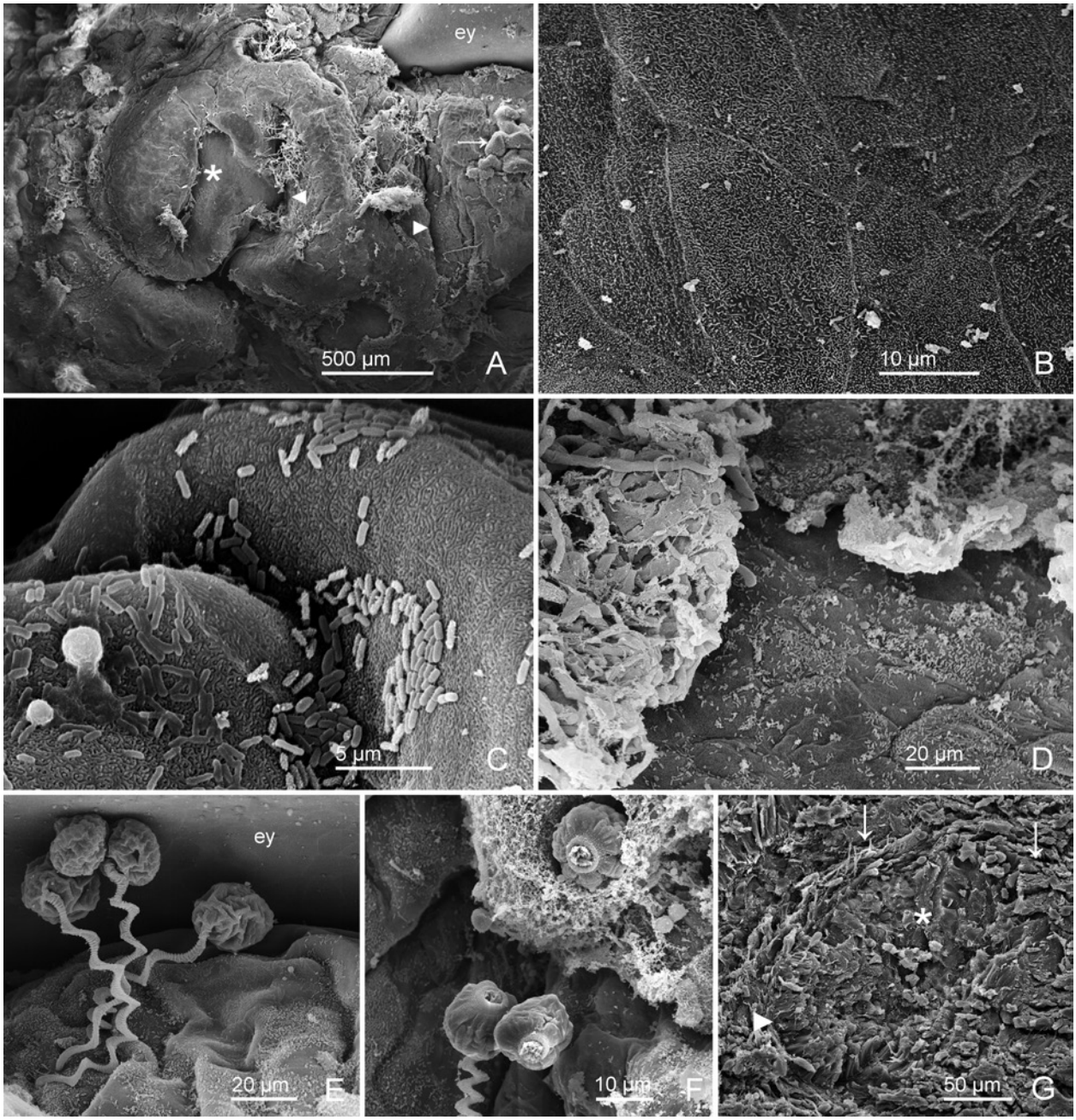


Fig. 2. SEM of the head of *Ichthyosaura alpestris* with a large papilloma. **A**: Overview; papilloma (asterisk) with a folded surface (arrowheads); note fungal hyphae; eye (ey). The amorphous materials are secretions of the granular glands (arrow). **B**: Surface of the middle part of the papilloma showing microridges of the pavement cells **C**: Surface below the eye with denser microridges and numerous bacteria. **D**: Fungal hyphae (left side) and numerous bacteria colonizing the surface of the papilloma. **E**, **F**: Peritrichous ciliates and bacteria on the lower eye lid; eye (ey). **G**: Fractured skin showing part of a bulbous papilloma (asterisk) with elongated basal keratinocytes (arrowhead); border of the bulb (arrows). Note adjacent bulbs.

Many papillomas exhibited cavities or cysts that reach considerable sizes. Two types could be distinguished. One type, preferably localized near the surface, was lined by flat cells and was filled with whirls of keratinized cell layers (Fig. 3 B–D). The second type, often very large (Fig. 3 A, 4 F), may occur in several numbers within a single bulb, if they are small (Fig. 3 A, E, F, 4 G), and may nearly reach the surface (Fig. 3 G). These cysts contained various amounts of cellular debris and PAS-positive material that obviously came from the cells lining the cavity. In our sections we did not find any fusion of the two types of cysts.

