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Formation of the secondary tongue in *Hynobius leechi* and *Ambystoma mexicanum* (Amphibia: Urodela) *

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* In memoriam Prof. Dr. Hermann Hartwig (* 1.1.1910; † 9.9.2012)

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Abstract

Tongue development in several developmental stages of the metamorphosing newt *Hynobius leechi* (Hynobiidae) and the paedomorphic *Ambystoma mexicanum* (Ambystomatidae) before and after artificially induced metamorphosis was studied by light microscopy (LM) and scanning electron microscopy (SEM). In *H. leechi* the anlage of the glandular field (lingual glands) of the secondary tongue appears under the free tip of the primary tongue and is clearly seen in late larvae (developmental stage approx. 65). The epithelium of the primary tongue is stratified and composed of epithelial cells, AB-positive goblet cells, some superficial ciliated cells, very few Leydig cells and typical taste buds. Later more or less radially arranged tubular glands (lingual glands) develop in the anterior portion of the prospective secondary tongue, which open in furrows lined in their upper region by the surface epithelium ("neck portion"). Fully developed glands are variously long, moderately branched and contain columnar secretory cells that are preferably AB-positive in their upper region, but AB- and PAS-negative in their terminal portions. Posteriorly the tubular glands become shorter in favour of the neck portion and then are abruptly replaced by a heavily ciliated area containing indentations of the epithelium interspersed with numerous goblet cells (crypts). This zone is considered as a modified remnant of the former primary tongue. Formation of the secondary tongue, often described as "fusion" of the glandular field with the primary tongue, is considered as a process levelling the free, probably regressive end of the primary tongue and the posterior part of the growing glandular field. The development of the secondary tongue of metamorphosing *A. mexicanum* follows the same pattern. However, the putative anlage of lingual glands in semiadult paedomorphic specimens may be considered as a further character indicating partial metamorphosis in this species. In the transformed axolotl we demonstrate the secondary tongue with lingual glands, epithelial folds with noticeable numbers of AB-PAS-positive goblet cells at the lower surface of the free tip of the secondary tongue, and, contrary to *H. leechi*, tubular glands immediately behind the dentary.

Kurzfassung

Wir haben die Bildung der Zunge bei verschiedenen Entwicklungsstadien des zur Metamorphose befähigten *Hynobius leechi* (Hynobiidae) und des artifiziell zur Metamorphose gebrachten pädomorphen *Ambystoma mexicanum* (Ambystomatidae) histologisch (LM) und rasterelektronenmikroskopisch (REM) untersucht. Bei *H. leechi* ist die Anlage des Drüsenfeldes (Lingualdrüsen) der Sekundärzunge bereits bei späten Larven (etwa Stadium 65) unter dem freien Ende der Primärzunge zu erkennen. Das Epithel der Primärzunge ist mehrschichtig und besteht aus „ normalen" Epithelzellen, Becherzellen, einigen Cilienzellen, Leydig-Zellen (sehr selten) und typischen Geschmacksknospen. Später entwickeln sich im vorderen Bereich der Sekundärzunge mehr oder weniger radiär angeordnete tubuläre, unterschiedlich lange und zum Teil mäßig verzweigt Drüsen (Lingualdrüsen). Diese münden in Furchen, welche im oberen Bereich noch vom Oberflächenepithel („Halsregion"), etwas tiefer aber von einem einschichtigen, prismatischen sezernierenden Epithel ausgekleidet sind, dessen Sekrete im oberen Teil AB-positiv, im unteren Teil aber AB-PAS negativ sind. Nach posterior werden die Tubuli insgesamt und ihr drüsiger Teil zugunsten der „Halsregion" kürzer; sie werden relativ abrupt von einer reich mit Cilien und Krypten, d. h. Einsenkungen des Epithels mit zahlreichen Becherzellen, abgelöst. Dieser Teil repräsentiert modifizierte Reste der ehemaligen Primärzunge. Die Bildung der Sekundärzunge, meist als Fusion des Drüsenfeldes mit der Primärzunge beschrieben, ist eher ein Vorgang, bei welchem durch das Höhenwachstum des Drüsenfeldes bei gleichzeitiger Reduktion des freien Raums zwischen der Spitze der Primärzunge und des hinteren nicht modifizierten Teils des Drüsenfeldes, beide Anteil auf gleiche Höhe gelangen. Die Entwicklung der Sekundärzunge bei *A. mexicanum* folgt dem gleichen Muster. Epitheliale Furchen im vorderen Bereich des Mundbodens semiadulter, nicht mit Thyroxin behandelter

Individuen deuten wir als Anlage der Lingualdrüsen und werten dies als weiteres Anzeichen für eine partielle Metamorphose dieser Art. Bei metamorphosierten Exemplaren des Axolotls finden sich zudem auf der Unterfläche der freien Spitze der Sekundärzunge Falten mit bemerkenswert zahlreichen AB-PAS-positiven Becherzellen sowie, im Gegensatz zu *H. leechi*, unmittelbar hinter dem Dentale tubuläre Drüsen.

Key words

Urodela, tongue development, lingual glands, metamorphosis, primary and secondary tongue.

Introduction

Studies on the development of the tongue in Urodela (= Caudata) are known for a long time (e.g., GEGENBAUR, 1894; KALLIUS, 1901; summarized in FAHRENHOLZ, 1937; STADTMÜLLER, 1938). The most thorough investigation to date comes from KALLIUS (l.c.), who described the tongue development in some salamandrids, but also added some notes on the histology of the tongue of the paedomorphic *Ambystoma mexicanum* (Ambystomatidae). His schematic drawings of the tongue development in *Salamandra salamandra* have been occasionally included in textbooks of comparative anatomy (e.g. STADTMÜLLER, 1938). Later, SEIFERT (1932) examined a larger spectrum of urodele species, described the various glands found in their buccal cavity and some variously organized primary tongues in paedomorphic species.

According to these classical studies, the primary tongue is formed at first as an anterior prominence of the hypoglossal apparatus. Around metamorphosis a glandular field (= lingual glands) develops in the anterior part of the floor, which later "merges" with the remnants of the former primary tongue. Both portions represent the secondary tongue.

In modern textbooks of comparative anatomy (see STARCK, 1982) and herpetology (see DUELLMAN & TRUEB, 1986) this issue is treated very short, if any. Generally, it is noted that during metamorphosis the primary tongue is replaced by the more or less moveable secondary tongue, and that the lingual glands of the secondary tongue originate from a median portion of the floor of the oral cavity. Among Urodela the organisation of the fully developed secondary tongue varies considerably ranging from simple pad- or trough-like structures with a limited ability to project (e.g. in ambystomatids) to highly projectile tongues (e.g. in some plethodontids). A relatively broad and pad-like tongue tightly attached to the floor of the mouth, which for example is present in terrestrial hynobiids and ambystomatids, and with partly free margins, may represent an ancestral condition (e.g. literature cited above; GIERSBERG & RIETSCHEL, 1986; DUELLMAN & TRUEB, 1986; WELLS, 2007).

Morphological studies following the above mentioned classical investigations have examined the urodele tongue, in particular the lingual glands, from various points of view such as histology, histochemistry and ultrastructure, or have focused on the fine structure and the presence of different gustatory organs in the primary and secondary tongue. In most of these articles tongue development and comparative aspects are touched only in passing (e.g., FÄHRMANN, 1974, 1975; ZYLBERBERG, 1973, 1977; KURABUCHI, 1986; MOTZEK *et al*., 1990; KURABUCHI *et al*. 1995; TAKEUCHI *et al*., 1997; KOBEGENOVA *et al*., 1998; WISTUBA & CLEMEN , 1998; WISTUBA *et al*., 1999; OPOLKA & CLEMEN , 1998; OPOLKA *et al*., 2001, 2003; ZUWALA & JAKUBOWSKI, 2001; ZUWALA *et al*., 2002). A more recent review on the tongue evolution in vertebrates completely neglects developmental aspects (IWASAKI, 2002).

