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DISSERTATION

Titel der Dissertation

Functionality and plasticity of turtle-feeding with special emphasis on oropharyngeal structures

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Für meine Eltern:

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Contents

1. Introduction

1.1. Origin and evolution of turtles

Turtles are the oldest known recent amniotes (e.g. Romer and Parsons, 1977; Starck, 1978). The origin and phylogenetic relationships of this clade has been discussed for decades. One main problem in reconstructing turtle evolution is the very old age of this clade (over 220 Ma), the second the fragmentary availability of fossil material. This has led to a range of alternative theories. While Gaffney (1980) suggested the turtle clade to represent the sister group of all other living amniotes, Werneburg and Sanchez-Villagra (2009) placed them as a sister group to all other sauropsid clades. Alternatively, turtles were discussed to represent the last descendents of anapsid parareptilia (Lee 1995, 1996, 1997; Laurin & Reisz, 1995) or they were grouped within the diapsid reptiles (Rieppel & deBraga, 1996; deBraga & Rieppel, 1997; Rieppel & Reisz, 1999; Müller, 2003; Hill, 2005). By contrast, some molecular biologists placed the turtles as a sister group of archosauria (Kumazawa and Nishida, 1999; Rest et al., 2003; Iwabe et al., 2005). Simplified models of these alternative theories are provided in Fig. 1. Consequently, the question where turtles come from is difficult to answer. Two main theories on the origin have been suggested based on their most striking symplesiomorphy: the turtle shell, consisting of the dorsal carapace and ventral plastron. The first theory is based on the "composite model", the second on the "de novo" one (Joyce et al., 2009). According to the composite model, the turtle shell derived from a series of intermediate forms with increasing amounts of dermal armour. Some elements of this dermal armour gradually fused with the internal skeleton to form both the dorsal and ventral turtle shell (Lee, 1996; Cebra-Thomas et al., 2005). By contrast, the "de novo model" argues the turtle shell to be an evolutionary novelty that developed through outgrows of the ribs from non-shelled forms, with no significant intermediates (Burke, 1989; Gilbert et al., 2001; Rieppel, 2001). The various highly contrasting phylogenetic hypotheses of turtle origin often directly favour one model over the other (Joyce et al., 2009). Those authors suggesting armoured groups of reptiles to represent turtle ancestors (Laurin & Reisz, 1995; Lee, 1996, 1997; Scheyer, 2007; Scheyer and Sander, 2007) provide support for the composite model, whereas those who claim turtles to be derived from non-armoured amniotes (deBraga & Rieppel, 1997; Rieppel & Reisz, 1999; Hill, 2005) favour the de novo model. The recent discovery of the ancestral *Odontochelys semitestacea* (Li et al., 2008) sheds new light on the discussion of turtle evolution. This so far oldest known turtle largely lacks a carapace but has a plastron. This condition supports evolutionary statements based on ontogenetic data from

turtle shell development (Burke, 1989; Li et al., 2008; Nagashima et al., 2009) and thus favours the "de novo model" (Li et al., 2008; Reisz and Head, 2008).

Whatever future studies on turtle origin and evolution will reveal, it is clear that turtles are 1) the oldest living amniotes and 2) unique amongst vertebrates in many respects.

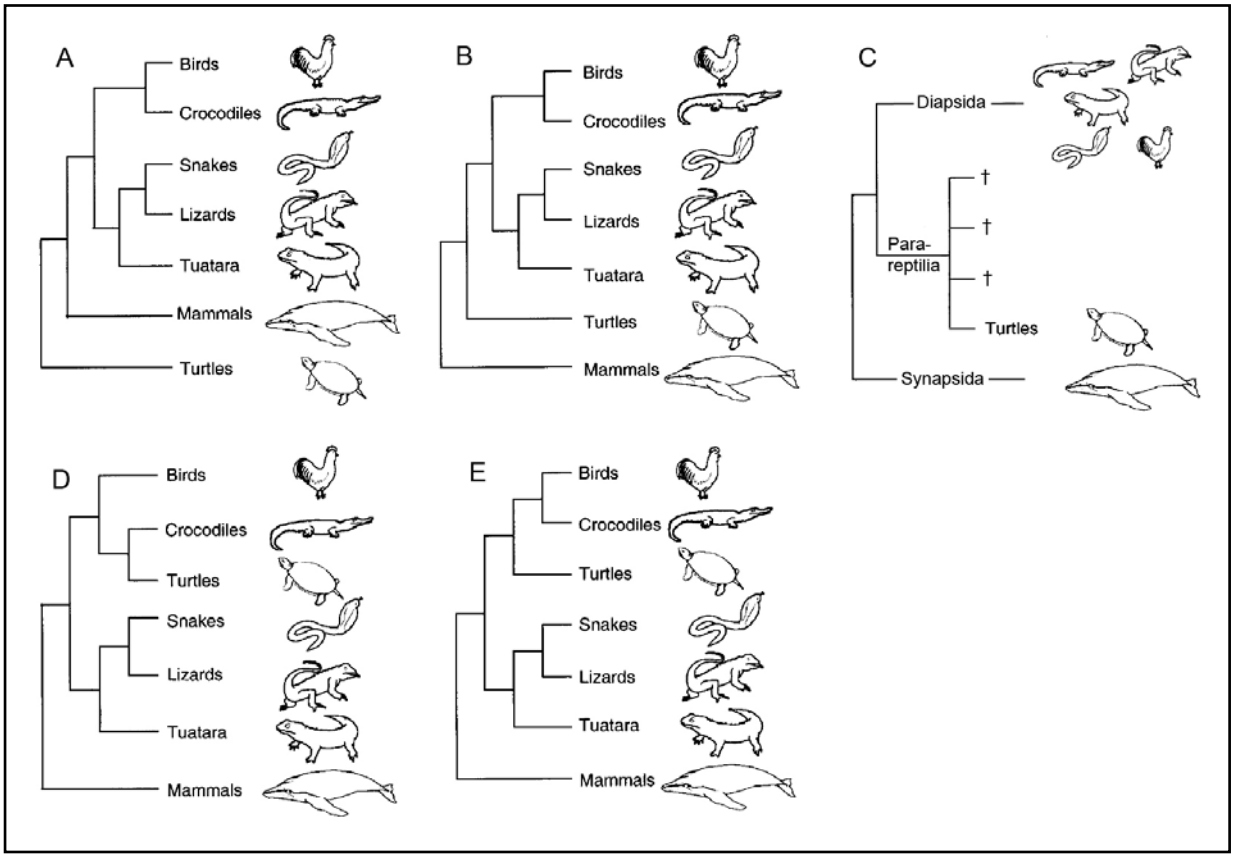

Fig. 1. Some examples of alternative hypotheses on the phylogenetic position of turtles within amniote vertebrates. A Turtles as sister group of all other amniotes. B Turtles as sister group of all other sauropsids. C Turtles as last descendents of the parareptilia. D Turtles within diapsid reptiles (all reptiles and birds). E Turtles as sister group of archosaurians (crocodiles and birds). Modified from Laurin and Reisz (1995) and Zardoya and Meyer (2001).

1.2. The paleoecological problem

The question whether turtle ancestors and ancestral turtles were aquatic or terrestrial has been – and remains – the subject of many paleoecological studies (for an overview see Joyce and Gauthier, 2004; Anquetin et al., 2009). Assessing the habitat preference of extinct turtles has been problematic. This is because of insufficient correlation between the habitats of living turtles and such commonly used indicators as shell morphology, and because of the blurry paleoecological interpretation of depositional environments (Joyce and Gauthier, 2004). For example, Gaffney (1990) assumed for the Upper Triassic cryptodiran *Proganochelys quenstedty* and the pleurodiran *Proterochersis robusta* a semiaquatic lifestyle. On the other hand, the Upper Triassic cryptodirans *Palaeochersis talampayensis* and the Lower Jurassic *Australochelys africanus* were proposed to have more terrestrial tendencies (Gaffney and Kitching, 1994; Rougier et al., 1995). Based on a morphometric study on the forelimbs of extinct and extant turtles, Joyce and Gauthier (2004) provided strong evidence for a purely terrestrial lifestyle of *P. quenstedti* and *P. talampayensis*. A histological investigation comparing turtle shell bone microstructures of extant turtles with different habitat preferences and extinct turtles supported the terrestrial lifestyle of *P. quenstedti* and provided further evidence for a similar habitat preference even in the pleurodiran *P. robusta* (Scheyer and Sander, 2007). These authors concluded their study in support of Joyce and Gauthier (2004), that: "the evolutionary history of turtles began in the terrestrial environment and the turtle ancestor was most probably a terrestrial animal." The discovery of *Odontochelys semitestacea* (Li et al., 2008), however, challenged that theory (see also Fig. 2). *O. semitestacea* is still the oldest recognisable turtle, with an estimated age of 235 Ma; it lived in a shallow marine habitat (Li et al., 2008; Reisz and Head, 2008). *Odontochelys* could therefore represent the primitive ecology, i.e. ancient turtles evolved in a marine environment. Alternatively, *Odontochelys* could represent the earliest turtle radiation from terrestrial environments into marine habitats (according to Li et al., 2008; Reisz and Head, 2008).

Whatever the habitat preferences of the stem turtles were, it is generally accepted that the ancestors of all living turtles (i.e. the "crown group" or "modern turtles") was aquatic (see Joyce and Gauthier, 2004; Scheyer and Sander, 2007). Early in their history, turtles then adapted to different environments and evolved different lineages (according to Scheyer and Sander, 2009).

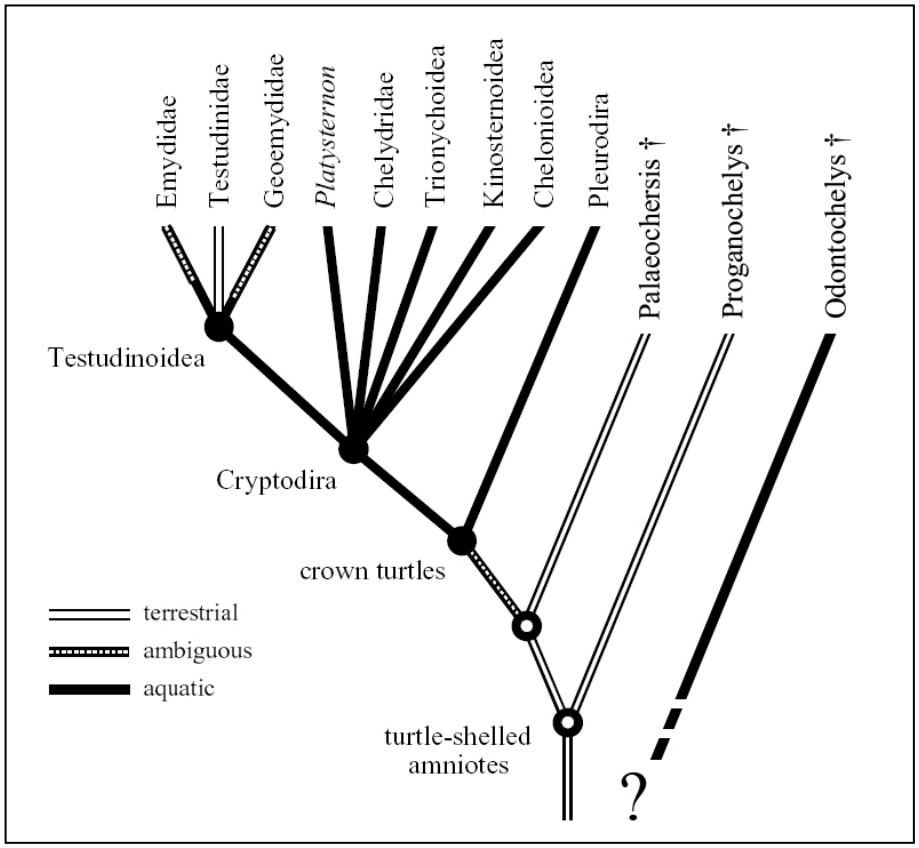

Fig. 2. Simplified cladogram showing the habitat preferences of the major turtle clades and their hypothetical ancestors. The term "crown turtles" refers to the clade that originates from the last common ancestor of all living turtles. The term "turtle-shelled amniotes" refers to amniotes with carapace and plastron homologous to those in living turtles. Even if there is little doubt that the turtle-shelled amniotes were terrestrial, the oldest known turtle-like amniote, *Odontochelys*, was definitely aquatic, obscuring again whether turtles developed from aquatic or terrestrial forms. Modified from Joyce and Gauthier (2004).

1.3. Short phylogeny and ecology of extant turtles

Today, approximatelly 316 species of extant turtles have been described (Bonin et al., 2006). This number, however, is subject to variations due to different phylogenetic approaches, new descriptions and extinctions. All living turtles are subdivided into two infraorders: Pleurodira and Cryptodira. The infraorder Pleurodira contains two families with 77 species, and their most striking synapomorphy is the lateral or horizontal plane of neck retraction. All extant Pleurodira are obligatory aquatic. The second infraorder, the Cryptodira, contains 11 families with 239 species, characterised by a neck vertically retracted into the carapace. Due to extensive radiation, cryptodirans have evolved many different forms which are adapted to a large variety of environments (Bonin et al., 2006). As noted above, however, the ancestors of extant turtles were most probably aquatic, and today all pleurodirans and most cryptodirans are fully aquatic forms. The only truly terrestrial or semiaquatic forms can be found within the cryptodiran superfamily Testudinoidea, which contains the families Testudinidae, Emydidae, and Geoemydidae. While the habitat range of Emydidae and Geoemydidae is from fully aquatic to mainly terrestrial with many intermediate stages, all testudinids are fully terrestrial (Pritchard, 1979; Ernst & Barbour, 1989; Claude et al., 2003). As all families of the Testudinoidea had aquatic ancestors, Summers et al. (1998) hypothesised that the terrestrial lifestyle (including terrestrial feeding) evolved several times independently in cryptodiran turtles. This makes these turtles a particularly interesting clade in which to examine aquatic-terrestrial transitions and the evolution of terrestrial feeding biology.

1.4. An introduction to feeding biology in vertebrates

Feeding plays a critical role in survival and therefore the contribution of the feeding system to fitness is likely to be substantial (Schwenk and Wagner, 2001). As terrestrial vertebrates developed from aquatic forms, aquatic feeding is the primitive mode of prey capture in vertebrates (Lauder, 1985). The hydrodynamic properties of water (e.g. water is 900 times as dense as air and 80 times as viscous) have played a fundamental role in shaping the basic mechanism of aquatic feeding. According to Lauder (1985), suspension feeding ("filter feeding") is the most primitive feeding mode for chordates. Many of the earliest groups of agnathans and gnathostomes probably filtered out detritus and suspended microorganisms with a pharyngeal mucus filter apparatus (Lauder, 1985). Such a primitive feeding mode is still found in amocoetes larvae, and modified filter systems are widely used in higher aquatic vertebrates (e.g. some Chondrythyes, Actinopterygii, tadpoles). Most aquatic vertebrates,

however, use principally two major feeding modes: (i) “suction feeding” and (ii) “ram feeding” (see Liem, 1980; Norton and Brainerd, 1993).

(i) Suction feeding involves a rapid expansion of the mouth and oropharynx, creating a pressure drop inside the oral cavity and causing food-laden water to flow into the mouth (see Van Leeuwen, 1984; Lauder, 1985; Aerts and De Vree, 1993; Ferry-Graham and Lauder, 2001; Wainwright and Day, 2007). The main movements involved in oropharyngeal expansion are similar among most suction feeders: rapid mouth opening, neurocranial lifting, depression and abduction of the hyoid and suspensoria in most suction-feeding fishes (Muller, 1987, 1989; Aerts, 1991; Aerts and De Vree, 1993), and rapid mouth opening combined with hyoid depression in most suction-feeding tetrapods – from amphibians up to mammals (e.g. Weisgram, 1985a, 1985b; Bramble and Wake, 1985; Lauder and Shaffer, 1985; Deban and Wake, 2000; Lemell et al., 2000, 2002; Marshall et al., 2008; Heiss et al., 2009; Kane and Marshall, 2009). In most vertebrates, the ingested water can then escape through the gill slit(s), while the prey is retained within the oral cavity and further transported to the oesophagus. This unidirectional water flow however is restricted to species with gill slit openings, such as fishes along with most larval and some adult urodeles (e.g. neotenous forms, *Cryptobranchus* sp.). Aquatic-feeding vertebrates without gill slits are incapable of inducing unilateral water flow and the whole amount of water sucked in has to flow out of the mouth again, while the prey item is retained. In a pure suction strike, the prey moves and the predator does not.

(ii) In ram feeding, the predator engulfs the prey by rapid acceleration of the whole body, or at least head and neck. During a pure ram strike the predator moves and the prey does not (Norton and Brainerd, 1993). The main problem in ram feeding is that the forward movement of the feeder pushes the water column anterior to its snout forward as well, creating a “bow wave”, that pushes the prey away. To solve this problem, most underwater feeders compensate the risk of pushing the food item away by highly coordinated expansion of the oropharyngeal (and in fishes opercular) cavities (Van Damme and Aerts, 1997). The mentioned head cavities therefore act as expanding reservoirs that ensure inward flow relative to forward movement. Thus, most feeders that move toward their prey use a combination of “suction feeding” and “ram feeding”. Van Damme and Aerts (1997). This has led to the suggestion to use the term “compensatory suction” when the bow wave is actively compensated and to reserve the term “ram feeding” for feeding events with a nearly unrestrained through-flow. The latter can be achieved in some fishes by keeping both the mouth and opercula wide open and the gill arches expanded while moving forward (Van

Damme and Aerts, 1997). Virtually no inertial and compensatory suction modes are used by moray eels (Metha and Wainright, 2007a, 2007b; Metha, 2009). These anguimorph fishes approach and capture their (usually highly elusive) prey in about 500 ms, what is approximately 10 times slower than in a comparable fish (e.g. *Anguilla rostrata*: Metha and Wainwright, 2007a). These relatively slow approaching and feeding movements, along with morphological specialisations in the jaw- and head, apparently prevent a bow wave per se, and no compensatory suction is needed (Metha and Wainright, 2007a, 2007b; Metha, 2009). Similarly, aquatic-feeding snakes have no morphological features to induce a prey capture mode based on suction (Schwenk, 1994; Van Wassenbergh et al., 2010). Snakes feeding on fish are reported to strike at prey with widely gaping mouth by stretching their curved trunk (Alfaro, 2003; Bilke et al., 2006; Herrel et al., 2008; Van Wassenbergh et al., 2010) or their strike is laterally directed (Herrel et al., 2008). The underwater prey capture mode used by snakes, however, is considered to be suboptimal compared with other aquatic-feeding vertebrates, and they apparently cannot generate any effective suction forces by oropharyngeal expansion (see Van Wassenbergh et al., 2010 for overview).

Beside the above-mentioned exceptions, the generation of at least minimal suction force by fast oropharyngeal expansion seems to be fundamental for almost all aquatic-feeding vertebrates (Lauder and Shaffer, 1985). In sharp contrast to this, due to the low density and viscosity of air, suction can rarely be used on land: the generated drag forces are too small to overcome the combined static inertial and gravitational forces operating on food objects (Bramble and Wake, 1985). Consequently, in terrestrial feeding vertebrates, highly coordinated movements of the hyoid, tongue and head replace the hydrodynamic mechanisms used by (almost all) aquatic vertebrates. Substantially different morphological designs of the feeding apparatus are required because of the different dynamic behaviour of food particles in water versus air (Bramble and Wake, 1985). The perhaps most important morphological adaptations of the feeding apparatus during the transition of aquatic to terrestrial lifestyle in tetrapods include an increase in the amount of oral glandular tissue, the increase of keratinisation on the oral surface, and the reduction in size and mineralisation of the hyoid-apparatus (which is strongly associated with the development of a movable tongue). Terrestrial feeding in vertebrates evolved independently several times. Nonetheless, certain actiopterygians capture prey on land using highly modified mechanisms, such as the mudskipper *Periophthalmus koelreuteri* and the catfish *Channallabes apus* (see Sponder and Lauder, 1981 and Van Wassenbergh et al., 2006 for detailed information). However, the first Devonian tetrapods that developed from sarcopterygian fishes, were probably the first

vertebrates to invade terrestrial environments and were there faced with the new physical conditions (Daeschler et al., 2006). These early amphibians were most likely carnivorous (De Vree and Gans, 1989), but how they ingested their prey remains largely speculative. Recent land-dwelling tetrapods use two main mechanisms to get food into the oral cavity: (i) "lingual prehension" and (ii) "jaw prehension".

(i) During lingual prehension, the tongue is extended from the mouth and returns to the oral cavity with the food item attached to it. The adhesive bond between tongue and food item arises from two physical sources: wet adhesion and interlocking. Wet adhesion occurs when a thin layer of a bonding fluid is placed between two solid surfaces (Bramble and Wake, 1985). The bonding fluid in the oral cavity is mucus. The interlocking effect is mainly based on the roughness of two surfaces, i.e. the texture of the food item and that of the tongue (Bramble and Wake, 1985). To increase the interlocking effect, terrestrial tetrapods that rely on lingual prehension or lingual intraoral transport have a tongue studded with a variety of papillae – in sharp contrast to the usually small and smooth tongues in specialised aquatic tetrapods (Iwasaki, 2002). During lingual transport, the food item is elevated by the tongue and brought backwards by cyclical lingual retractions (see Schwenk, 2000a for overview). The development of a tongue-based mechanism of food ingestion and transport is thought to represent one of the key innovations associated with the evolutionary transition from an aquatic to a terrestrial lifestyle (Herrel et al., 2000).

The use of the tongue as the primary "food uptake organ" on land, however, requires major morphological modifications that can limit the ability to feed underwater (except in some urodels, see Deban and Wake, 2000 and Wake and Deban, 2000 for details). Lingual prehension is characteristic for terrestrial feeding in most frogs, salamanders, tortoises, many lizards, many birds, and many mammals (Schwenk, 2000a).

(ii) Other terrestrial vertebrates use their jaws to grasp food. This is, according to Schwenk (2000a), the most often used prehension mode among vertebrates and is characteristic for caecilians, many turtles, many lizards, all snakes, crocodilians, many birds, and many mammals. During jaw prehension, the actual point of contact with the food item is (usually) the anterior part of the jaws: teeth, beak and lips (in some mammals). If jaw prehension is equivalent to prey capture (i.e. in predatory tetrapods), morphological and behavioural specialisations are widely used to enhance and optimise the ability to catch (fast-) moving prey with the jaws. For example, an elongated neck used for rapid strikes is found in most snakes, some lizards, some turtles, and many birds. Additionally, some piscivorous birds have long and sharply pointed bills that, in combination with the lashing neck, help spear prey. To

transport the food to the oesophagus, terrestrial vertebrates use mainly two mechanisms: lingual and inertial transport, or a combination of both. During inertial transport in many birds, some lizards, crocodiles, and some larger carnivorous mammals, the head is jerked posterodorsally while opening the jaws. At that time, the food item is disengaged from the jaws and carried backwards to the pharynx (or directly to the oesophagus) by its inertia (Cleuren and De Vree, 2000). Additionally, a fast forward movement of the head further enhances that transport mode in some species. Modified inertial transport mechanisms are used by most snakes (Bramble and Wake, 1985; Cundall and Greene, 2000) and some birds (Tomlinson, 2000; Baussart et al., 2009).

Feeding cycles in most vertebrates (aquatic and terrestrial), show similar cyclical features of kinematic events of the jaws and hyolingual complex. Bramble and Wake (1985) therefore suggested that they derived from a common ancestral feeding mechanism utilizing the same (neuro-) motor pattern. Consequently, they proposed a generalised feeding cycle model comprising four phases: slow mouth opening (SO), fast mouth opening (FO) fast mouth closing (FC) and slow mouth closing-power stroke (SC-PS). During SO, the hyolingual complex rests in its initial position; it is rapidly retracted during FO and again protracted in the SC-PS phase. Although this model is supported by many studies, it still finds many critics arguing that feeding mechanisms in vertebrates are too diverse for such a generalisation (see Dullemeijer, 1994; Smith, 1994; Schwenk, 2000; Alfaro and Herrel, 2001; Natchev, 2009 for overview).

1.5. Feeding biology in turtles and the role of the oropharynx

Turtles show a great diversity regarding their ecology – spanning from fully aquatic to fully terrestrial and a full range of intermediate variations. This makes them especially interesting for studying the morphological, kinematical, and behavioural changes that have been taken place during the transition from water to land in the evolution of tetrapods (Schwenk, 2000b). In the last few years, technical improvements have yielded detailed information on the morphology, kinematics, and behaviour of the feeding system in aquatic, semiaquatic, and terrestrial turtles (see Schwenk, 2000a; Bels et al., 2008; Natchev, 2009 for overview).

Analogous to lower aquatic tetrapods, purely aquatic turtles, which rely on hydrodynamic feeding mechanisms, have a large, rigid, and well-ossified hyoid apparatus with massively developed musculature. This helps them create a fast intraoral pressure drop for an efficient water flow into the mouth through the gaping mouth (see also section 1.4). To direct the instreaming water to the very anterior gape, the mouth is often restricted laterally by skin

folds. Additionally, a small tongue with a simple surface topography minimises obstruction to smooth, high-velocity fluid flow within the oropharyngeal cavity (Bramble and Wake, 1985; Weisgram, 1985b; Lauder and Prendergast, 1992; Van Damme and Aerts, 1997; Lemell et al., 2000; 2002, 2010). In suction specialists, a large and movable tongue with rough (i.e. papillous) surface is not needed because the adhesive bond forces (wet adhesion and interlocking, see chapter above) that allow lingual feeding on land are by no means so effective underwater as they are on land. Consequently, aquatic turtles have only simple, single-celled oropharyngeal mucus glands located in the superficial layer of the oropharyngeal epithelium (Fahrenholz, 1937; Kochva, 1978; Winokur, 1988; Weisgram et al., 1989; Iwasaki, 1992; Iwasaki et al., 1996a, 1996b; Beisser et al., 1995, 1998, 2001a; Lemell et al., 2000; Iwasaki, 2002). Abundant mucus as a wet adhesive- or lubricating medium is not needed in purely aquatic turtles. Similarly, oropharyngeal keratinisation is mainly reduced to the upper and lower beak, the rhamphothaecae (see Winokur, 1988 for overview). Nonetheless, though completely aquatic, the marine turtles (families Cheloniidae and Dermochelyidae) show exceptions, probably due to their specialised trophic biology: Even if suction plays an important role in their feeding strategy (Bels and Renous, 1991; Bels et al., 1998), their tongues are relatively large (but with smooth surfaces) and the oropharyngeal mucosa shows large-scale keratinisation and almost no mucous glands (Winokur, 1988; Iwasaki et al., 1996c, 1996d). The well-developed oropharyngeal keratinisation is most likely an adaptation to salt water to maintain homeostasis and avoid dehydration (Iwasaki et al., 1996c, 1996d; Iwasaki, 2002). Another unique characteristic in marine turtles is the occurrence of large and numerous (especially in *Dermochelys coriacea*) keratinous oropharyngeal and oesophageal spines; they apparently provide mechanical protection from dangerous and abrasive food items (Parsons and Cameron, 1976; Meylan, 1988; Winokur, 1988). Non-keratinised oropharyngeal and oesophageal proturberances are also present in some freshwater turtles. Some pleurodire turtles, for example, have blunt, finger-like papillae in their pharynx and oesophagus that are used for food particle trapping in neustophagy (Belkin & Gans, 1968; Parsons & Cameron, 1976; Vogt et al., 1998). By contrast, tall, slender and branched oropharyngeal and oesophageal papillae in soft-shelled turtles have no function in feeding, but play a central role in aquatic respiration (Gage and Gage, 1886; Dunson, 1960; Girgis, 1961; Wang et al., 1989; Yokosuka et al., 2000).

Many turtles, even if actually aquatic, seek the banks of their home waters and are also found far away from any water (Pritchard, 1979; Ernst and Barbour, 1989; Bonin et al., 2006). Some are also known to search for food on land and to grasp it by the jaws. To swallow the food

item, however, they need to submerge their head underwater. Otherwise they are unable to apply the hydrodynamic transport mechanisms they rely on (see Pritchard, 1979; Bramble and Wake, 1985; Weisgram, 1985b; Ernst and Barbour, 1989; Bonin et al., 2006; Natchev, 2009). Other turtles live a truly amphibious lifestyle. The true amphibious (or "semiaquatic"; these two terms are used here as synonyms) turtles are well adapted to both media and are found within the families Emydidae and Geoemydidae. These species are usually omnivorous and can complete the whole feeding process including ingestion (initial food apprehension), processing, transport, and swallowing underwater as well as on land (see Bels et al., 1997; Summers et al., 1998; Natchev, 2009 for overview). Due to their ecological flexibility, amphibious turtles are not restricted to strict ecological niches but can shift between environments. As functional-morphological adaptations to optimised aquatic feeding often limit the food uptake potential on land (and vice-versa), their morphology represents a compromise between both extremes.

Today, truly terrestrial turtles are found only in one family, the Testudinidae or tortoises. All testudinids are reported to have a terrestrial lifestyle and to exclusively rely on terrestrial feeding mechanisms (Weisgram, 1985b; Wochesländer et al., 1999, 2000; Bonin, 2006; Bels et al., 2008). While aquatic and semiaquatic turtles use jaw prehension to grasp food items, testudinids only use their tongue for food uptake and transport (Bramble and Wake, 1985; Weisgram, 1985b; Wochesländer et al., 1999; Bels et al., 2008). Consequently, their tongues are large, muscular and movable and have a rough surface topography (Weisgram, 1985b; Wochesländer, 1999; Beisser and Weisgram, 2001). The lingual surface is studded with numerous tall and slender lingual papillae that allow an efficient interlocking effect between food item and tongue (see also previous section). Additionally, mucus as a bonding fluid (wet adhesion) between both surfaces (food and tongue) is abundant and produced by large and numerous oropharyngeal glands (Fahrenholz, 1937; Luppa, 1977; Kochva, 1978; Weisgram, 1985b; Winokur, 1988; Weisgram et al., 1989; Wochesländer et al., 1999; Beisser et al., 2004). Mucus also plays an important role in postlingual food transport (i.e. swallowing) as a gliding medium and to lubricate the oropharyngeal cavity to avoid dehydration. Another typical characteristic of the testudinids is the large-scale keratinisation of the oral cavity, probably an additional adaptation to terrestrial food uptake (Iwasaki, 2002). The large, rigid, and well-ossified hyoid apparatus associated with massively developed musculature typical in aquatic turtles is strongly reduced and modified in terrestrial forms. In testudinids the hyoid skeleton is relatively small, mainly cartilaginous, and more flexible than in aquatic turtles. It is adapted to the flexibility of the tongue and also serves as its support (Wochesländer et al.,

1999). Although testudinids definitely developed from an aquatic ancestor (Joyce and Gauthier, 2004), the morphological changes in the feeding apparatus in recent tortoises largely impede aquatic food uptake.

1.6. Aims of the thesis & working hypotheses

As mentioned above, studies on aquatic and terrestrial turtles show a correlation between phylogeny, feeding mode and oropharyngeal morphology (Winokur, 1973; Weisgram et al., 1989; Iwasaki et al., 1996a, 1996b; Lemell et al., 2000, 2002; Beisser et al., 2004; Richter et al., 2007; Natchev, 2009). Accordingly, the oropharyngeal anatomy both enables and constrains the food uptake potential. While most studies on turtle feeding kinematics and oropharyngeal morphology have focused on aquatic species (Nalavade and Varute, 1976; Bramble, 1978; Weisgram, 1985a, 1985b; Bels and Renous, 1991; Lauder and Prendergast, 1992; Iwasaki, 1992; Iwasaki et al., 1992, 1996a, 1996c, 1996d; Beisser et al., 1995, 1998; 2001; Lemell and Weisgram, 1997; Van Damme and Aerts, 1997; Bels et al., 1998; Lemell et al., 2000, 2002, 2010; Aerts et al., 2001), limited information is available for semiaquatic (Iwasaki, 1996b; Bels et al., 1997; Summers, 1998; Beisser et al., 2004) and purely terrestrial turtles (e.g. Fahrenholz, 1937; Kochva, 1978; Weisgram, 1985b; Wochesländer, 1999, 2000; Bels et al., 2008). Information on the morphology of the oropharyngeal structures and the kinematics that enable terrestrial food uptake, transport and swallowing is still scarce. This thesis provides a detailed investigation on the form and function of the feeding apparatus in five turtle species: one aquatic kinosternid that spends much time on land (*Sternotherus odoratus*), three truly semiaquatic geoemydids (*Cuora amboinensis*, *C. galbinifrons* and *C. flavomarginata*), and a member of the most primitive recent testudinids (*Manouria emys emys*) that (still) shows a high affinity to water (ecological background according to Pritchard, 1979; Obst, 1986; Ernst and Barbour, 1989; Bonin, 2006). These species were chosen to cover the transition from water to land with one aquatic species (frequently spending time on land), three members of the genus *Cuora* (that show different habitat- and trophic preferences) and a testudinid that could still bear characteristics typical for aquatic feeders. Detailed micro- and macro-anatomical data of the feeding apparatuses are provided and discussed in the functional and evolutionary context. Microanatomy (light microscopy and scanning electron microscopy) provides information on the general design of the oral mucosa, regional specialisations and interspecific variations. Similarly, differences are expected in the design of the skulls and lower jaws with associated muscles, as well as of the hyoid

apparatuses in terms of ecological shifts (according to section 1.4). A small tongue with relatively smooth surface, simple single-celled mucus glands and scarce keratinisaton are expected in the aquatic turtle (*S. odoratus*). On the other hand, this species is also known to capture food on land. Therefore, tongue, oral glands and keratinisation could also be better developed – as a primordial adaptation to a (partly) terrestrial lifestyle. A higher evolved situation (compared with *S. odoratus*) is expected for the semiaquatic *Cuora* species. In contrast, *Manouria emys* probably shows a large tongue with tall and muscular papillae, large and complex glands, and large-scale keratinisation, as known for other testudinids (Winokur, 1988). However, *Manouria* is the most primitive recent member of the only exclusive terrestrial turtle family, and terrestrial forms evolved from aquatic ones. Therefore, some primitive features could be retained in the oropharyngeal design of that species, reflecting the aquatic to semiaquatic ancestor of testudinids.