Compared to the "normal" epidermis, in which no mitoses were seen in over 50 sections (German samples), mitotic figures were detected at all levels in the papilloma (Fig. I, J). Some of them could not be clearly assigned to a specific stage (aberrant mitoses?). Further, cells characterised by a clear cytoplasm and numerous inclusions, considered as macrophages (Fig. 4 J) and/or other 'leucocytes', were distributed in the neoplastic tissue.

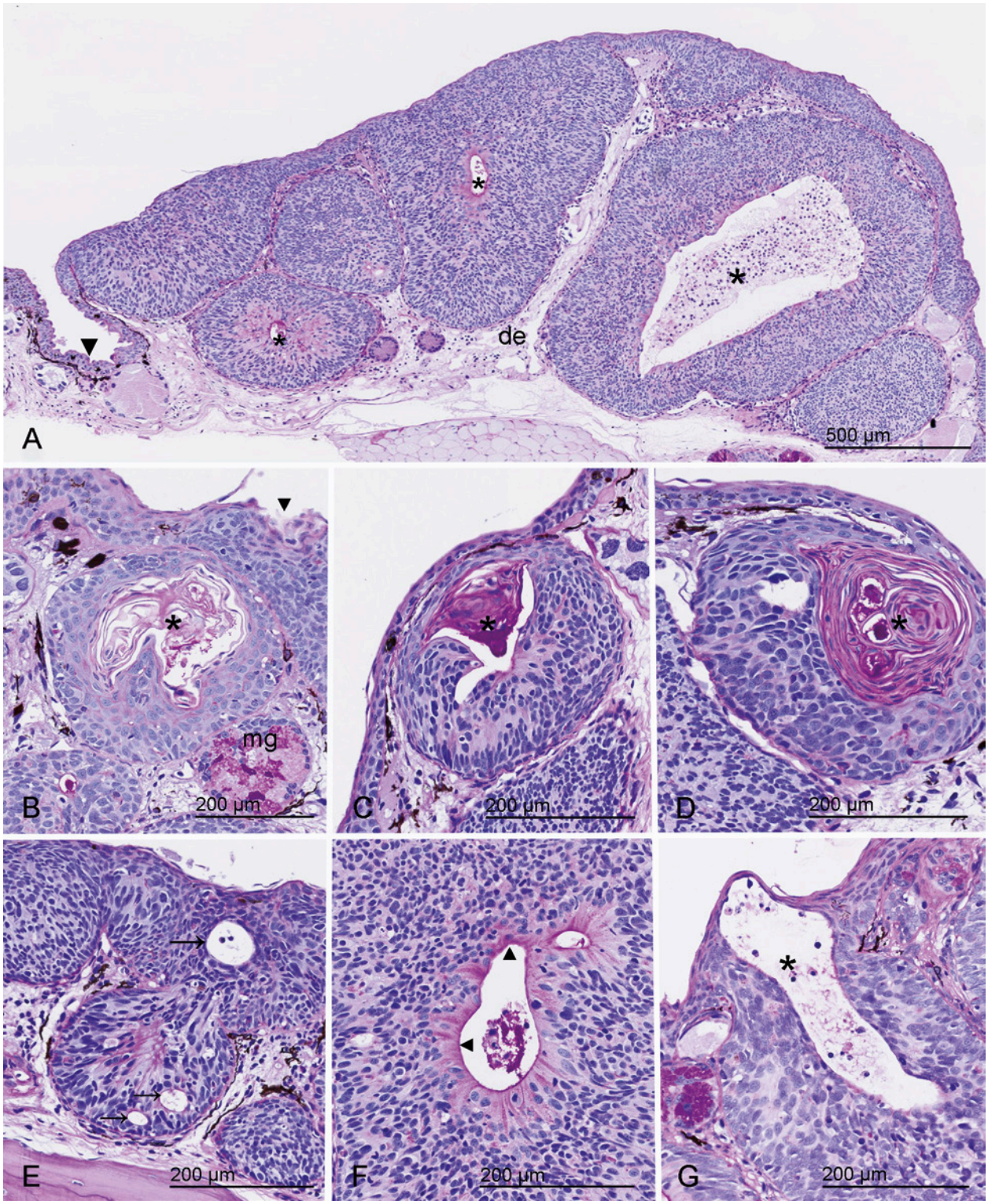


Fig. 3. Light micrographs from papillomas of *Ichthyosaura alpestris* (samples from Austria; Paraffin-sections. **A**: Overview of a large papilloma on the head. Note various bulbs, large and small cavities (asterisks) filled with cellular debris, and the thin obviously unaffected epidermis (arrow head; de = dermis. **B**, **C**, **D**: Cavities in papillomas filled with keratinized cell layers; note lesion in B (arrowhead); mg = mucus gland: **E**: Papilloma with multiple small cavities (arrows). **F**: Cells lining the cavity and the cavity itself contain PAS-positive (stained red) material. **G**: Cavity (asterisk) approaching the surface of the papilloma.

In contrast to the to stratum spongiosum of the dermis, which revealed irregularly arranged collagen fibres bundles interspersed with chromatophores, axons, elongate fibrocytes, and blood vessels (e.g. 4 A), the thin papillae separating the single bulbs from each other and a small zone of connective tissue underlying papillomas contained a large numbers of cells (some of them undergoing mitosis) that did not reveal the typical elongated

appearance of mature fibrocytes; these cells represent fibroblasts and cells of the immune system (Fig. 4 A, B).