We herein describe some stages of tongue development in a member of the phylogenetically basal Hynobiidae more thoroughly than before (see SCHMALHAUSEN, 1968; 1994; TAKEUCHI *et al*., 1997; KOBEGENOVA *et al*., 1998) and include some findings on the tongue development of the paedomorphic *Ambystoma mexicanum* after artificially induced metamorphosis, which were not appropriately considered in a previous article (see WISTUBA & CLEMEN, 1998).

Material and methods

All specimens used herein were anaesthetized with MS 222 (3-aminobenzoeacidethylester; Sigma) before decapitation and then processed for light microscopy (LM) and scanning electron microscopy (SEM).

Hynobius leechi. 15 specimens of various developmental stages of the Korean salamander *H. leechi* were available. We distinguished (1) "late larvae" (5 specimens), total length 3.5 to 4.2 cm, which were characterized by fully developed digits, gills, a single row of premaxillary teeth, developing maxillae, polystichously toothed palatines that were still connected with the pterygoid, polystichously toothed vomeres (approximately stage 60 according to IWASAWA & YAMASHITA, 1991), (2) specimens

in an "early stage of metamorphosis" (4 specimens, one specimen of 5.3 cm total length was on land since 2 d), total length 4.9 to 5.8 cm, characterized by the regression of the dorsal fin, the labial fringes and the gills, greatly reduced dentition of the palatine, palatines separated from the pterygoids, inner posterior outgrowth of the vomeres, and vomeres with a single row of teeth (stages 64 – 67), (3) specimens in an advanced stage of metamorphosis (4 specimens) two to four weeks on land, total length 5.3 to 5.8 cm, with remnants of gills or totally reduced gills, larger dentate maxillae and in some total loss of the palatinal dentition (stage 67/68) , and (4) 2 adult females, 9.8 and 10.4 cm (for the use of the dentition to stage urodele larvae see Clemen & Greven 1994).

For LM the lower skull of one specimen per stage was fixed in Bouin's fixative. For SEM lower skulls of each stage were fixed in 2.5% glutaraldehyde, pH 7.2) in 0.1 M cacodylate buffer for at least 2 h, dehydrated, critical point dried (CPC 020; Balzers Union) and sputtered with gold. Samples were glued with tempfix (Neubauer) to aluminium stubs, sputtered with gold and viewed in a scanning electron microscope (Hitachi S-530). After SEM-examination the primary tongue of the youngest stage was removed to expose the mouth floor, sputtered again and examined by SEM.

The lower skulls of the other specimen were fixed in 4% neutralized formol after Lillie and then macerated for some days in a Na-tetraborate-pancreatin-mixture (30 ml saturated Na-tetraborate in 70 ml aqua dest. plus 1 g pancreatin (Fa. Sigma)) to remove muscles and epithelial tissue (Eun-Ho & Dong Soo, 1984) and processed for SEM as described.

Ambystoma mexicanum. We used 12 paedomorphic semiadults (length 12 – 15.6 cm) characterized by bicuspid teeth on the premaxillae (see Clemen & Greven, 1977). Six of these specimens (semiadults, approximately 10 and 13 cm with some bicuspid teeth on the premaxillaries) get a single intramuscular injection of 25 µg L-thyroxin-sodium in 50 µl injection solution (Fa. Henning, Berlin) and were decapitated 28 and 54 days later (AZ 57/96, RP Münster). A 2.5 year old, artificially transformed male was available from a previous experiment after artificially induced metamorphosis (AZ 80/93, RP Münster).

For LM halves of the lower skull (from the thyroxin-treated specimens) and entire lower skulls were fixed in Bouin. Each second half was fixed in glutaraldehyde or formalin, dehydrated and processed for SEM as described above.

Embedding and staining. For LM, specimens were embedded in Paraplast and sectioned sagittally at 7 µm. We used several stainings noted in the legends of the figures (for staining procedures see Mulisch & Welsch (2009).

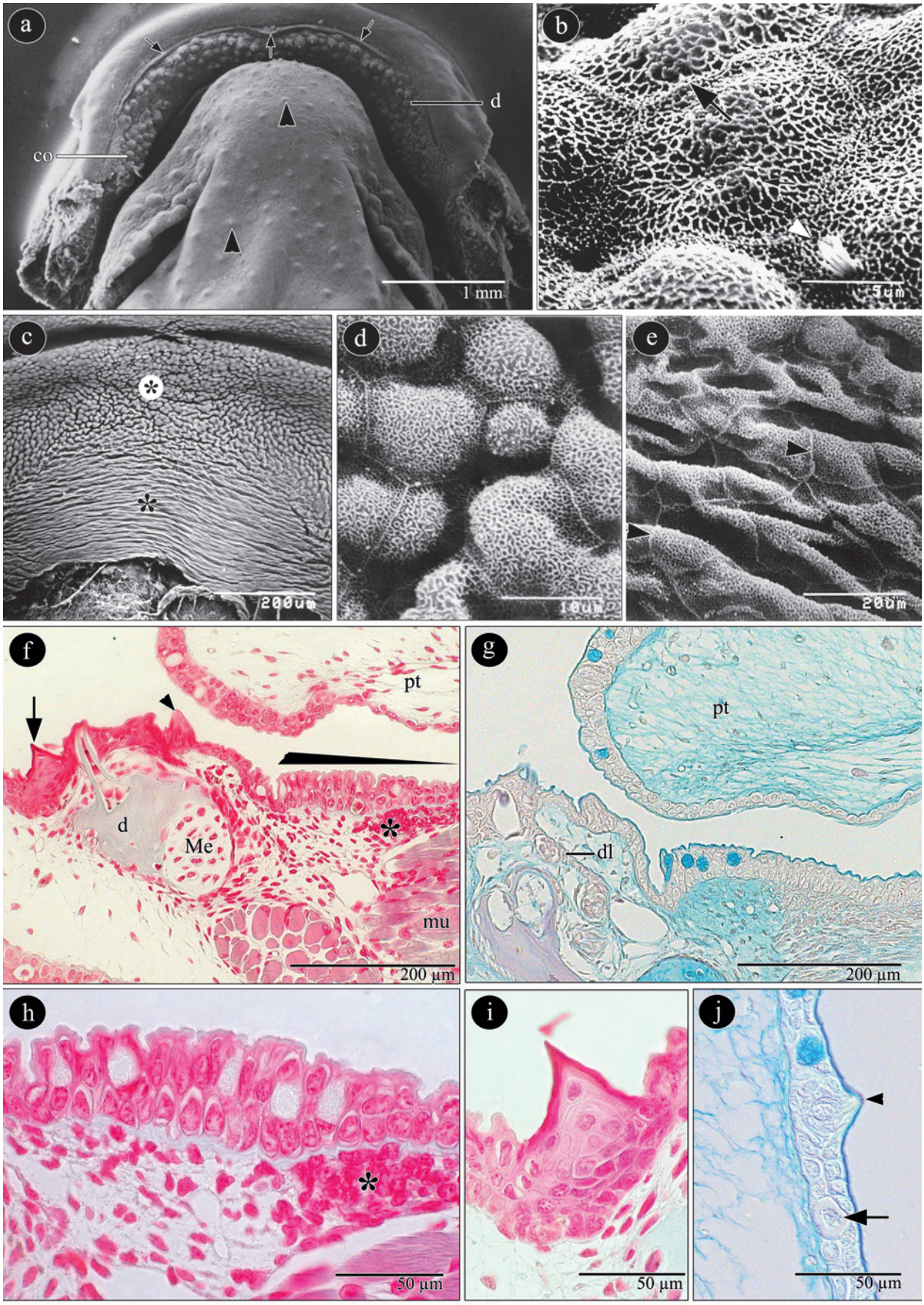
Terminology. The mouth cavity of Urodela is equipped with a variety of glands and secretory cells, which can be distinguished by their location, histochemistry of their secretory products, and ultrastructure. We distinguish herein single secretory cells (collectively named mucocytes or goblet cells), indentations (crypts) of the stratified surface epithelium of the tongue, in which secretory cells predominate that also may form the "neck" of tubular glands, and tubular glands, which are lined by a continuous single-layered secretory epithelium (largely adopted from Fahrenholz, 1937).