In addition to morphological data, kinematical analyses will shed new light on turtle feeding strategies. Some theoretical concepts on the evolution of feeding behaviour were suggested in the past, although only a very small percentage of gnathostomous species had been studied (Herrel et al., 2001). One of the lively discussed concepts is the generalised feeding cycle model proposed by Bramble and Wake (1985) (see also section 1.4). These authors predicted that the neuro-motor control of the (transport) feeding cycles are conserved in the evolution of lower tetrapods. This theory found supporters as well as opponents and everything in between (see Lauder and Shaffer, 1988; Schwenk and Throckmorton, 1989; Reilly and Lauder, 1990; Dullemeijer, 1994; Smith, 1994; Bels and Goosse, 1990; Lauder and Prendergast, 1992; Bels et al., 1997; Schwenk, 2000a; Alfaro and Herrel, 2001; Bels et al., 2008; Natchev, 2009 for overview). This generalised cyclic model, however, could not always be supported for turtles (Lemell and Weisgram, 1997; Natchev, 2009) because transport modes in turtles seem to be very variable, reflecting ecomorphological diversity. In the framework of this thesis, this theory will be tested for aquatic and terrestrial feeding turtles. Another important functional factor with impact on feeding behaviour is the feedback-response. Sauropsids (reptiles and birds), for example, show a considerable variation in food uptake between different food types within one individual. Ingestion and the transport strategy are apparently influenced by mobility, texture, size, toughness and taste of the food item (Berkhoudt, 1977; 1985; Zweers et al., 1977; Dullemeijer, 1994; Herrel et al., 1999; Vincent et al., 2006; Schaerlaeken et al., 2007; Montuelle et al., 2009). As a fast detection of the chemical composition of food may be crucial to avoid harmful items, this thesis will test for potential correlations between the distribution of taste organs (i.e. taste buds) and feeding behaviours in turtles.

In conclusion, an attempt is made to provide a theoretical, step-by-step model explaining the morphological and behavioural patterns that have taken place during the transition from water to land in turtles – based on the evolution of terrestrial feeding in this unique clade.

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2. Submitted articles

2.1. The fish in the turtle: On the functionality of the oropharynx in the common musk turtle *Sternotherus odoratus* (Chelonia, Kinosternidae) concerning feeding and underwater respiration.

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Abstract

In tetrapods, the oropharyngeal cavity and its anatomical structures are mainly, but not exclusively, responsible for the uptake and intraoral transport of food. In this study we provide structural evidence for a second function of the oropharynx in the North American common musk turtle, *Sternotherus odoratus*, Kinosternidae: aquatic gas exchange.

Using high-speed video, we demonstrate that *S. odoratus* can grasp food on land by its jaws, but is afterwards incapable of lingual based intraoral transport; food is always lost during such an attempt. Scanning electron microscopy and light microscopy reveal that the reason for this is a poorly developed tongue. Although small, the tongue bears a variety of lobe-like papillae, which might be misinterpreted as an adaptation for terrestrial food uptake. Similar papillae also cover most of the oropharynx. They are highly vascularized as shown by light microscopy and may play an important role in aquatic gas exchange. The vascularisation of the oropharyngeal papillae in *S. odoratus* is then compared with that in *Emys orbicularis*, an aquatic emydid with similar ecology but lacking the ability of underwater respiration.

Oropharyngeal papillae responsible for aquatic respiration are also found in soft-shelled turtles (Trionychidae), the putative sister group of the kinosternids. This trait could therefore represent a shared, ancestral character of both groups involving advantages in the aquatic environment they inhabit.

Introduction

Morphological investigations on the oropharyngeal mucosa in chelonians have demonstrated the correlation between the design of the oropharyngeal cavity and the feeding mode (Beisser et al., 1995; 1998; 2001; 2004; Iwasaki, 2002; Heiss et al., 2008, Natchev et al., 2009). Tortoises have fleshy tongues with numerous tall and slender lingual papillae (Winokur, 1988; Wochesländer et al., 1999; 2000; Beisser et al., 2004). This form of the dorsal tongue surface promotes the interlocking effect in lingual food prehension and food transport (see Natchev et al., 2009 for overview). Aquatic turtles ingest and transport food using hydrodynamic feeding mechanisms, in which the tongue plays a subordinate role (Bramble and Wake, 1985) and the lingual papillae are moderately sized (Beisser et al., 2001) or completely absent (Beisser et al., 1995; Lemell et al., 2002, 2010).

Kinosternids are reported to be exclusively aquatic feeders (Ernst and Barbour, 1989; Rogner, 1996; Schilde, 2004), although they occasionally emerge on land. The kinosternid *Sternotherus odoratus* almost permanently lives in the water as an adult, but juveniles spend time on land, among other things searching for food. Therefore, we predict that, at least, juveniles of the musk turtle may be able to feed on land. The ability to feed on land is highly contingent on oropharyngeal morphological adaptations (see Schwenk, 2000; Heiss et al., 2008 and Natchev et al., 2009). However, as no detailed information is available on the morphology of the oropharyngeal mucosa in kinosternids we explore whether and if the mucosal structure observed in kinosternids is related to feeding behavior.

The oropharyngeal morphology not only influences the feeding mode (i.e. aquatic versus terrestrial feeding), but also impacts other ecological potential, such as oropharyngeal gas exchange. Another goal of this study is therefore to search for oropharyngeal organs that are potentially responsible for aquatic gas exchange by using scanning electron microscopy and histological methods. The capability of *S. odoratus* to remain submerged for prolonged periods has been the object of many physiological studies (see Saunders et al., 2000 for overview). According to Root (1949), Pritchard (1979) and Stone et al. (1992), common musk turtles mainly use their papillous skin for oxygen uptake while submerged. Additionally, Root (1949) suggested that even if oropharyngeal gas exchange in *S. odoratus* happens, it may only contribute insignificantly to the ability to remain submerged. Bagatto et al. (1997), however, demonstrated that the cutaneous surface area may not be the main factor for aquatic respiration in kinosternids and that other factors must be sought. According to this, Belkin (1968) predicted, based on behavioral and physiological studies, that oropharyngeal ventilation is a respiratory response to prolonged submersion in *Sternotherus minor*. A highly

vascularized oropharyngeal mucosa is known to be the key organ responsible for aquatic gas exchange in the sistergroup of kinosternids (according to Gaffney and Meylan, 1988): the soft-shelled turtles, or Trionychidae (Gage and Gage, 1886; Dunson, 1960; Girgis, 1961; Wang et al., 1989; Yokosuka et al., 2000). We expect that a similar organ enables gas exchange in submerged *S. odoratus*. The main aim of the present study is therefore to examine whether or not the oropharyngeal specializations of *S. odoratus* exhibit a dual functionality combining both feeding on land and under water and aquatic gas exchange by providing new morphological data on this species.

Material and Methods

Sternotherus odoratus (Latreille, 1802), the stinkpot or common musk turtle, is a small-sized but abundant species ranging from southern Canada to the eastern half of the USA (Bonin et al., 2006). This species inhabits a wide range of aquatic habitats: rivers, lakes, swamps, cattle tanks, canals – and even fast-flowing creeks with rocky bottoms (Pritchard, 1979; Rogner, 1996; Schilde, 2004; Bonin et al., 2006). *Sternotherus odoratus* are reported to be omnivorous with a strong tendency to carnivory, feeding in the wild on various plants, worms, molluscs, crayfish, insects, tadpoles, fishes and their eggs; they can also take bites of flesh from dead animals (Ernst and Barbour, 1989; Schilde, 2004).

For the present study, five juveniles of unknown sex, and four subadult and three adult female *S. odoratus* ranging in size (straight carapace length) from 25.6 mm to 37.2 mm in juveniles, 61.5 mm to 69.3 mm in subadults and 93.4 mm to 114 mm in adults were used. The classification in the three groups was based on age differences at the time studies were performed. Juveniles ranged between two and six months, subadults between one and two years, and adults were older than three years. The turtles were obtained commercially and kept in a 360 liter tank (40 x 150 x 60 cm) with 20 % land and 80 % water, and a 12 h dark/12 h light cycle. The animals were fed with earthworms, fish pieces and turtle-food pellets from the pet trade. Animal care and treatment was in accordance with the Austrian national Protection of Animals Act (TSchG 2004**)**.

For filming terrestrial feeding in all twelve individuals, food items were offered in front of the animals on the bottom of a glass aquarium measuring 19 x 7 x 19 cm without water. To film animals, all turtles were fed with small fish pieces, which were apparently their preferred food measuring approximately 4 x 4 x 6 mm. They were filmed in lateral view with the digital high-speed camera Photron Fastcam-X 1024 PCI (Photron limited; Tokyo, JP) at 250 fr/s, with a reference grid in 1 x 1 cm as a background.

For morphological investigations, five juvenile and two subadult animals were anesthetized by intraperitoneal injection of sodium pentobarbital and, after deep narcosis, decapitated. The heads were immersed immediately in fixation solution. For scanning electron microscopy (SEM), two heads of juvenile turtles were immersed for 24 h at room temperature in modified Karnovsky solution consisting of 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer (Karnovsky, 1965). After rinsing in 0.1 M cacodylate buffer, the lower jaw with all the ventral oropharyngeal structures was removed from the head in order to get better views from both ventral and dorsal surfaces of the oropharyngeal cavity. Then, samples were

postfixed in buffered, 1% osmium tetroxide for 2 h at 37 °C, washed in distilled water, and treated with 25% HCl at 40 °C for 15 min to remove the mucus from the surface. After repeated washing in distilled water, the samples were dehydrated in a graded ethanol and acetone series and dried in a critical point drying machine (Polaron: Watford, UK). The dried samples were then coated with gold in an AGAR B7340 Sputtercoater (Agar Scientific Ltd, Stansted, UK) and observed in a Philips XL-20 scanning electron microscope (Philips, Eindhoven, NL).

For paraffin-based histology, two juvenile and two subadult turtles were used. The heads and two biopsies of the dorsal and ventral neck were immersed in Bouin's fixative (Romeis, 1989) for 30 days, changing the solution twice a week. After complete fixation and decalcification, the upper jaw was removed from the rest of the head and the cornified rhamphothecae were cut off. Then, the samples were dehydrated in a graded ethanol-isopropanol series and embedded in paraffin. After polymerization, 7-µm-thin serial-sections were made on a Reichert-Jung 2030 rotary microtome (Reichert-Jung, Bensheim, GER). The sections were mounted on glass slides and, after removing the paraffin, stained with Haematoxylin – Eosin (H-E), periodic acid Schiff (PAS) - Haematoxylin and Alcian blue (AB) - Haematoxylin (after Romeis, 1989; Kiernan, 2003). The preparations were documented by digital photography under a Nikon Eclipse 800 light microscope (Nikon, Tokyo, JP).

For semi-thin sectioning, one head of a juvenile turtle was fixed in the above- (for SEM) described modified Karnovsky solution for 48 h, washed three times in 0.1 M cacodylate buffer, postfixed for 2 h at room temperature in buffered 1% osmium tetroxide, and decalcified in EDTA (ethylenediaminetetraacetic acid) for 30 days. Afterwards, the lower jaw was removed from the rest of the head and the rhamphothecae were cut off. This procedure was followed by dehydration in a graded ethanol and acetone series and embedding in Agar 100 Resin (Agar Scientific, Stansted, UK). After polymerization at 65 °C for 15 h, semi-thin (1 µm) sections were made on a Reichert Ultracut S microtome (Leica Microsystem, Wetzlar, GER) using histo diamond knives (Diatome AG, Biel, CH). The sections were mounted on glass slides, stained with Toluidine blue (TB) and documented as described above for histological sections.

For morpho-functional comparision, sections of oropharyngeal papillae of the European pond turtle, *Emys orbicularis,* were kindly provided by Mr. Stefan Kummer, University of Vienna. *Emys orbicularis* is highly aquatic and inhabits similar environments as *S. odoratus* – but in Europe. The tissue preparation, staining and digital imaging were the same as described above for the paraffin-based histology of *S. odoratus*.

Results

Feeding behavior

In all cases where food items were offered on the land part of the aquarium, the prey was captured by juvenile or subadult individuals and brought immediately to water for further transport, manipulation and swallowing. Adults showed no interest in the food items presented on land. Behavioral observations, documented videographically, showed that *S. odoratus* employed hydrodynamic mechanisms to feed underwater (data not shown). Prey capture on land involved jaw prehension (Fig. 1). When access to water was hindered, none of the tested animals was able to transport the food through the oropharyngeal cavity, despite repeated efforts (Fig. 1).

Morphology

Scanning electron microscopy (SEM) of juvenile *S. odoratus* revealed the blunt, massive and highly keratinized rhamphothecae (Fig. 2A). The surface of the palatal mucosa was relatively flat and smooth, in contrast to the ventral side of the oral cavity, which showed a multiplicity of structures. The posterior part of the ventral rhamphotheca passed into the almost unkeratinized and triangular floor of the mouth (Fig. 2A). The floor of the mouth itself was hidden posteriorly by the tongue. The tongue of *S. odoratus* was small with a flannel-like appearance and was covered with relatively large, flattened, lobe-like lingual papillae (Fig. 2A, B). Posteriorly adjacent to the tongue lay a narrow and small groove, the glottis. The glottis itself was surrounded by oropharyngeal fold-like papillae, which closely resembled the lingual papillae (Fig. 2A, B, C). Posterior to the glottis, the fold-like papillae increased in number and length, often overlapped, resembling a blunt, rocky landscape (Fig. 2C). In this region, similar structures were also present in the dorsal part of the oropharyngeal cavity. The pharyngeal papillae were oriented longitudinally relative to body axis (Fig. 2A, C).

Histological sections indicated that the hypoglossum and the hyobranchial apparatus were cartilaginous, and the intrinsic musculature of the tongue was poorly developed (Fig. 3A, B, C). The lingual papillae, which are extensions of the lingual mucosa, were broad in transverse sections (Fig. 3A) on the anterior part of the tongue, becoming more slender posteriorly (Fig. 3B, C; 4A). These slender and lobe-like papillae sometimes covered the glottal slot (Fig. 3B). On the root of the tongue and behind the tongue, the papillae became more numerous and elongated. The highest density occured posterior to the glottis, in the pharyngeal cavity. These papillae were relatively short and simple in juvenile turtles (Fig. 3C) but tall and branched in subadults (Fig. 4A). Higher magnification showed the high degree of vascularization of the

oral mucosa in *S. odoratus* (Fig. 4B, C). In the deeper lamina propria, large blood vessels ran parallel to the surface and gave rise to vessels to the superficial layer, where they formed an extensive capillary network (Fig. 4B, C). These capillary vessels ran immediately subjacent to the basement membrane and were most dense in the pharyngeal papillae (Fig. 4C).

The oropharyngeal mucosa consisted mostly – if not completely – of a non-keratinized stratified cuboidal to columnar epithelium and an underlying connective tissue containing loosely (superiorly) to densely (in deeper regions) packed collagen fibers (Fig. 3A). Keratinization occured exclusively on the dorsal and ventral interfaces with the rhamphothecae. The oropharyngeal epithelium consisted of 2 to 5 cell layers and the appearance of the cells varied according to their function. While the oral epithelium of the palate, floor and tongue contained many columnar cells with mucus, these were scattered in the pharyngeal epithelium, where cuboidal cells were prevalent. The thickness of the oropharyngeal epithelial layer varied between 10 and 35 µm. No multicellular glands were found.

Compared with the oropharyngeal mucosa, the superficial layer of the dermis of the outer skin taken from the neck region contained fewer blood vessels and a well developed capillary network was absent (compare Fig. 4C and Fig. 5A), although larger veins and arteries were present in the deeper dermis. The epithelium of the outer skin consisted of 2-3 basal cell layers plus at least 2-4 flattened superficial keratinocytes (Fig. 5A), which were eosinophilic (Fig. 5A). The whole width of the epithelium of the outer skin varied between 20 and 50 µm.

The oropharyngeal papillae in the European pond turtle *E. orbicularis*, compared with those of *S. odoratus*, were flat and rare, and capillaries were scarce (Fig. 5B).

Discussion

The common musk turtle, *S. odoratus*, is highly aquatic, although occasionally found on banks of its home waters (Pritchard, 1979; Ernst and Barbour, 1989; Rogner, 1996; Schilde, 2004). Our observations of feeding behavior are consistent with this tendency to an amphibious lifestyle. When food was offered in the water, all of juvenile, subadult, and adult individuals immediately grabbed and swallowed it. Prey capture and transport occurred, as in certain other aquatic cryptodirans, via hydrodynamic mechanisms (see Bramble and Wake, 1985; Lauder and Prendergast, 1992; Bels et al., 1997; Summers et al., 1998; Aerts et al., 2001; Natchev et al., 2009). As juvenile and subadult animals sometimes climbed out onto the land part of the aquarium, we tested the hypothesis that they may also be able to feed on land in contrast to adult individuals that rarely left the water. Food items offered on land were immediately grasped by juveniles and brought to the water. Subadults sometimes showed a similar behavior, but adults never did. When access to water was hindered, the juvenile and subadult individuals grasped the prey successfully but failed in all cases to transport it toward the esophagus. All those turtles studied so far that feed exclusively (terrestrial) or occasionally (semiaquatic) on land use their tongue for terrestrial food transport (Weisgram et al., 1989; Wochesländer et al., 1999, 2000; Natchev et al., 2009); their tongues are fleshy and papillated with abundant mucous glands (Nalavade and Varute, 1976; Iwasaki, 1992; Iwasaki et al., 1992, 1996; Beisser et al., 2004). In contrast, exclusive aquatic feeders have a small and smooth tongue with sparse glandular tissue (Bramble and Wake, 1985; Winokur, 1988; Iwasaki, 1992; Iwasaki et al., 1992, 1996; Weisgram et al., 1989; Beisser et al., 1995, 1998, 2001; Lemell et al., 2000, 2002, 2010).

Interestingly, the common musk turtle does not fit into that dichotomy, as the morphological investigations revealed a weak and small tongue (typical for aquatic feeders) but with numerous papillae (expected for terrestrial feeders). All animals tested lost the food item when fed on land presumably because they were unable to fix the food to the palate with their tiny tongues during the first attempted transport cycle. Therefore, the presence of lingual papillae in *S. odoratus* cannot be explained as an adaptation for occasional terrestrial feeding. Furthermore, their orientation would not promote the interlocking effect between tongue and food. Finally, longer branched fold-like papillae are present posterior to the lingual root, around the glottis and throughout the pharyngeal cavity. Pharyngeal papillae in reptiles are known to have at least four different functions: First, non-keratinized mucus secreting oral, pharyngeal and esophageal papillae are used for protection from dangerous prey in horned lizards (*Phrynosoma*) (Sherbrooke & Schwenk, 2008). Second, keratinized pharyngeal and

esophageal papillae are found in some marine turtles and are believed to provide mechanical protection from dangerous or abrasive food items (Parsons & Cameron, 1976; Meylan, 1988; Winokur, 1988). Third, non-keratinized pharyngeal and esophageal papillae are used for food particle trapping in neustophagy found in some pleurodire turtles (Belkin & Gans, 1968; Parsons & Cameron, 1976; Vogt et al., 1998). Fourth, non-keratinized oropharyngeal papillae are described for some soft-shelled turtles (Trionychidae) and were shown to function in underwater gas exchange (e.g. Gage and Gage, 1886; Dunson, 1960; Girgis, 1961; Wang et al., 1989; Yokosuka et al., 2000). While the first three possible functions of pharyngeal papillae cannot be true for *S. odoratus* due to ecological or structural reasons, the latter function found in trionychids seems more likely.

The Trionychidae are the putative sistergroup (according to Gaffney and Meylan, 1988) of the Kinosternidae. Trionychids practice gas exchange underwater through pharynx and skin while hibernating and diving (see Gage and Gage, 1886; Dunson, 1960; Girgis, 1961; Wang et al., 1989; Yokosuka et al., 2000). Interestingly, physiological investigations revealed that *S. odoratus* oxygenates its blood under water like soft-shelled turtles do. The common musk turtles can remain underwater at 10 °C for more than 100 days (Jackson et al., 1984; Ultsch et al., 1984) and at 3 °C for at least 150 days (Ultsch, 1985, 1988; Ultsch and Wasser, 1990; Ultsch and Cochran, 1994; Ultsch and Jackson, 1995) without discernible ill effects. While submerged, the turtles remain aerobic as evidenced by the relatively small increases in plasma lactate (Ultsch and Cochran, 1994; Ultsch and Jackson, 1995). Three organs are predicted to be involved in aquatic gas exchange in chelonians, namely the cloacal bursae, the skin and the oropharyngeal mucosa. Cloacal gas exchange has been demonstrated in some pleurodiran turtles (see King and Heatwole, 1994; Gordos and Franklin, 2002; Clark et al., 2008). All kinosternids, however, lack cloacal bursae (Dunson, 1960; Peterson and Greenshields, 2001) and their skin is thick, strongly keratinized (especially plastron and carapace) and lacks an extensive capillary network (see Fig. 5A). This excludes those two potential modes of gas exchange for *S. odoratus*. In contrast to the latter, the surface-amplifying oropharyngeal papillae are highly vascularized. Histologically, those structures are very similar to the viliform oropharyngeal papillae described for the soft-shelled turtle *Trionyx sinensis japonicus*, in which the papillae definitely play a central role in gas exchange underwater (Yokosuka et al., 2000). The oropharyngeal papillae of *T. sinensis japonicus* are slender, tall and branched. In contrast to this, the papillae of *S. odoratus* are lobe-like folds and oriented longitudinally relative to the body axis. A moderate to extensive capillarization, coupled with cutaneous surface amplification, is a strong indicator for cutaneous respiration in vertebrates

(according to Feder and Burggren, 1985). Within tetrapods, cutaneous gas exchange contributes significantly to tissue respiration in almost all amphibians, some reptiles and certain mammals. In *S. odoratus* the density of capillaries just beneath the thin oropharyngeal epithelium is comparable (if not even higher) to that of lungless salamanders (Plethodontidae), which exclusively rely on cutaneous respiration (see Feder and Burggren, 1985). Lungless salamanders can cover their demand for gas exchange throughout life by this way. Oropharyngeal respiration in trionychids and kinosternids can ensure survival while diving at a decreased activity level (Dunson, 1960) or during hibernation (Ultsch and Jackson, 1995; Yokosuka et al., 2000). At high metabolic rates, these animals can no longer cover their oxygen demand in this manner and die if prevented from reaching the water surface to breathe (Dunson, 1960).

The high degree of vascularization and capillarization in the oropharyngeal papillae of *S. odoratus* becomes more apparent if compared with the oropharyngeal mucosa in *E. orbicularis* belonging to Emydidae. The ecology and feeding behavior of this aquatic European turtle are similar to those of the common musk turtle. *Emys orbicularis* has a prolonged hibernation, but it has a far lower capacity for lengthy submergence than *S. odoratus*. *E. orbicularis* must periodically seek the water surface to breath during hibernation (Bonin et al., 2006) and *S. odoratus* need not (Ultsch and Cochran, 1994; Ultsch and Jackson, 1995). The oropharyngeal surface in *E. orbicularis* is flat with few and small papillae that contain some blood vessels but lack a well-developed capillary network. Such a design limits the potential for gas exchange through the oropharyngeal mucosa.

We assume that the oropharyngeal papillae in *S. odoratus* are morpho-functional adaptations for gas exchange underwater. Their design should not significantly affect the potential of this species to suction feed. The oropharynx in this turtle, therefore, exhibits a dual functionality concerning feeding and breathing underwater. Future studies will examine the feeding behavior and oropharyngeal structures of other kinosternids to determine whether the dual functionality of the oropharynx found in *S. odoratus* is shared with other members of the family. Until we document the distribution of this trait within the Kinosternidae, we cannot determine whether the presence of respiratory oropharyngeal papillae and -folds is an ancestral character shared with the Trionychidae, or evolved independently in both groups.

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Figures

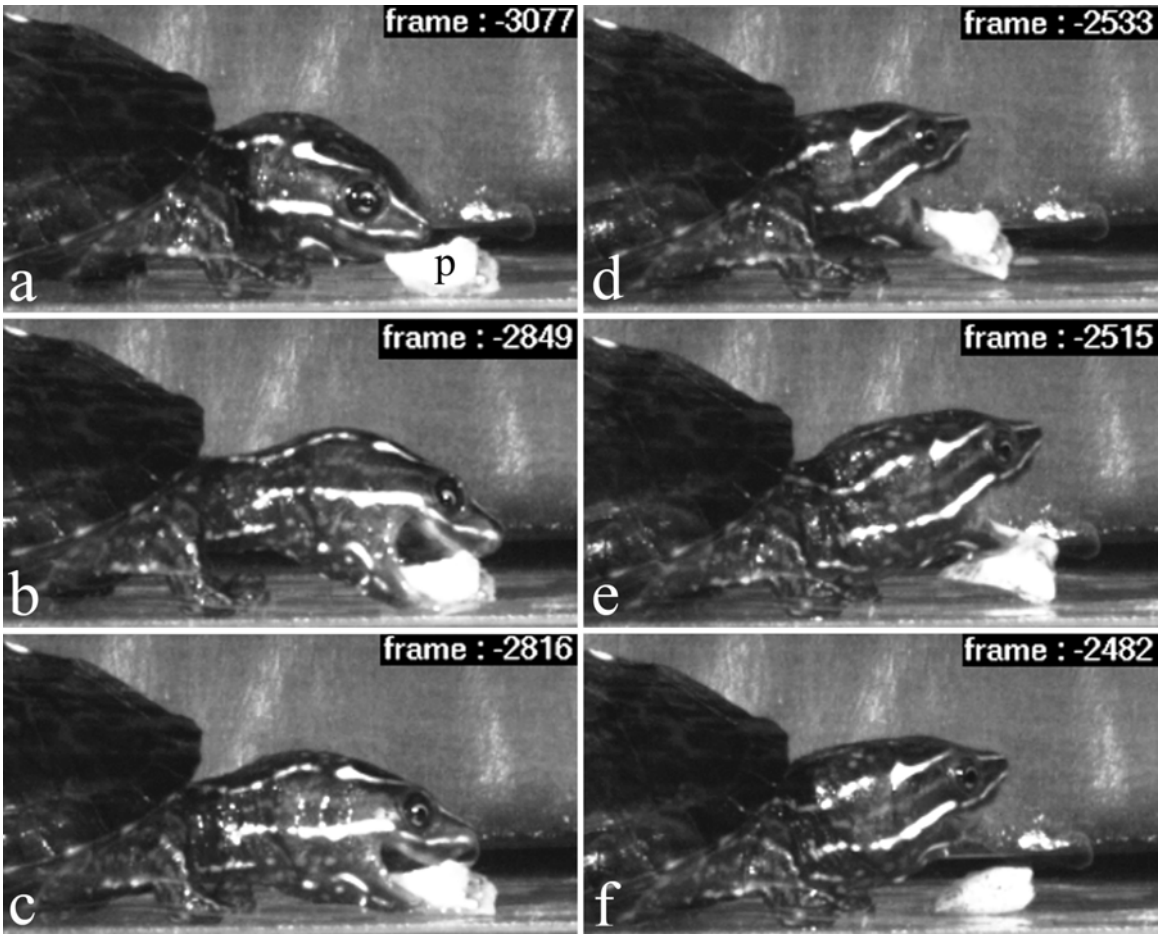

Fig. 1. Selected video frames showing a juvenile *S. odoratus* attempting to feed on land (recorded at 250 fr/s). The animal approaches (a) the prey item (P) grabs it with the jaws (b, c) but fails to transport it through the oral cavity (d-f).

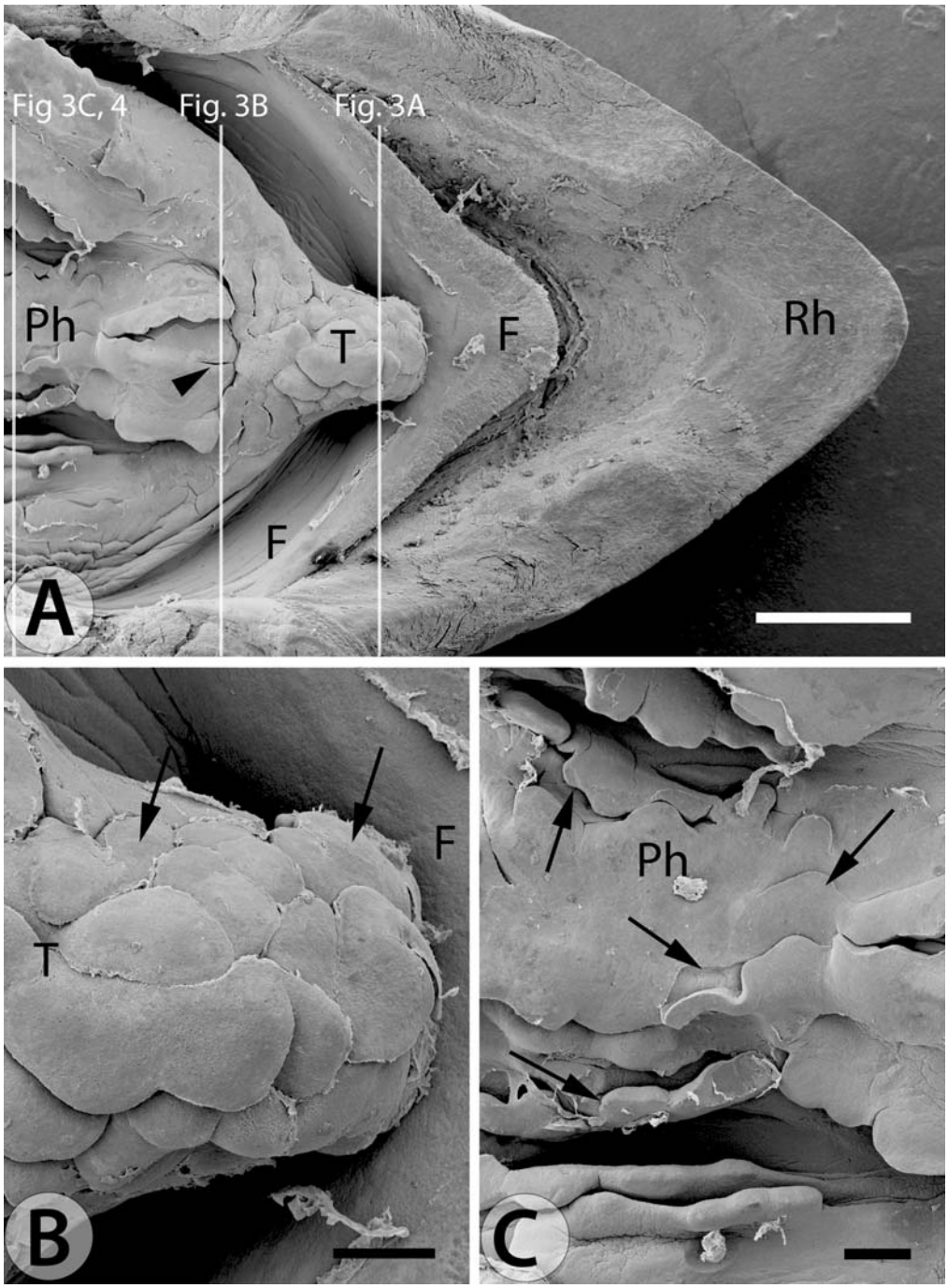

Fig. 2. Scanning electron micrographs showing the ventral surface of the mouth of a juvenile *S. odoratus*. A Overview, showing the massively keratinized rhamphotheca (Rh), which presents the ventral part of the "beak", the lightly keratinized floor of the mouth (F), the small tongue (T), the glottis (indicated by arrowhead) and the pharynx (Ph). Note that the tongue (details shown in B) and the pharynx (details shown in C) are studded with flattened, floppy papillae (arrows). The white, vertical lines in micrograph A indicate where the histological sections (see Fig. 3 and Fig. 4) were taken. Scale bars: A 1 mm; B and C 200 µm.

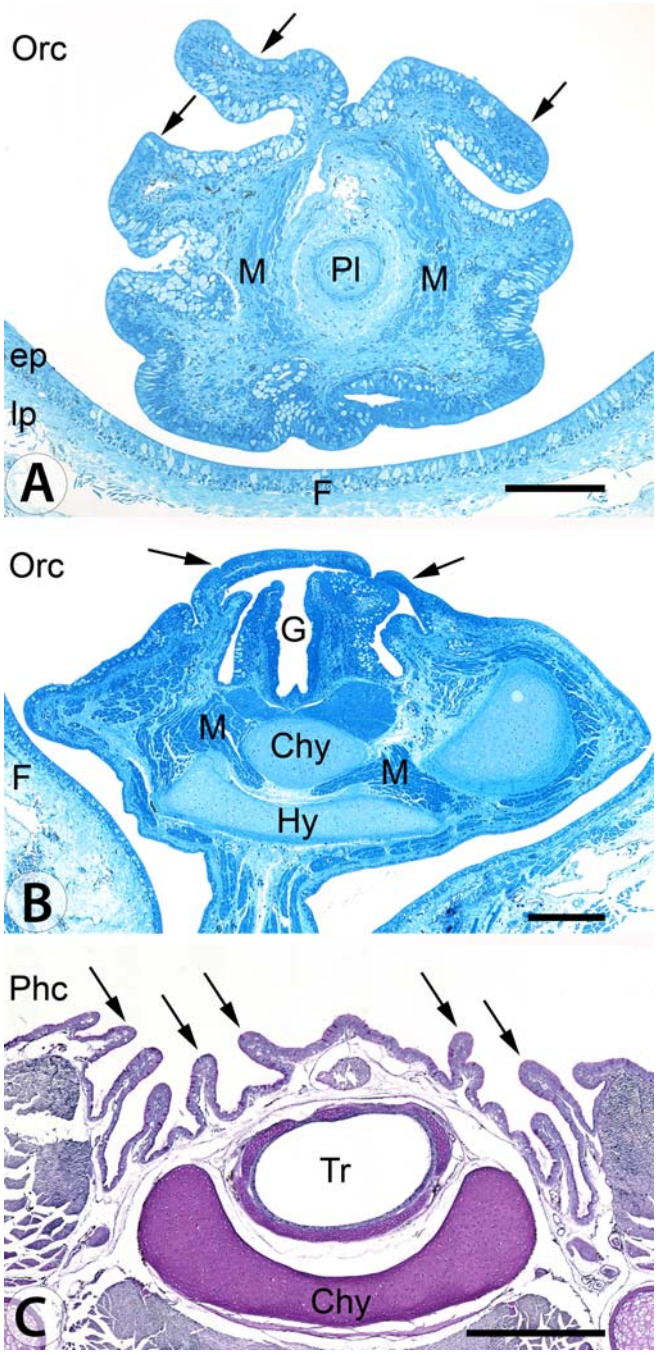

Fig. 3. Light micrographs of cross sections of the ventral oral cavity of a juvenile *S. odoratus*. For a better orientation, the white, vertical lines in the scanning electron micrograph of Fig. 2A indicate where the sections were taken. A Anterior section showing the tongue with some flannel-like papillae (arrows) and the floor of the mouth (F). Note the thin epithelium (ep) and the scarcely developed intrinsic musculature (M). B Slender floppy papillae (arrows) can cover the glottal slot (G). The hyolingual skeleton is well developed and cartilaginous. C Floppy papillae are also abundant posterior to the glottis: in the pharynx. Chy, Corpus hyoidei; Hy, hypoglossum; lp, lamina propria; Orc, oral cavity; Phc, pharyngeal cavity; Pl, processus lingualis; Tr, trachea. Scale bars: A and B 200 µm; C 500 µm. A and B semithin sections stained with Toluidine blue; C paraffin section stained with PAS-H.