Ultrastructure

The organisation of the stratum corneum covering the neoplastic tissue, as well as the underlying replacement layer was similar to the corresponding layers in unaffected regions (Fig. 5 A). However, the stratum corneum, consisting mostly of two layers, appeared not fully keratinized and in part desquamated (Fig, 5 B, C). These underlying keratinocytes showed signs of degradation (Fig. 5 C). The bulk of keratinocytes seemed less differentiated (Fig. C – G). The width of the intercellular space varies from large (Fig. 5 C, D, E) to very narrow (Fig. 5 F). Adjacent cells were connected by small desmosomes (maculae adhaerentes), often somewhat obscured by the staining with potassiumferrocyanide, but very conspicuous in less impregnated parts of the papilloma (Fig. 5 B) and in the samples from Austria, which were not treated with potassiumferrocyanide. Although not counted, desmosomes appeared reduced in number compared to the adjacent normal epidermis. Also the amount of tonofilaments appeared to be smaller than in the normal tissue and filaments are tightly packed. Cytoplasmic organelles and membrane systems did not show conspicuous abnormalities, but seemed to be present in a lesser amount than in the unaffected epidermal cess. Basal cells are attached to the basal lamina by hemidesmosomes (Fig. 5 H). Only once we saw a breakthrough in the basal lamina penetrated by a small projection of a keratinocyte (Fig. 5 H).

Macrophages, not connected with adjacent keratinocytes by desmosomes, were scattered throughout papillomas. Apart from the usual cell organelles, they contained membrane-bound granules, large vacuoles with inclusions (secondary lysosomes), a polymorphic nucleus and some 'lamellipodia' (Fig. 5 E), but other 'leucocytes' with inclusions, but without lamellipodia (granulocytes) may be present (Fig. 5 F).

Nuclei of keratinocytes are large, oval or ellipsoid, sparsely lobated, and in varying proportions eu- and heterochromatic (see above, Fig. 5 A – G). Papilloma cells of Austrian and German samples often contained nucleolus-like inclusions with a highly specific, sometimes hexagonal organisation, to our knowledge not desribed as yet (Fig. 6 A; see also Fig. 5 C – F). We did not find virus-like particles either in the nuclei or in the cytoplasm.

Cysts with cellular debris and PAS-postive substances were lined with keratinocytes (Fig. 6 B – D) that typically showed apically small microvilli or microridges and contained small bundles of tonofilaments (Fig. 6 C). However the apical portions of their cytoplasm were rich in vacuoles with granular and floccular material, which they discharge in the cyst lumen (Fig. 6 D).

Concerning the dermal papillae and the dermis underlying papillomas, TEM largely confirmed the light microscopical findings. Both contain fibroblast-like cells and various cells of the immune system, i.e. 'leucocytes' tentatively classified by morphological criteria, but not further specified (e.g. Fig. 5 G, 6 E – G).