Results

Hynobius leechi

Stage 1 (late larvae). The mouth floor is largely covered by the primary tongue that bears numerous taste buds (Fig. 1 a); elements of the hyobranchial skeleton, e.g. the copula, do not reach the most anterior portion of the tongue. Typically, all superficial cells bear microridges at their surface (Fig. 1 b). Removal of the primary tongue exposes the surface of the mouth floor showing the anlage of the glandular field of the prospective secondary tongue. Here, two distinct surface patterns can be recognized (Figs. 1 c, d, e). Anteriorly, the surface shows pavement cells bulging out slightly (Figs. 1 c, d), posteriorly, however, the surface is transversally folded (Figs. 1 c, e). Histological sections reveal that the anlage is covered by a stratified three-layered epithelium, most distinct in the median plane. Anteriorly, it is cuboidal and is composed of basal cells, pavement cells, and goblet cells stained preferably blue after AB-PAS (Fig. 1 g). The height of the epithelium decreases posteriorly and finally continues with the very thin epithelium of the lower surface of the primary tongue, where goblet cells are missing (Fig. 1 f). Below the epithelium of the anlage a conspicuous aggregation of nuclei (anlage of the *musculus genioglossus*) and some fibres of the *m. geniohyoideus* are seen (Figs. 1 f, h). The epithelium of the tip and the surface of the primary tongue are bi- to tri-layered, approximately 30 µm thick, and principally contains the same cell inventory as the anlage of the glandular field (Fig. 1 f). In addition, typical taste buds (Figs. 1 a, j) and very seldom Leydig cells are present (Fig.1 j). Immediately behind the dentary a keratinized fold arises (Figs. 1 f, i).

Stage 2 (early metamorphosis). The anterior part of the primary tongue is considerably reduced. The anlage of the glandular field is separated from the anterior part of the primary tongue by a transversal bulge at the mouth floor. Between the bulge and the anterior part of the primary tongue an indentation is seen following the course of the tip of the primary tongue (Fig. 2 a). The surface of the primary tongue bears taste buds and a considerable number of ciliated cells (Fig. 2 a, inset).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Ciliated cells lie also at the margin of the glandular field (Fig. 2 b). Pancreatin treatment reveals the architecture of the lamina propria showing more or less radially oriented furrows devoid of the epithelium (Fig. 2 c). Anteriorly the glandular field is separated from the lower jaw arcade by a deep groove (Fig. 2 d). Tubular glands seen as epithelial cones without or with a lumen arise anteriorly and progressively develop in posterior direction (Fig. 2 e). All goblet cells in this stage are preferably blue after AB-PAS, whereas the secretory epithelium of the developing tubules remains largely unstained (not shown).

Stage 3 (advanced metamorphosis). In specimens one month after going on land the glandular area has been further grown. After pancreatin treatment, furrows in the connective tissue are more regular than in the stage before. They arise at the margin of the tongue and terminate at the above mentioned occasionally bulged area of the mouth floor (Fig. 2 f). Small septa within the furrows separate the devloping tubular glands of each other (Fig. 2 g, inset). Most posterior these furrows are abruptly replaced by an area that is characterized by indentations (crypts) of variable shape and depth (Fig. 2 h). Anteriorly the glandular field of the secondary tongue is well developed and most of the superficial pavement cells are elongated (= villus cells) (Fig. 2 i). The glandular field is now equipped with taste disks (instead of taste buds), which doe not occur in the area of crypts (Fig. 2 j).

Stage 4 (adult): The secondary tongue has its final shape. The anterior mushroom-like portion contains the lingual glands. It ends in a stalk that continues in the folded pharynx. The anterior part of the stalk represents remnants of the primary tongue (Fig. 3 a). Furrows of connective tissue are shortened at the margin of the tongue, but show transversal septa (Fig. 3 b, c). The groove between the glandular field and the upper jaw arcade shows aggregations of preferably AB-positive goblet cells after AB-PAS-staining (Fig. 3 d). Thickness and extension of the glandular field has increased considerably. Glands lie close to the tip of the tongue (Fig. 3 e). In the midst of the field tubular gland reach their largest depths and are slightly branched at their term (Fig. 3 f). After AB-PAS staining of the secretory epithelium clearly decreases top down, with cells preferably AB-positive near the surface and cells practically unstained at the bottom (Fig. 3 g). Posteriorly located tubules decrease in depth and finally will be abruptly replaced by crypts (Fig. 3 h). Beyond the last tubular gland the number of ciliated cells increases remarkably and the lamina propria becomes noticeably thicker (Figs. 3 h, i). Also here goblet cells are preferably blue after AB-PAS (Fig. 3 j).

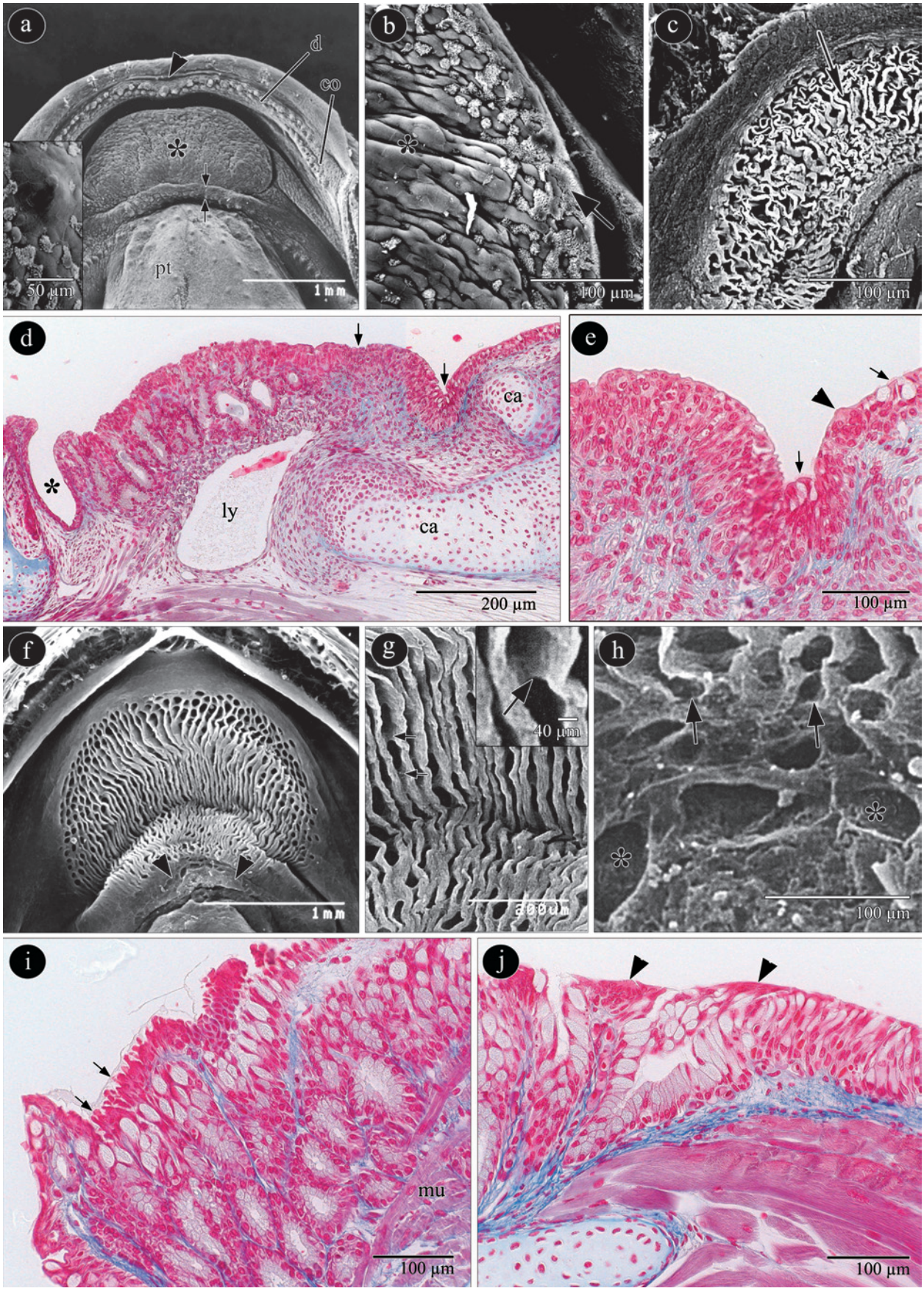
Ambystoma mexicanum

The organisation of the tongue of paedomorphic and transformed *Ambystoma mexicanum* has been described previously (s. Introduction). Therefore, we focus here on the presence of prospective lingual glands before metamorphosis, the posterior end of the glandular field in transformed specimen and a gland-like area at the inner face of the lower jaw arcade.