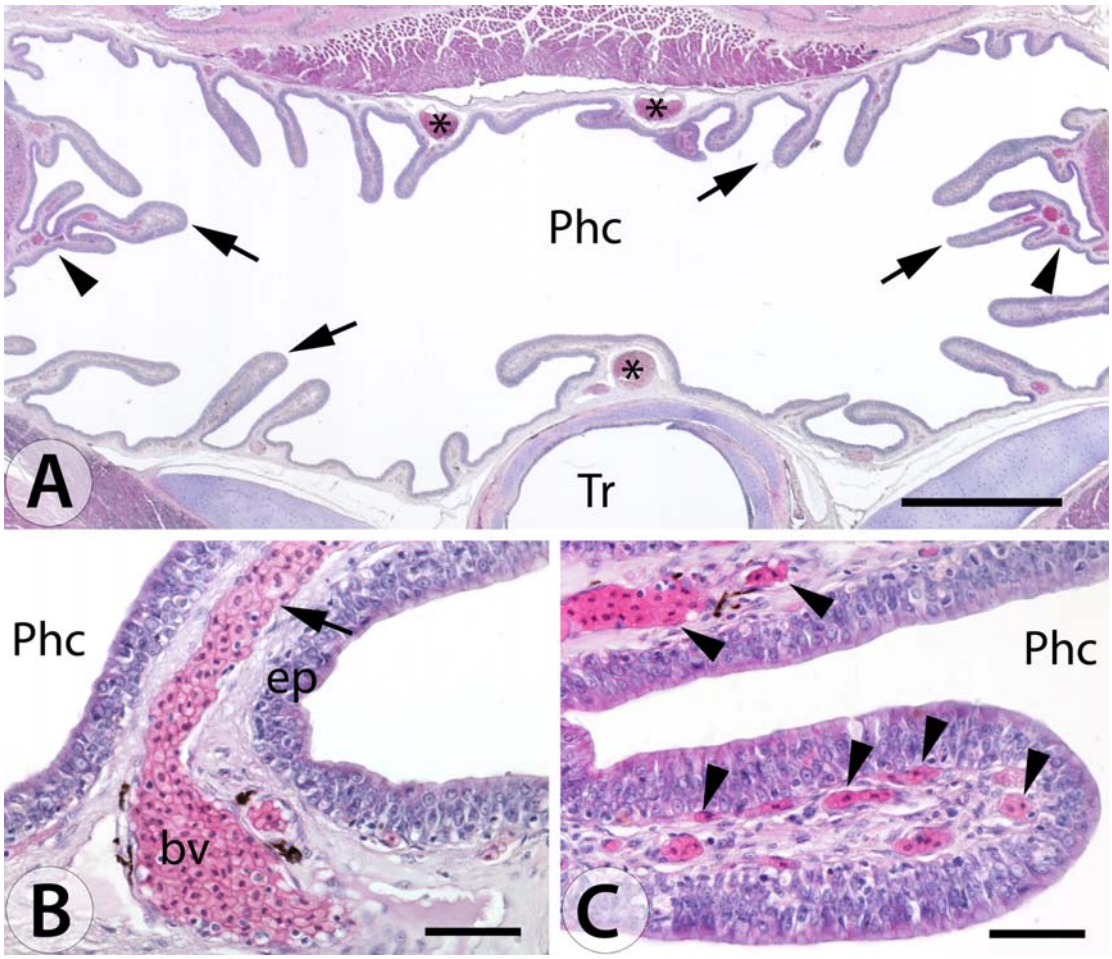

Fig. 4. Light micrographs of cross sections of the pharynx of subadult *S. odoratus*. A Overview showing the large and sometimes branched pharyngeal papillae (arrows). The arrowheads point to branched papillae. The asterisks mark some large blood vessels that supply the capillaries. B Larger blood vessel (bv, filled with erythrocytes) branches into a papilla (arrow). C Numerous small capillaries filled with erythrocytes, run immediately subjacent to the epithelium (arrowheads). ep, epithelium; Phc, pharyngeal cavity; Tr, trachea. Scale bars: A 1 mm; B and C 50 µm. All sections H-E stained.

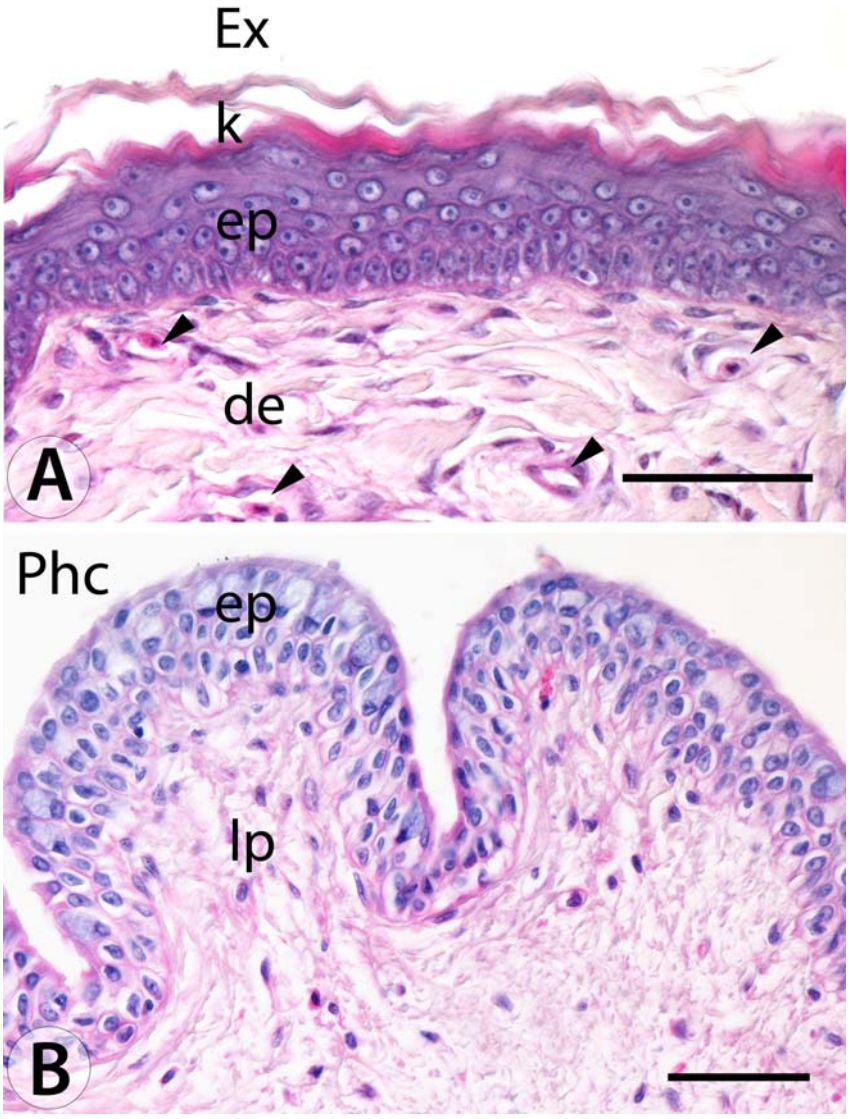

Fig. 5. Light micrographs of (A) longitudinal section of the skin of the neck of a subadult *S. odoratus* and (B) of a transverse section of two postglottal papillae of *E. orbicularis*. A Note the superficial keratin-layer (k) and the lack of a well developed capillary network in the skin of *S. odoratus*. Arrowheads point to small vessels. B Also the small and rare papillae in the pharynx of *E. orbicularis* do not show a well-developed capillary network, in contrast to those of *S. odoratus* (see Fig. 4). Both integument surfaces shown here are not suitable for life-supporting cutaneous respiration. de, dermis; ep, epithelium; Ex, external space; lp, lamina propria; Phc, pharyngeal cavity. Scale bars: 50 µm. Both sections are H-E stained.

2.2. Structure and Function of the Feeding Apparatus in the Common Musk Turtle *Sternotherus odoratus* (Chelonia, Kinosternidae)

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Abstract

The present study examined the kinematic patterns of prey capture, prey transport, pharyngeal packing and swallowing in the common musk turtle *Sternotherus odoratus*. These data are supplemented by morphological descriptions of the skull and the hyolingual complex. Although the hyoid is mainly cartilaginous, *S. odoratus* still use exclusively hydrodynamic mechanisms in prey capture and prey transport. The tongue is relatively small, with weakly developed intrinsic musculature. We propose that the elasticity of the hypoglossum and the hyoid body impacts the capability of *S. odoratus* to suction feed, but allows these turtles to effectively re-position the food items within the oropharyngeal cavity during transport, manipulation and pharyngeal packing. We standardised conditions in all feeding events by using food items of the same consistence and size, and by always offering the food at the same position at the bottom of the aquarium. Nonetheless, the measured kinematic values varied considerably. The duration of prey capture and prey transport cycles were relatively long in *S. odoratus* compared to other freshwater turtles studied so far. The onset of jaw opening relative to the initiation of hyoid retraction can be modulated not only in prey capture but also in prey transport cycles. In the common musk turtle, the jaw and hyoid movements apparently have a low level of integration, and the motoric system of the feeding apparatus is permanently adjusted during a particular feeding situation.

Introduction

In tetrapods the feeding process consists of four main phases: “prey capture” or “ingestion”; “food transport”; “pharyngeal packing”; and “swallowing” (Schwenk, 2000) (or “mammalian deglutition” (Smith, 1992)). In turtles, aquatic prey capture has been studied in pleurodirans (Van Damme and Aerts, 1997; Lemell and Weisgram, 1997; Lemell et al., 2002) – and also in marine (including one estuarine) cryptodirans (Bels and Renous, 1992; Bels et al. 1998). To date, food uptake kinematics have been analyzed in only three freshwater cryptodirans – *Chelydra serpentina* (Lauder and Prendergast, 1992), *Terrapene carolina* (Summers et al., 1998) and *Cuora amboinensis* (Natchev et al., 2009). Information on the kinematics of all other main phases is scarce (Bels et al., 2008).

The oldest known chelonian was an aquatic animal (Li et al., 2008). Some other stem turtle groups were terrestrial (Joyce and Gautier, 2004; Scheyer and Sander, 2007). Anyhow the feeding apparatus of the recent chelonians is secondarily adapted to aquatic feeding (Lauder and Prendergast, 1992) – turtles have developed aquatic feeding convergently with feeding systems in anamniotes. Lauder and Prendergast point to kinematic similarities in prey capture modes in some bony fishes, salamanders and the turtle *C. serpentina* (Lauder and Prendergast, 1992). These authors explain the analogy in the underwater food uptake motoric by hydrodynamic constraints placed on prey capture due to the physical properties of the water as feeding media. The prey capture mechanism of *C. serpentina* was defined as “ram feeding” – the prey is not sucked up into the oral cavity, but engulfed by the jaws in a rush forward strike of the cranocervical complex (Lauder and Prendergast, 1992). In tetrapods utilising “bidirectional feeding” (Reilly and Lauder, 1992), any oropharyngeal volume expansion will entail a pressure decrease within it. Van Damme and Aerts introduced the terms “compensatory suction” and “inertial suction” for turtles, retaining the term “ram feeding” only for feeding systems with unrestrained water through-flow (Van Damme and Aerts, 1997). According to Summers and colleagues, the term “compensatory suction” describes exactly the prey capture mode in cryptodirans (Summers et al., 1998), so the present study will not use the term “ram feeding” for food uptake.

The neuromotor program of the underwater food transport is predicted to be conserved in the evolution of gnathostome feeding systems (Reilly and Lauder, 1990). In the cyclic model proposed by these authors for anamniotes, the jaw cycle is divided into a “fast open” and “fast close” phase, as hyoid retraction (expansion phase) starts simultaneously to the begin of jaw opening. The coincidence between hyoid retraction and jaw opening is regarded as a uniform pattern throughout tetrapods. This hypothesis is not always supported for turtles – the

transport modes in turtles seem to be extraordinarily variable (Lemell and Weisgram, 1997; Lemell et al., 2002; Natchev et al., 2009; Natchev et al., 2010 in press). According to Aerts and colleagues the underwater transport in chelonians combines "compensatory suction" and "inertial suction" (Aerts et al., 2001). Both mechanisms are termed "intraoral-aquatic hyoid transport" (Bels et al., 2008). Nonetheless, some chelonians, even completely aquatic species, use tongue-based transport, which is termed "intraoral-aquatic lingual transport" (Bels et al., 2008).

Information on the feeding kinematics in kinosternid turtles is completely lacking. In this study, we describe the kinematics of the neck, jaws and the hyoid complex based on high-speed film analysis during the whole aquatic feeding process in the omnivorous kinosternid *Sternotherus odoratus*. These data are supplemented by morphological descriptions of the skull and the hyolingual complex. Although *S. odoratus* has a highly papillaeted dorsal tongue surface (Heiss et al., 2010 in press), we hypothesise that the common musk turtle uses exclusively hydrodynamic mechanisms for food transport underwater. We investigate statistically which variables of food uptake and food transport kinematic profiles exhibit similarities. We also test whether the kinematic patterns differ on an inter-individual level. Special focus is devoted to analysing the coordination (*sensu* Wainwright and colleagues (Wainwright et al., 2008)) between the neck, hyoid and jaw movements. This is designed to test a predicted strength correlation between the movements of the elements of the feeding apparatus in underwater food transport in tetrapods (Reilly and Lauder, 1990).

Material and Methods

The 25 recent kinosternid species are divided in four genera: *Claudius*, *Staurotypus*, *Kinosternon* and *Sternotherus* (Pritchard, 1979). The common musk turtle or stinkpot – *Sternotherus odoratus* (LATREILLE, 1802) (the name *Kinosternon odoratum* is still used as a synonym (Bonin et al., 2006)) – is an entirely aquatic species. It is widely distributed in the eastern USA, south to the Mexican border and north up to Canada (Ernst and Barbour, 1989). This omnivorous to carnivorous species feeds on carrion, insects, molluscs, crayfish and fish on the bottom of rivers, lakes and swamps (Pritchard, 1979; Bonin et al., 2006).

The animals investigated here were obtained commercially and kept in a 360 l tank with 20 % land and 80 % water, and a 12 h dark/12 h light cycle. They were fed with earthworms, fish pieces and turtle-food pellets from the pet trade. Animal care and treatment was in accordance with the "Austrian National Protection of Animals Act" (TSchG 2004**)**.

For morphological analysis, the turtles were anesthetized by intraperitoneally injecting sodium pentobarbital (Nembutal) and, after deep narcosis, decapitated. The heads were immersed immediately in fixation solution.

For computed tomography (CT), the heads of one subadult (carapace length: 69.3 mm) and one adult animal (carapace length: 114 mm) were immersed in 4% formaldehyde for two weeks prior to storage in 70% ethanol. The 3D data were generated using industrial X-ray Computer Tomography. During measurement projection, images were grabbed using an a-Si matrix detector at several angular positions. Depending on the density, the atomic number and the irradiation length, different gray values occurred in these 2D images. A full 360 degree rotation typically yielded 720 images. Table 1 lists the parameters used for the ct- scans. Surface and volume reconstructions were made using Amira 4.1 (Mercury Computer Systems, Chelmsford, MA, USA).

For scanning electron microscopy, two heads of juvenile turtles were immersed for 24 h at room temperature in modified Karnovsky solution (2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer; Karnovsky, 1965). After rinsing in 0.1 M cacodylate buffer, the lower jaw was removed. Then, samples were postfixed in 0.5% osmium tetroxide for 2 h at 37 °C, washed in distilled water, and treated with 25% HCl at 40 °C for 15 min to remove surface mucus. After repeated washing in distilled water, the samples were dehydrated in a graded ethanol and acetone series and dried in a critical point drying machine (Polaron: Watford, UK). The dried samples were then coated with gold in an AGAR B7340 Sputtercoater (Agar Scientific Ltd, Stansted, UK) and observed in a Philips XL-20 scanning electron microscope (Philips, Eindhoven, NL).

For histological analysis, two juvenile and two subadult turtles were used. The heads were immersed in Bouin's fixative (Romeis, 1989) for 30 days, changing the solution twice a week. After complete fixation and decalcification, the upper jaw was removed from the rest of the head and the cornified rhamphothecae were cut off. Then, the samples were dehydrated in a graded ethanol - isopropanol series and embedded in paraffin. After polymerisation, 7 µm thin serial-sections were made on a Reichert-Jung 2030 rotation microtome (Reichert-Jung, Bensheim, GER). The sections were mounted on glass slides and, after removing the paraffin, stained with Haematoxylin – Eosin, periodic acid Schiff (PAS) – Haematoxylin and Alcian blue – Haematoxylin (Romeis, 1989; Kiernan, 2003). Digital photographic documentation was made using a Nikon Eclipse 800 light microscope (Nikon, Tokyo, JP).

For filming aquatic feeding, the food items (pieces of epaxial musculature of fish) were offered in front of the animals on the bottom of a glass aquarium (19 x 7 x19 cm) with a water depth of 12 cm. The food size was calibrated to the distance between the tip of the lower jaw and the point "A" (Figure 1) at the jaw articulation of every single turtle tested. The food type and size were chosen to allow recording of the whole feeding process – from food uptake to swallowing. Three subadult specimens (carapace length: 61.5 - 69.3 mm) were filmed in strict lateral view with a reference grid (1 x 1 cm) as background, using the digital high-speed camera Photron Fastcam-X 1024 PCI (Photron, Tokyo, JP) with 500 fr/s. For a total of 19 films, the horizontal (on the X-axis) and vertical (Y-axis) coordinates of each landmark on Figure 1 were recorded frame by frame using "SIMI-MatchiX" (copyright (c) by SIMI Reality Motion Systems, Unterschleisheim, Germany). "Time zero" for our marker tracking was the moment of the first detectable hyoid elevation prior to "jaw opening". Based on landmark displacement in the bi-directional level, we calculated: a) the gape amplitude – distance between the tips of the upper and lower jaw; b) ventral hyoid movement – distance between point "S" on the squamosal and point "H" at the origin of the ceratobranchiale II; c) neck extension and retraction – the distance between the point "S" on the squamosal and the anterior tip of the carapace; d) food item movement relative to point "N".

Statistical tests were performed by using SPSS 11.5 (IBM, Chicago, USA) software. The 13 variables used to compare feeding kinematic patterns are listed in Table 2. A MANOVA was performed to test differences regarding repeated measurements in the three individuals, differences between individuals, and differences between ingestion and transport cycles. The model residuals (MANOVA residuals) were checked for normal distribution by the K-S-test and showed normal distributed for each variable. Food uptake cycles from all specimens were aligned at reaching peak gape, and the mean profiles (±SEM) of the movements of the gape,

hyoid and neck were calculated and plotted. Mean transport kinematic profiles (±SEM) of gape, hyoid and neck were calculated separately for every individual, as the cycles were aligned at reaching peak gape.

Additionally, a canonical centroid plot was performed to demonstrate individual kinematic differences in the transport mode. Correlations between movements of head, jaws and hyoid were analyzed in both ingestion and transport modes; only significant correlations > 0.3 are presented and considered to truly impact the material examined.

Results

The skull in *S. odoratus* was relatively flat and elongated with a prominent supraoccipital ridge and wide and high temporal arches. The rhamphothecae were well developed and, as typical for most kinosternids, the edges were blunt. The palate formed no dorsal flexure (Figure 2c). The tongue was relatively small with weakly developed proper musculature (Figure 2e). In subadults the hypoglossum, hyoid body, epibranchials I and the second hyoid horn were completely cartilaginous. Only the ceratobranchials I were ossified. The CT analysis demonstrated that the hyoid complex remains mainly cartilaginous even in older individuals (Figure 2b). There were only two pairs of ossifications on the hyoid corpus (at the basis of ceratobranchials I and II).

Scanning electron microscopy revealed the occurrence and distribution of taste buds (tb). Tb's were identified based on their typical taste pore with large microvilli in the centre (Figure 2d, f). Tb's were found throughout the oropharyngeal cavity: from the well-keratinized very anterior mouth region to the very posterior pharynx. Most tb's in juvenile and subadult turtles were counted on the anterior palate (anterior to the choanae) and on the anterior floor of the mouth.

Light microscopy revealed the barrel- to onion shape of tb's. They consisted of 30 to 60 specialized, slender epithelial cells. The nuclei of the tb-cells were located mainly in the basal half to two-thirds of the tb. While the descending cell processes contacted the basement membrane, the microvilli of the slender cytoplasmatic processes of the apical region reached freely into the tastepore (Figure 2d,f).

The splanchnocranial components and the hypoglossum of the hyolingual complex were predominantly cartilaginous and the intrinsic musculature of the tongue was poorly developed. The dorsal surface of the tongue was amplified due to the formation of floppy papillae (Figure 2e).

When feeding on small pieces of fish, the feeding process included the prey capture cycle, zero to four (2.054 ± 1.08.) transport cycles, two to six (4.1 ± 1.3) pharyngeal packing cycles, followed by swallowing. The food items used in our experiments were offered at the bottom of the aquarium at a fixed position in front of the animals. The turtles swam slowly toward the prey and stopped their forward locomotion when the tip of the lower jaw was at 0.48 ± 0.17 cm from the fish. Food uptake started with hyoid elevation followed by jaw opening. The body was almost entirely motionless. In most of our sequences (14 of 19) we were able to detect a separation of "slow jaw open (SO)" and "fast jaw open (FO)" phases. In 5 other films, the gape increased gradually and no discrete phases were recognized prior to reaching

"peak gape". During jaw opening the head rotated ventrally, and the rotation was attended (except in 1 case) by neck extension. In 5 of our sequences the hyoid retraction started prior reaching peak gape (Figure 4b). In 14 cycles the first detectable retraction of the hyoid complex started during a static gape phase where the jaw amplitude remained at its maximum for a period of time (here termed MG-phase) (Figure 4c). The MG-phase (present in all 19 "prey capture" events) was followed by fast jaw closing. The food item was actively sucked up into the mouth due to the pharyngeal expansion, but was often pushed slightly forwards during jaw closure (Figure 3). In 2 of the 19 food uptake events, the prey was ingested entirely within the oropharyngeal cavity and the animals closed their jaws completely.

Prey transport cycles started with hyoid protraction followed by jaw opening. In 28 of 39 food transport events, the neck extended rapidly during jaw opening. The magnitude and the velocity of the hyoid retraction was rather variable, still in all sequences the process started prior reaching peak gape (Figurs 3, 5). MG- phase was detected in 7 of our sequences. In transport cycle where the prey was positioned almost entirely within the mouth, the neurocranium remained motionless or was ventrally rotated rather than protracted.

During pharyngeal packing, the prey was not visible. The jaw and hyoid displacement amplitudes were quite small (sometimes less than one millimetre). In most cases the jaws remained fully closed (Figure 3).

The 13 kinematic variables used (Table 2) revealed no significant differences between repetition of measurements within the single individuals (MANOVA Wilk's λ, F=0.88, P=0.785), or between individuals in food ingestion (MANOVA Wilk's λ, F=3.486, P=0.116). Highly significant differences were detected between individuals in the transport-phase (MANOVA Wilk's λ, F=79,919, P<0.001) and between ingestion and transport mode (MANOVA Wilk's λ, F=15.653, P<0.001). This means that even if individuals show similar kinematic patterns, the differences between individuals are significant (at least in transport); the feeding patterns between ingestion and transport also differed significantly.

Individual kinematic differences in food transport were additionally demonstrated through a conical centroid plot (Figure 6). Variables t_D and t_{FO} loaded positively and variables V_{FC}, t_{HFOE} and V_{HR} loaded negatively to the first canonical axis, while variable t_{HFOB} loaded positively and variables V_{NE} and t_{HR} loaded negatively to the second canonical axis. Together, the axes explained 100% of the variance among individuals.

When testing for correlation between movements of head, gape and hyoid during ingestion, highly significant correlations were found between head and hyoid in animal 1 and 2; between head and gape in none; and between hyoid and gape in animal 1 and 3. When testing the same

parameters for correlation in the transport mode, only the head and hyoid movements in animal 3 were highly significantly correlated. All other correlations were not significant or below 0.3 (i.e. below real relevance).

In contrast to prey capture and transport, in pharyngeal packing the hyolingual complex was slightly elevated rather then protracted at the beginning of every cycle. The subcutaneous pharyngeal compressor muscles clearly contracted. During pharyngeal packing the head remained permanently retracted. Pharyngeal packing was followed by swallowing. Swallowing was clearly delimitable from the pharyngeal packing cycle because the head was always elevated and the contraction of the constrictor muscles induced a very typical “bent” on the ventral side of the oesophagus.

Discussion

According to Bever (2007; 2008 in press), the skull features associated with the feeding apparatus in *S. odoratus* are the most variable cranial structures for both continuous and discrete characters. One explanation for the postnatal morphological variations in the continuous characters of the skull is a potential dietary shift during ontogenetic development (Pritchard, 1979; Bonin et al., 2006; Bever, 2007, 2008 in press). Our morphological investigations based on CT and histological analysis demonstrate no major change in the design of the hyoid complex during ontogeny. The hyoid corpus remains mainly cartilaginous even in adult common musk turtles. A heavily ossified hypoglossum and hyoid body are predicted in all turtles using predominantly suction feeding under water (Bramble, 1973; Bramble and Wake, 1985; Van Damme and Aerts, 1997; Lemell et al., 2000; Lemell et al., 2002; Lemell et al., 2010). A cartilaginous hyoid body (or lingual processus) in chelonians is associated with a greater role of the tongue in feeding (Bramble and Wake, 1985; Wochesländer et al., 1999; Natchev et al., 2009; Natchev et al., 2010 in press). *S. odoratus* utilises exclusively hydrodynamic mechanisms in prey capture and prey transport, so one could expect more rigid hyoid complex. Our interpretation is that the elastic hyoid body allows *S. odoratus* to increase the mobility of its relatively small tongue and to increase the efficiency of the aquatic food transport and pharyngeal packing. Turtles with large and fully ossified hyoids generate strong suction forces during prey ingestion, but their food transport involves so-called “slow suction”. The time between two successive transport cycles is prolonged by up to half a minute and the transport gape cycles durations are up to 10 times longer than in prey capture (Lemell et al., 2002).

Some of our prey capture sequences revealed that the common musk turtles actively sucked up the food items by somewhat expanding the oropharyngeal cavity. In one film, the food uptake even resembled pure “inertial suction” – the neurocranium remained fully static as the prey moved into the mouth. Kinematic patterns of the gape and neck varied considerably. We therefore conclude that *S. odoratus* adjusts its prey capture behavior to every single feeding situation, depending on the prey position relative to the jaws. As the pieces of fish used in our experiments often remained under the jaws prior to initial jaw opening, the animals apparently have no direct visual contact with the food items. Perhaps the motoric of the feeding apparatus is coordinated mainly in response to olfactory feedback and to the tactile “cirri” (Winokur, 1982).

In contrast to the most cryptodiran turtles studied to date (Lauder and Prendergast, 1992; Bels et al., 1998; Summers et al., 1998; Bels et al., 2008), hyoid retraction in the common musk

turtle starts shortly before or even after reaching peak gape during food uptake (Figures 3, 4). This behavior probably reflects the relatively poor capacity of *S. odoratus* to suction feed. The abrupt retraction of the relatively small and elastic hyoid complex cannot produce the high suction forces found in e.g. *C. fimbriatus* (Lemell et al., 2002). The common musk turtle lacks the skinny "cheeks" lateral to the gaping mouth, as found in some turtles specialized in suction feeding (Lemell et al., 2002), so the water flow cannot be directed so precisely toward the oropharynx. In our interpretation, the largest possible gape during the initiation of suction and the start of head protraction increases the grasping success.

When feeding on fish pieces, the common musk turtle transported the food items using "intraoral-aquatic hyoid transport". In 72 % of our sequences, prey transport involved "compensatory suction". As the neurocranium remained fully static in the other food transport events, the turtles apparently relied on pure "inertial suction" in these cases. Our film sequences reveal no discrete slow phase during the jaw opening, but there is variability in the delay of the start of hyoid retraction to the start of gape increase (see Table 2; Figure 5). The beginning of pharyngeal expansion does not correspond to the start of the jaw open phase as predicted from the model of Reilly and Lauder (1990). In some turtles that can feed under water and have relatively well-developed tongues, the hyoid retraction starts shortly before or even after reaching peak gape (Natchev et al., 2009, 2010 in press). The amboina box turtle *Cuora amboinensis* use its tongue to fix the prey against the palate during jaw opening, whereas the Indochinese box turtle *Cuora galbinifrons* fixes relatively large prey to the dorsal tongue surface during jaw opening (Natchev et al. 2010 in press). This enables the two species to hold the food items within the oral cavity even at maximum gape. The common musk turtle has a weak intrinsic lingual musculature and the tongue papillae are involved in aquatic gas exchange rather than in food transport (Heiss et al., 2010) – so the tongue cannot be used to fix the prey. *S. odoratus* uses exclusively hydrodynamic mechanisms for prey transport. Hyoid retraction must start after the prey is released from the rhamphothecae during jaw opening, but before the prey can escape or float out of the oral cavity. Due to the numerous taste buds on the oropharyngeal surfaces (Figures 2 d, f), we propose that besides mechanoreceptor input, the chemosensorial feedback helps coordinate the movements of the feeding apparatus during transport. Tb's are most highly concentrated in the anterior palate and the anterior floor of the mouth. This anterior concentration enables a fast motoneural response of the type "eat it or leave it" (Heiss et al., 2008) because the first prey contact occurs there. In the natural environment, the negative response (rejection) may be crucial: the

benefit of avoiding harmful food is self-evident (Schwenk, 1985; Berkhoudt, 1985; Berkhoudt et al., 2001; Heiss et al., 2008).

The omnivorous aquatic cryptodiran turtles studied to date have a very flexible (*sensu* Wainwright et al., 2008) feeding behavior (Lemell and Weisgram, 1997; Bels et al., 1998; Lauder and Prendergast, 1992). *S. odoratus* also exhibited plasticity in their food uptake kinematics. It feeds on a variety of non-elusive prey and approaches the food items to very close distance prior to ingestion. We conclude that the morphology of the hyolingual complex constrains this turtle's capacity to ingest prey via suction, but benefits its ability to re-position and manipulate food within the oropharynx. The present study demonstrates that common musk turtles modulate their feeding behavior even in successive feeding events involving food items which have exactly the same consistence, size and position. The food transport kinematics differed significantly within the three individuals: one of the turtles needed about half as many transport cycles prior to pharyngeal packing as the other two. Interestingly, that was the only individual showing significant correlations between the movement of the hyoid and the head. Further studies will reveal whether this can be interpreted in the context of the learning capacity of cryptodiran turtles (Wilkinson et al., 2007, 2009, 2010 in press). Plasticity in the cognitive capacity of *S. odoratus* could strongly affect its ecological potential.

Abbreviations list

cbI - ceratobranchiale I;

cb II - ceratobranchiale II,

ch - corpus hyoidei;

hg - hypoglossum;

lj - lower jaw;

uj - upper jaw;

CT - computed tomography;

FC - fast close;

FO - fast open;

kV – Kilovolt;

MG phase - maximum gape phase;

µm - micrometre;

SD - standard deviation;

oss I - island of ossification at the basis of cb I;

oss II - island of ossification at the basis of cb II;

protr. - protraction;

retr. - retraction;

SD - standard deviations;

SEM - standard error of the mean;

SO - slow open;

tb - taste bud;

t_D - total duration;

t_{FO} - fast opening;

t_{FC} - fast closing duration;

t_{HFOB} - hyoid retraction delay to fast opening begin;

t_{HFOE} - time interval of hyoid retraction onset relative to fast opening end;

t_{HR} - hyoid retraction duration;

t_{MG} - MG-phase duration;

t_{NE} - neck extension duration;

t_{PG} - time to peak gape;

V_{FC} - fast closing velocity;

V_{FO} - fast opening velocity;

V_{HR} - hyoid retraction velocity;

V_{NE} - neck extension velocity;

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Tables

Tab. 1

Unit	kV		µm	min	
Parameter	Voltage	Projections	Voxel size	Duration	Filter
Value	120	990	27	60	none

Table 1. Parameters used for the ct-scans. Measurements were made at the Upper Austrian University of Applied Sciences, Campus Wels, using the Microfocus-tube of the device RayScan 250 XE by RayScan Technologies GmbH (Meersburg, Germany).