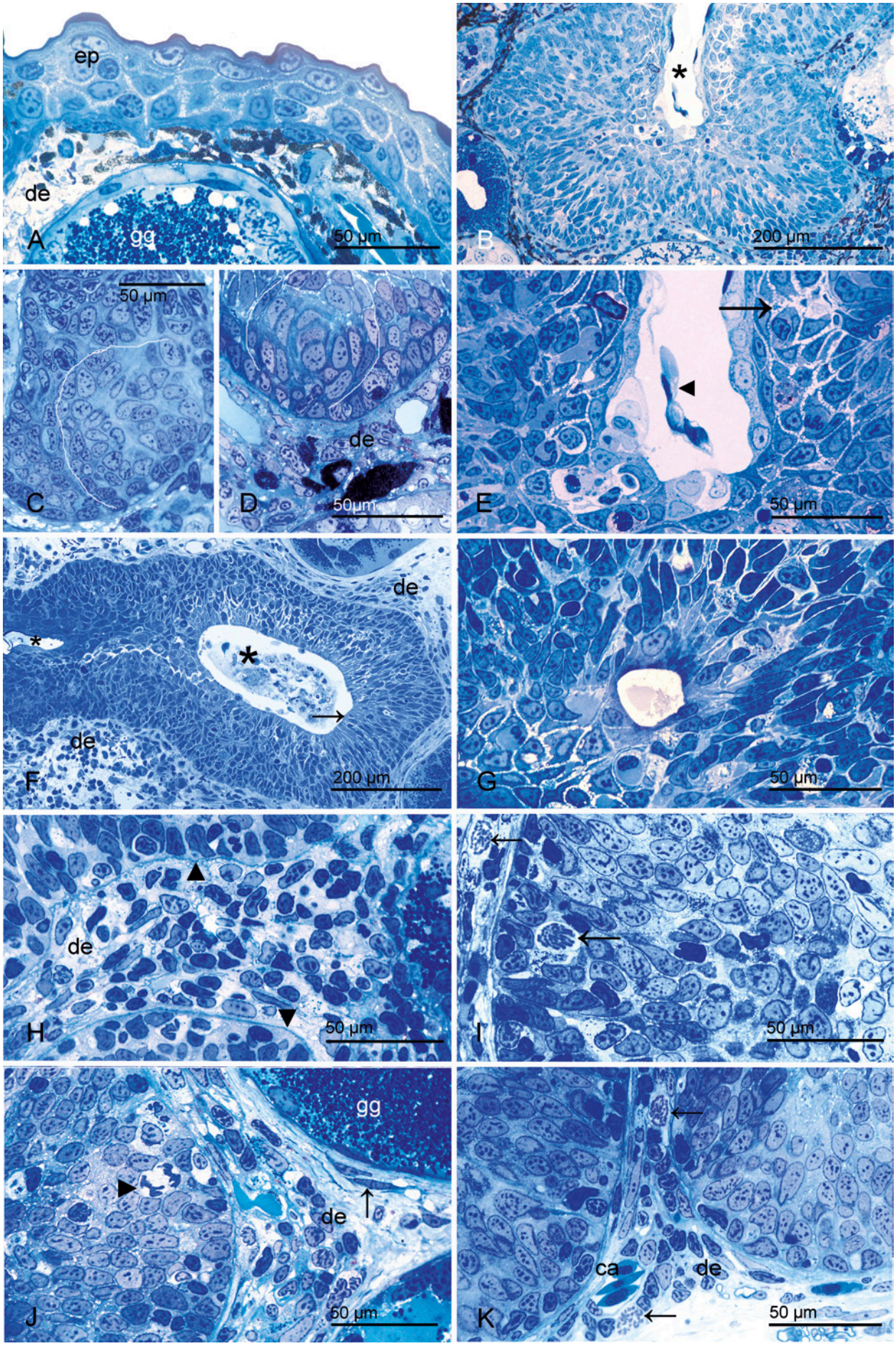
Discussion

External appearance, histology and ultrastructure of the tumors studied herein prove them to be epidermal papillomas resembling skin tumors known from other newts. However, apart from the broad studies with *Cynops pyrrhogaster*, which contain data on morphology and epidemiology (i.e. seasonality, thermal effects, resistance to infections, ultrastructural demonstration of viruses etc.), epidermal papillomas in newts and other amphibians appeared still strikingly unexplored. All other studies on spontaneous epidermal papillomas in newts are case reports. To our knowledge the data situation concerning European newts is even scarcer (*Triturus cristatus*: Seilern-Aspang *et al*., 1966; *Triturus* (today *Ichthyosaura*) *alpestris*: Champy & Champy 1935; Darquenne & Matz, 1971 fide Asashima & Meyer-Rochow 1988; Hachtel & Greven, 2014), and only a few papillomas have been diagnosed by more than simply taking a look at the external appearance (Champy & Champy 1935; Seilern-Aspang *et al*., 1966; Hachtel & Greven, 2014).

Histology and ultrastructure

Papillomas described herein, typically formed large bulbs of tightly packed keratinocytes that appeared to have a relatively high mitotic rate at least in the samples from Germany. Except for a single case (see below) bulbs were well-demarcated and separated from the underlying tissue by a basal lamina, which was continuous with the basal lamina of adjacent parts of the seemingly unaffected epidermis (see for example the histological description of epidermal papillomas in newts Bryant, 1973; Pfeiffer *et al*., 1979; Rose, 1981; Asashima & Meyer-Rochow, 1988; Harshbarger *et al*., 1989 fide Stacy & Parker, 2004; Trauth *et al*., 2002; Hachtel & Greven, 2014).

The only detailed study on this subject addressing the ultrastructure (TEM) was conducted by Pfeiffer *et al*. (1989). In the neoplastic tissue of *Cynops pyrrhogaster* authors found (1) undefined material (membrane fragments, floccular and granular material) in the intercellular space; (2) modifications of stratum corneum cells (filled with electron lucent granules; not seen in the present study) that were occasionally buried within the stratum granulosum (probably pre-stages of the cysts filled with keratinized cells); (3) loss of intercellular bridges and desmosomes in the space between the stratum corneum and the subjacent replacement layer (not seen in our samples); (4) widening of the intercellular spaces (see below); and (6) in about 40% of 25 tumors examined virus-like particles in the cytoplasm (see also the few TEM images in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Pfeiffer *et al*.,1979; Asashima *et al*., 1982; not seen in the present study). Authors concluded "The fine structure of hyperplastic stratum granulosum cells, which constituted the greater mass of the epitheliomas, was generally normal…" (Pfeiffer *et al*., 1989, p. 663).

Regarding intercellular spaces; their widening has been emphasized in epidermal papillomas of other newts (e.g.; Trauth *et al*., 1992), but such widened intercellular spaces were not apparent in the German material, whereas moderate to large intercellular spaces were seen in the Austrian material. These differences should not be overemphasized, as the amphibian epidermis is highly susceptible to various parameters including fixation.

Pfeiffer *et al*. (1989) as well as Trauth *et al*. (1992) stated, somewhat misleading, that the tumors they described were derived from the intermediate layer(s) of the epidermis, which they equate with the stratum granulosum in the epidermis of higher vertebrates (for a somewhat modified terminology see Lavker, 1972; Fox, 1986, 1994). In a previous article Pfeiffer *et al*., 1979) stated that proliferation occurred throughout the epidermis. We think that mainly cells of the stratum intermedium (and surely of the stratum basale) are involved. Mitotic figures were mostly seen in lower areas of the papillomas.