Semiadult: The paedomorphic, semiadult specimen possesses a typical primary tongue with a stratified epithelium that is dorsally thicker than ventrally. Ventrally the epithelium continues with the epithelium of the mouth floor, which exhibits a moderate number of goblet cells (Fig. 4 a). In anterior direction the epithelium increases in thickness and most anteriorly, in front of a prominent bulge, epithelial cones with a considerable number of apical PAS-positive goblet cells are seen. This organisation resembles anlagen of tubular glands (Fig. 4 b). Removal of the epithelium in this area exposes the *lamina propria* showing a prominent bulge that follows the lower jaw arcade and in front of this bulge an area with parallel furrows. The elongate furrows, in which smaller septa become inserted, are longest in the middle of the area and shortest at the posterolateral end of the area (Figs. 4 c, d). Anteriorly, on the other side of bulge, a thick epithelium with numerous goblet cells covers the inner face of the premaxilla (Fig. 4 e).

28 days after thyroxin application. The glandular field is well-developed showing tubular glands of considerable depth. The deeper the tubule, the more intensely the secretory epithelium is stained. Tubules, however, do not reach the tip of the tongue. (Fig. 5 a). The groove be-

Fig. 1 a–j. *Hynobius leechi*, late larva, SEM (a–e), LM (f–j; f, h. i : H.A. (Heidenhain´s azan trichrome stain); g: AB (alcian blue) (pH 2.5)-PAS (periodic acid Schiff stain), j: AB (pH 2.5)-PAS; Nomarski). **a** Primary tongue with tase buds (arrowheads). Coronoid (co), dentary (d);. keratinized fold (arrows). **b** Surface (pavement) cells of the primary tongue with a network of microridges; ciliated cell (arrowhead), cell boundary (arrow). **c** Surface of the anlage of the lingual glands; anterior part (asterisk, white), posterior part (asterisk). **d** Epithelial surface of the anterior part of the anlage. **e** Ditto; posterior part of the anlage; cell boundary (arrowheads). **f** Lower skull and primary tongue (pt), parasagittal section. Anlage of the glandular field (pointed bar). Keratinized tooth-like fold (arrow; see fig. 1 a) and taste bud (arrowhead); aggregation of nuclei (asterisk) and the extending *m. geniohyoideus* (mu). Dentary (d); Meckelian cartilage (Me). **g** Ditto; goblet cells are stained blue. Dental lamina (dl), primary tongue (pt). **h** Detail of the anterior part of the anlage with a cuboidal epithelium and goblet cells. Aggregation of nuclei (asterisk). **i** Keratinized fold in front of the dentary. **j** Leydig cell (arrow), goblet cell (blue) and taste bud (arrowhead) in the epithelium of the primary tongue.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tween the lower jaw and the anterior end of the tongue is broad exhibiting some folds. The epithelium of the inner face of the lower jaw is remarkably thickened (Fig. 5 a). Posteriorly, tubules became shorter and were abruptly replaced by crypts. This part is underlain by a strong *lamina propria* (Fig. 5 b).

54 days after thyroxin application. The glandular field has increased showing radially arranged furrows and ridges (Fig. 5 c), which is reflected in pancreatin-treated preparations that expose the connective tissue framework of the glandular field. Furrows exhibit transversal septa lying somewhat deeper than the ridges. The free margin of the tongue is smooth (Fig. 5 d). In both preparations the zone that joins the furrows and ridges show less deep holes, i.e. largely crypts (see above). Posteriorly the thickness of the glandular field is reduced. Tubules appear to have a reduced glandular portion, are of less depth and lack the heavily stained end section (Fig. 5 e). Folding of the area between the anterior end of the glandular field and the lower jaw arcade has increased; the epithelium covering the inner face of the lower jaw appears considerably thick (Fig. 5 f).

2.5 years after metamorphosis. The glandular field now is large and well-developed. The figures 6 a-h show the histological changes of the glandular field from the tip of the tongue (Fig. 6 a) until the area, where the glandular field abruptly ends (Figs. 6 f, g) joining the modified remnant of the primary tongue (Figs. 6 g, h). Here the *lamina propria* is remarkably strong (Fig. 6 g). As shown already in the stage described before, secretory cells in the terminal end of the tubular glands are stained more intensely after H.E. than in the upper regions (Fig. 6 a, c). After AB-PAS a clear change is seen from a mixed colour (bluish-purple) to blue with decreasing intensity towards the bottom of the glands (Figs. 6 b, d). Tubular glands become shorter towards the posterior end of the glandular field having relatively long necks. (Fig. 6 e, f, g). They are replaced by crypts with various PAS- and AB- positive goblet cells showing various bluish-purple shadings (Fig. 6 f, h). At the height of the last gland, number of cilated cells increases significantly.

The folds immediately before and below the free margin of the secondary tongue already seen 54 days after thyroxin application are now prominent exhibiting high numbers of AB- and PAS-positive goblet cells (Figs. 6 a, j). The thickened epithelium seen at the inner face of the lower now exhibits tubules with a continuous secretory epithelium at least in their deeper regions (Fig. 6 i). Tubules contain epithelial cells that are stained very weakly, if, any after AB-PAS, whereas goblet cells again show various bluish-purple shadings (Fig. 6 j).