Tab. 2

	ingestion				transport				P^3
variables	animal 1 (n=7)	animal 2 (n=6)	animal 3 (n=6)	P^1	animal 1 (n=15)	animal 2 (n=16)	animal 3 (n=8)	P^2	
t_{FO} (s)	0.055±0.008	0.055±0.005	0.048±0.024	0.695	0.046±0.014	0.041±0.015	0.034±0.008	0.009*	0.031*
t_{PG} (s)	0.425±0.159	0.358±0.308	0.201±0.105	0.12	0.092±0.064	0.144±0.156	0.085±0.102	0.148	0.001*
t_P (s)	0.034±0.0062	0.016±0.007	0.015±0.010	0.002*	0.007±0.013	0.000±0.000	0.005±0.008	0.161	<0.001*
t_{FC} (s)	0.056±0.022	0.049±0.03	0.048±0.017	0.71	0.071±0.04	0.054±0.029	0.046±0.013	0.002*	0.48
t_D (s)	0.515±0.169	0.423±0.34	0.265±0.112	0.101	0.162±0.09	0.198±0.182	0.131±0.095	0.015*	0.001*
t_{HR} (s)	0.035±0.011	0.033±0.014	0.033±0.005	0.448	0.038±0.015	0.033±0.018	0.044±0.016	0.025*	0.103
t_{NE} (s)	0.093±0.048	0.07±0.038	0.075±0.018	0.886	0.056±0.03	0.04±0.013	0.06±0.022	0.125	0.37
t_{HFOB} (s)	0.060±0.007	0.062±0.006	0.047±0.021	0.176	0.023±0.007	0.021±0.01	0.017±0.004	0.154	<0.001*
t_{HFOE} (s)	0.006±0.007	0.003±0.008	0.001±0.007	0.321	-0.022±0.01	-0.14±0.004	-0.017±0.006	0.007*	<0.001*
V_{FO} (cm/s)	4.795±1.082	5.89±4.747	4.851±3.081	0.453	7.195±3.31	15.316±5.159	16.587±7.29	0.008*	0.127
V_{FC} (cm/s)	9.408±3.587	14.517±5.37	9.18±1.543	0.015*	3.659±3.365	11.769±4.027	7.067±2.295	<0.001*	0.003*
V_{HR} (cm/s)	10.642±3.932	8.554±3.292	8.694±2.77	0.505	5.477±2.511	12.869±7.62	8.285±1.958	0.001*	0.56
V_{NE} (cm/s)	4.933±2.742	7.89±1.449	9.663±1.811	0.91	1.876±4.42	4.819±6.927	6.753±2.019	<0.001*	0.187

Table 2. Values are means ± SD with associated significance values. t_{FO} - fast opening; t_{PG} - time to peak gape; t_{MG} - MG-phase duration; t_{FC} - fast closing duration; t_D - total cycle duration; t_{HR} - hyoid retraction duration; t_{NE} -neck extension duration; t_{HFOB} - hyoid retraction delay to fast opening begin; t_{HFOE} – time interval of hyoid retraction onset relative to fast opening end; V_{FO} - fast opening velocity; V_{FC} - fast closing velocity; V_{HR} - hyoid retraction velocity; V_{NE} - neck extension velocity; *Significant differences (α=0.05) among individuals in the ingestion phase (P^1), among individuals in the transport phase (P^2) and between ingestion and transport mode (P^3).

Figures

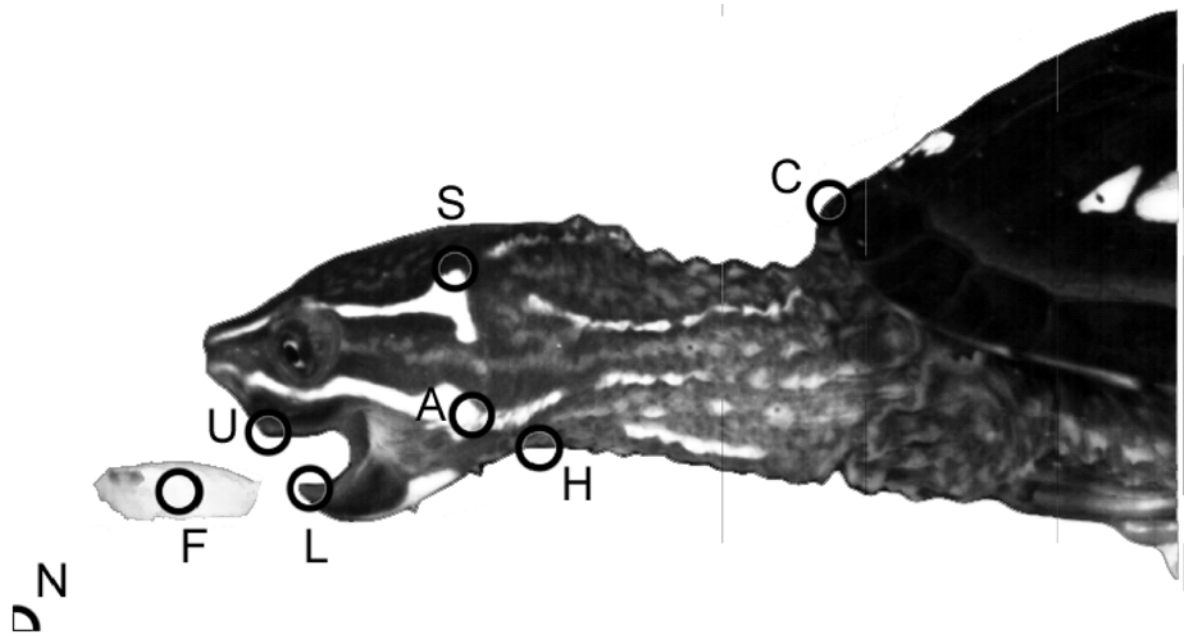

Fig. 1. Points used for kinematic analyses of feeding cycles of *Sternotherus odoratus*. A, ventral margin of tympanum (jaw articulation); C, anterior tip of carapace; F, centre of mass of the feeding items; H, basis of CB II on hyoid; L, anterior tip of lower jaw; N, point "zero" on the measurement board; S, dorsal margin of tympanum (the most dorsal point of the squamosal); U, anterior tip of upper jaw.

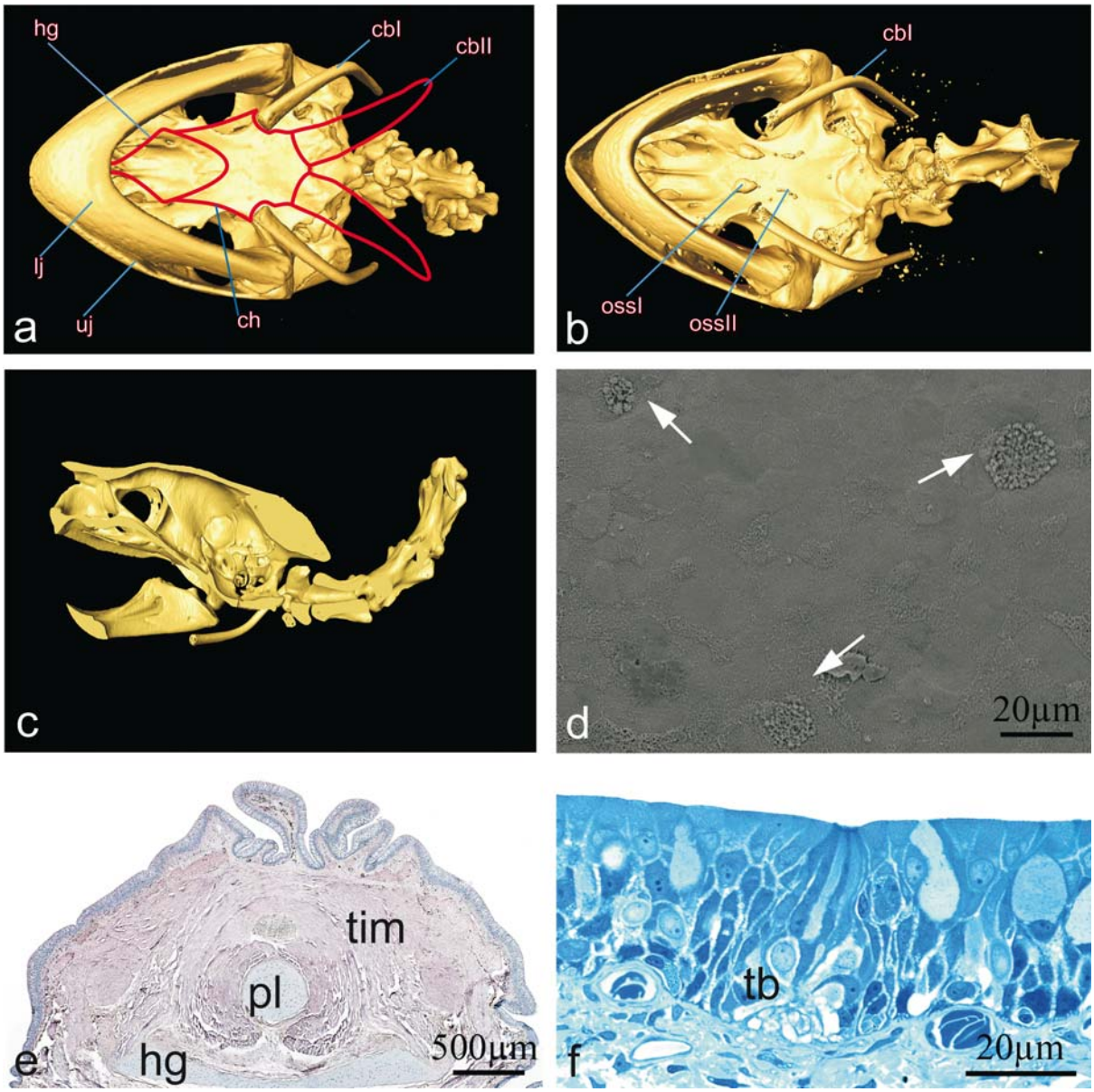

Fig. 2. *Sternotherus odoratus*, head morphology. a, CT-scan after Amira 4.1 reconstruction from ventral with schematic illustration of hyoid complex (subadults): cbI, ceratobranchiale I; cb II, ceratobranchiale II, ch, corpus hyoidei; hg, hypoglossum; lj, lower jaw; uj, upper jaw; b, CT-scan after Amira 4.1 reconstruction from ventral (adults): cbI, ceratobranchiale I; oss I, island of ossification at basis of cb I; oss II, island of ossification at basis of cb II; c, CT-scan after Amira 4.1 reconstruction sagittal section (subadults); d, Scanning electron micrograph at medium magnification showing three neighbouring taste buds (tb). tb's are recognized by their taste pores containing large microvilli (arrows); e, Light micrograph cross-section of the tongue of a subadult *S. odoratus* slighly anterior to the glottis. Note the cartilaginous hyoidal "support" elements and the weakly developed intrinsic musculature: hg, hypoglossum; pl, processus lingualis; tim, tongue intrinsic musculature; f, Light micrograph

showing detailed cross section of a typical taste bud (tb) of *S. odoratus* from the anterior floor of the mouth.

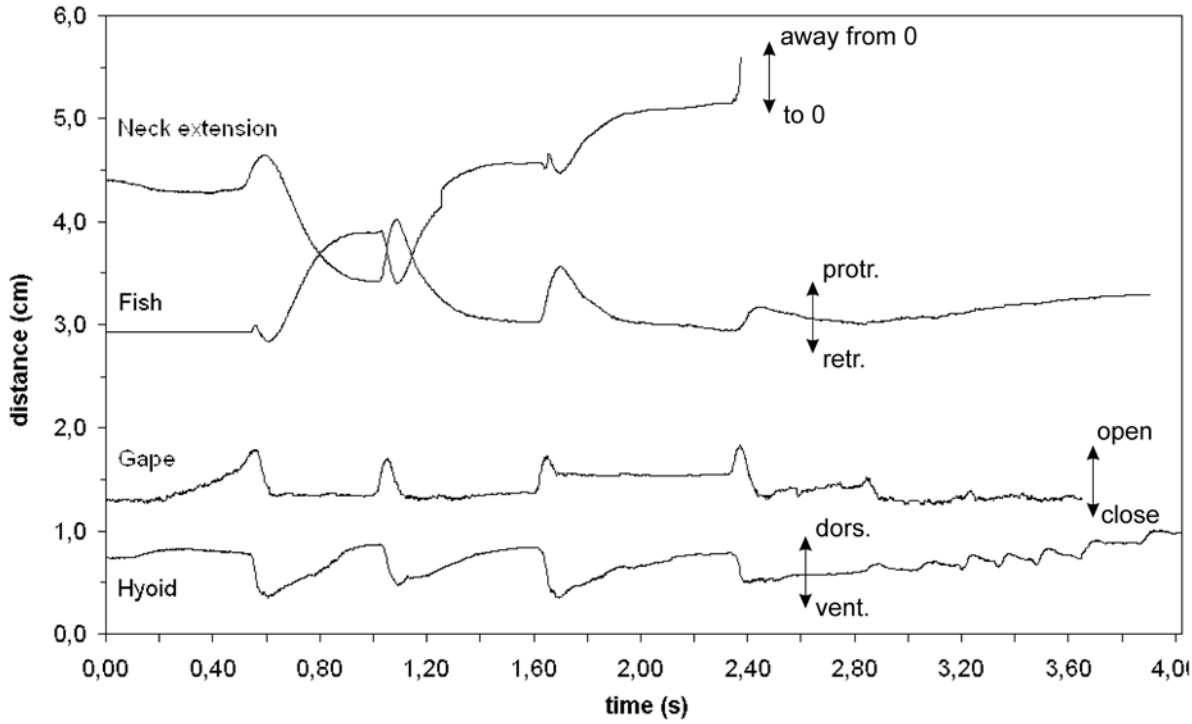

Fig. 3. Kinematic profiles from an aquatic feeding event in *S. odoratus*, including one food uptake, three food item transport, six pharyngeal packing and two swallowing cycles based on high-speed film (500 fr/s): protr., protraction; retr., retraction; dors., dorsal; ventr., ventral.

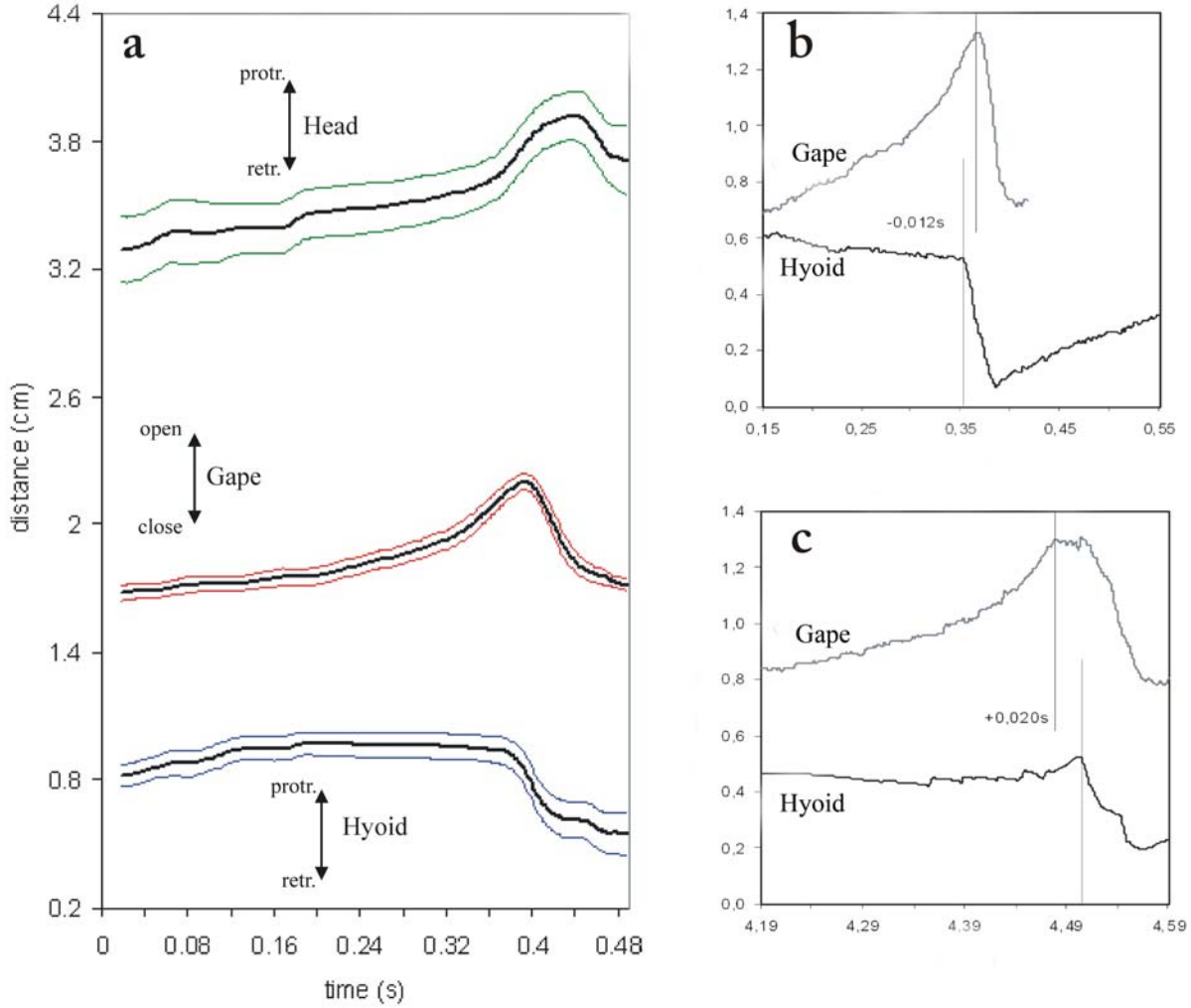

Fig. 4. Kinematic patterns in aquatic food uptake in *S. odoratus*. a, kinematic profiles from aquatic food uptake cycles (n=19) in three *S. odoratus* specimens based on high-speed films (500 fr/s). Black lines: mean kinematic profile. Coloured lines: standard error of mean (±SEM), calculated from all other profiles, aligned synchronised on the first peak gape frame; b, food uptake – minimum delay of hyoid retraction to reaching peak gape (hyoid retraction starts prior to reaching peak gape); c, food uptake – maximum delay of hyoid retraction to reaching peak gape (hyoid retraction starts during maximal gape phase "MG - phase"); protr., protraction; retr., retraction.

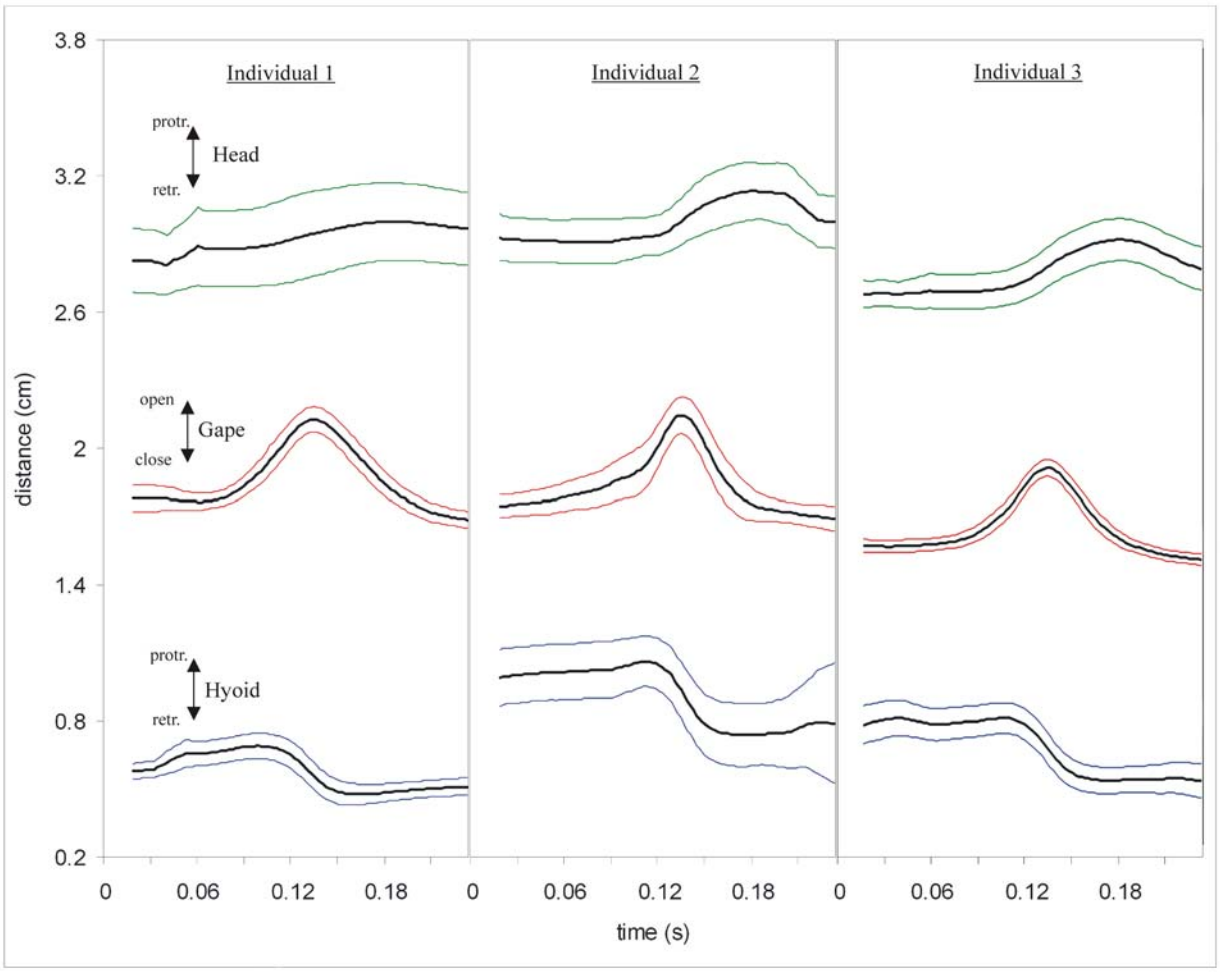

Fig. 5. Kinematic patterns in aquatic food transport in three *S. odoratus* specimens based on high-speed films (500 fr/s). Black lines: mean kinematic profile. Coloured lines: standard error of mean (±SEM), calculated from all profiles per individual, aligned synchronised on the first peak gape frame; protr., protraction; retr., retraction.

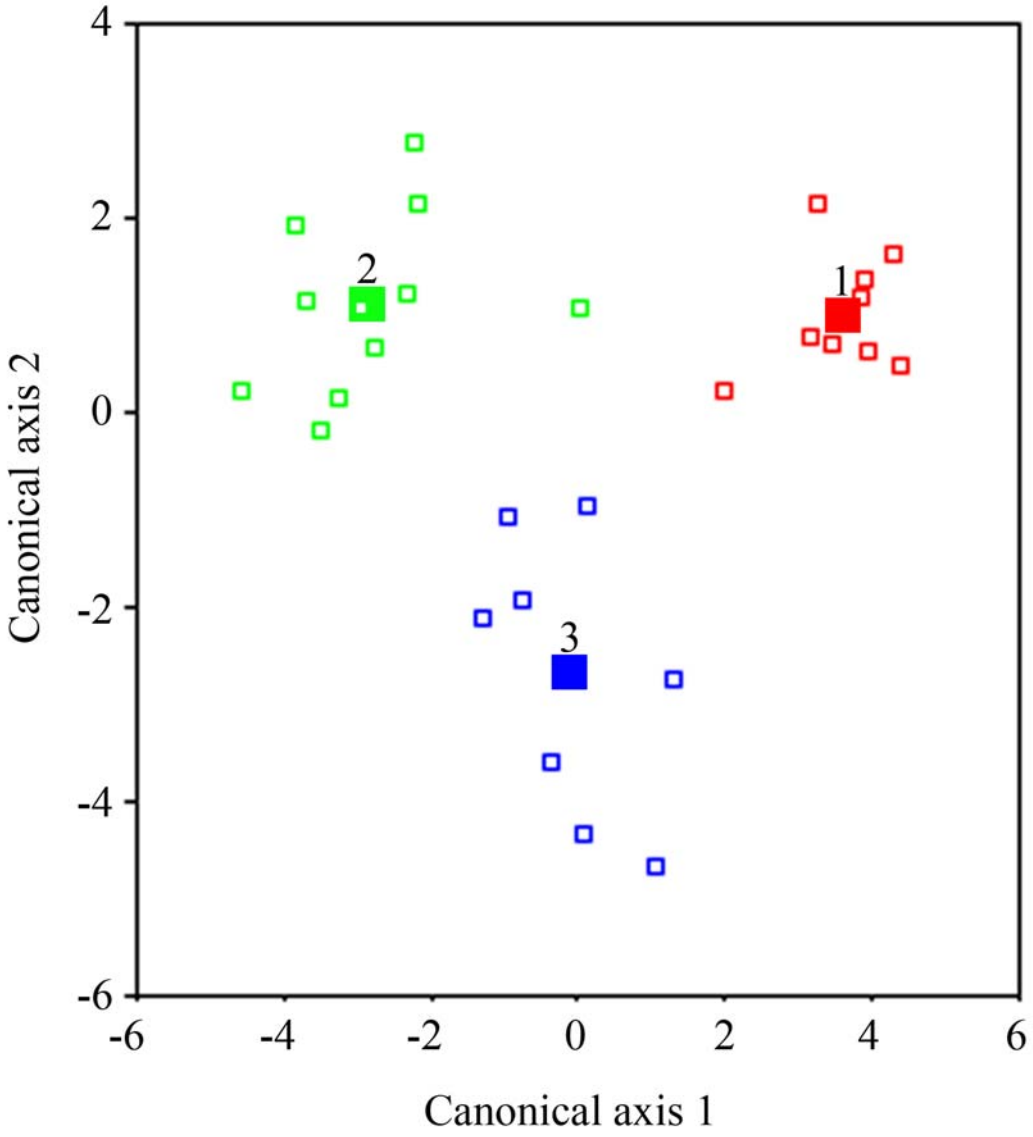

Fig. 6. Conical centroid plots of kinematic variables of food transport in three *S. odoratus* specimens. Centroids plotted for each individual (filled squares) and measurement repetition (unfilled squares).

2.3. Microanatomy of the Palatal Mucosa of the semiaquatic Malayan Box Turtle *Cuora amboinensis*, and Functional Implications

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Abstract

Scanning Electron Microscopy (SEM) revealed that the palate of *Cuora amboinensis* has a flat surface with keratinized and non-keratinized regions. Keratinization is reflected in disc-shaped keratinized dead cells with rough microplicae on the surface, and is concentrated close to the rhamphotheca. The surface of the non-keratinized hexagonal epithelial cells is dotted with microvilli and sometimes with cilia. Taste buds are present both in lightly keratinized and non-keratinized regions and exhibit a crater-like shape. Light microscopy shows the different tissue layers of the oral mucosa and the different epithelial structures. In keratinized regions, keratinocytes mature from basal to superficial, where they build up keratin layers of varying thickness. In non-keratinized regions, the epithelial cells are arranged in a stratified fashion, and cuboidal to cylindric cells form a superficial layer. Goblet cells appear to be diffusely distributed, but are often organized in goblet cell fields which can be folded into crypts. Taste buds consist of slender epithelial cells, exhibit the typical barrel-like shape and are specially concentrated in the anterior, praechoanal palate. This anterior concentration of taste buds is shown by kinematographic analysis to correlate with the food prehension mode in *Cuora amboinensis*. The lamina propria of the palatal mucosa consists of loose connective tissue with inflammatory cells between capillaries. All these structures of the oral mucosa act as a functional entity and help determine how successfully an organism adapts ecologically to the environment.

Introduction

The covering of the oral cavity in vertebrates, the oral mucosa, is a specialized form of integument (Squier and Finkelstein, 2003). It reflects a variety of functional adaptations and includes integumentary derivatives such as glandular tissue (from single secretory cells to monostomatic glands; Fahrenholz, 1937; Nalavade and Varute, 1976; Iwasaki et al., 1992, 1996; Beisser et al., 2004), keratinized cells (Winokur, 1988; Putterill and Soley, 2003) and taste buds (Korte, 1980; Uchida, 1980; Schwenk, 1985; 2000a; Berkhoudt, 1985; Berkhoudt et al., 2001; Putterill and Soley, 2003; Song et al., 2007). The oral mucosa plays an essential role in feeding mechanisms, and feeding is clearly a crucial factor in the success of an animal in its adaptation to the environment (Schwenk, 2000a; Schwenk and Wagner, 2001; Iwasaki, 2002). Accordingly, structures and specializations of the oral cavity define the mode of life and thus the mode of feeding in tetrapods. Their feeding mechanisms depend on the physical constraints of the surrounding media: water or air (Bramble and Wake, 1985; Roth and Wake, 1989; Deban and Wake, 2000; Schwenk, 2000a; Aerts et al., 2001; Schwenk and Rubega, 2005). Within tetrapods, turtles are of special interest in studying feeding because they span the whole range from fully terrestrial to fully aquatic, with numerous intermediate representatives. The systematic position of the turtles within the sauropsids remains unclear. Hedges and Poling (1999) and Rest et al. (2003) placed chelonians as a sister group of the archosaurians, and Benton (2005) suggested turtles to be the ‘‘crown group’’ of the parareptilian anapsids. However, they are an ancient group that might retain ancestral amniote features in some aspects of their feeding system. This makes their further study all the more desirable (Schwenk, 2000b). In aquatic feeding of vertebrates, the main force in ingestion is a rapid rush of water into the oro-pharyngeal cavity caused by a rapid pharyngeal expansion: socalled ‘‘suction feeding’’ (Lauder, 1985). Most aquatic tetrapods combine suction feeding with ‘‘ram feeding’’ (fast approach to the prey plus jaw prehension; Bramble and Wake, 1985; Bels et al., 1998; Schwenk, 2000a; Lemell et al., 2002; Schwenk and Rubega, 2005). Fully (nonmarine) aquatic turtles have a strongly reduced tongue with a flat surface (Winokur, 1988; Iwasaki, 1992; Iwasaki et al., 1992, 1996; Beisser et al., 1995, 1998, 2001, 2004) to save space that can be used for volumetric expansion during underwater suction feeding (Bramble and Wake, 1985; Lemell and Weisgram, 1997; Lemell et al., 2000, 2002; Natchev et al., 2007). They lack multicellular glands on the tongue and on the palate: an extensive, sticky or lubricating mucus film is unnecessary under water. Tetrapods living and feeding on land use two main strategies for food uptake: jaw prehension (ingestion) and lingual prehension because of the low density and viscosity of air (Bramble and Wake, 1985; Bels et

al., 1997; Summers et al., 1998; Weisgram et al., 2001). Fully terrestrial turtle species practice lingual prehension (Weisgram et al., 1989; Wochesla¨nder et al., 1999, 2000) and have a well-developed, beefy and papillated tongue with many well-developed mucus glands (Nalavade and Varute, 1976; Iwasaki, 1992; Iwasaki et al., 1992, 1996; Beisser et al., 1995, 1998, 2001, 2004), which are also numerous on the palate (Fahrenholz, 1937; Kochva, 1978). Semiaquatic turtles are generalists that can feed both in terrestrial and in aquatic environments (Bels et al., 1997; Summers et al., 1998). Their oral anatomical adaptations are intermediate between the former described extremes and depend mainly on the degree of tendecy to aquatic or terrestrial life (Winokur, 1988). Unfortunately, except for one short abstract (Heiss et al., 2007), no (micro-) anatomical description of the palatal mucosa of a semiaquatic turtle species is available in the literature. Therefore, the present study describes the ultrastructural and histological features of the palatal mucosa of the semiaquatic turtle species *Cuora amboinensis*. It uses scanning electron microscopy and light microscopy and compares these results with those described previously for the oral (and whenever possible palatal-) mucosa of other turtles and (sauropsid) tetrapods. In addition, we discuss the functional meaning of certain microanatomical structures and combine some of them with experimental observations done using high-speed video.

Materials and Methods

The Malayan box turtle, *Cuora amboinensis* (Daudin, 1802), is a well known and abundant species from Tenasserim, Thailand, Cambodia, Vietnam, Malaysia and Indonesia. This species, although a poor swimmer, inhabits ponds, marshes, and flooded rice paddies. It can also be found far away from water, which reflects the semiaquatic lifestyle. It is reported to be herbivore, but in captivity it becomes omnivorous and quite voracious (Pritchard, 1979). Two female and two male *C. amboinensis* were used in the present study. The adult animals were obtained commercially with body weights from 657g to 967g. In captivity, they were kept in a 250 liter tank with ¼ land and ¾ water, and a 12 h dark / 12 h light cycle. The turtles were fed with vegetables, fish and earthworms.

For investigation, the animals were anesthetized by intraperitoneal injection of a lethal dose of sodium pentobarbital and decapitated. Immediately after decapitation, the entire roof of the oral cavity was removed and immersed in fixative solution.

For SEM, two palates were fixed overnight in modified Karnovsky-solution (Karnovsky, 1965) (2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer). After rinsing in 0.1M cacodylate buffer, samples were postfixed in buffered 1% osmium tetroxide at 37 °C for 2 h. Then, samples were treated with 25% HCl at 60 °C for 15 min in order to remove the mucus from the surface. This procedure was followed by dehydration in a graded ethanol series, HMDS (hexamethyldisilazane) drying and gold coating in the Sputtercoater AGAR B7340. The palates were observed in a Philips XL-20 scanning electron microscope.

For light microscopy, samples were fixed in buffered 4% formaldehyde overnight. After washing out the formaldehyde with tap water, the samples were partly decalcified with EDTA (ethylenediamine tetraacetic acid). This procedure was followed by dehydration in a graded ethanol series and embedding in methylmetacrylate resin (Merck). Every 200 µm, ten 5 µm thick cross sections were made on a Jung Reichert K-microtome for hard tissues in order to document the changes in the epithelial structures along the anterior-posterior-axis. After resin removal, the sections were stained with Toluidine blue, Giemsa-solution and PAS-Alcian blue (Plenk, 1989). Observations and photographic documentations were made under a Nikon Eclipse 800 light microscope.

To demonstrate the correlation between the distribution of taste buds and prehension mode, we used selected kinematogrphic frames, generously provided by Patrick Lemell and Robert Wochesländer. For kinematographic studies, *C. amboinensis* and *Testudo hermanni* were filmed while feeding on common turtle-pellets from pet trade. Feeding events in *C. amboinensis* were recorded at 250 frames per second using a NAC color H-S-V-1000 high-

speed videorecorder. Feeding events in *T. hermanni* were recorded using an ARRI-16 SR camera at 25 frames per second.

Results

SEM

General Structure of the Epithelial Surface

The topology of this turtle's palate is shown in Fig.1. The anterior (praechoanal) part is flat, with ridges and epithelial flaps only around and between the inner openings of the choanae. Here, the epithelium is more or less keratinized. Posterior to the choanae, only the mucosa next to the rhamphotheca (upper part of the horny beak) is keratinized, while the remaining part is not keratinized and also flat, sometimes showing openings of intraepithelial mucus glands.

Partially removing the covering mucus film reveals the keratinized parts of the anterior palate: the hexagonal to round keratinocytes become visible. They lack microvilli and cilia, but show microplicae, typical maze-like-shaped structures on their surface (Fig. 2A and Fig. 2B). Microplicae are rougher on the heavily than on the lightly keratinized cells (compare Fig. 2A and 2B). The outline of the apical surface of typical non-keratinized epithelial cells is hexagonal (Fig. 3A), most of these cells showing a multiplicity of microvilli (Fig. 3B, C). Cilia, clearly distinguishable by their larger size from microvilli, are often located at the openings of the intraepithelial glands next to the choanae (Fig. 3B).