Gross morphology and histology suggest several putative developmental stages of papillomas. We think that in an early stage a slight skin elevation is covered with an intact stratum corneum with a coloration determined by the chromatophores underlying the epidermis. Proliferation of the hyperplastic epidermal cells leads to an expansion upward, downward (= endo- and exophytic growth) and laterally. In later stages papillomas may be still covered by the stratum corneum or the underlying neoplastic cells layers may be exposed to the environment (papillomas without a s. corneum have also been mentioned by Trauth *et al*., 1992) either by mechanical abrasion of the outer layer(s) or by desquamation due to other reasons.

Despite the overall similarity with previously studied epidermal papillomas of newts, we herein show some morphological traits hitherto undescribed or only marginally considered.

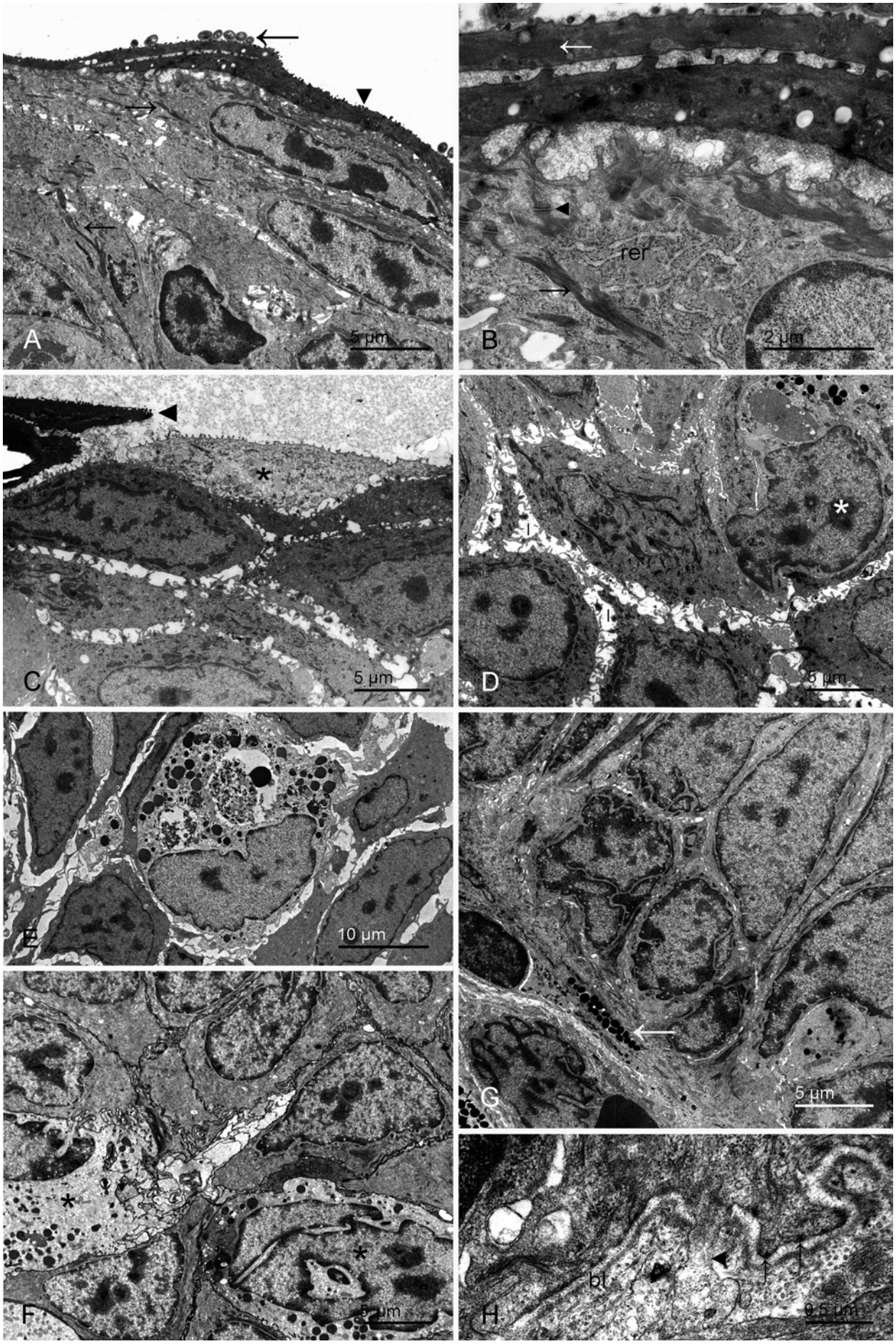
(1) Presence of immigrant mesenchymal cells, mainly of the immune system in the neoplastic tissue: Such cells like "blood cells, fibrocytes, mesenchymal macrophages, melanocytes" appear to be common in the "normal" transformed newt epidermis and dermis (see Fox, 1986, tab. 1, and p. 97; 1994), but have to our knowledge not sufficiently characterized in the amphibian epidermis. We classified most of the large cells scattered throughout the papilloma tissue as macrophages due to their largeness, they variable appearance, their "lamellipodia" (for ultrastructure of newt macrophages see Curtis *et al*., 1979; Reyer, 1990 a, b) and the fact that they were not connected with adjacent cells by desmosomes, but certainly, other 'leucocytes' may be present in the neoplastic tissue. Currently we do not know, whether their presence has to do directly or indirectly with the tumor. It is however known, that amphibian macrophages are involved in viral clearance, and that macrophages may be vectors of an aviral persistance (for review see Grayfer & Robert, 2016).