Discussion

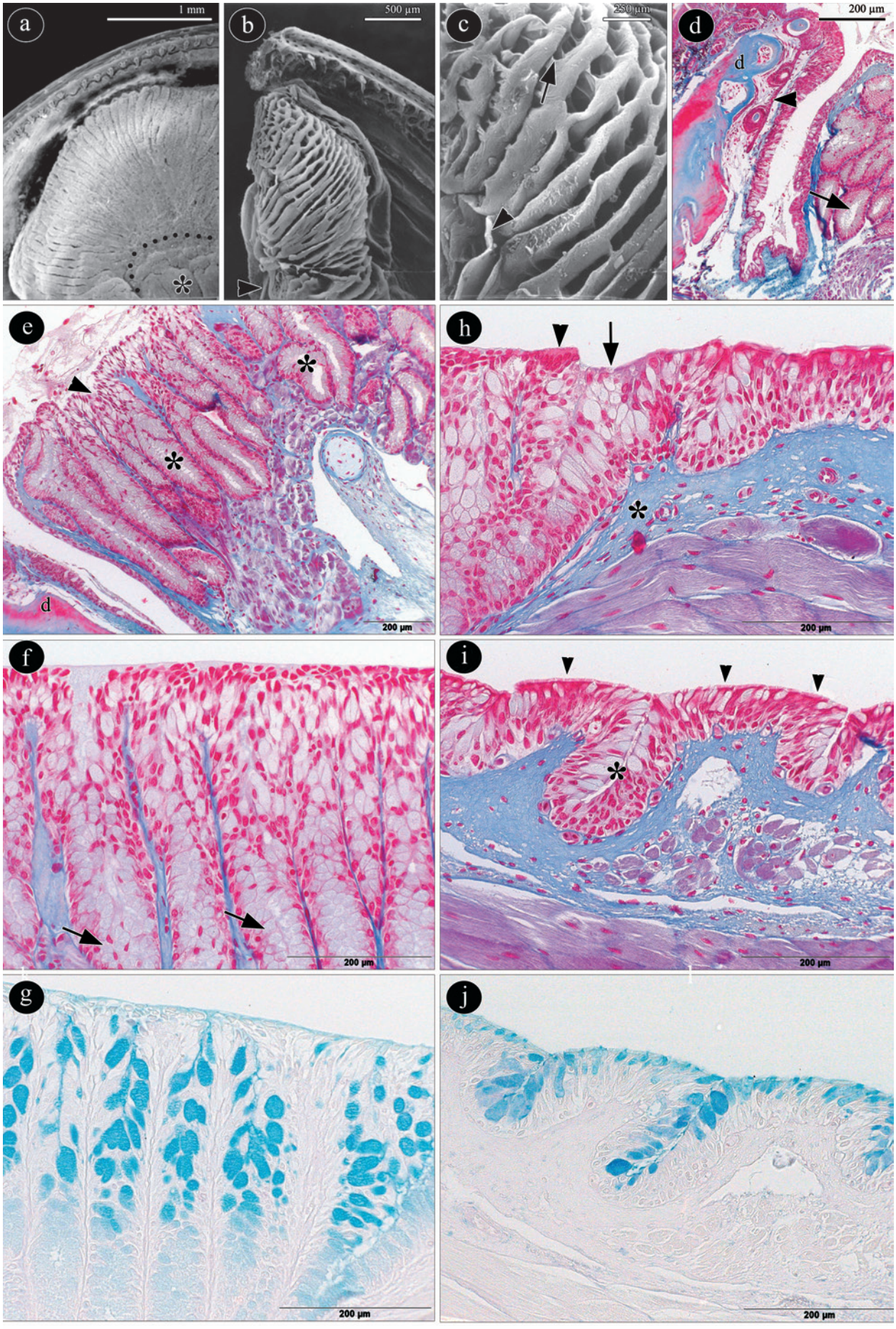
General aspects

Our study broadens the few hitherto published notes on tongue development in hynobiids (*Hynobius retardatus*: Takeuchi *et al*., 1997; *Ranodon sibiricus*: Kobegenova *et al*., 1998; *Hynobius dunni*: Zuwala *et al*, 2002) and confirms the basic pattern of tongue development in Urodela so far studied (e.g. Gegenbaur, 1894; Kallius, 1901; Opolka & Clemen, 1998). This pattern includes the development of a glandular pad beneath the free anterior margin of the primary tongue, an obvious regression of the latter, and finally the "fusion" of the glandular field with the anterior part of the former primary tongue (see literature cited in the Introduction). Unsurprisingly, this pattern is also observed in the axolotl in the course of an artificially induced metamorphosis (see Wistuba & Clemen, 1998).

According to Takeuchi *et al*. (1997) "taste disk-like cell masses" develop on the surface of the glandular field of the secondary tongue in transformed *Ambystoma mexicanum* and *H. retardatus*, whereas more posteriorly taste buds are formed which resemble the "barrel-shaped taste buds" typical for the primary tongue, but somewhat larger (Takeuchei *et al*., 1997, figure 9, p. 543). Obviously the latter reflect the less dramatic remodelling of the primary tongue during metamorphosis.

The various relations of the tongue muscles especially of the fibres of the *musculus genioglossus* are known

Fig. 2 a–j. *Hynobius leechi*, early (a–e) and middle (f–j) metamorphosis; SEM (a–c, f–h), LM (d, e, i, j: H.A.) **a** Developing glandular field (asterisk). Bulge (arows) in front of the primary tongue (pt), coronoid (co), dentary (d). Inset: Cilated cells on the primary tongue. **b** Margin (arrow) of the developing glandular field (asterisk) with ciliated cells. **c** Irregular radial furrows (arrow) in the *lamina propria* of the glandular field, pancreatin. **d** Parasagittal section; groove between the lower jaw and the thickened glandular field (asterisk). Elements of the cartilaginous hyobranchial apparatus (ca). Lymphatic space (ly). Bulged part of the developing glandular part (arrows; see fig. 2 a). **e** Sector of figure d showing the posterior portion of the seondary tongue with developing tubular glands (left side). Depression (boundary between the glandular field and primary tonge) with ciliated cells (arrows); taste bud (arrowhead). **f** Anterior part of the glandular field with furrows; remnants of the primary tongue (arrowheads), pancreatin. **g** Furrows (small arrows) connective tissue septa delimit the glandular tubules (Inset, arrow), pancreatin. **h** Furrows (arrows) of the glandular field and crypts (asterisks) of the primary tongue, pancreatin. **i** Anterior part of the glandular filed with tubular glands and apically elongated pavement cells (arrows); muscles (mu). **j** Border (beyond the taste disks, arrowheads) of the glandular field and the primary tongue (right side).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(see Gegenbaur, 1894). In *Hynobius leechi* and *A. mexicanum* fibres of the *m. genioglossus* extend between the tubules reaching almost the surface of the glandular field, whereas for example in *Salamandra salamamdra* the mass of this muscle is situated below the tubular glands (Gegenbaur, 1894; Kallius, 1901; Seifert, 1932; Fahrenholz, 1937). The development of the glandular field of the secondary tongue appears to start at the beginning of metamorphosis in the species examined herein. However, compared to *Salamandrella keyserlingii*, Kobegenova *et al*. (1998) noted a considerably delayed onset in *R. sibiricus* suggesting species-specific differences that have not studied in detail yet.

The epithelia of the primary and secondary tongue

Primary and secondary tongues are covered by a non-keratinized stratified epithelium. Appearance of the surface cells largely depends on the developmental stage and their location on the tongue. In the examined species, we did not find evidence of pseudostratification (often mentioned in the classical literature) of the epithelium either of the tongue or of the oral cavity (see also Elkan, 1955; Clemen, 1984, 1985 a, b).

The thin two- to three-layered cuboidal epithelium of the primary tongue is composed of pavement or superficial cells, basal cells and intermediate cells (if more than two layers are present). Interspersed are roundish goblet cells, which are preferably stained blue after AB-PAS in *Hynobius leechi*, and typical taste buds. As expected, a largely identical inventory of cells can be found in the tongue of the paedomorphic *Ambystoma mexicanum*, where goblet cells appear not to be exclusively stained blue after AB-PAS (see Wistuba & Clemen, 1998; Wistuba *et al*., 1999) as well in the epithelium that lines the entire oropharyngeal cavity.

We occasionally noticed the presence of Leydig cells (LC) in the tongue epithelium of larval *Hynobius leechi*. Here, they have a similar size as the goblet cells, but contrary to these cells, LC were AB-PAS negative. In addition, their presence was unequivocally demonstrated by transmission electron microscopy (not shown). These enigmatic and usually very large cells are typical for the epidermis of larval Urodela (for a more recent survey see Gerling *et al*., 2012). As yet, their occurrence in the epithelium of the primary tongue (and in the oropharnygeal epithelium) has been reported only in semiadult and adult paedomorphic *A. mexicanum*, where they occur in considerable numbers (Kantorek & Clemen, 1990, 1991; Toyoshima *et al*., 1992; Wistuba & Clemen, 1998). LC have not been found yet in the tongue epithelium either of young larval axolotls or of other larval Urodela. Reasons for this irregular occurrence among taxa are unknown.

Also noticeable is the keratinized fold in front of the dentary teeth in the late larva of *H. leechi.* In sagittal sections they strikingly resemble the keratinized labial teeth of the jaw sheaths (also called a ‘‘beak’’) in anuran tadpoles (e.g., Altig & McDiarmid, 1999). In later stages this fold becomes flat; its keratinized outer layer melts into the *stratum corneum* of the epidermis.

In the glandular field superficial cells may become largely modified being elongate in the vertical axis and having “free” apices. Such cells were called “büschelförmige Zellen” (Seifert, 1932) or “villus cells” (Opolka *et al*., 2001) and some taste discs (instead of taste buds) dispersed in the epithelium (Takeuchi *et al*., 1997; Zuwala & Jakubowski, 2001).