Taste buds (TB) are spread all over the palate, but are most concentrated in the anterior, praechoanal part (Fig. 3D-F). In the keratinized epithelium, TBs can be recognized by the TB-microvilli and the keratinocytes, which lack microvilli and surround the taste pore (Fig. 3D). Within non-keratinized epithelium, TBs can be recognized by the typical surrounding small epithelial cells, forming the taste pore, and by their longer and sometimes branched microvilli arising from the apices of the TB-cells (Fig. 3E).

Light Microscopy

General Structure of the Mucosa

The epithelial layer of the palatal mucosa of *C. amboinensis* consists of a stratified epithelium (Fig. 4A, B) and the underlying lamina propria with the periosteum of the bony roof of the oral cavity (Fig. 4A). At the interface between epithelium and connective tissue lies a distinct, 0.5-1µm thick basement membrane. This interface can be flat and regular (Fig. 4B, C), or irregular due to single connective tissue papillae (compare Fig. 4A with Fig. 4C).

The epithelium is stratified and squamous (Fig 4B), cuboidal or columnar (Fig. 5A). It consists of three cell layers: the basal layer, the intermediate layer (subdivided into basal intermediate and superficial intermediate), and the superficial layer (Fig. 4B). The cells of the basal layer are large, rounded or cuboidal with a large round to oval nucleus in the center; they directly overlie the basement membrane. The basal intermediate cell layer is composed of 2-3 more or less oval cells. The cells of the superficial intermediate layer are flat and pass into the superficial cell layer. The cells of the superficial layer are highly specialized and vary in shape. They are flattened (Fig. 4A and 4C), cuboidal or columnar (Fig. 5A, B). Gender-related dimorphisms were not found within this species.

Keratinized Epithelium

Apart from the heavily keratinized rhamphotheca, keratinization of the turtle's palate is also visible in the anterior, praechoanal region and in the lateral parts. The epithelium is interdigitized with connective tissue papillae, except in heavily keratinized regions (Fig. 4C). The basal cells show mitotic activity and exhibit distinct cytoplasmatic protrusions where they interdigitate with the basal lamina (Fig. 4D). In the intermediate layer, cells have a greater periplasmatic percentage and show spinous processes. Heavily or lightly keratinized keratinocytes are present in the superficial layer (Fig. 4B and Fig. 4C). The thickness of this keratinized layer varies among the epithelial regions (compare Fig. 4B and Fig. 4C) and is usually thicker in the lateral and anterior parts, close to the rhamphotheca.

Secretory Cells and Glands

Secretory cells are distributed all over the palate epithelium, but largely lacking in the anterior, praechoanal part, where keratinized cells are prevalent. Where secretory cells are prevalent, the epithelium is no longer stratified and squamous, but mostly stratified and columnar. Active secretory cells appear as morphologically unspecific but PAS-positive, superficial cuboidal epithelial cells, or as specialized either PAS-, AB-, or as both PAS- and AB-positive goblet cells (Fig. 5B). Typical goblet cells are bottle-shaped with granular contents, and an irregularly shaped nucleus lies at the basal cell pole (Fig. 5A, B). Goblet cells arise from the intermediate cell layer of the epithelium, but reach the surface. Multiple goblet cells are sometimes organized into glandular cell-fields (Fig. 5A). These goblet cell-fields (or intraepithelial glands) can be folded into crypts with large openings into the oral cavity (Fig. 5B). Single glandular crypts are located in the area between the choanae and in the lateral

part, next to the rhamphotheca; they are also present in the postchoanal region. In the postchoanal palatal mucosa, the density of single goblet cells, goblet cell-fields and glandular crypts clearly increases.

Taste Buds

Taste buds (TBs) in *C. amboinensis* occur principally in regions of stratified, lightly keratinized squamous epithelium of moderate to greater thickness. They are rare within secretory epithelia and absent on heavily keratinized surfaces. TBs extend over the whole thickness of the oral epithelium (Fig. 6), consist of specialized epithelial cells and are easily distinguishable from the surrounding cells by their vertically clustered slender cells.

TBs are distributed in various concentrations all over the palatal epithelium, but are most dense in the anterior, praechoanal part of the turtle's palate (25-42 TBs per mm²). Fewer TBs are present in the region between the inner choanae and rhamphotheca; the lowest concentration is in the area between the choanae and in the postchoanal part, where secretory cells are prevalent. The only TB-type in this species is a barrel- to onion-shaped structure consisting of 30 to 60 specialized, slender epithelial cells. The nuclei of the TB-cells are located mainly in the basal half to two-thirds of the TB. While the descending cell processes contact the basement membrane, the slender cytoplasmatic processes of the apical region reach freely with their microvilli into the tastepore (Fig. 6).

Lamina propria

Based on morphology and different staining properties, the lamina propria of the turtle's palate can be divided into two different tissue layers: a loose, cell-rich superficial layer underlying the epithelium, and a deeper, more fibrous layer (mucoperiosteum) connecting with the periosteum of the bony roof of the oral cavity (fig. 4A). After AB-PAS-staining, the superficial layer stains PAS-positive, and the deeper layer AB-positive.

The superficial, loose connective tissue layer incorporates Mucosal-Associated Lymphoid Tissue (MALT) with inflammatory cells (lymphocytes, occasional plasma cells, and mast cells). It also contains blood vessels and nerve fibers embedded in the differently staining ground substance. Like the epithelial layer, the lamina propria shows regional variations in the proportion of its elements.

In the deeper lamina propria, larger arteries run parallel to the surface, giving rise to vessels to the superficial layer, where they form an extensive capillary network. These capillary vessels

run immediatelly subjacent to the basal epithelial cells, especially in the connective tissue papillae.

Preliminary Kinematographic Studies

Kinematographic analyses of terrestrial feeding events of *C. amboinensis* show the first contact with the food item to occur in the jaws (Fig. 7A, B). In contrast, the tortoise *Testudo hermanni* uses its tongue for food prehension and the first contact with the food takes place there (Fig. 8).

Discussion

The term palatal mucosa describes the oral integument, comprising both epithelium and the underlying lamina propria. All the structures of the epithelium and the lamina propria act as a functional unity, promoting secretion, immunological response, and metabolism, and also have important sensory and mechanical properties. These functions, in combination, play a key role in the feeding system and help determine an organism's adaptation success in the environment. Parsons (1968) described the gross anatomy of the palatal mucosa and compared the choanal structures of over 4,000 specimens representing most of the recognized genera of living turtles. Parsons found certain morphological relationships between families and described, for the genus *Cuora*, flaps and ridges around the choanae; these structures were also found in the present study. Weisgram et al. (1989) reported massive ridges in areas surrounding the choanae in the tortoise *Testudo hermanni*. The authors supposed that these ridges play a role by holding back the first piece of food when the tongue is extended out of the mouth to seize a second piece. In *C. amboinensis*, these structures were less prominent than described for *T. hermanni*. We, therefore, hypothesize that such palatal ridges are more essential for terrestrial feeding turtles. The palatal epithelium in *C. amboinensis* is stratified squamous or cuboidal, with three different cell layers. According to Iwasaki et al. (1996) and Beisser et al. (2004), a basal layer, an intermediate layer (subdivided into basal intermediate and superficial intermediate), and a superficial layer can be described in the present study. However, in this turtle's palatal epithelium, the distinction of the intermediate layer into a basal and a superficial one is not always possible. This finding is due to the varying thickness of the epithelial cell layers and the type of epithelia visible at the limited resolution of light microscopy. In the present surface investigations, higher SEM magnification reveals the round to hexagonal shape and maze-like microplicae of the squamous keratinocytes. These microstructures are very similar to the cell surface features on the keratinized crocodylian palate (Putterill and Soley, 2003) and seem to have mechanical and mucus adherence functions. On the other hand, the surface of the nonkeratinized cylindric or cuboidal cells is studded with microvilli. Microvilli on oral epithelia have been described for tetrapods by many authors (e.g., Meyer and Prutkin, 1974; Iwasaki, 1990, 1992, 1996, Iwasaki et al., 1992, 1996, 1997; Beisser et al., 1995, 1998, 2001, 2004). Their function is clearly to amplify the cellular surface to adhere the mucus-film produced by secretory cells. Keratinization in the oral epithelium of tetrapods is a common and well-known phenomenon (Berkhoudt, 1985; Schwenk, 1985; Toubeau et al., 1994; Iwasaki et al., 1997; Schwenk, 2000a; Putterill and Soley, 2003; Squier and Finkelstein, 2003). The thickness of the keratinized layer varies

within the palatal epithelium of *C. amboinensis*. In mechanically stressed regions, such as the areas directly medial to the rhamphotheca and the praechoanal part, the keratinized layer is very thick and hard. Only in these areas with heavily keratinized epithelium is the basal cell layer interconnected with the underlying basal membrane by cytoplasmic protrusions. This provides stronger mechanical adhesion to the lamina propria. Keratinization in the oral epithelium clearly serves as a mechanical protection for the soft epithelium. Even if oral keratinization is common within tetrapods, it specially evolved within turtles (and birds). The sharp, cornified rhamphothecae replace the teeth – lacking in turtles – and play an important role in prey capture and food manipulation (Pritchard, 1979; Ernst and Barbour, 1989), as well as in breeding behavior and individual defense.

The present study revealed single secretory cells, flat glandular fields and glandular fields folded into crypts in the palatal epithelium of the semiaquatic *C. amboinensis*. These relatively shallow and nonbranched crypts lie intraepithelially between the choanae, in the postchoanal area and laterally along the rhamphotheca. Compared with other reptiles (Fahrenholz, 1937; Kochva, 1978; Schwenk, 2000a) these crypts are rare. In or close to the intraepithelial glandular openings, especially near to the choanal area, lie ciliated cells. Ciliated cells are also very dense on the choanal epithelium. They probably function in distributing and moving the mucus film (Spungin and Silberberg, 1984; Gheber and Priel, 1989). Cilia are clearly distinguishable from microvilli based on their larger size. The first appearance of oral secretory cells and glandular tissue in vertebrates was probably connected with the emergence of amphibians onto land and with the need for a lubricating substance to help catch and swallow prey in the new environment. Fahrenholz (1937) reported the structure of oral glands in amphibians to be the most primitive, with sauropsid glands representing the next step in complexity and mammalian glands being the most highly evolved form. Kochva (1978), referring to Fahrenholz (1937), suggested that oral glands in pretetrapods and primitive tetrapods were mainly represented by single secretory cells, differentiated in the oral epithelium. First, secretory granules appeared in the stratified epithelium and, second, the single secretory cells (usually of the goblet type) developed into organized glandular fields. These glandular fields can be folded into crypts, extending into deeper areas. Such extended and branched glands open through several excretory ducts (polystomatic glands) or through a single duct (monostomatic glands). The latter represent the most complex form. The oral glands of reptiles, like those of mammals, show the entire range of glandular developement (Fahrenholz, 1937; Kochva, 1978). Those authors reported that the palatal glands are well developed among tortoises in *Testudo* sp., *Gopherus* sp. and

Geochelone sp. Palatal glands were described as small tubular mucus glands that form glandular fields both anterior and posterior to the choanae. In the aquatic turtles, however, palatal glands were described as being absent or poorly developed (Weisgram et al., 1989). The classification of the single secretory cells within reptiles is difficult and disputed within the literature (e.g., Fahrenholz, 1937; Nalavade and Varute, 1976; Kochva, 1978). The present histological staining reactions enabled the distinction between PAS-positive, AB-positive and both AB- and PAS-positive goblet cells. Carbohydrate histochemistry suggests that AB-positive cells contain acidic mucus with sialoglycoproteins exhibiting carboxyl and sulfate-ester groups. The both PAS- and AB-positive cells contain lightly acidic mucus and (sialo-) glycoproteins, and the PAS-positive cells produce only neutral mucosubstances containing hexose and sialicacid. The acidic mucosubstances represent the viscous (sticky) part, and the neutral mucosubstances the more fluid part of the mucus. Proteins, usually strongly associated with carbohydrates, probably liquefy the viscous mucus to create the optimal consistence of the mucus film. The main function of the mucus film covering the oral epithelium is probably to lubricate the oral epithelium to protect it from dehydration and to improve food ingestion, oropharyngeal transport and swallowing (Bramble and Wake, 1985). Mucus may also capture olfactory and gustatory molecules and make them available for olfactory sensitive cells and taste buds (Berkhoudt, 1985). Another important role of the mucus film is to defend the delicate epithelium against noxious influences of prey and damage from acidic or alkaline substances (Fahrenholz, 1937). According to the latter author, the protective function against pathogens is mainly mechanical. The bacteriocidal effect of reptile mucus is disputed within the literature (Kochva, 1978). In conclusion, the structural organization of the epithelium, described in the present study of *C. amboinensis*, may represent a secondary simplification of glandular oral epithelial tissue due to previously aquatic to semiaquatic feeding.

Taste buds serve as a chemosensory organ for closely related chemical stimuli (Schwenk, 1985; Berkhoudt, 1985; Manteifel et al., 1992; Berkhoudt et al., 2001). Experimental studies on sauropsid feeding have shown that unpalatable prey is rejected only after prey has been attacked or held in the jaws (Schwenk, 1985; Berkhoudt, 1985; Berkhoudt et al., 2001). Berkhoudt (1985) reported that the sense of taste encourages the ingestion of nutrients, discriminates between the available food items and possibly enables avoidance of toxic items. In the natural environment, the negative response (rejection) may be crucial for the individual; the biological benefit of avoiding harmful food is self-evident. While TBs in mammals are mainly found on the tongue epithelium and are there restricted to specialized fungiform, circumvallate and folliate papillae (e.g., Murray, 1973; Jackowiak and Godyniki, 2007), this

seems not to be true for other tetrapods (Schwenk, 2000a). The presence of TBs, but not their local distribution (except Schwenk, 1985 for iguanid lizards), has been described in the oral epithelium of all major sauropsid groups: turtles (Korte, 1980; Uchida, 1980; Weisgram et al., 1989; Beisser et al., 1995, 1998, 2001, 2004), crocodiles (Bath, 1905; Putterill and Soley, 2003), lizards (Schwenk, 1985, 2000c; Iwasaki, 1990; Iwasaki and Kobayashi, 1992; Young, 1997; Herrel et al., 1998), snakes (Kroll, 1973; Young, 1997; Berkhoudt et al., 2001), and birds (Berkhoudt, 1985, 1992; Rubega, 2000; Song et al., 2007). Most sauropsids seem to have at least three chemosensory systems: vomeronasal, olfactory and gustatory. Within turtles, the vomeronasal organ is strongly reduced and poorly investigated. The main chemosensory systems are thus olfactory and gustatory. While the first one operates over large distances, responding to chemicals of higher volatility, the latter acts only when a potential food item is in the mouth; the animal's response can then be either further oral processing or rejection (Schwenk, 1985). While Berkhoudt (1985) described three different TB-types in birds, the reptile TBs reportedly represent a common TB-type: the barrel- or onion-shaped one (Uchida, 1980; Korte, 1980; Schwenk, 1985). Berkhoudt (1985) reported avian TBs to be highly associated with glandular openings. We cannot confirm this localization for our species. TBs were found in the entire palatal mucosa of *C. amboinensis* – the highest density in the anterior, praechoanal part. To relate our (micro-) anatomical findings of the palate to turtle feeding behavior, we used significant frames from kinematographic studies. We analyzed terrestrial feeding events to demonstrate the possible correlation between TB-distribution and the prehension mode in our semiaquatic turtle. *C. amboinensis* uses jaw prehension for food uptake on land (Fig. 7A, B) and a similar approach under water (Natchev et al., 2007). The first contact to the food item occurs usually in the tip of the jaws. Because of the scissor-like construction of the rhamphothecae, the lower jaw presses the prey against the very anterior part of the palate during jaw closing (Fig. 7B). Therefore, we propose that the feedback response of the TBs, which are notably concentrated in the praechoanal area, strongly impact further feeding behavior (''eat it or leave it''). Schwenk (1985) described the highest TB-density on the tip of the tongues in iguanid lizards. They practice lingual food prehension (Delheusy et al., 1994; Schwenk, 2000c; Meyers et al., 2002; Schwenk and Rubega, 2005; Schaerlaekan et al., 2007). Thus, the tongue tip is the area of first food contact. Accordingly, the oral region that first contacts food apparently incorporates the highest TB-distribution. Lingual prehension is also found within chelonians, for example, *Testudo hermanni* (Wochesländer et al., 1999) (see also Fig. 8). A focus of further investigations might be whether the distribution and density of lingual TBs is

correlated with lingual food prehension in purely terrestrial turtles. The lamina propria can clearly be divided into two layers based on different staining properties in *C. amboinensis*. The relatively thin superficial layer is composed of loosely arranged reticular and collagen fibers embedded in a ground substance containing glycosaminoglycans and serum-derived proteins (Junqueira et al., 1998). This layer stains PAS-positive. The more fibrous underlying tissue stains AB-positive because of the localization of proteoglycans and hyaluronic acids. The lightly PAS-positive interface lies at the border between these two layers in the present study. The fibers and the ground substance of the connective tissue are produced by the fibroblasts. Fibroblasts are more abundant in the superficial layer of the lamina propria than in the underlying layer. The defense role of mucosal lymphoid connective tissues in the organism is related to phagocytic (e.g., macrophages, neutrophils) and immunocompetent cells (lymphocytes, etc.), as well as to the cells that produce antibodies or immunoglobulins (plasma cells), which are important in modulating inflammation (Junqueira et al., 1998). We also found lymphatic follicles within the superficial lamina propria, as described by Bianchi et al. (1990) in the esophageal and intestinal lamina propria of turtles, but less frequently. Functionally, the described layers have different fiber arrangements, chemical components and densities to protect the superficially lying epithelium from mechanical stress during prey capture or oropharyngeal food transportation. The loosely arranged collagen fibers of the superficial lamina propria are embedded in the highly hydrated ground substance and stain PAS-positive. Thus, the superficial part of the lamina propria acts like a pillow and enables deformation, reducing the pressure acting on the epithelium. The deeper layer, in turn, is tougher. This mucoperiosteum contains abundant proteoglycans to help stabilize the palatal form, including the above-described choanal flaps and ridges. The epithelium is interdigitized with connective tissue papillae, except in heavily keratinized regions. Villiform processes in the pharyngeal and palatal epithelium, which improve gas exchange under water – as described by Yokosuka et al. (2000) for the aquatic turtle *Trionyx sinensis japonicus* – were not found in the present study. However, because the lamina propria is highly vascularized, a minimum gas exchange under water cannot be excluded.

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Figures

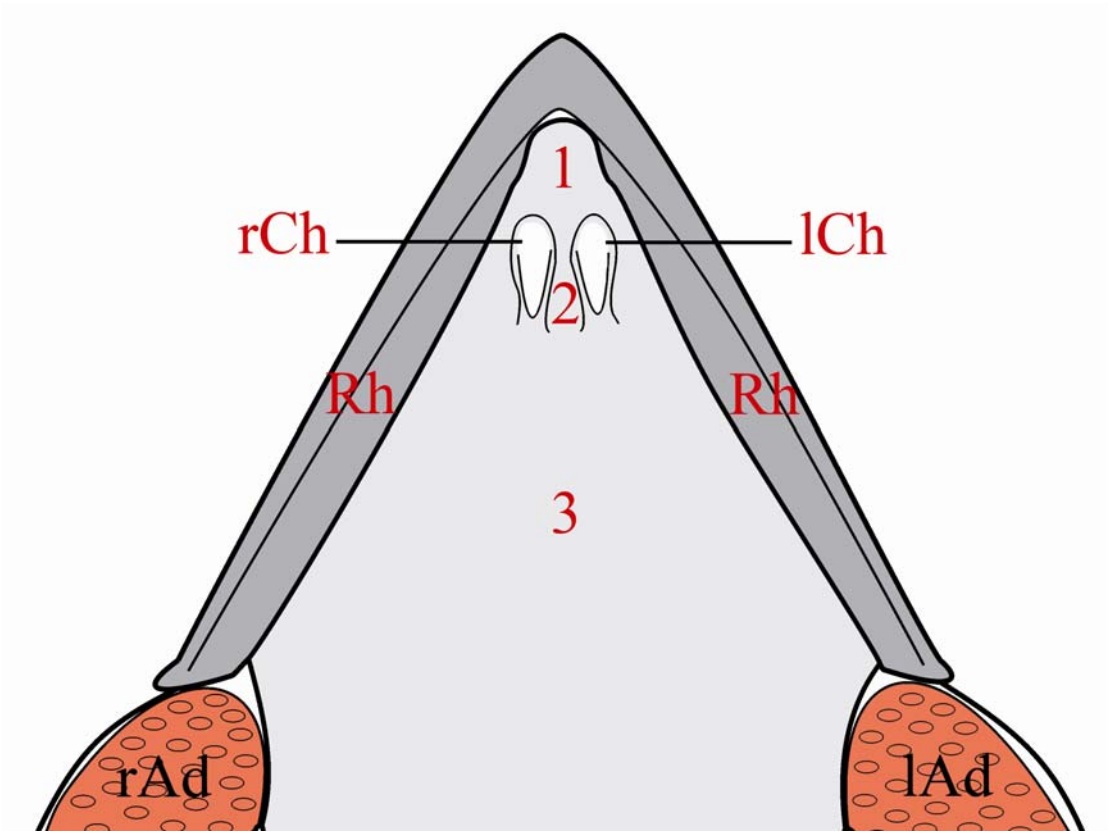

Fig. 1. Schematic drawing showing the topology of the turtle's palate from ventral after removal of the lower jaw. For that purpose, both Mm. adductores mandibulae (rAd, right M. adductor mandibulae; lAd, left M. adductor mandibulae) are cut horizontally. rCh and lCh, right and left choana; Rh, upper rhamphotheca (upper part of the horny beak). 1, praechoanal region; 2, interchoanal region, 3 postchoanal region.

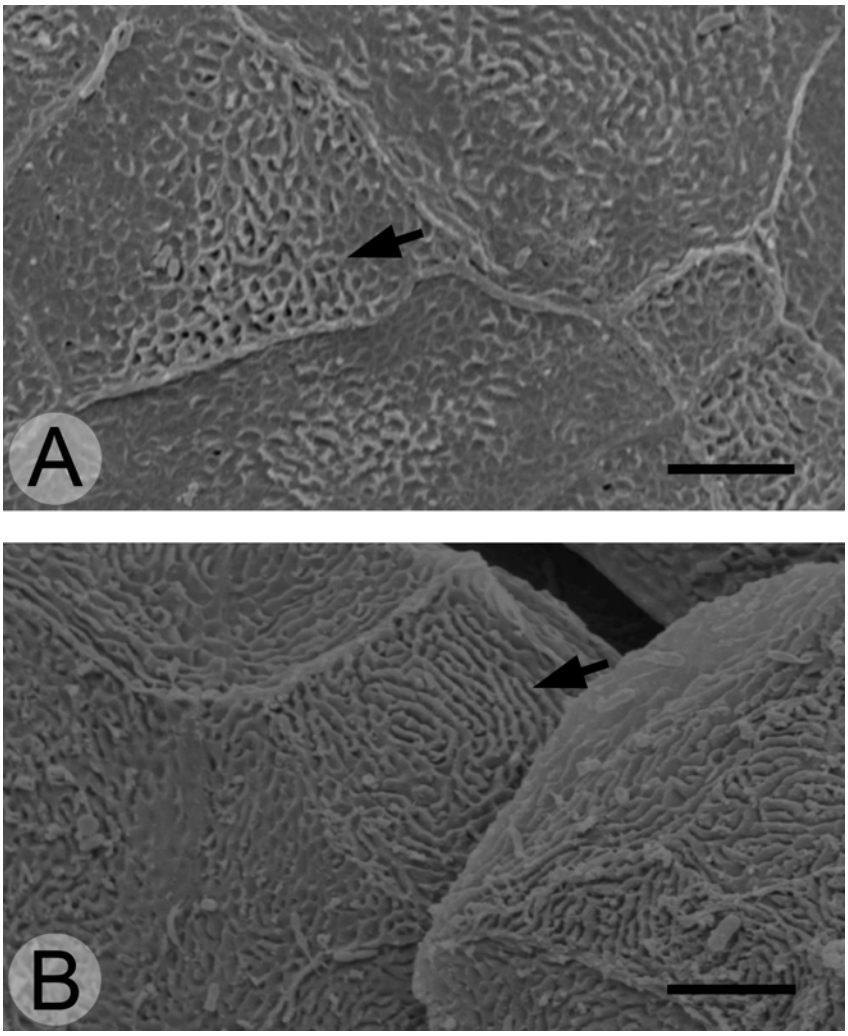

Fig. 2. Scanning electron micrographs of keratinized epithelial cells. A: Surface of lightly keratinized cells. B: Surface of heavily keratinized ones. Note difference in the relief of the microplicae (indicated by arrows) between lightly (A) and heavily (B) keratinized cells. Scale bars = 5 μm.

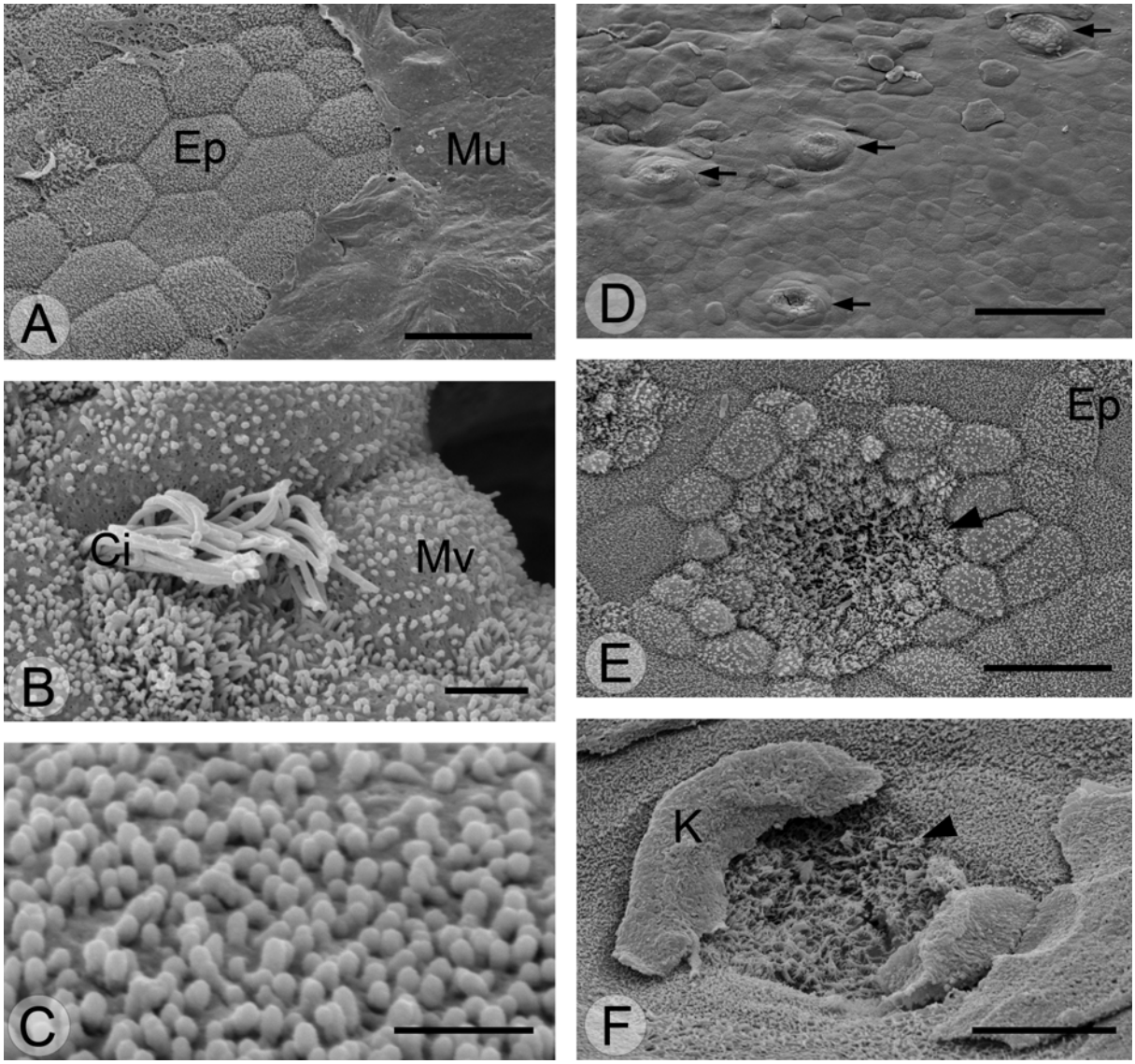

Fig. 3. Scanning electron micrographs of the epithelial surface. A: Hexagonal epithelial cells (Ep) studded with microvilli. The mucus film (Mu, on the right) covers the epithelial surface and is here partly removed (left). The cilia (Ci) in B are clearly distinguishable from microvilli (Mv) by their larger size. C: Palatal epithelial surface at high magnification. Note the abundant microvilli. D: Abundant crater-shaped taste buds (arrows) in lightly keratinized (top left) and nonkeratinized (bottom right) epithelium. E: Taste bud of the nonkeratinized palatal epithelium (Ep). Note the typical, relatively small cells surrounding the taste pore (arrowhead) which are absent in F, and the larger size of taste bud microvilli versus normal epithelial microvilli. F: Detail taste bud surface in a keratinized region. Note the keratinocytes (K) surrounding the taste pore (arrowhead). Scale bar = 10 μm in A, E, F, 2 μm in B, 1 μm in C, 50 μm in D.

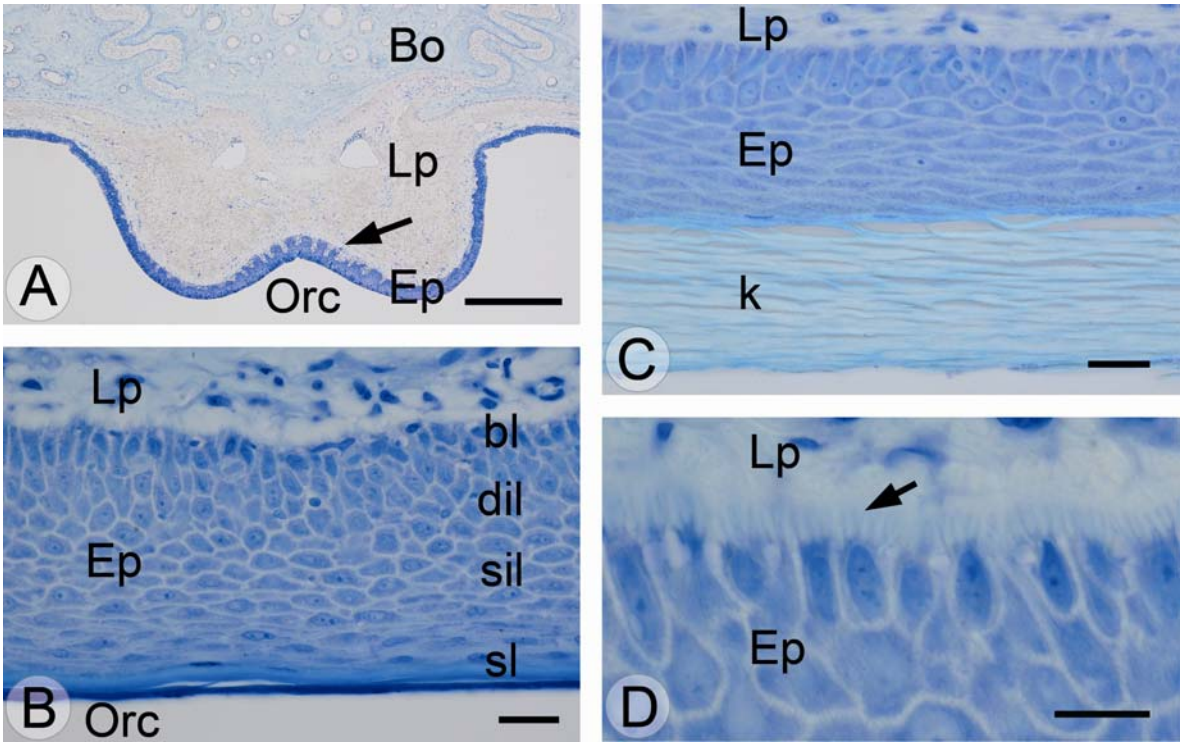

Fig. 4. Light micrographs of cross-sections of the turtle's palate. A: General structure of the interchoanal roof of oral cavity consisting of a bony- (Bo) proprial- (Lp) and epithelial (Ep) layer. The latter is interdigitized with connective tissue papillae (arrow). Orc, oral cavity. Giemsa-staining. B: Organization of the lightly keratinized, stratified squamous epithelium. Note the lack of connective tissue papillae, which are visible in A. Ep, epithelium; Lp, lamina propria; Orc, oral cavity; bl, basal layer; dil, deep intermediate layer; sil, superficial intermediate layer; sl, superficial layer. Toluidine blue-staining. C: Comparatively thin epithelial stratum with a very thick keratinized layer (k). Note the lack of nuclei there. Ep, epithelium; Lp, lamina propria; Orc, oral cavity. Toluidine blue-staining. D: Basal region of the heavily keratinized epithelium. The cytoplasmatic protrusions of the basal epithelial cells (arrow) improve adhesion and are only found in regions of heavy keratinization. Ep, epithelium; Lp, lamina propria. Toluidine blue-staining. Scale bar = 500 µm in A, 20 µm in B, C, 10 µm in D.