(2) Connective tissue between and beneath the bulbs unusually rich in fibroblast-like and other, occasionally mitotic, cells: An "increased cellularity … beneath tumors was occasionally observed" by Pfeiffer *et al.* (1979, p. 1905; see also Champy & Champy, 1935). This proliferation seems to be common in various other epitheliomas and may among other things be associated with an infiltration of inflammatory cells, i.e. various 'leucocytes" such as macrophages, granulocytes, lymphocytes. Some of these cells were classified by their morphology (see Castellani, 1968; Tooze & Davies, 1968 Curtis *et al*., 1979; Turner, 1988), but they should be characterized by specific stainings in future. Acute and chronic inflammatory response of the stroma was also reported from mammalian neoplastic skin lesions including humans (e.g., Yanofsky *et al*., 2011; Wassef *et al*., 2012).

(3) Presence of more or less ovoid clusters of keratinocytes within a given bulb: These clusters may indicate focal multiplication of single or few infected cells more than once in a growing papilloma, but direct evidence for that is missing.

(4) Cysts (or cavities) of variable sizes containing either masses of keratinized cell layers or cellular debris and PAS-positive secretions: In our sections we did not find evidence that these cysts communicate with the external surface or that they merge with each other. To our knowledge only Champy & Champy (1935, p. 209.) distinguished "tumeurs de type cavitaire" and "de type compact" in newts and described their variable size, but did

← **Fig. 4.** Light micrographs from papillomas of *Ichthyosaura alpestris* (A, B, E, F, G samples from Austria; C, D, H, I, J, K; samples from Germany; semithin sections of papillomatous bulges and the underlying connective tissue. **A**: Unaffected skin; note thinness of the epidermis (ep); the dermis (de) has relatively few cells. gg = granular gland. **B**: Small papilloma with fold (asterisk). **C**, **D**: Cells are often arranged in clusters (outlined in white). **E**: Detail of B showing widened intercellular spaces (arrow), shedding of stratum corneum cells (arrowhead) and apoptotic cells (e.g. close to the scale bar). **F**: Larger bulge with fold (small asterisk) and large cavity containing cellular debris. Note prismatic cells lining the cyst (arrow) and the cell-rich dermis (de) adjacent to the bulge. **G**: Small cyst and surrounding elongate cells (sequential section of B). **H**: Cell-rich dermal papilla (de) between two bulges that are sharply demarcated (arrows). **I**: Mitoses of a basal cell (large arrow) in the bulge and in the dermal papilla (small arrow). Cells with heavily stained nuclei are probably apoptotic. **J**: Basis of a bulge with mitosis (arrowhead). Clear cells are mainly 'leucocytes'. Note cell rich dermal papilla; typical elongate fibrocytes (arrow). **K**: Mitoses in the cell-rich dermal papilla (de) between two bulges (arrows). ca = capillary.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not mention cysts with keratinized cell layers indicating a kind of hyperkeratosis. The PAS-positive substances, in the second type of cysts were secreted by keratinocytes. Especially in the mid and upper regions of the 'normal' epidermis of newts (*Triturus cristatus*) Lavker (1972) demonstrated small PAS-postive membrane bound granules that he interpreted as mucus granule (see also Fox, 1984). Production of mucus appeared considerably enhanced at least in some of the neoplastic keratinocytes; in addition also its composition might be altered as suggested by their size and ultrastructure.

The herein described papillomas share some features described for mammalian skin lesions including human, such as 'inverted follicular keratosis' and 'irritated seborrheic keratosis', i.e. benign skin lesions of mammals including humans, which are sharply demarcated, may show endophytic and exophytic growth, and contain keratin-filled pseudocysts, and (micro)cysts lined by non-keratinized squamous cells scattered with mucus secreting cells and/or filled with neutrophils (e.g. Wassef *et al*., 2012; see also Digital Pathology Project digital.path.utah.edu/).

The rich colonisation with bacterial, fungal hyphae and peritrichous ciliates, studied in only a single specimen, may indicate its overall weakening probably due to the large papillomas. Bacteria, surely in part indigenous, were found in a moderate number also on the seemingly unaffected skin (not shown).

Comments on epidemiology

Prevalence, causes (i.e. aetiology) and pathogenesis of epidermal papillomas in newts are by no means well established (Robert, 2010). Although the herein presented findings do not contribute much to these questions, a few notes shall be added.

It is assumed that epidermal tumors in newts may be caused by chemical agents and/or viruses.