Secretory cells in the tongue epithelium excepting tubular glands

There are various cell types in the tongue epithelium, which produce secretions: 1) the non-keratinized superficial cells probably including the ciliated cells, 2) variously shaped, secretory single cells (goblet or mucous cells), and 3) highly secretory cells that form the single-layered epithelium arranged in the lingual glands.

Apart from the lingual glands (see below) all produce largely acidic and/or neutral glycoconjugates, which can be demonstrated by various techniques (e.g. Mulisch & Welsch, 2010). In the present study we used AB-PAS-staining, which roughly differentiates between acid (AB-positive, tinted blue) and neutral “mucosubstances” (PAS-positive, tinted magenta), or a mixture of acidic or neutral glycoconjugates (tinted either blue-red, or red-blue). We did not consider various slight shades of blue and magenta to distinguish between various mucocytes

Fig. 3 a – i. *Hynobius leechi*, adult secondary tongue. SEM (a, b, c); LM (d, e, f, h, i: H.A.; g, j: AB (pH 2.5)-PAS). **a** Secondary tongue and putative boundary (dotted line) of the former primary tongue (asteriks). **b** Halved tongue showing the connective tissue framework of the glandular field; border of the glandular field (arrowhead); pancreatin. **c** Ditto; border (arrowhead), septa between tubular glands (arrow); pancreatin. **d** Groove between the glandular field and the upper jaw arcade with numerous goblet cells. Dental lamina (arrowhead); tubular glands (arrow). Dentary (d). **e** Anterior portion (arrowhead) of the glandular field with tubules (asterisks). Dentary (d). **f** Midst of the glandular field. Note goblet-like cells in the upper portion of the tubules; secretory epithelium (arrows) **g** Ditto. Staining of secretory cells of the tubular decreases top down. **h** Posteriorly the area of tubular glands (left side) ends relatively abruptly (arrow); taste disk (arrowhead). Strong *lamina propria* (asterisk). **i** Area posterior to the tubular glands showing a multilayered ciliated epithelum (arrowheads) and crypts (asterisk) resting on the thick *lamina propria* . **j** Ditto. Goblet cells of the epithelium and the crypts are preferably stained blue.

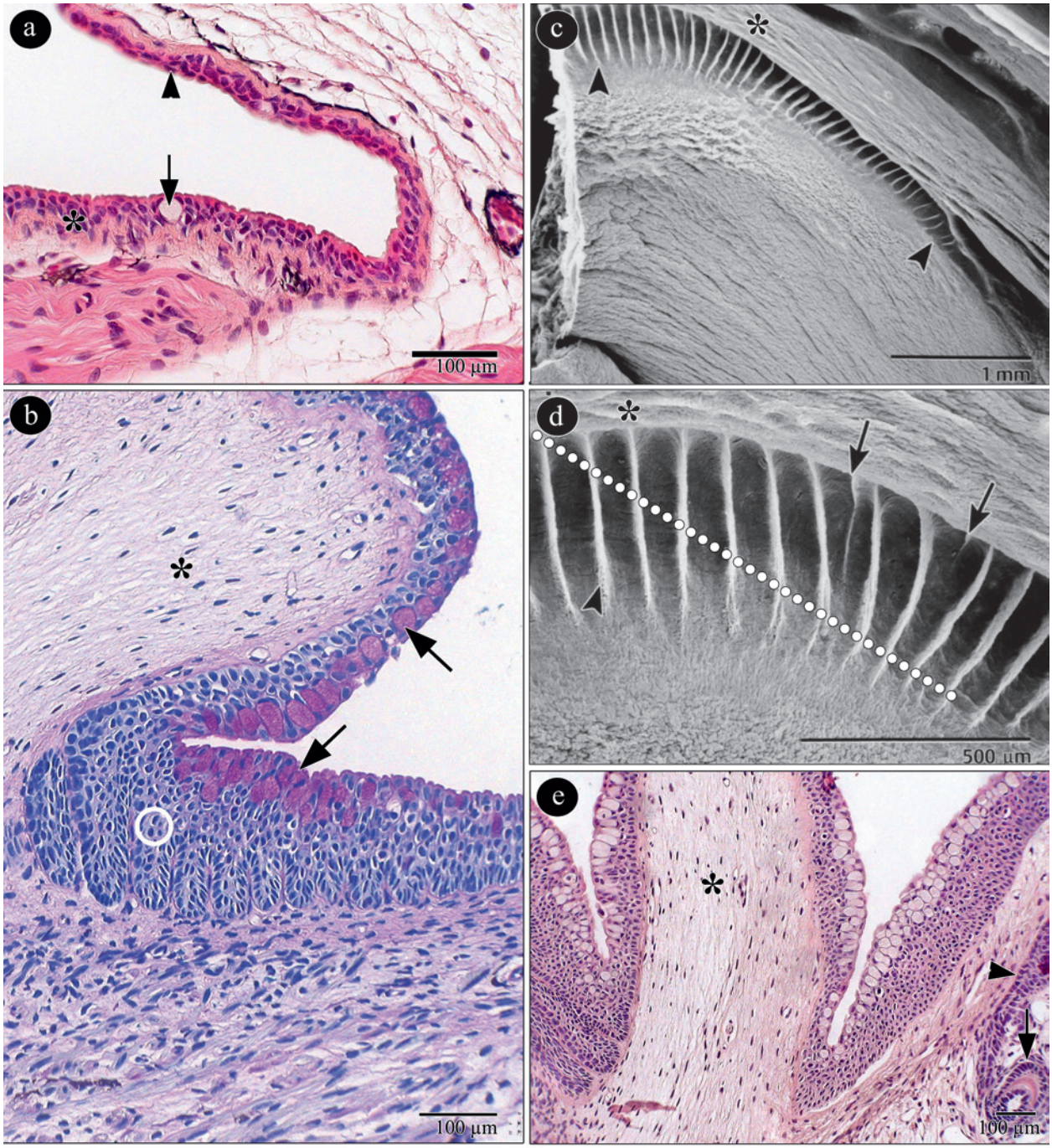


Fig. 4. *Ambystoma mexicanum*, semiadult. LM (a, e: H.E. (hematoxylin-eosin); b; PAS-hematoxylin); SEM (c, d). **a** Undersurface of the primary tongue (arrowhead) and epithelium of the mouth floor (asterisk); goblet cell (arrow). **b** Mouth floor showing epithelial cones, i.e. anlagen of tubular glands (circle) in front of a prominent bulge (asterisk). Goblet cells (arrowheads). **c, d** Framework of the connective tissue in the anterior mouth floor in front of a bulge (asterisk) with thick (arrows) and thin (arrows) septa; pancreatin. The dotted line indicates the level of the section in figure b. **e** Nearly sagittal section showing the anlagen of the tubular glands (left side), the bulge (asterisk), and the thick epithelium covering the inner face of the lower jaw arcade (right side). Dental lamina (arrowhead), tooth bud (arrow).

(see Wistuba *et al*., 1999), but employed this technique only to describe clearly visible differences or changes in the various secretory cells during development of the tongue, all the more as other techniques including transmission electron microscopy will give more detailed information (e.g., Zylberberg, 1977; Wistuba *et al*., 1999; Opolka *et al*., 2001).

In *Hynobius leechi* both the non-keratinized superficial cells of the epithelium that cover primary and secondary tongue as well as the various goblet or mucous cells dispersed throughout the epithelia predominantly produce AB-positive secretions, i.e. acidic glycoconjugates. Obviously this does not hold for *Ambystoma mexicanum*. Here already in young larvae pavement cells and goblet cells have changing portions of PAS- and AB-positive secretions that seem to be shifted to a larger portion of neutral glycoconjugates during development including artificially induced metamorphosis (Wistuba *et al*., 1999). Concerning the transformed specimens, our results do not show such a clear shift, and goblet cells in the former primary tongue and crypts as well as in the folds between the upper jaw and the tip of the secondary tongue show various shades from exclusively magenta to blue. We did not follow up this matter, but AB- and PAS-staining and especially transmission-electron microscopy revealed a sequence of several types of goblet-like cells in the oral cavity and the tongue from the larva (only AB-positive goblet cells) to the terrestrial transformed *Salamandra salamandra* (AB-positive and two types of PAS-positive goblet cells clearly differing in ultrastructure). These changes have been discussed in the context of the terrestrial habit after metamorphosis (Clemen, 1984, 1985 a, b; Opolka *et al*., 2001). Further comparative studies concerning this matter are missing.