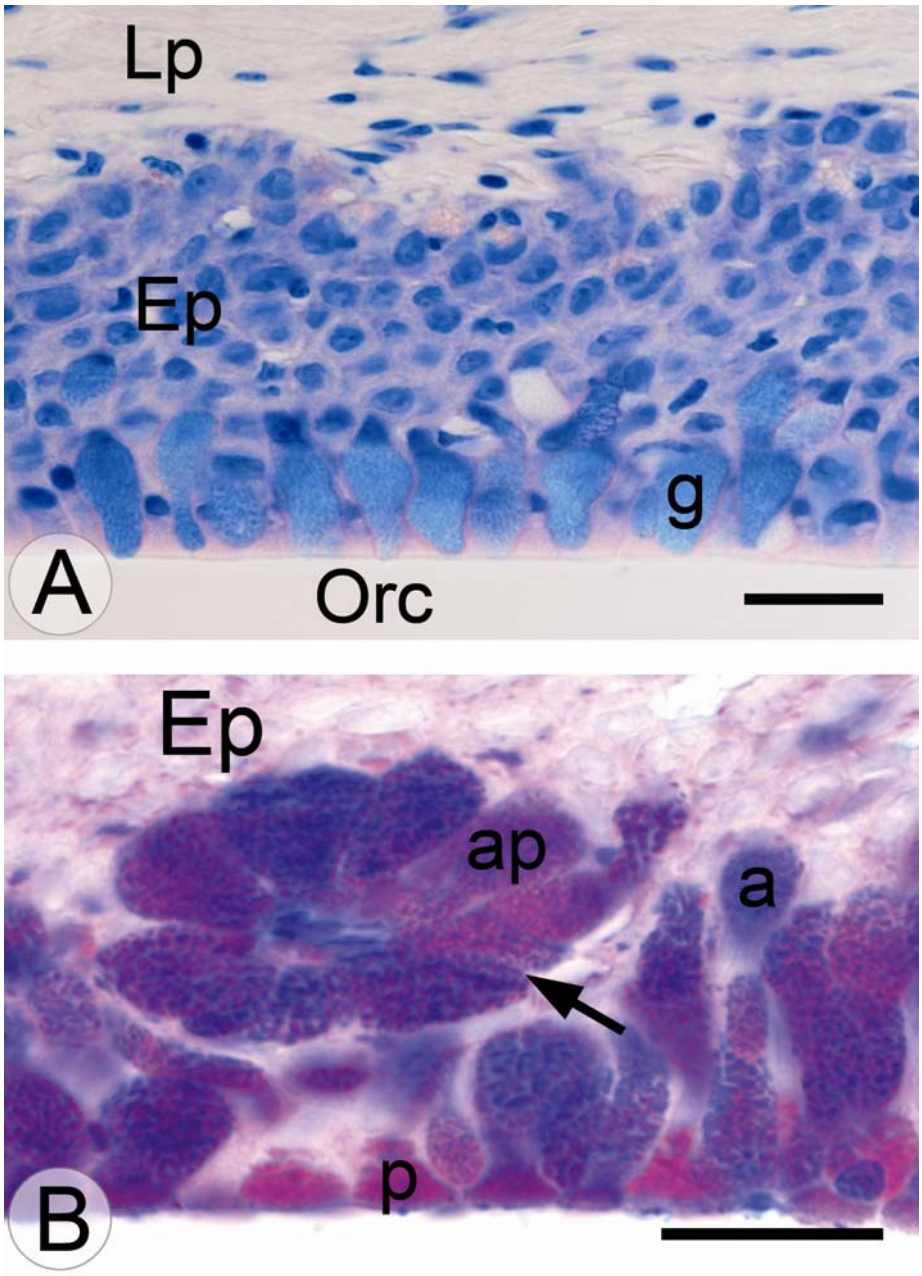

Fig. 5. Light micrographs of the turtle's palatal epithelium in a postchoanal region. A: Goblet cell field organized as a stratified columnar epithelium. The goblet cell field itself lies amongst stratified cuboidal epithelium. Ep, epithelium; g, goblet cell; Lp, lamina propria; Orc, oral cavity. Giemsa-staining. B: An intraepithelial glandular crypt (arrow) which communicates with the oral cavity. Note the three types of goblet cells shown by different staining properties: The AB-positive cells (a, blue), the PAS-positive cells (p, pink) and the both AB- and PAS-positive cells (ap, purple/blue). Ep, epithelium. AB-PAS-staining. Scale bar = 20 μm in A, 10 μm in B.

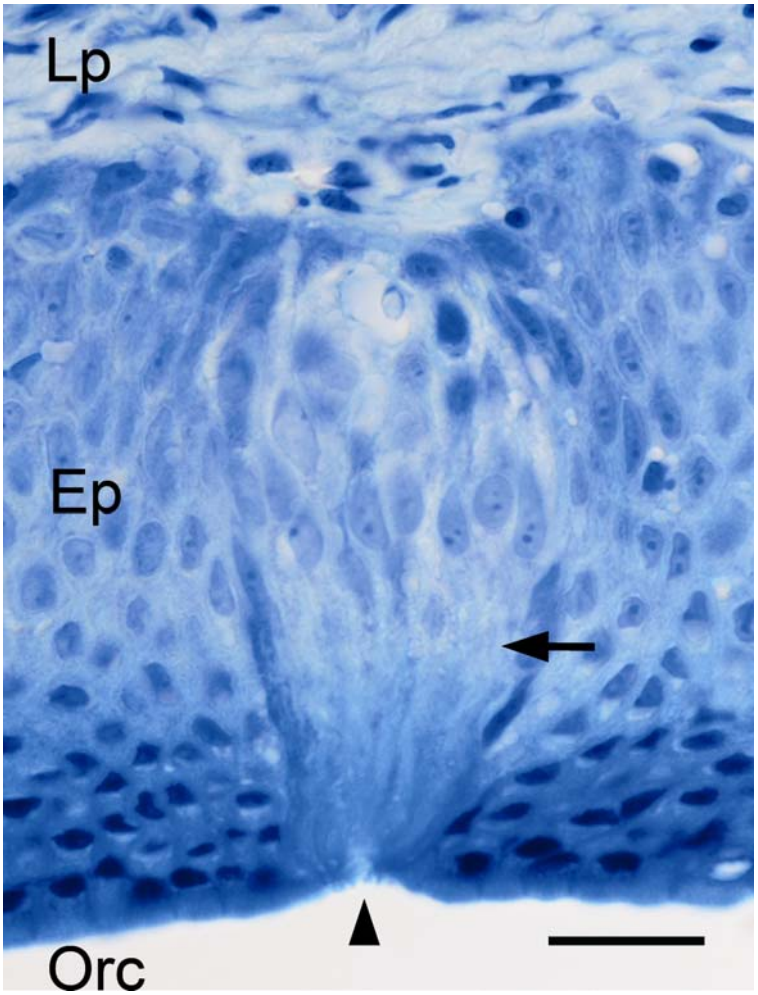

Fig. 6. Light micrograph of a typical taste bud. Note the nuclei in the basal two thirds and the slender cytoplasmatic processes (arrow) in the superficial third of the taste bud. The arrowhead indicates the taste pore. Ep, epithelium; Lp, lamina propria; Orc, oral cavity. Toluidine blue-staining. Scale bar = 20 μm.

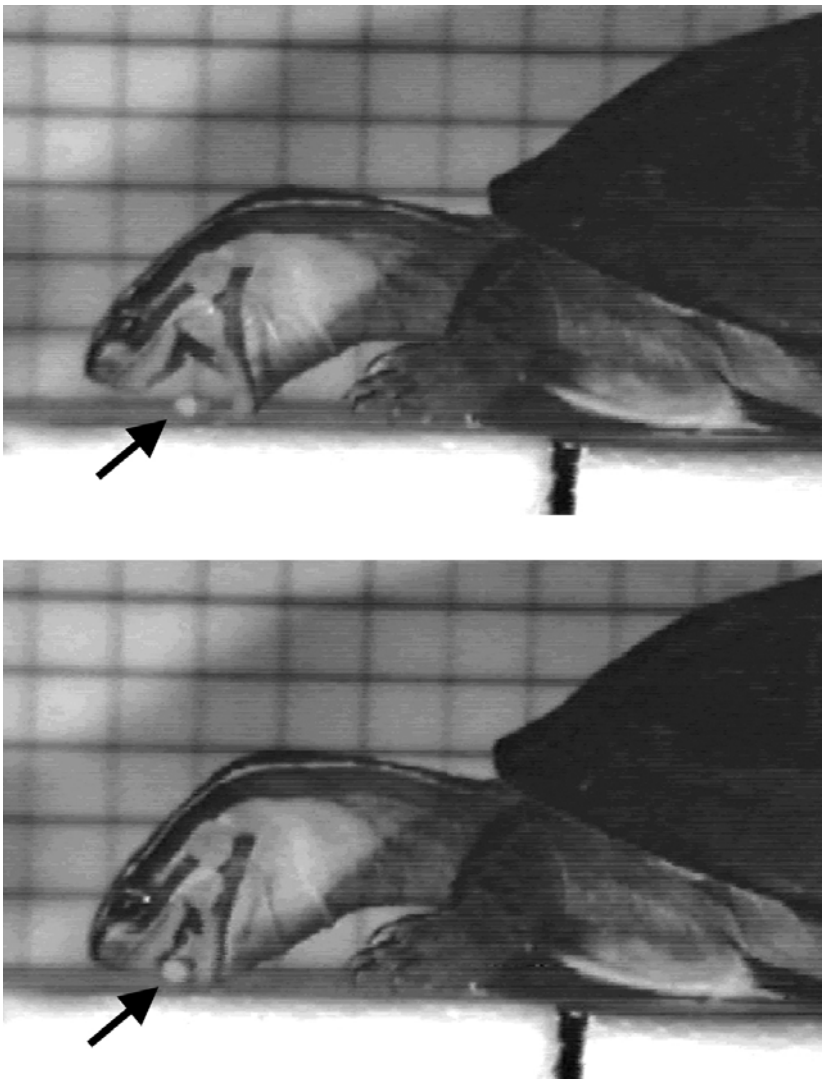

Fig. 7. Two single frames of a high-speed video recording showing *C. amboinensis* grabbing a food pellet (arrow) by the jaws (jaw prehension) on land.

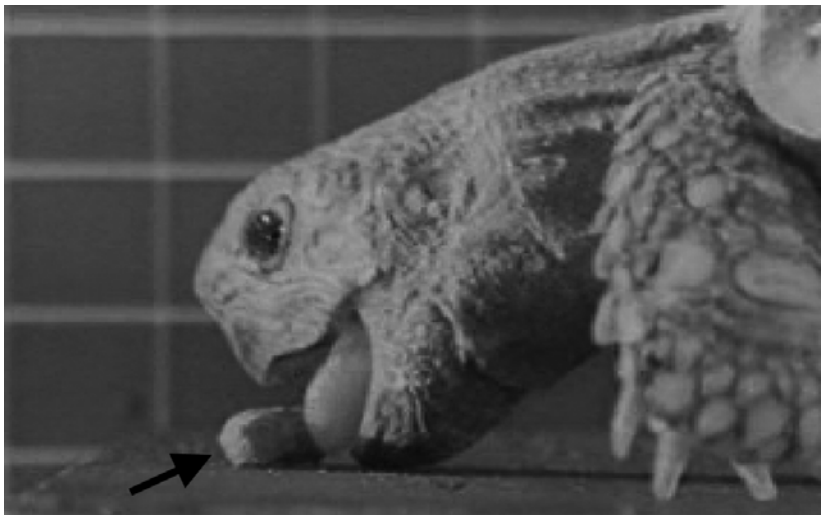

Fig. 8. A single frame of an analogue recording showing the tortoise *T. hermanni* grasping a food pellet (arrow) by its beefy tongue (lingual prehension).

2.4. Analysis of prey capture and food transport kinematics in two Asian box turtles, *Cuora amboinensis* and *Cuora flavomarginata* (Chelonia, Geoemydidae), with emphasis on terrestrial feeding patterns.

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Abstract

This study examines the kinematics and morphology of the feeding apparatus of two geoemydid chelonians, the Malayan (Amboina) box turtle (*Cuora amboinensis*) and the yellow-margined box turtle (*Cuora flavomarginata*). Both species are able to feed on land as well as in water. Feeding patterns were analysed by high-speed cinematography. The main focus of the present study is on the terrestrial feeding strategies in both Asian box turtles, because feeding on land has probably evolved *de novo* within the ancestrally aquatic genus *Cuora.* During terrestrial feeding (analysed for both species), the initial food prehension is always done by the jaws, whereas intraoral food transport and pharyngeal packing actions are tongue-based. The food uptake modes in *Cuora*s differ considerably from those described for purely terrestrial turtles. Lingual food prehension is typical of all tortoises (Testudinidae), but is absent in *C. amboinensis* and *C. flavomarginata*. A previous study on *Terrapene carolina* shows that this emydid turtle protrudes the tongue during ingestion on land, but that the first contact with the food item occurs by the jaws. Both Asian box turtles investigated here have highly movable, fleshy tongues; nonetheless, the hyolingual complex remains permanently retracted during initial prey capture. In aquatic feeding (analysed for *C. amboinensis* only), the prey is captured by a fast forward strike of the head (ram feeding). As opposed to ingestion on land, in the underwater grasp the hyoid protracts prior to jaw opening. The head morphology of the investigated species differs. In contrast to the Malayan box turtle, *C. flavomarginata* exhibits a more complexly structured dorsal lingual epithelium, a considerable palatal vault, weaker jaw adductor muscles and a simplified trochlear complex. The differences in the hyolingual morphology reflect the kinematic patterns of the terrestrial feeding transport.

Introduction

Terrestrial feeding has evolved several times among vertebrates in connection with the shift from water to land: e.g. Actinopterygii *vs.* Tetrapoda (Sponder and Lauder, 1980; Van Wassenbergh et al., 2006); terrestrial feeding also probably evolved independently within turtles (Summers et al., 1998). The stem turtles were terrestrial organisms, but the common ancestor of all living forms lived in fresh water (Joyce and Gauthier, 2004; Scheyer and Sander, 2007).

Aquatic feeding in chelonians is well studied (Bramble, 1978; Weisgram, 1985; Bels and Renous, 1991; Lauder and Prendergast, 1992; Bels et al., 1997; Lemell and Weisgram, 1997; Van Damme and Aerts, 1997; Bels et al., 1998; Summers et al., 1998; Lemell et al., 2000; Aerts et al., 2001; Lemell et al., 2002). Two main mechanisms for underwater food uptake and transport have been recognised so far – compensatory suction and inertial suction (*sensu* Van Damme and Aerts, 1997; Aerts et al., 2001).

In contrast to aquatic feeding, only few studies treat the issues of terrestrial feeding kinematics in turtles (Bels et al., 1997; Summers et al., 1998; Wochesländer et al., 1999, 2000, Bels et al., 2008). There are two methods of food uptake on land: lingual and jaw prehension (see Bels et al., 2008). The terrestrial intraoral food transport in tortoises is lingual-based. Highly stereotypical, coordinated hyolingual and jaw movements convey the food to the pharynx (Wochesländer et al., 2000). A slow jaw opening phase prior to reaching peak gape is present in all terrestrial turtles (Bels et al., 2008).

Our study focuses on species that are able to complete the whole feeding process, including ingestion (initial prey capture), processing, transport and swallowing (see Schwenk, 2000), on land and in water. Our general question was how both investigated Asian box turtles handle the different physical requirements of the feeding media (high density and viscosity of water compared to air; Gans, 1969). In water the food items are buoyant, but when feeding on land, the turtles have to handle the weight of the prey. If the tongue is not large or movable enough to ensure the lingual prey fixation in the buccal cavity during jaw opening, the food cannot be transported (terrestrial "inertial" food transport is not found in chelonians). Laboratory experiments on the Mexican mud turtle, C*laudius angustatus,* confirm that prey caught on land can be processed only by using hydrodynamic mechanisms, when the head is put below the water level (Weisgram, 1985). Bramble and Wake (1985) also reported that some aquatic chelonians can take food on land but are unable to handle the items and to transport them through the oral cavity.

Among living chelonians, species with terrestrial habitat preferences are found only within the three testudinoid families: Emydidae, Testudinidae and Geoemydidae. The Malayan box turtle and the yellow-margined box turtle belong to a subtaxon within the geoemydid genus *Cuora* (see Stuart and Parham, 2004). *Cuora amboinensis* is closely related to water, but *Cuora flavomarginata* is a "terrestrial omnivorous generalist" (see Winokur, 1988). Within the superfamily Testudinoidea the form of the skull is closely correlated with ecological factors, but "phylogeny seemed to constrain only localized features of the skull and remained of minor influence …" (Claude et al., 2004). Considering the diversity of habitat and food types exploited by the different *Cuora* species, we propose that the construction of the hyolingual complex and the jaw adductors can also vary considerably within the genus. A comparative morphological investigation of the head should answer how the environmental shift is reflected by the "Bauplan" of the feeding apparatus in the investigated species.

The common ancestor of the testudinoids was an aquatic form (see Joyce and Gauthier, 2004, for overview and cladogram). Currently, within Testudinoidea, species exist that feed terrestrially (all tortoises), aquatically (most emydids and geoemydids), or both (few emydids and geoemydids). We have analysed the terrestrial feeding kinematics in two geoemydid chelonians to compare their food prehension modes to those described for tortoises and for the semi-terrestrial turtle *Terrapene carolina*. All testudinids studied to date obligatorily use lingual food prehension (Wochesländer et al., 1999; Bels et al., 2008). Jaw prehension is typical for turtles that feed in water. *Terrapene carolina* belongs to a primitively aquatic clade within Emydidae (see Summers et al., 1998). Terrestrial food uptake in this species involves the jaws (Bels et al., 1997). The genus *Cuora* is ancestrally aquatic (see Claude et al., 2003 for overview). We propose that the Asian box turtles, analogous to *T. carolina*, use jaw prehension when feeding on land. Nonetheless, we expect kinematic differences in the prey capture modes of emydids and geoemydids because terrestrial feeding presumably evolved secondarily within these groups.

The present study is also designed to determine the differences in the strategies used for aquatic and terrestrial feeding in *Cuora amboinensis*. Some semi-aquatic turtles are able to modulate their feeding kinematics in response to prey capture in different media (Summers et al., 1998). We predict the same for the food transport.

Material and methods

Both of the Southeast-Asian box turtles examined in this study, *Cuora amboinensis* (Daudin, 1802) and *Cuora flavomarginata* (Grey, 1863), live in semi-aquatic habitats and are omnivorous. *Cuora amboinensis* prefers shallow, stagnant or slow-flowing waters, although adult animals also wander far from any water source. In the literature these turtles are regarded as primarily herbivorous in the wild (Ernst and Barbour, 1989). In captivity, *C. amboinensis* is highly carnivorous and feeds even on fast-swimming prey such as frogs and fish. The home range of *Cuora flavomarginata* includes southeast China, Taiwan and the Ryukyu Islands of Japan. This species is less closely associated with water than *Cuora amboinensis*. It has been found in rice paddies and in forested hills (Ernst and Barbour, 1989).
The animals used for the present study were kept in glass aquariums at 25 °C constant temperature with 12h/12h light/dark photoperiod and were fed vegetables (bananas, cucumbers, apples, tomatoes, etc.), small invertebrates (mealworms, snails and land worms), but also fish and pieces of liver.
Anatomical observations were made on three *C. amboinensis* specimens – two females and one male (carapace lengths 16.4, 15.9, and 16.9 cm) – and three female *C. flavomarginata* specimens (carapace lengths 16, 16.5, and 17.2 cm.). The animals were killed by intraperitoneal injection of a lethal dose of sodium pentabarbitol. Immediately after death the skin was removed and the morphology of the skeletal elements and the cranial, hyoidal and cervical muscles were investigated using a Wild M5A dissecting microscope. The anatomical research focused on the structures involved in feeding. For both species we investigated the "Bauplan" of the cranium, the hyolingual complex and the head musculature. The description of the musculature and the tendon systems is based on the terminology of Schumacher (1973).
The tongue, part of the oesophagus and the trachea were fixed using Bouin's solution for histological investigations. The material was embedded in paraffin and the tongue was sectioned longitudinally. For light microscopic observation, the sections (8 μm) were stained with Azan after Heidenhain and were observed and photographed using a Nikon Eclipse E800 light microscope.

High-speed videos

Four subadult *Cuora amboinensis* and three subadult *Cuora flavomarginata* were used to investigate terrestrial feeding. The animals were filmed in lateral view in an empty aquarium (size 40 cm x 16 cm x 25 cm) with a reference grid (1 cm x 1 cm) in the background using a NAC-1000 high-speed video system at 250 frames per second. We offered the food items

(one by one, or arranged in rows) on the bottom of the aquarium in front of the animals. From each specimen, a minimum of 15 feeding events were filmed. The food items were pellets, mealworms, earthworms and bananas. A total of 36 video sequences of *C. amboinensis* and 27 of *C. flavomarginata* feeding on pellets or small mealworms were selected for qualitative and quantitative analyses. We detected two different transport modes here termed as "prey positioning" and "lingual transport". We quantified their occurrence by recording the number of times each individual utilised a pure form of prey positioning or lingual transport and calculated the mean percentage occurrence for each species. For our transport kinematics analysis we selected 36 sequences per transport type in *C. amboinensis* and 27 for *C. flavomarginata* (the same number as for prey capture for both species). The transport cycles in both investigated turtles could well be differentiated from "pharyngeal packing" cycles, because in pharyngeal packing the gape is quite modest and the head is slightly elevated.

For filming aquatic feeding, the aquarium was filled with 15 cm of water and the food items were suspended in the water on a thin thread in front of the animals. The turtles were fed with pieces of fish. Three specimens of *C. amboinensis* were filmed in lateral view (10 films per specimen), and 21 videos were selected for analysis. Our *C. flavomarginata* specimens refused to feed under water under the strong light needed for filming.

The videos were digitised using Adobe Premiere, and horizontal and vertical coordinates of specific anatomical landmarks (Fig. 1) were matched using an AVI- digitiser (© Peter Snelderwaard, University of Leiden, Leiden, The Netherlands). Based on the landmarks' displacement in the bi-directional level, we were able to calculate: a) the gape amplitude – distance between the tips of the upper and lower jaw; b) ventral hyoid movement – dorso-ventral height of the head at the level of the origin of the ceratobranchiale II; c) the anterior-posterior movements of the tongue – the distance between the tip of the tongue and the line connecting the tips of the upper and lower jaw; d) the extension and retraction of the neck – the distance between the point on the parietal surface of the cranium and the anterior tip of the carapace; e) the distance predator to prey – the distance between the centre of mass of the prey and the tip of the upper jaw; f) the maximal gape angle – the angle between the jaws at maximum gape. These data enabled us to calculate the duration of the different feeding stages, the time at which the peak gape was reached, the duration and the velocity of hyoid retraction, the delay between peak hyoid depression and peak gape, the time of pro- and retraction of the head, and the total duration of the cycles.

Results

Anatomy

In both species the skull is anapsid and akinetic. The temporal roof is ventrally and dorsally open due to emarginations (see Fig. 2). The temporal arch is complete and consists of three elements: os postorbitale, os jugale and os quadratojugale. An os quadratojugale was also found in *C. flavomarginata* (in contrast to Wermuth and Mertens, 1961 and Ernst and Barbour, 1989). No direct connection between the jugal and the quadratojugal bones was detected. The supraoccipital bone bears a crista supraoccipitalis. The dorsal ridge is straight and extends behind the foramen magnum occipitale in *C. amboinensis* (Fig. 2C). In *C. flavomarginata*, the crista supraoccipitalis is shorter, convex and has fenestrations (Fig. 2A). The jaw articulation, as typical of the Cryptodira, allows no lateral mandible movements. The trochlear process is formed only by the quadrate bones. The palate is flat in *C. amboinensis* but concave in *C. flavomarginata* (see also Heiss et al., 2008). The palatines are bright bony plates, they are robust in the Malayan box turtle but thin and transparent in *C. flavomarginata.* The hyoid is relatively large compared to cranium length and only partially ossified (see Fig. 3C, D). The hyoid corpus is square and lacks fenestrations. The lingual process is elongated, cartilaginous and elastic. The branchial horns I (CB I) are caudo-dorsally extended and ossified. The epibranchialia I are cartilaginous. On the origin of the cornu branchiale II (CB II) on the hyoid corpus there are two islands of ossification. The branchial horns II are shorter than CB I, and their caudal ends are strongly laterally divided in *C. amboinensis*. The hypoglossum (Fig. 3C, D) has one caudal and one rostral process. It is a relatively big (compared to *Testudo hermanni;* see Wochesländer et al., 1999) and flat cartilaginous plate without any ossifications. *C. flavomarginata* exhibits two typical fenestrations in the rostral part.

A schematic illustration of the most important jaw and hyoid muscles is shown in figure 3A and B. The m. adductor mandibulae externus fills the whole upper temporal fossa. The mm. add. mand. ext. pars superficialis and pars profunda are strongly developed in *C. amboinensis* (Fig. 2D) and relatively small in *C. flavomarginata* (Fig. 2B). The external tendon system is well developed and has a myovector-changing function (see Bramble, 1974; Jordanskii, 1990). *C. amboinensis* possesses a large transiliens bulge (3.4 ± 0.3 mm in diameter) within the external adductor tendon. The m. adductor mandibulae posterior has no insertion on the temporal bone, and we were unable to detect a margin splitting that muscle into caudal and rostral portions (see Schumacher, 1956). No clear morphological separation of the m. add. mand. internus into a pars pseudotemporalis and pars pterygoideus was possible.

The tongue in both species is developed differently. The lingual glands in *C. flavomarginata* are concentrated anteriorly on the origins of the high and slender lingual papillae. The lingual papillae are well vascularised, and the blood vessels form lacunae (Fig. 4). The tongue of *C. amboinensis* is smaller and the ridge-like lingual papillae are broader and shorter (Beisser and Weisgram, 2001). Lingual papillae on the ventral side of the tongue were found in *C. flavomarginata*.

Kinematics

Terrestrial feeding

In 83.3 % of our film sequences of *Cuora amboinensis*, the plastron contacted the bottom of the aquarium after the forwards locomotion of the body stopped. The limbs were stretched to the side (see Fig. 5B). Throughout the feeding process the animals "lay" on the ground. *Cuora flavomarginata* exhibited different behaviour. Its plastron never contacted the ground during ingestion (Fig. 6B).

The static phase of the "final head fixation" was taken as "time zero" for our kinematic analyses. All kinematic patterns during ingestion and oropharyngeal transport were analysed frame by frame (4 ms steps). Jaw opening is characterised by lower jaw depression, which coincides with neck protraction and head depression. No protraction of the hyoid complex was recorded. The gape opened gradually (Figs. 5A, 6A). During jaw opening, before reaching maximum gape, the lower jaw can contact the ground. This occurred in 100 % of the prey capture sequences of *C. amboinensis* (Fig. 5B) but only in 14.8 % of *C. flavomarginata*.

The peak gape was reached in 0.267 ± 0.041 s in *C. amboinensis* and in 0.360 ± 0.026 s in *C. flavomarginata*. Even at maximal gape amplitude (the maximal gape angle measured for *C. amboinensis* was 58°), the large tongue was not visible in lateral view (see Figs. 5B, 6B). The diameter in the larynx region of the neck increased. After reaching maximum gape the whole head rotated ventrally. During head rotation, the jaw amplitude remained constant. A static gape phase of maintaining the maximal jaw amplitude (here termed MG phase = maximal gape phase) was detected in all cycles of prey capture and oropharyngeal transport. The duration of the MG phase during ingestion was 0.068 ± 0.008 s in *C. amboinensis*, but shorter by almost half in *C. flavomarginata*: 0.042 ± 0.008 s. The gape closing was followed by a neck retraction and the hyoid was then lifted to its zero position.

In *Cuora flavomarginata* (number of transport cycles per food item: 5.22 ± 1.23) and in *C. amboinensis* (number of transport cycles per food item: 6.94 ± 2.47), we detected two

different transport pattern modes. In the first mode the lower jaw depressed gradually. Peak gape was reached in 0.148 ± 0.030 s in *C. flavomarginata* and in 0.169 ± 0.021 s in *C. amboinensis*. The neck remained motionless (Fig. 7) and was permanently retracted, which hindered precise observation of the hyoid movements. Discrete gape phases lacked prior peak gape. We have termed such transport cycles "lingual transport".

Symptomatic of the second detected mode was that the neurocranium and the neck did not remain static. After the food item was pressed to the upper jaw by the tongue, the lower jaw depressed slowly to approximately half the maximal gape. A static gape phase in which the jaw amplitude remained constant followed. The duration of that static phase was variable (see Tables 1 and 2), not only in the two species and the different specimens, but in some cases also between two successive transport cycles. In the next phase, the gape increased rapidly. The end of the fast jaw opening phase coincided with the start of a rapid retraction of the hyolingual complex (velocity in *C. amboinensis* V = 9.6 ± 1.4 cm/s). Almost simultaneously a neck extension occurred (Fig. 8A). The food item was moved forward and did not lose contact with the tongue. The MG phase was relatively short (0.018 ± 0.002 s) in *C. flavomarginata* and 0.024 ± 0.003 s in *C. amboinensis*). The MG phase was followed by rapid mouth closing (Tables 1 and 2). We recorded such transport cycles (up to 12 for some feeding events in *C. amboinensis*) as "prey positioning".

In *C. flavomarginata*, from a total of 141 transport cycles in 27 film sequences, 61.8 % were recognised as "lingual transport". In *C. amboinensis*, from a total of 250 transport cycles in 36 film sequences, 66.8 % were "prey positioning". *C. flavomarginata* used lingual transport immediately after prey capture in 7 of our sequences (n = 27), or after 1 to 4 (average = 2.00 ± 1.39) prey positioning cycles. In *C. amboinensis* (n = 36) we detected 4.64 ± 2.41 prey positioning cycles prior to the first lingual transport cycle. In only two sequences was lingual transport used directly after prey capture.

Aquatic feeding

Aquatic feeding modes were investigated only in *C. amboinensis*. Six kinematic variables are represented in Table 3. The turtles approached the food item slowly. When the tip of the upper jaw was at 1.32 ± 0.34 cm from the fish, the forward locomotion stopped. The body and the head remained static. The prey capture started with a hyoid protraction and lifting, followed by neck extension (Fig. 9A, B). Jaw opening started during the fast neck extension. Upon reaching peak gape, the jaws were moved over the prey. No division into different gape phases was detected during mouth opening. The extension of the neck continued after the

peak gape was reached. The gape remained for a maximum of 0.012 ± 0.002 s and, during this phase, the hyoid retraction started. Hyoid retraction lasted 0.042 ± 0.006 s. The peak ventral hyoid depression was reached during the jaw closing phase. Jaw closing phase was very short: 0.033 ± 0.004 s. During ingestion, the prey remained static. No inertial suction effect was detected (Fig. 9B2, B3). The first contact with the prey always occurred at the jaws. Due to the impulse of the fast forward strike of the head, the grasped food item moved in the same direction. Ingestion ended with head retraction. The gape could not be completely closed because the prey was not entirely in the oral cavity. The water volume taken up during prey capture was expelled by protracting the hyoid complex to its resting position.

The following cycle starts the transport phase (Fig. 10). The neck is retracted at the beginning of the cycle. The oropharyngeal transport was hydrodynamic and occurred without reduction; no crushing actions were recognised. The prey was freed shortly from the jaws (Fig. 10B2) during the mouth opening (duration: 0.028 ± 0.004 s). The time to peak gape was nearly twice as short as the same phase during prey capture. The end of jaw opening coincided with head protraction and hyoid retraction (duration: 0.048 ± 0.008 s). The rush neck extension and the abrupt hyoid depression help reposition the food deeper within the oral cavity. Hyoid depression reached its peak at the end of gape closing. In transport cycles where the prey was almost completely taken into the oral cavity, neck extension was smaller, but the hyoid depression was still considerable.

Discussion

Previous research has shown that feeding modes and head morphology are well correlated in turtles (Bramble, 1973; Claude et al., 2004). Although *C. amboinensis* feeds aquatically as well as terrestrially, our morphological study points to a predominantly aquatic lifestyle within this ecomorphological framework. The tongue, with short and broad lingual papillae, and the flat roof of the mouth cavity indicate inefficient food transport on land. The strongly developed jaw adductors, the large transiliens tendon bulge and the hard palatinal bones correlate with the feeding ecology and the diet of the species – the Amboina box turtles need to bite hard to hold elusive prey. The elongated and stilted hyoid horns support the enlargement of the anterior oesophagus in aquatic feeding with a bidirectional flow system (Lauder and Shaffer, 1986; Van Damme and Aerts, 1997; Aerts et al., 2001). Both investigated species have a relatively large hyoid complex to produce sufficient suction when feeding under water. Nonetheless, the elasticity of the anterior part of the hyoid body enables the tongue to be highly mobile. The tongue movements in *C. flavomarginata* are additionally facilitated by the considerable dorsal vault of the mouth roof.

The tongue morphology in turtles can vary considerably (Winokur, 1988; Weisgram et al., 1989; Iwasaki, 1992; Beisser et al., 1995, 1998, 2001, Beisser and Weisgram, 2001; Beisser et al., 2004). The tongue of specialised suction feeders such as *Chelus fimbriatus* is tiny and lacks lingual papillae or glands (Lemell et al., 2002). Terrestrial species possess a large, fleshy tongue with numerous large lingual papillae (Winokur, 1988). The secretion of the well-developed lingual glands in *C. flavomarginata* promotes oropharyngeal food transport on land. Most lingual glands are concentrated at the base of the anterior papillae, as opposed to *Testudo hermanni* (Wochesländer et al., 1999), whose glands are concentrated at the posterior surface of the tongue; this is also different from *Rhinoclemmys pulcherrima incisa*, where the lingual glands are distributed over the whole dorsal lingual surface (Beisser et al., 2004).

The geoemydids originated in the Eocene (Claude et al., 2003) as an aquatic turtle lineage and are considered to be a sister group of the testudinids (Claude et al., 2004) or the emydids (Joyce, 2007). In the two geoemydid species examined here, the tongue is not used for food uptake in terrestrial feeding, as opposed to tortoises (Wochesländer et al., 1999; Bels et al., 2008). Although *C. flavomarginata* and *C. amboinensis* possess highly mobile tongues that can be extended outside the oral cavity, initial prey capture is never tongue-based. We found no exceptions concerning the different food types. During mouth opening and the MG phase in ingestion, a strong depression rather than protraction of the hyoid complex occurs. We propose that this is correlated to the mechanism of gape opening. The simultaneous

contraction of the m. coracohyoideus, m. depressor mandibulae and m. branchiomandibularis, the low activity of the m. geniohyoideus, m. genioglossus and m. Intermandibularis, and the deep depression of the anterior tip of the head help explain the extraordinary hyoid position during the MG phase. Posterior fixation of the hyoid during jaw opening has not been reported for chelonians that are able to feed on land. Presumably the increased impact of the m. coracohyoideus in jaw opening promotes a larger gape. Bels et al. (1997) report a maximal gape angle of 40° in *T. carolina.* The maximum angle measured in *C. amboinensis* was almost 60°.