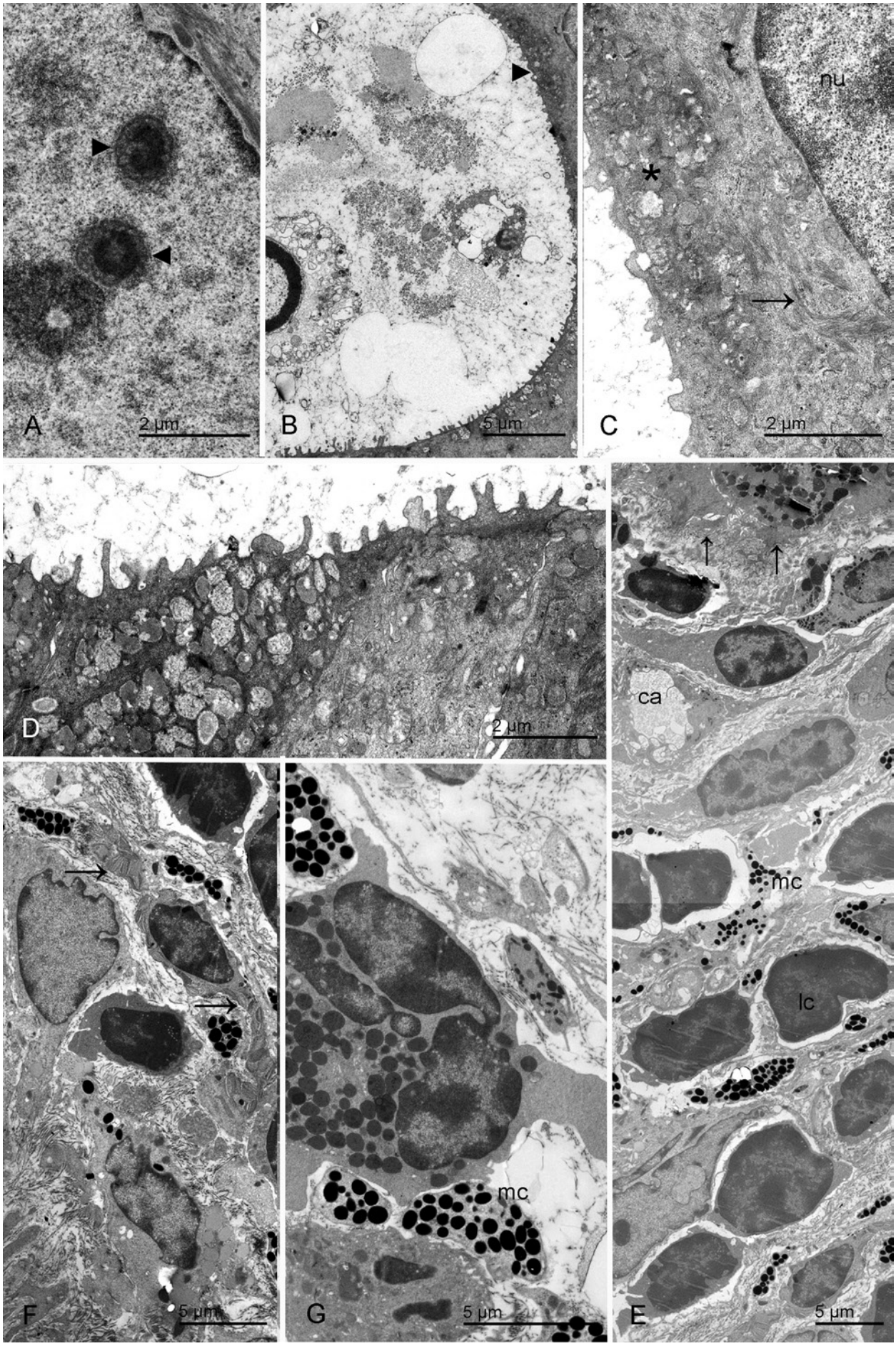
Champy & Champy (1935) believed that the causative agent of the tumors they found in *Ichthyosaura alpestris* was a virus showing species specificity (among others as other newt species were not affected). Rose & Harshbarger (1977) and Rose (1981) did not find viruses in epidermal papilllomas of *Ambystoma tigrinum* from seawage ponds, whereas Asashima *et al*. (1982) and Pfeiffer *et al*. (1979, 1989) found virus particles in nearly the half of tumors in *Cynops pyrrhogaster* noting a (morphological) similarity to herpes-type viruses, but did not further pursue this matter.

In the neoplastic tissue examined by us, we could not find viral particles either in the nucleus or in the cytoplasm of the keratinocytes. This might have to do with the fact (1) that our sample was too small, i.e. that viruses were not present in all cells, (2) that presence of papillomas and viruses was highly temperature dependent – for example in wild-caught *C. pyrrhogaster* percentage of newts with papillomas was highest in autum (Asashima *et al*., 1982, 1986) and growth of these tumors was highly temperature dependent; in adenocarcinomas of frogs virus particles were absent in summer, but present in wintertime (Granoff, 1973; Asahima & Koyama , 1986) –, or (3) that viruses were not the disease agent (which does not necessarily exclude their presence in these tumors).

Strictly speaking, we cannot exclude any of the three options. Skin lesions of Urodela and Anura are attributed mainly to iridoviruses and herpes-like viruses. Iridoviruses, i.e. the highly virulent host specific ranaviruses cause ulcerations, edema, and hyperkeratosis in the hyperplastic and hypertrophic epidermis, but simultaneously affect other tissues causing severe disease followed by mass mortality (for review see Duffus, & Cunningham, 2010; Miller *et al*., 2011, Rector & van Ranst, 2013; see also Geng *et al*., 2011, and for histological images Bollinger *et al*., 1999). The likewise host-specific herpesviruses are known to cause adenocarcinomas (Lucké tumor) (for review see van Beurden & Engelsma, 2012). A single article reports on epidermal hyperplasia with enlarged nuclei in frogs associated with herpesvirus-like particles (Bennati *et al.*, 1994).