Lingual glands and other gland-like complexes

Our histological findings and previous notes show that in the prospective glandular field more or less radially arranged epithelial furrows develop, which contain the tubular glands (e.g. Kallius, 1901; Wistuba & Clemen, 1998; Opolka & Clemen, 1998). The pancreatin-treated preparations allowed a closer look on the three-dimensional architecture of the *lamina propria* showing that tubules are encircled by small septa of connective tissue. Septa lie somewhat deeper as the furrows (devoid of the epithelium) indicating the size and depths of the "neck" of the tubular glands, which is still lined by the stratified tongue epithelium. In *Hynobius leechi* the upper smaller part of the tubular glands exhibits preferably AB- positive secretory cells (goblet cells), occasionally interspersed with non-secretory cells followed by the continuous single-layered glandular epithelium. The latter is characterized by cells with an AB-PAS- negative granular secretion. Contrary to these findings, upper goblet cells in the related *Hynobius tokyoensis* are AB- and PAS-positive, whereas the terminal portions are preferably AB-positive, i.e. mucous in character. This was also confirmed by the ultrastructure of the secretory granules that revealed a rather electron-lucent and structure-less content (Kurabuchi *et al*., 1995) possibly indicating differences also in closely related species.

Secretory products of lingual glands in Urodela are heterogeneous glycoconjugates ranging from "mucous" (more or less acidic) to "seromucous" (with a proteinaceous fraction) and often there is a more or less gradual change between both "types" from the surface (mucous) towards the bottom (seromucous). In many species tubules may contain also intermediate types of secretory products. Especially in species that live in terrestrial habitats after metamorphosis secretions of the lingual glands appear to be more rich in proteins**,** and secretory granules of such glands are often characterized by a highly complex ultrastructure, e.g. in salamandrids, but also in plethodontids. This has been repeatedly revealed by histochemistry and transmission electron microscopy (for review see Zylberberg, 1973, 1977; see also Fährmann, 1974, 1975; Kurabuchi, 1986; Opolka *et al*., 2001). The herein presented findings only confirm histochemical differences between the near-surface secretory cells and the more terminal cells in the lingual glands of transformed *Hynobius leechi* and *Ambystoma mexicanum* (for the latter species see some notes in Wistuba *et al*. (1999), which in view of the present results can not be reproduced in every detail). Ultrastructural details are missing.

Further, we have shown that semiadult paedomorphic *A. mexicanum* possess an epithelial thickening at the posterior margin of the mouth roof with epithelial cones and numerous goblet cells, which closely resembles the anlage of a glandular field. Currently, no own observations are available concerning the further development of this structure. However, Kallius (1901) pointed to a field of "crypts", i.e. non-glandular epithelial indentations, in the anterior part of the mouth floor of an adult axolotl, to which parts of the *musculus genioglossus* extend. Unfortunately he does not illustrate this finding, but already discussed his observation in terms of a partial metamorphosis in this species (e.g. Clemen & Greven, 1977; Wiens *et al*., 2005).

Two further distinctive features are noticeable, which can be recognized clearly in the adult transformed axolotl. In the folds of the groove between the lower jaw and the tip of the tongue there are conspicuous accumulations of AB- and PAS-positive goblet cells. Certainly, they do not form "true" glands, but seem to correspond to the various sublingual or infralingual glandular elements (*sensu* Fahrenholz, 1937) seen in many Amphibia. These elements also include the sublingual gland of some plethodontids with protrusible tongues, e.g. *Eurycea* spp. This gland was described as a group of simple tubules with granular secretory cells (Seifert, 1932; Fahrenholz, 1937) or, more recently, as a group of short tubules with numerous "goblet cells" producing an acidic secretion (Opolka *et al*., 2003).