Descriptions of prey capture kinematics in emydids are restricted to a single species, *T. carolina* (Bels et al., 1997; Summers et al., 1998). As the Malayan box turtle and the yellow-margined box turtle, *T. carolina* uses jaw prehension when feeding on land. The kinematic patterns of the jaws and the hyolingual complex in both Asian box turtles diverge notably from those in *T. carolina*. As discussed above, hyoid protraction is absent in *Cuora*s. In *T. carolina* the hyolingual complex protracts prior to peak gape, and slow and fast jaw opening phases are determinable (Bels et al., 1997). Based on our results, we conclude that at least three different modes of terrestrial food uptake can now be recognised within the superfamily Testudinoidea: 1. Lingual prehension, which is obligatory in testudinids (Wochesländer et al., 1999, 2000; Bels et al., 2008); 2. Jaw-based prehension involving hyolingual protraction (found in the emydid *T. carolina;* Bels et al., 1997); 3. Prey capture via jaw prehension without hyolingual protraction (in the geoemydids studied here). It remains open whether any species exhibits facultative tongue prehension as stated by Bels et al. (1997) and Summers et al. (1998). Because of the limited information on the prey capture modes in emydids and geoemydids, we are unable to determine if there is a phylogenetic signal concerning the terrestrial food uptake kinematics in testudinoids.

As opposed to prey capture on land, in the underwater grasp the hyoid is protracted prior to jaw opening. The hyoid depresses considerably during prey capture. The anterior part of the oesophagus in *C. amboinensis* is distensible and serves as a reservoir for the swallowed water; still we were not able to detect inertial suction effects like in ingestion modes of *Chelodina longicollis* (Van Damme and Aerts, 1997) and *Chelus fimbriatus* (Lemell et al., 2002). *C. amboinensis* seems to be predominantly a ram-feeder using a similar aquatic prey capture strategy as *Chelydra serpentina* (see Lauder and Prendergast, 1992). As presented in table 4, the strike of the Malayan box turtle is slower than that of the specialised aquatic predators. It is faster than the strikes in the less specialised, purely aquatic, carnivorous forms *Pelusios*

castaneus (Lemell and Weisgram, 1997), *Malaclemys terrapin* and *Dermochelys coriacea* (Bels et al., 1998).

For terrestrial food transport, the kinematic patterns in tetrapods have been proposed to adhere to a generalised cyclic model (Bramble and Wake, 1985) of five phases: "slow open I" (SO-I), "slow open II" (SO-II), "fast open" (FO), "fast close" (FC) and "slow close-power stroke" (SC-PS). The terrestrial transport modes described in turtles to date correspond with the generalised cyclic model concerning gape, neck and hyoid kinematics (see Bels et al., 1997, Wochesländer et al., 2000; Bels et al., 2008). Discrete SO-I, SO-II and FO gape phases are well determinate. Fast jaw closing starts immediately after the peak gape is reached. Ventral head rotation starts at the end of SOII and the hyoid retraction begins early in the FO phase.

In *C. amboinensis and C. flavomarginata* we have detected certain deviations from the proposed conservative kinematic feeding patterns (see Alfaro and Herrel, 2001): during lingual transport, the neurocranium remains almost completely motionless. In contrast to tortoises (Bels et al., 2008), there is no clear kinematic distinction between SO and FO phases (Fig. 7A). We propose that food transport is effected predominantly by intrinsic lingual movements without major hyoid displacement. Such a transport mechanism is facilitated in *C. flavomarginata* because of the large, movable tongue and the well-developed dorsal lingual papillae. The gape kinematic profiles in prey positioning are almost similar to those predicted by the generalised cyclic model of Bramble and Wake (1985). The main difference is the occurrence of a static MG phase after fast opening. The hyoid and the neck kinematic patterns in prey positioning are quite different from those proposed by the cyclic model. The retraction of the hyoid complex starts at the beginning or during the static MG phase rather than at the beginning of FO, and is abrupt and rapid. The end of fast jaw opening is correlated with extension of the neck. The head and the food are moved forwards. Despite the rapid neck protraction, we cannot categorise the process as cranial inertial feeding (Bramble and Wake, 1985) because the food is accelerated together with the head (no inertial moment). The lack of posterior head movement immediately before neck extension does not allow the transport mode to be defined as “It”-type (*sensu* Elias et al., 2000) either. Prey positioning cycles seem to be used by the turtles to position the prey on the dorsal tongue surface. The further transport of the food item toward the oesophagus involves mainly lingual transport.

When feeding on fish, the aquatic food transport in *C. amboinensis* involves compensatory suction. Hyoid retraction starts shortly prior to reaching peak gape, or during the MG phase. The food displacement is promoted by forward movement of the head. There are certain kinematic similarities between the aquatic transport and terrestrial prey positioning in *C.*

amboinensis. The start of the neck extension, as well as the start of the hyoid retraction, correlate with the same gape phases. Nevertheless, the duration of all phases measured in aquatic transport is shorter than in terrestrial transport. The difference in the duration of the gape cycles is more than four-fold. In underwater transport the slow opening gape phases are absent.

Most of the measured parameters varied considerably. The food transport in the investigated *Cuora* species seems to be permanently modulated, fine-tuned and optimised by sensory feedback. The sensorimotor feedback impacts on prey capture are sufficiently studied for lower tetrapods (for references see Deban et al., 2001; Schaerlaeken et al., 2007). The existence of modulations in reptilian feeding transport is evidenced in lizards (Bels and Baltus, 1988; Schwenk and Throckmorton, 1989; Herrel and De Vree, 1999, Herrel et al., 1999; Schwenk, 2000; Herrel et al., 2001; Lappin and German, 2005; Ross et al., 2007) and snakes (Kardong and Berkhoudt, 1998) as well as in aquatic feeding in turtles (Lauder and Prendergast, 1992; Lemell and Weisgram, 1997; Bels et al., 1998).

The formation of high and slender dorsal tongue papillae, cranial flexure and a palatinal vault which are morphological features typical of tortoises are present in *C. flavomarginata* but almost absent in *C. amboinensis*. Our results therefore support the statement of Claude et al. (2004) that differences in the habitats correspond to important and rapid morphological changes within Testudinoidea.

Jaw prehension is obligate in both investigated species when feeding on land and in water. Typical of terrestrial prey capture in *C. amboinensis* and *C. flavomarginata* is the occurrence of a prolonged maximal gape phase (MG phases) and the absence of hyoid protraction. Such kinematic patterns have not been detected in other turtles to date. A topic for further investigation will be the range in which this prey capture mode is distributed within those geoemydids able to feed on land. The jaw, neck and tongue movements during terrestrial food transport are not stereotypical in the investigated *Cuora*s. In both Asian box turtles examined in this study, sensory feedback plays an important role in feeding, and the process itself is very flexible.

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Tables

Table 1. Mean ± standard deviation for nine kinematic variables (measured in seconds) from terrestrial feeding events of *Cuora flavomarginata*. SO-I = slow open I; SO-II = slow open II; FO = fast open; MG = maximal gape; FC = fast close.

Variable	Capture (N=27)	Lingual Transport (N=27)	Prey Positioning (N=27)
Duration of gape cycle	0.444 ± 0.046	0.242 ± 0.042	0.454 ± 0.086
Time to peak gape	0.360 ± 0.026	0.148 ± 0.030	0.414 ± 0.064
Duration of SO-I phase			0.096 ± 0.008
Duration of SO-II phase			0.264 ± 0.062
Duration of FO phase			0.036 ± 0.005
Duration of MG phase	0.042 ± 0.008	0.016 ± 0.002	0.018 ± 0.002
Duration of FC phase	0.042 ± 0.004	0.038 ± 0.004	0.040 ± 0.004
Duration of hyoid retraction			0.050 ± 0.004
Peak hyoid delay to end of MG phase			0.036 ± 0.008

Table 2. Mean ± standard deviation for nine kinematic variables (measured in seconds) from terrestrial feeding events of *Cuora amboinensis*. SO-I = slow open I; SO-II = slow open II; FO = fast open; MG = maximal gape; FC = fast close.

Variable	Capture (N=36)	Lingual Transport (N=36)	Prey Positioning (N=36)
Duration of gape cycle	0.429 ± 0.078	0.225 ± 0.022	0.510 ± 0.096
Time to peak gape	0.267 ± 0.041	0.169 ± 0.021	0.424 ± 0.088
Duration of SO-I phase			0.086 ± 0.009
Duration of SO-II phase			0.308 ± 0.066
Duration of FO phase			0.040 ± 0.004
Duration of MG phase	0.068 ± 0.008	0.020 ± 0.002	0.024 ± 0.003
Duration of FC phase	0.077± 0.012	0.045 ± 0.006	0.045 ± 0.005
Duration of hyoid retraction			0.048 ± 0.008
Peak hyoid delay to end of MG phase			0.038 ± 0.006

Table 3. Mean ± standard deviation for six kinematic variables (measured in seconds) from aquatic feeding events of *Cuora amboinensis*. MG = maximal gape; FC = fast close.

Variable	Capture (N=21)	Transport (N=21)
Duration of gape cycle	0.120 ± 0.015	0.073 ± 0.009
Time to peak gape	0.067 ± 0.014	0.028 ± 0.004
Duration of MG phase	0.012 ± 0.002	0.014 ± 0.002
Duration of FC phase	0.032 ± 0.004	0.031 ± 0.002
Duration of hyoid retraction	0.042 ± 0.006	0.048 ± 0.008
Peak hyoid delay to end of MG phase	0.024 ± 0.008	0.028 ± 0.012

Table 4. Comparison of gape cycle duration in prey capture of four aquatic feeding specialists: *Chelus fimbriatus, Chelodina longicollis, Dermochelys coreacea* and *Chelydra serpentina*; two aquatic generalists*: Pelusios castaneus* and *Malaclemys terrapin*; and the semi-aquatic generalist *Cuora amboinensis.* cr, crab; f, fish; m, pieces of meat; mf, mussel flesh.

Species	Prey capture cycle duration (s) Mean ± SD N=Number of feeding events	Prey type
Cuora amboinensis	0.120 ± 0.015 (N=21)	f
Chelydra serpentina	0.078 ± 0.002 (N=15)	f
Chelus fimbriatus	0.083 ± 0.020 (N=20)	f
Chelodina longicollis	0.110 ± 0.015 (N=8)	m
Malaclemys terrapin	0.300 ± 0.154 (N=10)	cr
Dermochelys coriacea	0.615 ± 0.196 (N=10)	mf
Pelusios castaneus	0.280 ± * (N=4)	f

Data from Lauder and Prendergast (1992), Van Damme and Aerts (1997), Bels et al. (1998), Lemell and Weisgram (1997); Lemell et al. (2002).

* No SD given by the authors (Lemell and Weisgram, 1997).

Figures

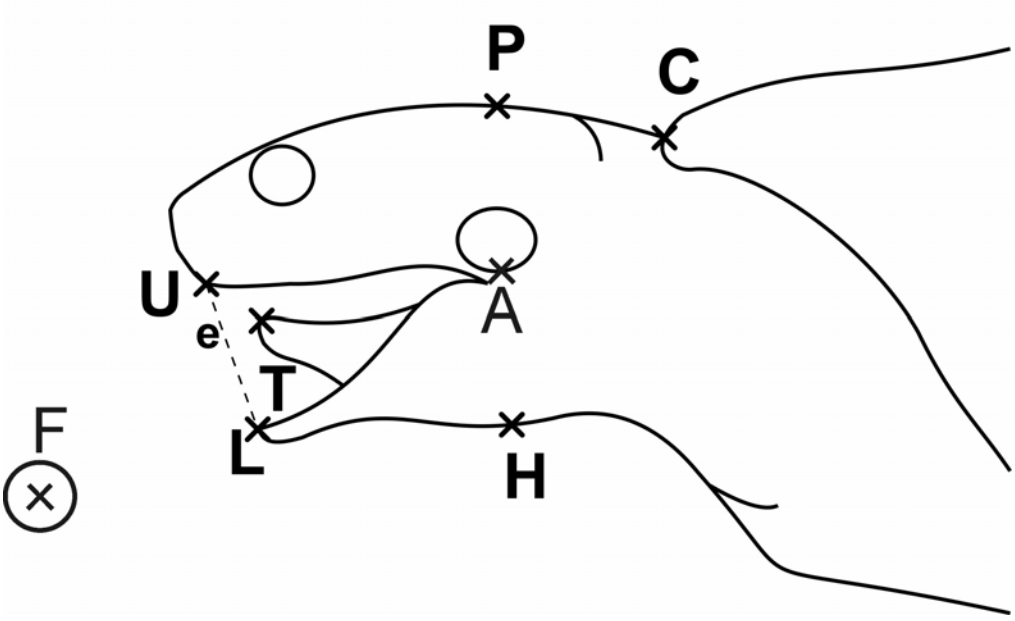

Fig. 1. Points used for kinematic analyses of feeding cycles of *Cuora amboinensis* and *Cuora flavomargina*ta. A, ventral margin of tympanum; C, anterior tip of carapace; F, centre of mass of the feeding items; H, hyoid; L, anterior tip of lower jaw; P, parietal bone; T, anterior tip of tongue; U, anterior tip of the upper jaw; e, imaginary line connecting U and L.

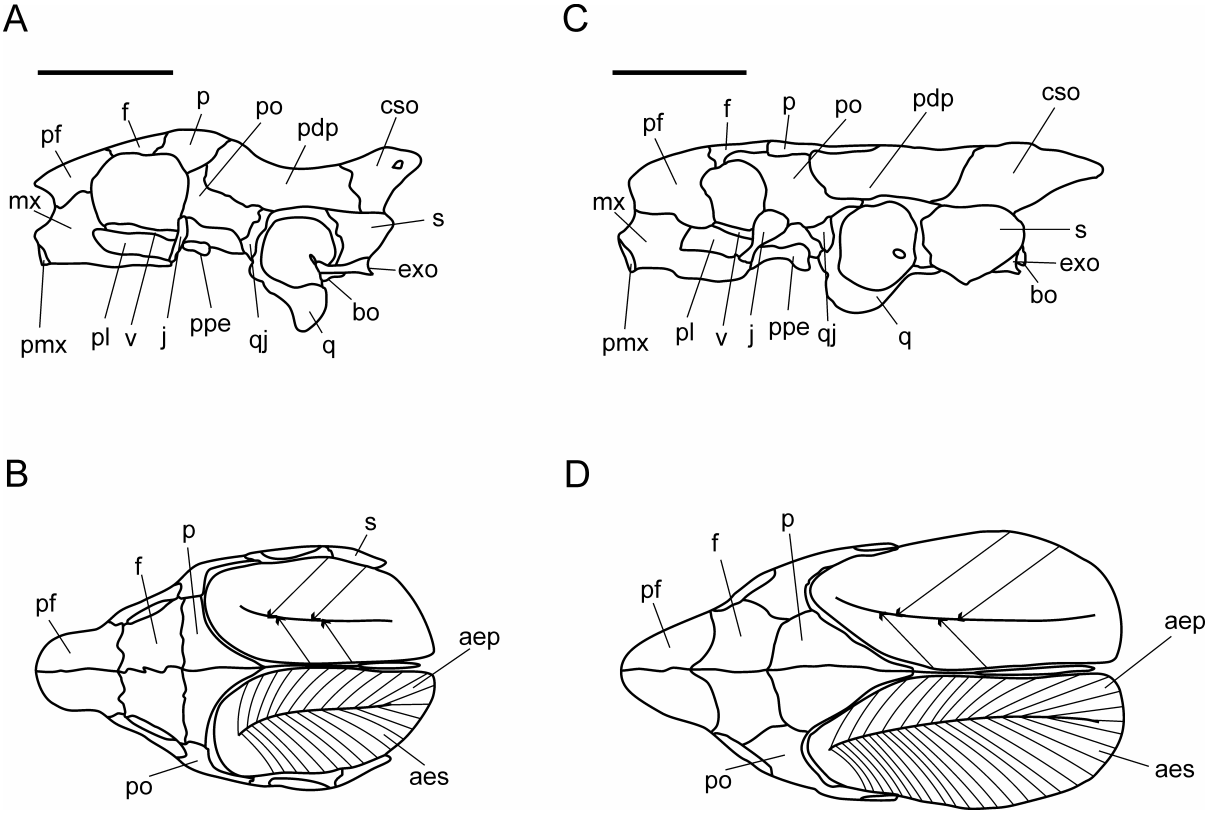

Fig. 2. Head morphology of *C. flavomarginata* and *C. amboinensi*s. (A) *Cuora flavomarginata,* cranium lateral view; (B) *Cuora flavomarginat*a, m. adductor mandibulae externus dorsal view; (C) *Cuora amboinensi*s, cranium lateral view; (D) *Cuora amboinensis*, M. adductor mandibulae externus dorsal view. Scale bar = 10 mm. aep, m. add. mand. externus pars profunda; aes, m. add. mand. externus pars superficialis; bo, os basioccipitale; cso, crista supraoccipitalis; exo, os exoccipitale; f, os frontale; j, os jugale; mx, os maxillare; q, os quadratum; qj, os quadratojugale; p, os parietale; pdp, processus descendens ossi parietale (dorsal part); pf, os praefrontale; pl, os palatinum ; pmx, os praemaxillare ; po, os postorbitale; ppe, processus pterigoideus externus (vertical plate); s, os squamosum; v, os vomer.

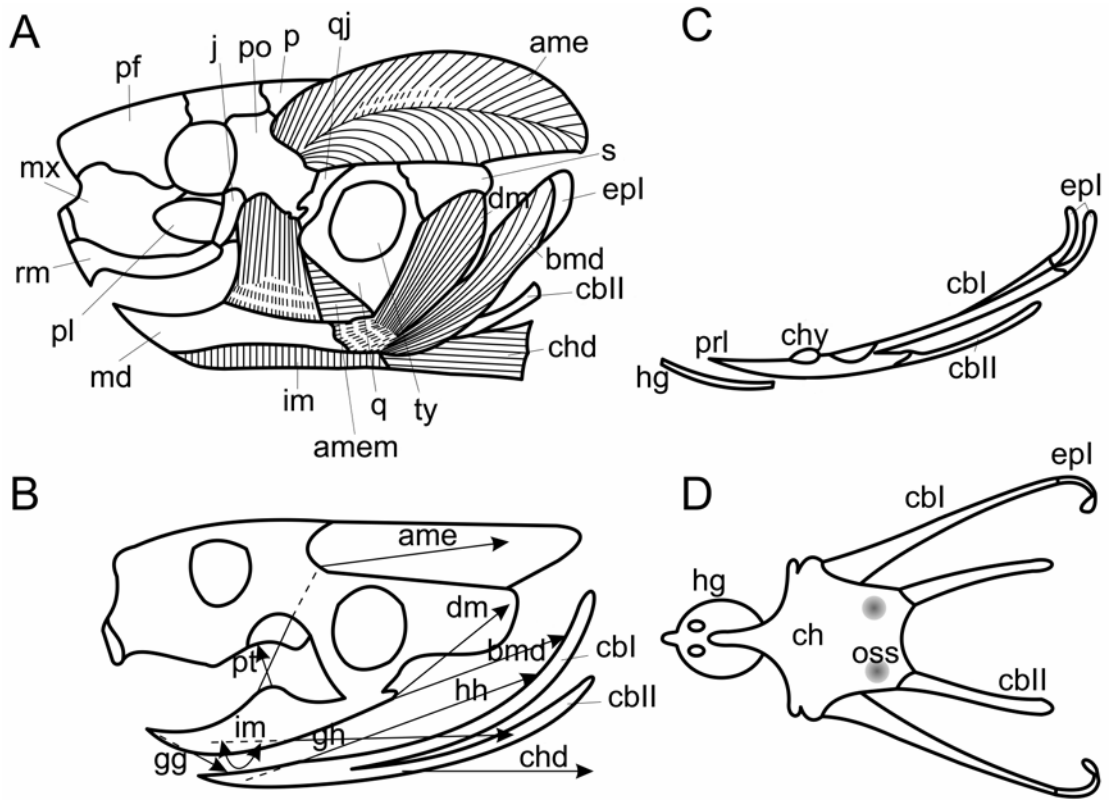

Fig. 3. *Cuora sp*., schematic illustration of head morphology. (A) Osteology and myology of the head; (B) direction of forces during contraction of the jaw and hyoid muscles; (C) hyoid and hypoglossum, lateral view; (D) hyoid and hypoglossum, dorsal view. ame, m. add. mand. externus; amem, m. add. mand. externus pars media; bmd, m. branchiomandibularis; cbI, cornu branchiale I; cbII, cornu branchiale II; ch, corpus hyoidei; chd, M. coracohyoideus; chy, cornu hyale; dm, m. depressor mandibulae; epI, epibranchiale I; gg, m. genioglossus; gh, m. geniohyoideus; hg, hypoglossum; hh, m. hyohyoideus; im, m. intermandibularis; j, os jugale; md, mandibula; mx, os maxillare; oss, ossifications on the hyoid corpus; p, os parietale; pf, os praefrontale; pl, os palatinum; po, os postorbitale; prl, processus lingualis hyoidei; pt, m. add. mand. internus pars pterygoideus; q, os qudratum; qj, os quadratojugale; rm, ramphotheca; s, os squamosum; ty, tympanum.

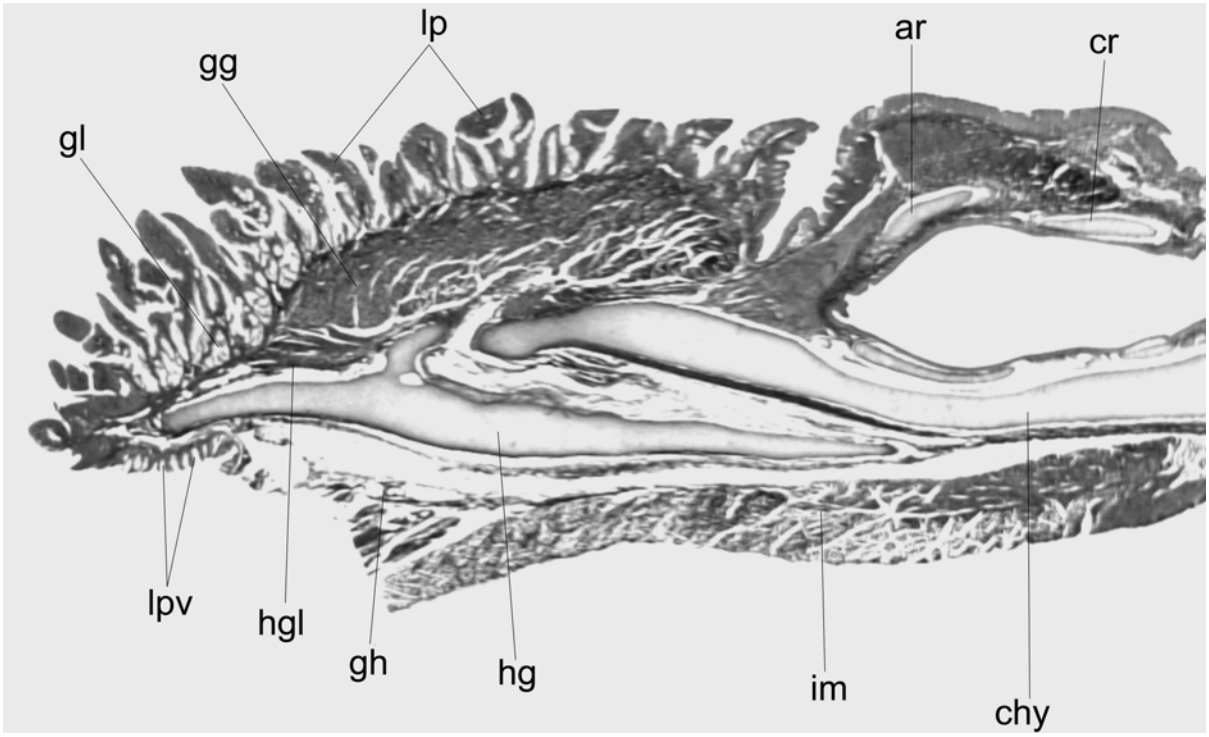

Fig. 4. *Cuora flavomarginata,* light micrograph; tongue and hyoid, median-longitudinal cut, 8 µm; Azan staining. ar, cartilago arytaenoidea; chy, corpus hyoidei; cr, cartilago cricoidea; gg, m. hypoglossoglossus; gh, m. geniohyoideus; gl, glandulae linguales; hg, hypoglossum; hgl, m. hyoglossus; im, m. intermandibularis; lp, lingual papillae (dorsal); lpv, lingual papillae (ventral).

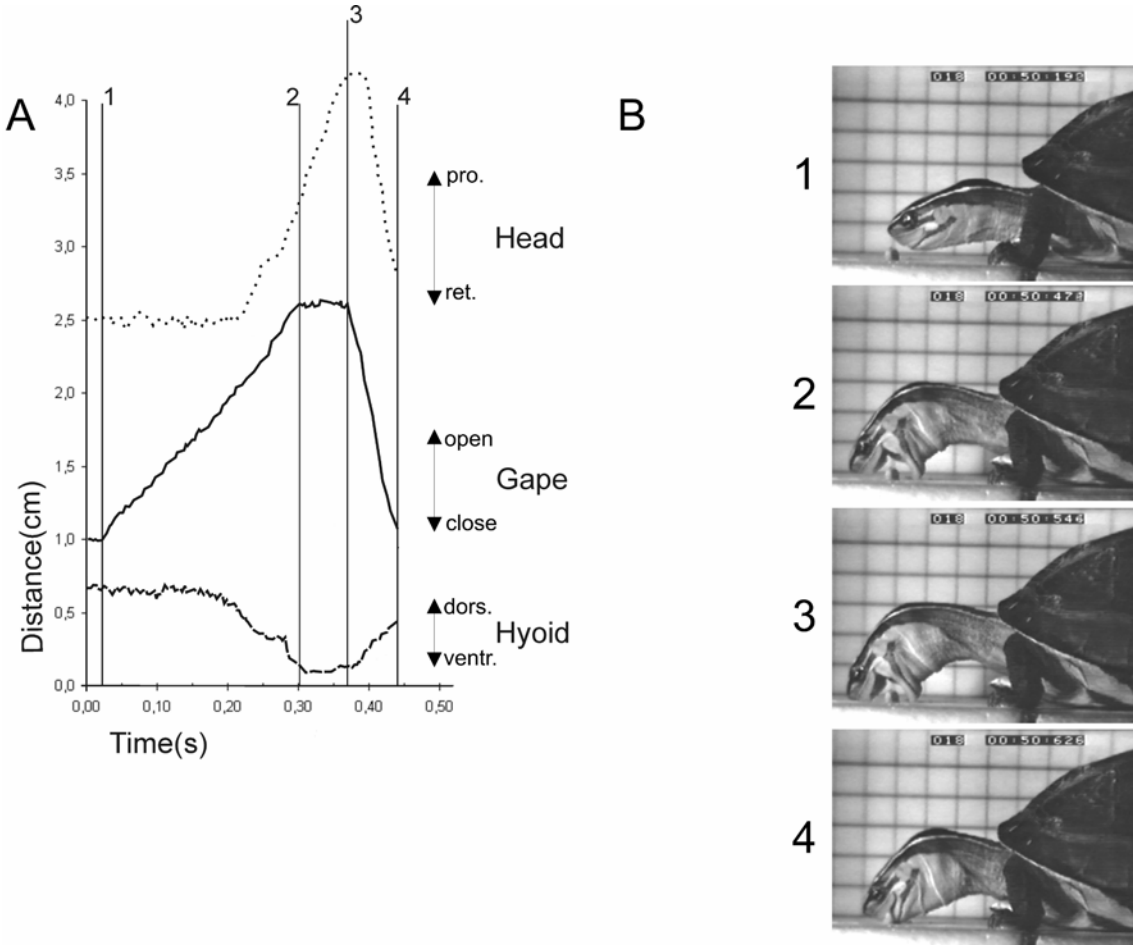

Fig. 5. (A) Kinematic profiles from a terrestrial food ingestion event in *Cuora amboinensis*, based on cinematography (250 fr/s). Top to bottom: first line = kinematics of head extension; second line = gape; third line = dorsoventral hyoid movements. (B) Video frames from a terrestrial food ingestion event in *Cuora amboinensis* based on cinematography (250 fr/s); B1, final head approach; B2, beginning of MG phase; B3, end of MG phase; B4, end of fast closing phase.

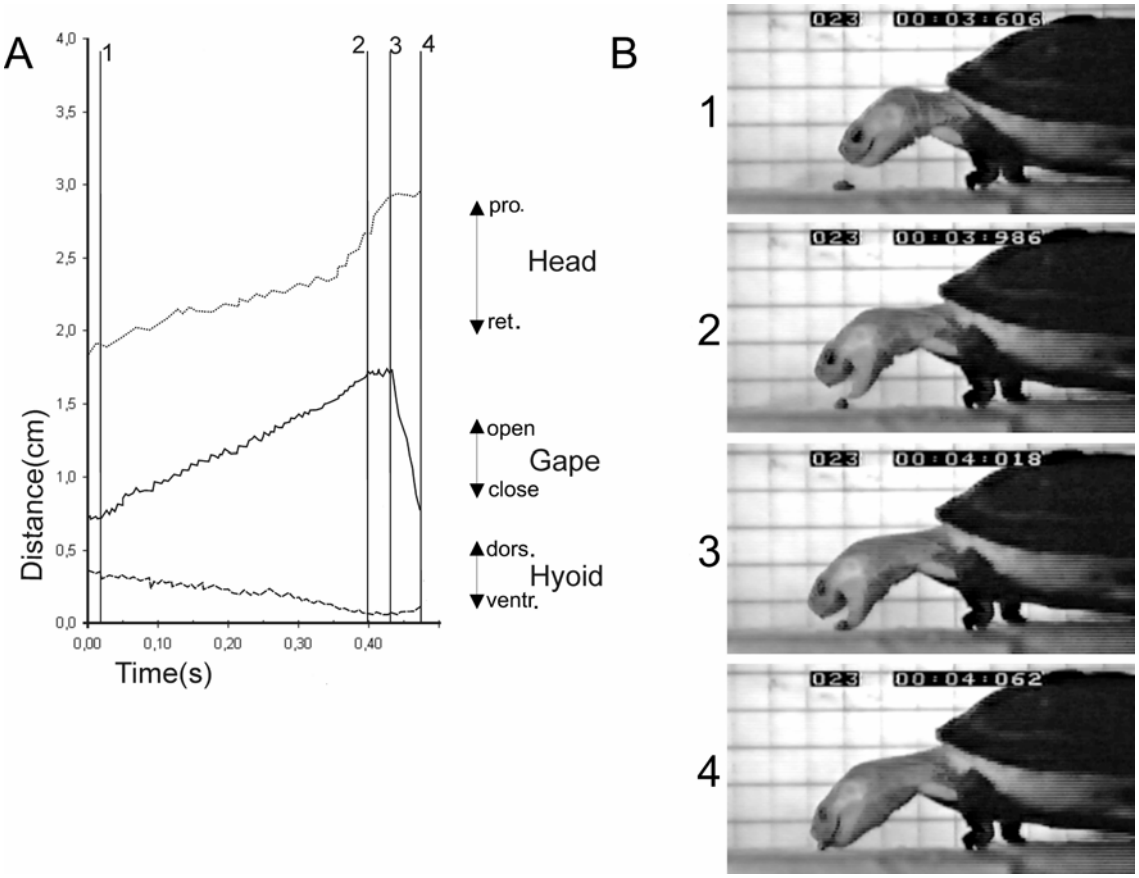

Fig. 6. (A) Kinematic profiles from a terrestrial food ingestion event in *Cuora flavomarginata* based on cinematography (250 fr/s). Top to bottom: first line = kinematics of head extension; second line = gape; third line = dorsoventral hyoid movements. (B) Video frames from a terrestrial food ingestion event in *Cuora flavomarginata* based on cinematography (250 fr/s); B1, final head approach; B2, beginning of MG phase; B3, end of MG phase; B4, end of fast closing phase.

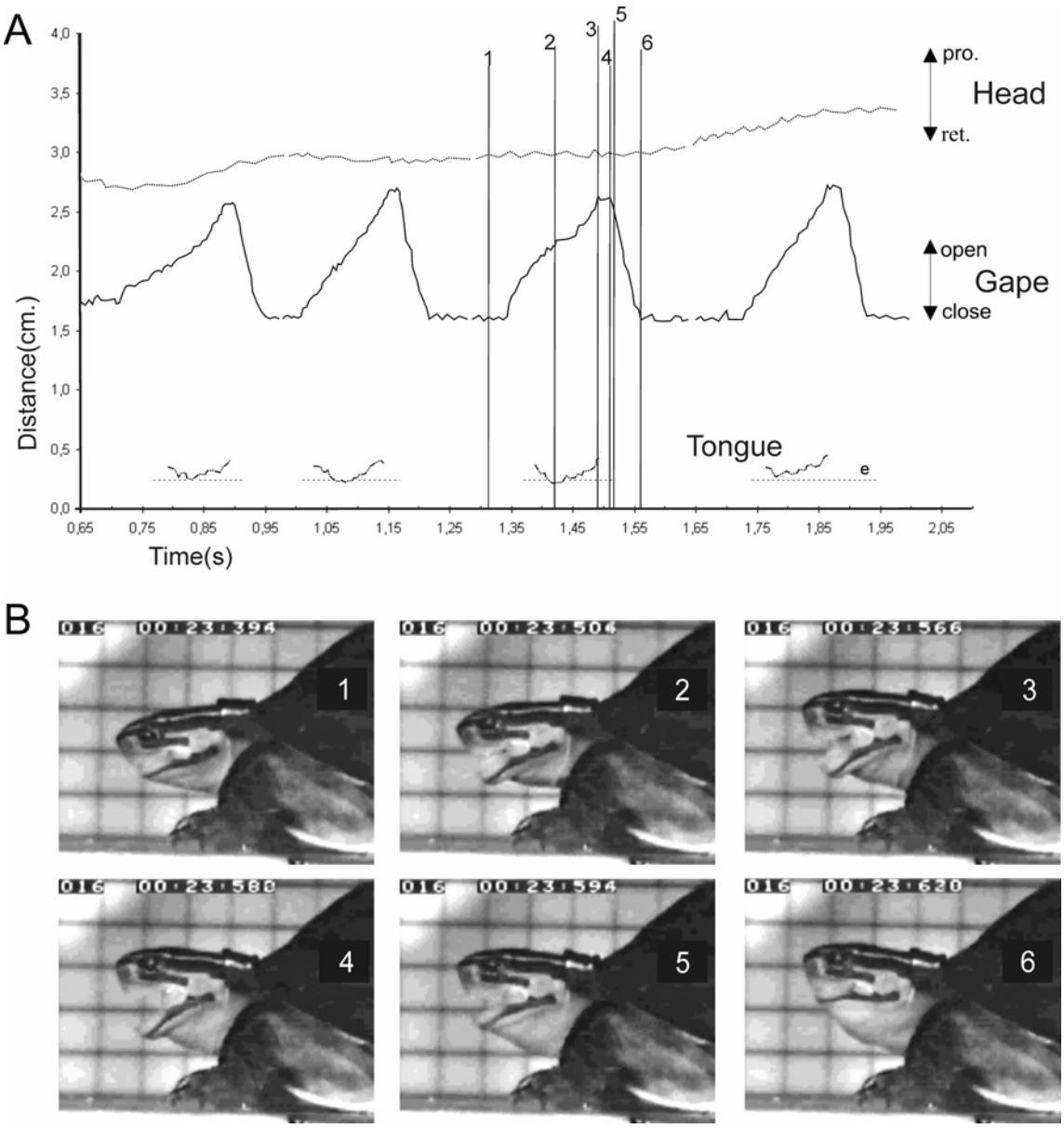

Fig. 7. (A) Kinematic profiles from a terrestrial pure lingual food transport ("lingual transport") event in *Cuora amboinensis* based on cinematography (250 fr/s). Top to bottom: first line = kinematics of head extension; second line = gape; third line = anterior/posterior movement of tongue related to imaginary line connecting U and L; e, imaginary line connecting U and L. (B) Video frames from a terrestrial pure lingual food transport (lingual transport) event in *Cuora amboinensis* based on cinematography (250 fr/s); B1, prior to jaw opening; B2, jaw opening; B3, beginning of MG phase; B4, end of MG phase; B5, jaw closing; B6, end of transport cycle.