Generally, these lesions and pathologies of these virus-induced diseases are not comparable to those of the epidermal papillomas described herein and by others (see citations above). As yet, however, further diagnostic techniques were not used for these papillomas. One might also suspect that epitheliotrophic host-specific viruses such as papillomaviruses may be involved, which often infect squamous epithelia of a variety of vertebrates including humans when cells are exposed by wounding. However, contrary to some suspections, especially in summarizing articles (e.g. Schmidt & Reavill, 2015), papillomaviruses have (so far) not been found in amphibians (e.g. Bravo *et al*., 2010; Doorbar *et al*., 2016). Therefore, it was speculated that the host range of papillomaviruses is restricted to amniotes (see Rector & van Ranst, 2013).

← **Fig. 5.** TEM of the papilloma. **A**, **C**, **D**, **E**, **G**, **H**: samples from Austria; **A**, **F**: samples from Germany. **A**: Apical region with stratum corneum (arrowhead) and underlying cell layers with bundles of tonofilaments (small arrows). Note bacteria on the surface (arrow). **B**: Detail of the bilayered stratum corneum and the 'replacement' layer. Tonofilaments (arrow); desmosome (arrowhead); rer = rough endoplasmic reticulum. **C**: Bottom of a fold (see Fig.4 B and E). Shed stratum corneum-cells (arrowhead) und underlying, partly degenerating cells (asterisk) of the 'replacement layer'. **D**: Upper third of a papilloma with widened intercellular spaces (I); macrophage (asterisk). **E**: Macrophage between neoplastic keratinocytes. **F**: Keratinocytes from the middle of a papilloma; note the small intercellular space impregnated with potassiumferrocyanide and a putative eosinophil granulocyte (asterisk). **G**: Basal part of a papilloma with small intercellular spaces. Left bottom: dermal papilla with melanocyte (arrow) and immune cells. **H**: Suspected breakthrough of the basal lamina (bl) by a keratinocyte (arrowhead). Hemidesmosomes (arrows).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In the papillomas inspected during this study the basal basal lamina appeared intact preventing invasion of tumor cells into the dermis. Only once we found a small breakthrough of the basal lamina. This means that malignancy can not be entirely excluded simply due to the lack of a sufficient number of samples. For example, Trauth *et al* (2002) upgraded their first diagnosis of an epidermal papilloma in *Cryptobranchus alleganiensis*, after examining some more skin samples of the previously examined specimen, in which they found signs of dermal invasion (see Harishberger & Trauth, 2002). Pfeiffer *et al.* (1979) reported that squamous cell papillomas in *C. pyrrhogaster* occasionally continued to develop causing disease and either directly or indirectly death, but also often regressed spontaneously. Later authors emphasized the general absence of metastases, but the occasional presence of invasiveness (Pfeiffer *et al.,* 1989). Papillomas studied by us did not seem to affect the animals directly. At the time of capture the German specimens seemed neither emaciated nor otherwise weakened (Hachtel & Greven, 2014) and also the Austrian specimens, in part kept alive from April to October in a small tank outdoors, did not show any signs of impairment, and even reproduced successfully. In no case, however, a conspicuous regression of the tumors was observed.

In any case, these tumors run the risk of being damaged just because they are protruding and have cysts or cavities often near the surface. Therefore, these sites may be an entrance for various infections triggering among others inflammatory processes and viral diseases and/or sites releasing infectious tissue. Otherwise papillomas are often late effects of injuries of the epithelia involved (see above). In this context it is noteworthy that in our material injuries were often seen on the head and the tip of the snout (see also Hachtel & Greven, 2014) and that infection was heaviest on the male's head. However, in *C. pyrrhogaster* Pfeifer *et al*. (1979) did not find significant differences between the sexes and preferred sites of the body. Transmission and spread of putative infection(s) certainly requires physical contact with the substrate (e.g. when foraging), with conspecifics or with predators. However, due to the lack of reliable data, additional speculations would not make sense.

Conclusion

In brief, the bulk of the papillomas described herein for *Ichthyosaura alpestris* are characterized by exo- and endophytic growth of keratinocytes derived from cells of the stratum intermedium and stratum basale, and an immigration of cells of the immune system. The dermis just underneath shows a relatively dense layer of various cells that represent in largely an inflammatory infiltrate. These lesions seem to be mostly benign; their primary cause is unclear.

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← **Fig. 6.** Papilloma (samples from Austria). **A**: Conspicuously organised nucleolus-like inclusion of a papilloma cell (arrowheads). **B**, **C**, **D**: Small cavity (cyst) in the papilloma shown in Fig. 4 G (B) lined by secretory keratinocytes (arrowhead); (C) keratinocytes with subapical secretory granules (asterisk) and bundles of tonofilaments (arrow); nu = nucleus; (D) detail of the subapical zone of three adjacent cells; two of them contain subapical secretory vesicles. **E**, **F**, **G**: Inflammatory infiltration with various "leucocytes" in the connective tissue beneath a papilloma; (E) lymphocytes (lc) and fibroblast-like cells; note the highly contoured basal lamina of the papilloma (arrows); ca = capillary; mc = melanocyte; (F): Small portions of the cytoplasm of a basophil showing the substructure of its inclusions. (G): Eosinophilic granulocyte (?).

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Note added in proof

One year after the remediation of the Austrian pond, no newts with papillomas were found. However, there were significantly fewer newts than in the previous year. Further, HPV (Human Papilloma Virus) was detected neither by immunohistology nor by PCR kindly carried out by Profs Drs Rolf Graf, Dieter Zimmermann, and Daniela Lenggenhager, all University Hospital Zürich (Switzerland).