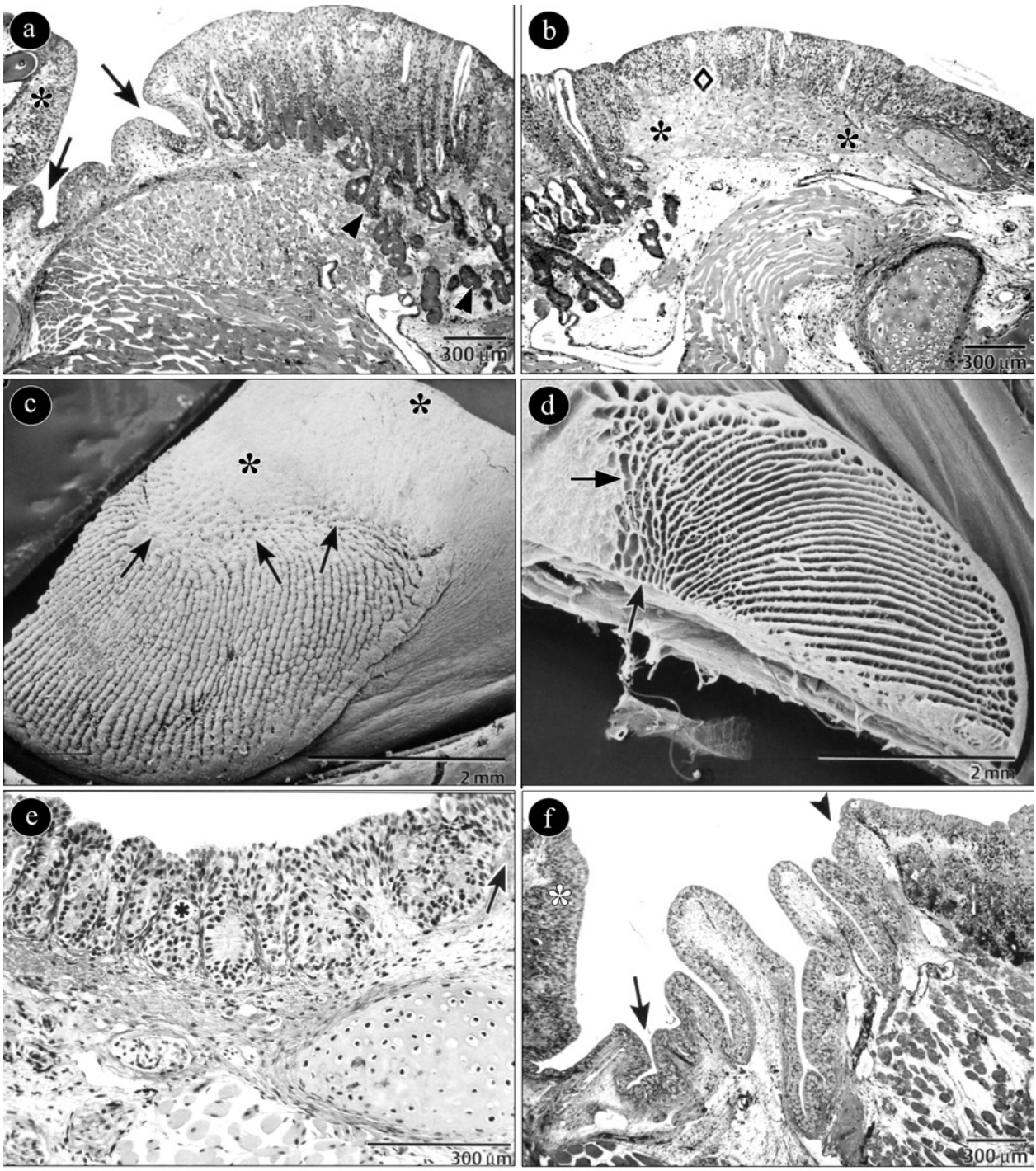


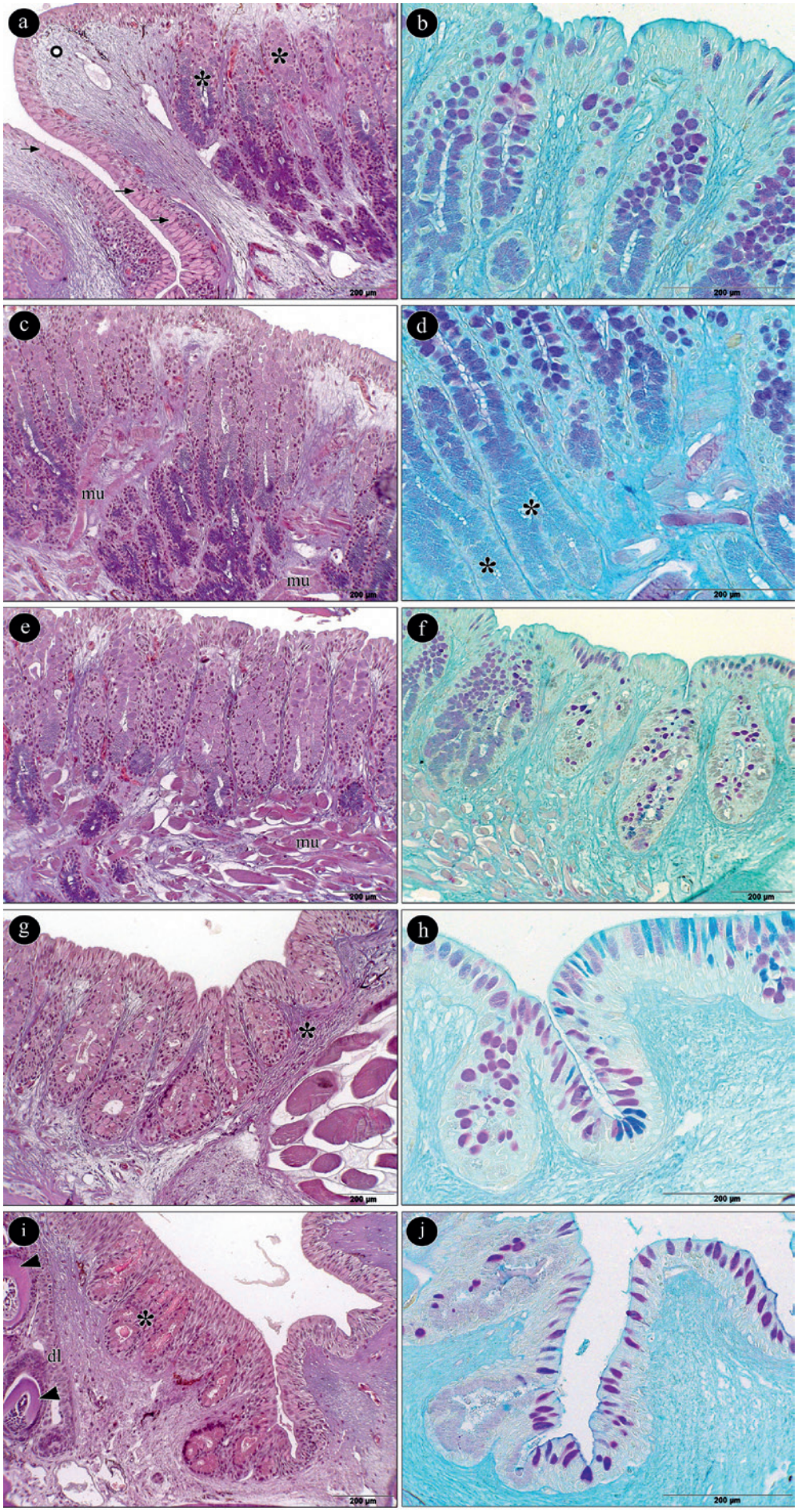
Fig. 5. *Ambystoma mexicanum*, 28 d (a, b) and 54d (c – f) after thyroxin application. LM (a, b, e, f: H.E.); SEM (c, d). **a** The glandular field shows tubular glands deeply embedded in the connective tissue (arrowheads). Folded area (arrows) between the posterior end of the glandular field and the thickened inner face of the lower jaw arcade (asterisk). **b** Posteriorly the epithelium abruptly decreases in height (rhomb) showing crypts only and a dense *lamina propria* (asterisks). **c** Secondary tongue with parallel furrows and ridges; boundary between the glandular field and remnant of the primary tongue (arrows). Smooth part of the primary tongue (asterisks). **d** Ditto, after pancreatin treatment; area of crypts (arrows). **e** Posterior zone of the glandular field (asterisk) showing short tubular glands; remnant of the primary tongue (arrow). **f** Tip of the secondary tongue (arrowhead), folds in front of the tongue (arrow) and thick epithelium at the inner face of the lower jaw arcade (asterisk).

The thickening at the inner epithelial face of the lower jaw in the axolotl specimen 54 days after thyroxin application indicates the development of further gland-like complexes, which later consists of tubules that are definitely lined (at least in part) with a continuous layer of largely AB-PAS negative secretory cells. However, based on our sections we are not quite sure, whether the epithelium is entirely single-layered. Fahrenholz (1937: 139) noted the common occurrence of mucous crypts ("Schleimkrypten") at the inner face of the lower jaw of Urodela and Anura, but did not further specify his note. Surely, this complex is lacking in transformed *S. salamandra* (see Opolka *et al.*, 2001) and *H. leechi*.

The zone of "fusion"

Kallius (1901) thoroughly described the "fusion" of the glandular field and the remnants of the former primary tongue in *Salamandra salamandra*. He noted that the posterior anlage of the glandular field, still without glands, approaches the ventral surface of the primary tongue and finally merges with the latter, and recognized a clear demarcation line consisting of a strand of cells in the subjacent connective tissue glands. His very schematic drawings do not allow further interpretation. In the species herein examined such a clear demarcation line was not observed.

We think the terms "fusion" or "merging" generally used for this process (Kallius, 1901 and all other citations) are misleading, as the two parts do not really fuse. Rather, the height of the glandular field increases considerably during growth successively approaching the level of the (regressive?) primary tongue. Simultaneously, the relatively large space between the anterior free end of the primary tongue and the posterior, largely undifferentiated portion of the glandular field gradually becomes reduced leaving a small groove, which may persist for a while in transformed specimens. In histological sections the boundary between the two portions is clearly indicated by the relatively abrupt thinning of the epithelium, the significant increase of the number of ciliated cells, the presence of epithelial crypts instead of tubular glands, which represent a conspicuous modification of the former primary tongue, and the strong subjacent *lamina propria*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Acknowledgements

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Fig. 6. *Ambystoma mexicanum*, transformed adult, 2,5 years old. LM (a, c, e, g, i: H.E.; b, d, f, h, j: AB (pH 2.5)-PAS). **a** Anterior portion of the secondary tongue (circle) lacking tubular glands. Goblet cells (arrows), tubular glands (asterisks). **b** Secretion of tubular glands change from top (blue-red) to bottom (blue with decreasing intensity). **c** Middle part of the secondary tongue with deep tubular glands. Note different staining of secretory cells; mu = muscles. **d** The terminal secretory cells of tubular are stained relatively weakly (asterisks). **e** Tubular glands decrease in length posteriorly; mu = muscles. **f** Posteriorly tubular glands (left side) are replaced by epithelial crypts (right side). **g** Posterior part; reduced tubular glands (left side) and some crypts (right side). Strong *lamina propria* (asterisk). **h** The area following the reduced tubular glands with crypts exhibit variously stained goblet cells. **i** Tubular glands (asterisks) in the epithelium covering the inner face of the lower jaw. dl = dental lamina, replacement teeth (arrowheads). **j** Ditto; contrary to the goblet cells, tubular glands (left side) are very weakly stained after AB-PAS.

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Note added in proof

Recently we became aware of a rarely cited article about tongue development in a hynobiid salamander. Despite intensive effort the journal, in which the study was published, was not available. However, for the sake of completeness and fairness, we give here the full citation (Yamasaki, S. (1956): Über die Entwicklung der Zunge von *Hynobius retardatus*. – Sapporo medical Journal, **10**: 1–34).