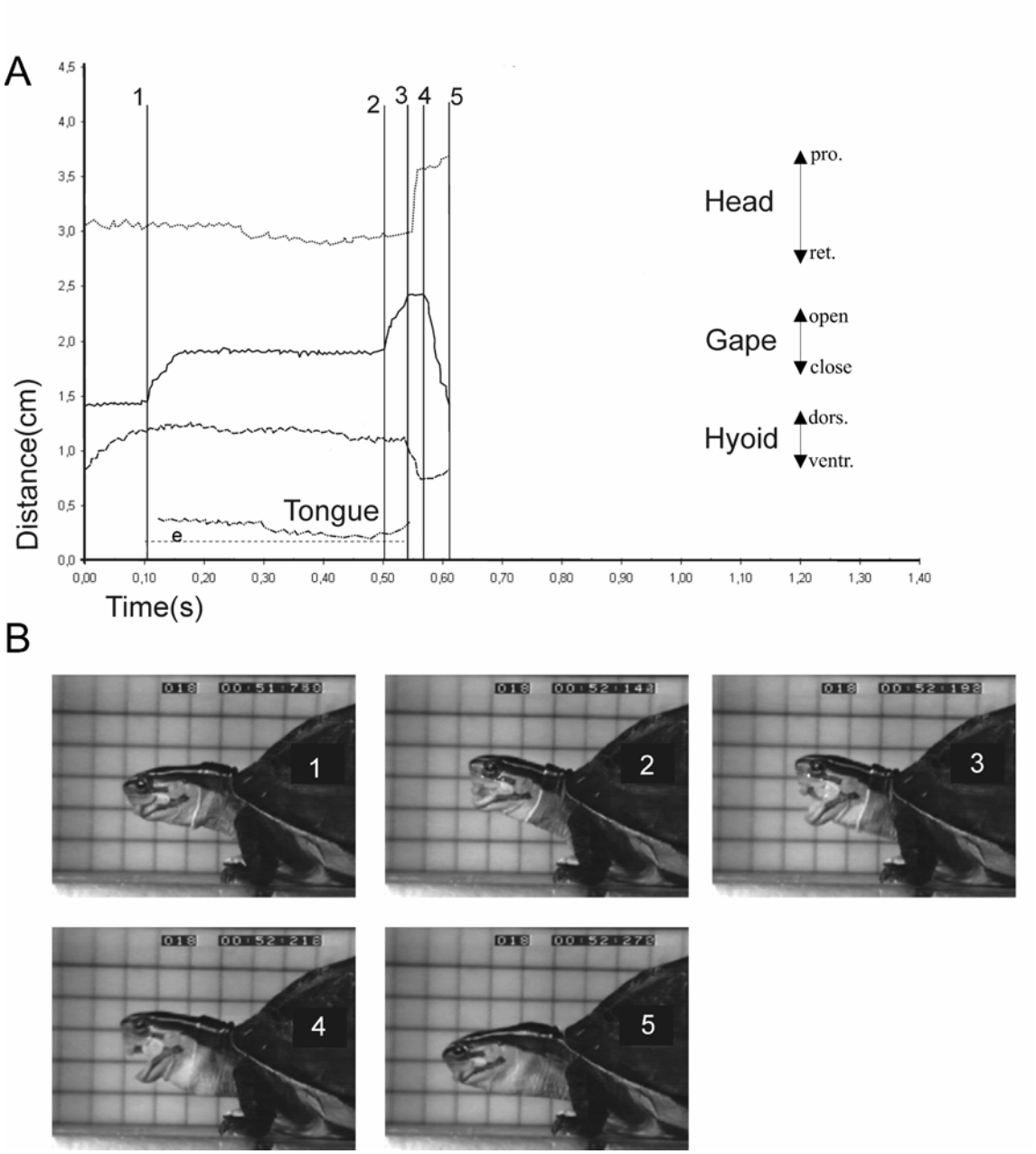

Fig. 8. (A) Kinematic profiles from a terrestrial food "prey positioning" event in *Cuora amboinensis* based on cinematography (250 fr/s). Top to bottom: first line = kinematics of head extension; second line = gape; third line = dorso/ventral hyoid movement; fourth line = anterior/posterior movement of tongue related to imaginary line connecting U and L; e, imaginary line connecting U and L. (B) Video frames from a terrestrial food "prey positioning" event in *Cuora amboinensis* based on cinematography (250 fr/s); B1, beginning of jaw opening; B2, end of slow opening phase; B3, beginning of MG phase; B4, end of MG phase; B5, end of fast closing phase.

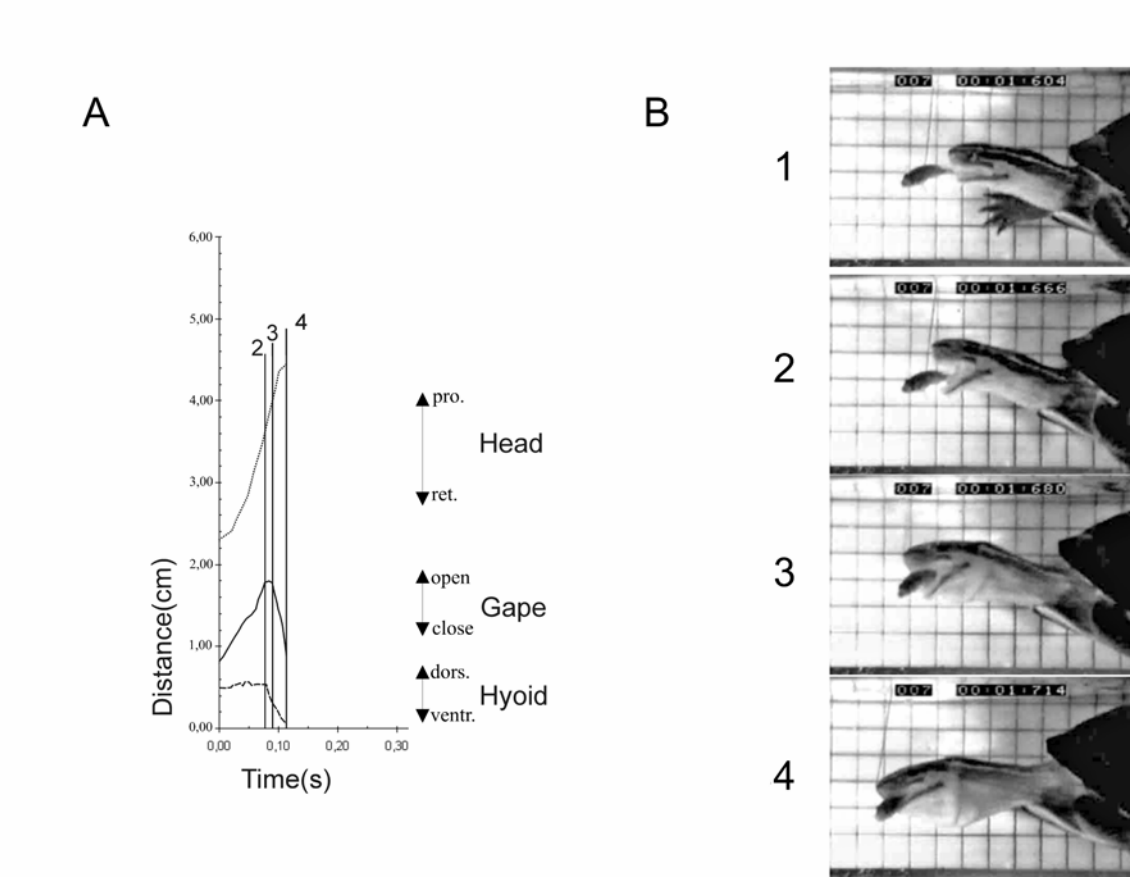

Fig. 9. (A) Kinematic profiles from an aquatic food ingestion event in *Cuora amboinensis* based on cinematography (250 fr/s). Top to bottom: first line = kinematics of head extension; second line = gape; third line = dorsoventral hyoid movements. (B) Video frames from an aquatic food ingestion event in *Cuora amboinensis* based on cinematography (250 fr/s); B1, final head approach (corresponds to the zero line on the x-axis); B2, beginning of MG phase; B3, end of MG phase; B4, end of fast closing phase.

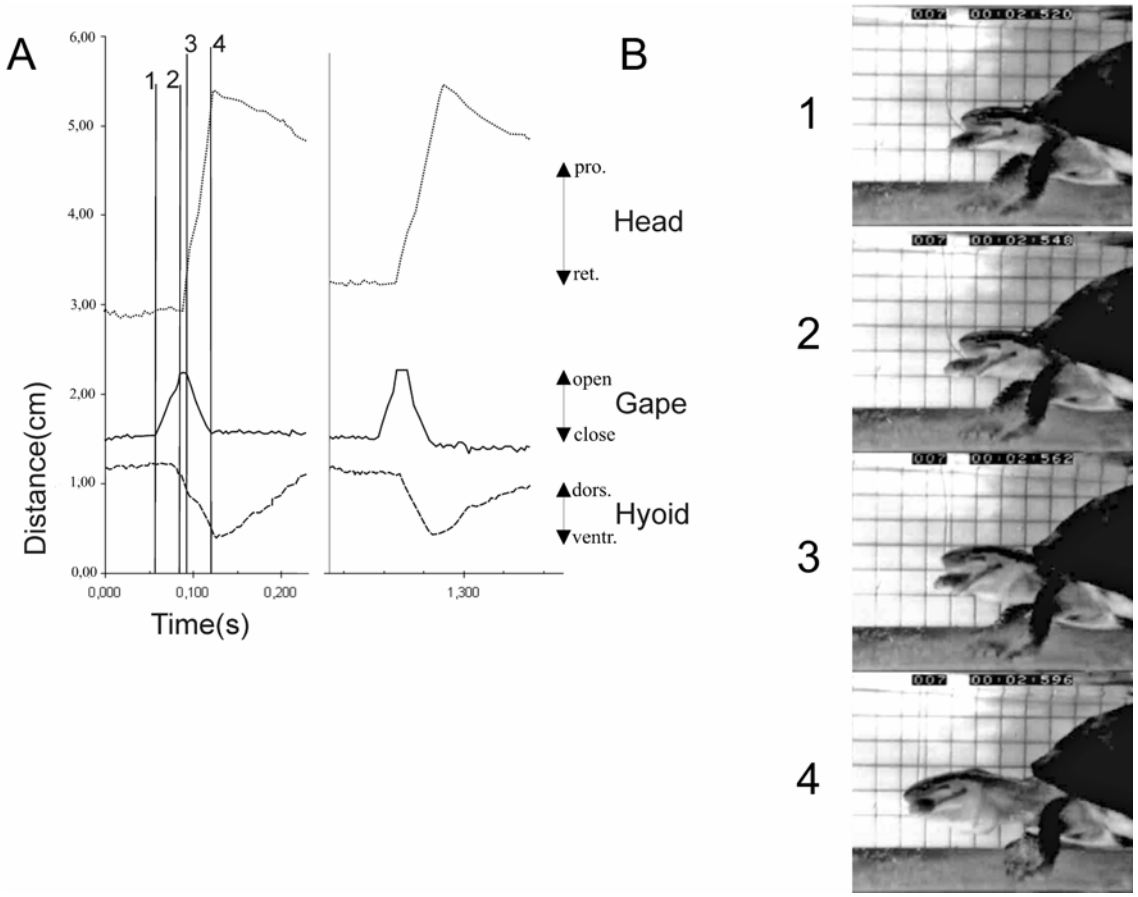

Fig. 10. (A) Kinematic profiles from an aquatic food transport event in *Cuora amboinensis* based on cinematography (250 fr/s). Top to bottom; first line = kinematics of head extension; second line = gape; third line = dorsoventral hyoid movements. (B) Video frames from an aquatic food transport event in *Cuora amboinensis* based on cinematography (250 fr/s); B1, beginning of jaw opening; B2, beginning of MG phase; B3, end of MG phase; B4, end of fast closing phase.

2.5. Aquatic feeding in a terrestrial turtle: a functional-morphological study of the feeding apparatus in the Indochinese box turtle *Cuora galbinifrons* (Testudines, Geoemydidae).

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Abstract

The Indochinese box turtle *Cuora galbinifrons* is regarded as a purely terrestrial species, but our results demonstrate that it can feed both on land and in water. The inverse relationship between the relative development of the hyoid apparatus and the tongue found in the most investigated chelonians is not valid in the Indochinese box turtle. Our morphological analysis of the feeding apparatus reveals that the palate shape and the design of the tongue are consistent with terrestrial feeders, but the construction of the hyoid complex is more characteristic of aquatic feeders. Previous studies have demonstrated that tongue enlargement negatively impacts the capacity of the turtles to suction feed. The present study focuses on the aquatic intraoral prey transport kinematic patterns. Our analysis is based on high-speed films with 250 fr/s and high-speed cineradiography with 50 fr/s. The aquatic intraoral food transport mechanisms differ depending on prey size: small items are transported predominantly by "inertial suction", whereas larger items are moved by the tongue - normally a clear terrestrial strategy. As the genus *Cuora* is ancestrally aquatic, the use of lingual food transport in the aquatic environment is presumably an aberrant modus typical only for the most terrestrial among the Asian box turtles.

Introduction

According to Bramble (1973), feeding in turtles is media dependent. Thus, several chelonian species can capture the food on land, but to continue the feeding process they have to go under water (Bramble and Wake 1985; Weisgram 1985; Lemell and Weisgram 1997). Within the largest recent turtle group – Testudinoidea – one can distinguish fully aquatic and completely terrestrial forms, as well as species with amphibious live mode (for overview see Heiss et al. 2008; Natchev et al. 2009). Some emydid and geoemydid species can handle the different requirements connected to the high density and viscosity of water compared to air (Gans 1969), they can complete the whole feeding process both on land and in water (Summers et al. 1998; Natchev et al. 2009). The Indochinese box turtle, *Cuora galbinifrons*, is a terrestrial geoemydid species (Richter et al. 2007) and is one of the least aquatic species within the genus *Cuora* (Ernst and Barbour 1989; Ernst et al. 2000). Studying the construction of the hyoid apparatus in 25 geoemydid species, Richter et al. (2007) provided evidence that terrestrial geoemydids have cartilaginous or partially cartilaginous hyoid apparati. The high degree of ossifcation of the hyoid corpus in *C. galbinifrons* is predicted to be an ancestral character. Our experiments will demonstrate that the Indochinese box turtle is able to feed in both media. An important aim of the present study is to investigate the bauplan of the feeding apparatus in *C. galbinifrons* and to analyse how the opportunistic "feeding ecology" of the species is reflected by the head design. Within the Testudinoidea, the skull form is closely correlated with ecological factors (Claude et al. 2004). As *C. galbinifrons* is a highly terrestrial turtle (see Pritchard 1979; Ernst and Barbour 1989), we can expect the head morphology to exhibit features similar to those found in tortoises (see Bramble 1973; Wochesländer et al. 1999). All chelonian species studied to date (Bramble 1978; Weisgram 1985; Bels and Renous 1992; Lauder and Prendergast 1992; Lemell and Weisgram 1997; Van Damme and Aerts 1997; Bels et al. 1998; Summers et al. 1998 Aerts et al. 2001; Lemell et al. 2000; Lemell et al. 2002, Bels et al. 2008; Natchev et al. 2009) use hydrodynamic mechanisms for aquatic food uptake (see Van Damme and Aerts 1997; Aerts et al. 2001). Under water, the tongue is not used in food prehension. Aquatic prey transport in turtles is poorly analysed (see Bels et al. 2008 for overview). According to Aerts et al. (2001), this transport involves a combination of "compensatory suction" and "inertial suction". Nonetheless, some purely aquatic turtles like *Malaclemys terrapin* (Schöpff 1793) and *Dermochelys coriacea* (Vandellli 1761) transport food by the tongue (Bels et al. 1998). The gape kinematical patterns of the aquatic food ingestion and the transport cycles in these two species adhered exactly to the kinematical patterns proposed in the "generalized cyclic

model" of Bramble and Wake (1985). The movements of the hyoid complex were not analysed (Bels et al. 1998). Transport mechanisms in which suction plays the dominant role are termed according to Bels et al. (2008) "intraoral aquatic hyoid transport" (here abbreviated IAHT) and the lingual-based transport is termed "intraoral aquatic lingual transport" (here abbreviated IALT). The present study applies high-speed cinematography (250 fr/s) to investigate whether the *Cuora galbinifrons* uses suction or tongue-based transport when feeding under water. The genus *Cuora* is ancestrally aquatic (see Claude et al. 2003), so IAHT can be predicted for these geoemydids. For the Indochinese box turtle, as the most terrestrial species within this genus, we predict – as typical for all highly terrestrial turtles (see Winokur 1988) – a large and movable tongue. Tongue enlargement and greater lingual mobility negatively impacts the capacity to suction feed (see Lemell et al. 2000). To obtain information on the exact movement of food items within the oropharyngeal cavity, we used high-speed cineradiography with 50 fr/s. We hypothesise that the Indochinese box turtle is able to use IALT, but that the kinematical pattern will show differences to *M. terrapin* and *D. coriacea* because aquatic lingual transport has evolved de novo independently within *Cuora* sp.

Materials and methods

Cuora galbinifrons galbinifrons BOURRET 1939 (the Indochinese box turtle) (Geoemydidae, Testudinoidea) is known from small areas including provinces in southern and northern Vietnam, eastern-central Laos as well as island of Hainan in southern China (Fritz and Havan 2007). Very little is known about its biology (Pritchard 1979). Ernst and Barbour (1989) reported that the species inhabits bushy woodlands and even forests at high elevations. Bonin et al. (2006) report that *C. galbinifrons* spends most of its time on land buried in moss. This species is primarily carnivorous but also feeds on fruits and vegetables. The animals used for our experiments were kept in an aqua-terrarium (120 x 70 x 70 cm) with a pool of water at 25°C constant temperature and a 12/12 h light/dark cycle. They were fed fruits and vegetables (bananas, cucumbers, apples, tomatoes, etc.), small invertebrates (mealworms, *Zophobas* sp. larvae, snails and earthworms) and food pellets. Our turtles frequently entered the water pool and spent prolonged periods in water, especially when food items were offered.

Anatomy

The anatomical investigations were conducted on four *C. galbinifrons* specimens – two female animals (obtained commercially; carapace length 14.4 and 12.9 cm) and two male animals (carapace length 16.9 and 17.6 cm). The female animals were killed by abdominal injection of a lethal dose of sodium pentobarbital (Nembutal). The two adult male specimens were preparations belonging to the collection of the Museum of Natural History in Vienna, where they were stored in 70% alcohol. The morphology of the skeletal elements and the jaw, hyoidal and cervical muscles were examined using dissection microscopes (WILD M5A and WILD 420). The terminology of Schumacher (1973) was used to describe the musculature and the tendon systems. For scanning electron microscopy (SEM), one tongue was fixed overnight in modified Karnovsky solution (2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer; Karnovsky 1965). After rinsing in 0.5% cacodylate buffer, the sample was postfixed in buffered 1% osmium tetroxide at 37°C for 2 h and treated with 25% HCl at 60°C for 30 min in order to remove the mucus from the surface. This procedure was followed by dehydration in a graded ethanol series, HMDS (hexamethyldisilazane) drying and gold coating in the Sputtercoater AGAR B7340. The specimen was observed in a Philips XL-20 scanning electron microscope.

Film recordings

For filming aquatic feeding events, the aquarium was filled with 5 cm water: the food items were offered at the bottom in front of the animals. The turtles were fed *Zophobas* sp. larvae (body length: 38–49 mm). Three subadult *C. galbinifrons* individuals (carapace length 14.9, 14.1 and 12.9 cm) were filmed in lateral view using a NAC-1000 high-speed video system at 250 fr/s. The first transport cycles in 24 videos (8 films per specimen) were selected for analyses. The horizontal and vertical coordinates of each landmark (Fig. 1) were digitised frame by frame using an AVI-digitiser (©P. Snelderwaard). Based on the landmarks' displacement in the bi-directional level, we calculated: (a) the gape amplitude – distance between the tips of the upper and the lower jaw; (b) ventral hyoid movement – dorso-ventral height of the head at the level of the origin of the Ceratobranchiale II (CB II); (c) the anterior-posterior movements of the tongue – the distance between the tip of the tongue and the imaginary line connecting the upper and lower jaw's tips; (d) the extension and retraction of the neck – the distance between the point "P" on the parietal cranial surface and the anterior tip of the carapace. These data enabled us to measure and represent graphically the duration of the different feeding stages, the time required to reach peak gape, the duration of hyoid retraction, the duration of jaw closing, the time required to pro- and retract the head and the duration of the total cycles. Since 8 values per individual and item are available, the distribution is not certifiable with statistical tests (e.g., by a Kolmogorov–Smirnov-Test for normal distribution). Thus, the nonparametric Kruskal–Wallis-Test is computed to compare the three individuals or the transport numbers.

For radiographic experiments, we used a U-matic video recorder Sony VO-5800 PS at 50 fr/s. A wire-grid (square size 7 x 7 mm) was used as a background. The turtles were fed mealworms (body length: 15–19 mm) and *Zophobas* larvae (body length: 38–49 mm). The food items were marked with contrast medium (Gastrografin) to demonstrate the movement of the food inside the oropharyngeal cavity and the oesophagus. One day prior to filming, lead markers were glued to the skull on the points used as relevant markers (see Fig. 1). A total of 12 aquatic feeding events were analysed. To calculate kinematic variables, only data derived from NAC video sequences were used.

Results

Anatomy

The skull in *C. galbinifrons* is relatively high and narrow, with well-defined ventral and dorsal emarginations of the temporal roof. Nonetheless, a slender temporal arch (see Fig. 2a, c), formed by the postorbital, quadratojugal and quadrate, is present (contrary to Wermuth and Mertens 1961). There is no direct connection between jugal and quadratojugal bones. Characteristic for *C. galbinifrons* is the convex prefrontals. The palatines are thin and transparent bony plates. The whole dorsal palate region is strongly vaulted. The vertical plates of the processus pterigoideus externus are small and rounded. The supraoccipital is slightly enlarged caudally, forming a short and fragile median supraoccipital ridge (Crista supraoccipitalis). The hyoid apparatus consists of the hyoid body, a pair of rudimental cornu hyale, two pairs of hyoid horns and a hypoglossum. The hyoid body is partially cartilaginous, caudally elongated and ventrally arched (see Fig. 2b). The lingual process is long, narrow and elastic. Ventro-rostrally to the lingual process is a cartilaginous hypoglossum. The branchial horns I (CB I) are caudo-dorsally elongated and ossified. Epibranchialia I remain cartilaginous. The branchial horns II are flattened, shorter, but wider than CB I, and their caudal ends are distinctly divided laterally. The posterior hyoid horns attach to the ventro-lateral wall of the anterior oesophagus. The tongue of *C. galbinifrons* is a large, triangular, beefy and highly movable organ. The dorsal surface shows a high number of densely packed lingual papillae. These lingual papillae are large, columnar and homogeneously distributed, but fuse together in the posterolateral region, forming relatively smooth, fleshy “horns” (hindtongue) ambilateral to the glottis (Fig. 3). The main jaw and hyobranchial muscles (see Schwenk and Rubega 2005) are shown in Fig. 2a. The M. adductor mandibulae externus fills the whole upper temporal fossa. The M. add. mand. ext. pars supeficialis and pars profunda are less voluminous in *C. galbinifrons* compared to other box turtles. The external tendon system has a myovector-changing function in turtles (see Schumacher 1956, 1973; Bramble 1974; Iordanskii 1990). In *C. galbinifrons,* no transiliens cartilage was present. The trochlear process is a tiny, rough-surfaced area on the quadrate (see Fig. 2c). The direction of forces during contraction of the intrinsic and extrinsic hyoid and lingual muscles are represented in lateral and dorsal view in Fig. 2e, f.

Aquatic food transport

Our sequences show that *C. galbinifrons* needs up to 14 transport cycles (mean = 10.4 ± 2.3) when feeding on large prey (*Zophobas* sp. larvae). The neck is in retracted position when the initial hyolingual protraction starts. The gape opens as the tongue slides under the prey, fixing it against the upper jaw. Using the terminology of Bramble and Wake (1985), we termed this phase SO I. At a certain point, the gape opening stops. That marks the beginning of the SO II phase. The durations of SO I and especially of SO II vary strongly influencing the duration of the whole feeding cycle (see Table 1). During the fast opening of the gape (FO phase), the prey loses contact with the palate region, sticking on the dorsal lingual surface (Fig. 4c). Maximum gape lasts 0.043 ± 0.008 s (termed here "Maximum Gape" – MG phase). During the MG phase, the tongue is rapidly retracted, dragging the prey further into the mouth. The movement of the prey corresponds exactly to the movement of the hyolingual complex (Fig. 4c, d). With the jaw closing (FC phase), the prey is fixed in its new position inside the oral cavity. The mean time of SO I is significantly lower in individual 1 than in the two other individuals (*P* value 0.036), as indicated by the Kruskal–Wallis-test, while all the other variables show no significant difference between individuals (see Table 1). The transport number of mealworms is furthermore always smaller than the transport number of *Zophobas* larvae – the results are identical for every individual per se (*P* value 0.012 for each of the three individuals). Smaller prey (mealworms) require fewer transport cycles (mean = 2.6 ± 0.9). In most cases (83%), the head and neck remain static. As the pharynx expands, the prey is carried deep within it (Fig. 5c, d). Prior to jaw closing, the prey is at the level of the second branchial horns (Fig. 5d). During the second cycle, the prey is fixed against the pharyngeal roof prior to peak gape (Fig. 5f). During hyoid retraction, the prey floats into the anterior oesophagus (Fig. 5g, h). During hyoid elevation, the food item once again moves rostrally, but remains behind the tongue in the pharyngeal chamber (Fig. 5i). The prey is swallowed by peristaltic contractions of the throat musculature, supported by neck extension and head elevation (Fig. 5j–m). In two of our X-ray films, we detect that the swallowed food was stored in the posterior third of the oesophagus (Fig. 6).

Discussion

Anatomy

The structural differences in the feeding apparatus of terrestrial and aquatic tetrapods can be explained by the specific physical properties of the two fluids – water and air (Bramble and Wake 1985). The aquatic testudinoids have a flattened skull with elongated squamosal and posterior cranial parts – compared to the maxillar region (see Claude et al. 2004). The dorsal vault of the palates is lacking, which helps prevent swirls in the water flow during suction (Bramble 1973). In herbivorous terrestrial testudinids, e.g. *Testudo* sp., *Gopherus* sp., the skull is higher and the cranial flexure is more prominent (see Bramble 1974; Wochesländer et al. 1999; Claude et al. 2004). In purely aquatic turtles, the massively developed hypobranchial musculature correlates with a large, well-ossified hyoid apparatus (see Van Damme and Aerts 1997; Lemell et al. 2002). The hypoglossum is large and can even ossify. In terrestrial testudinoids, the hyoid corpus and hypoglossal plates are small, mainly cartilaginous and more flexible than in aquatic forms. The transiliens cartilage is large and can even ossify to an Os transiliens (Bramble 1974). The tongue morphology in turtles can vary significantly (see Beisser et al. 1995, 1998, 2001, 2004; Iwasaki 1992; Weisgram et al. 1989; Winokur 1973, 1988). Purely aquatic turtles such as *Acanthochelys pallidipectoris* (Rhodin, Mittermeier and McMorris 1984) and *Chelus fimbriata* (Schneider 1783) have small tongues with a simple surface topography (Beisser et al. 1995; Lemell et al. 2002). Terrestrial testudinids possess a highly mobile, large and beefy tongue equipped with abundant high and slender lingual papillae (Winokur 1988). The head morphology in *Cuora galbinifrons* does not fit completely into the schema described above. The vaulted palate is necessary to provide space for the large and movable tongue. The dorsal tongue surface with numerous columnar lingual papillae in *C. galbinifrons* is structured quite similarly to testudinid species. This resemblance apparently reflects convergent adaptation to a predominantly terrestrial lifestyle. The inverse relationship between the relative development of the hyoid apparatus and the tongue found in the most investigated chelonians (see Lemell et al. 2000) is not valid in *C. galbinifrons*. In adult specimens, the hyoid complex can be well ossified (see Richter et al. 2007). The elongated form of the hyoid body and the elasticity of the cartilaginous lingual processes allow tongue protrusion even outside the mouth cavity in terrestrial feeding. The large hyoid body and the broad hypoglossum promote the oropharyngeal expansion during underwater feeding. A distensible anterior oesophagus is predicted in all vertebrates confronted with bidirectional waterflow (Lauder and Schaffer 1986). In *C. galbinifrons*, the remarkably spread CB II horns are attached laterally to the highly expandable anterior oesophagus wall, this

ensures large dilation during hyoid retraction (Fig. 4e). In most sequences of transport of small mealworms, the neurocranium remains fully static during hyoid retraction. The prey is drawn far into the pharynx by the water flow. We propose that this hydraulic mechanism is similar to the "inertial suction" mechanism (sensu Van Damme and Aerts 1997). This transport mode is highly effcient: within a few cycles (1–4), the prey can be positioned behind the tongue. The large tongue acts as a barrier holding the prey inside the pharyngeal area of the oral cavity when the swallowed water is expelled.

Kinematics

In *C. galbinifrons*, the kinematic patterns of pharyngeal packing differ from those of transport and swallowing. During pharyngeal packing, hyoid protraction is modest but its elevation is distinct (see Fig. 5f). We hypothesise that the dorsal shift of the hyoid is affected mainly by activating the intermandibular muscles. One possible mechanism of muscle activity is proposed here: first, the most anterior part of the M. intermandibularis contracts. During gape amplitude increases, the caudal parts of the M. intermandibularis are activated. The tongue remains elevated and the combined activity of the intrinsic lingual muscles and the intermandibular muscles forces the food item caudally into the pharynx and anterior oesophagus. The tongue in *C. galbinifrons* has a well-developed hindtongue (Fig. 3), which makes pharyngeal packing highly effective (see Schwenk 2000). This explains the lack of a discrete "pharyngeal compression phase" (see Smith 1984; Herrel et al. 1996). A characteristic feature is the food storage in the posterior third of the oesophagus - here termed "esophageal packing" (Fig. 6). The reason for this behaviour remains unclear. Histological investigations on the oesophageal epithelium in *C. galbinifrons* reveal no pepsin-secreting regions (Scheidel 2002). Nonetheless, pre-digestion cannot be completely excluded because gastric digestive enzymes may be present in the most posterior oesophageal segments. The hydrodynamic effects typically used by aquatic tetrapods for food transport apparently play a subordinate role when *C. galbinifrons* feeds on larger prey. The pharynx expands by reaching peak gape and "according to the continuity principle any change of this volume (head and neck) must generate a flow *relative* to the turtle's head, that is, any increase in volume must be filled instantaneously with water" (Aerts et al. 2001). We predict that these suction forces created by hyoid retraction are not strong enough to lift the *Zophobas* sp. larvae from the surface of the tongue and to accelerate them towards the anterior oesophagus. The food item does not lose contact with the tongue surface, and the posterior movement of the prey correlates exactly with the magnitude of the hyoid retraction. On land, the connection between

the tongue and prey is due to wet adhesion and the interlocking effects (McDowell 1972; Schwenk 2000). Under water, only surface interlocking seems to be relevant in large prey transport in *C. galbinifrons*. X-ray films showed that the meal worms lost contact with the dorsal tongue surface and were sucked up with the instreaming water deep within the pharyngeal section. Using the terminology of the general feeding cyclic model (Bramble and Wake 1985), we can formally recognise a separation of the jaw opening cycles into SO I, SO II and FO in IALT cycles in *C. galbinifrons*. SO I and SO II prior to FO gape phases are found in terrestrial food transport in *Testudo hermanni* (Gmelin 1789) (Wochesländer et al. 2000) and *Terrapene carolina* (Linnaeus 1758) (Bels et al. 1997), but also in the IALT cycles in *Malaclemys terrapin* and *Dermochelys coriacea* (Bels et al. 1998). Bels et al. (2008) propose that the intraoral transport cycles are similar for all terrestrial turtles and for turtles using IALT. In that hypothetical generalised cycle, tongue protraction starts early in the SO stage and retraction occurs at the beginning of the FO phase. Our kinematical analyses do not support that theory for *C. galbinifrons*. When feeding on *Zophobas* sp. larvae, hyoid retraction started during a static gape phase (here termed MG phase) and correlated with the initiation of neck extension. We propose that neck extension improves control over the direction of the prey being moved towards the pharynx.

Evolutionary perspective

The ancestor of *Cuora* was an aquatic animal (for an overview see Claude et al. 2003; Joyce and Gauthier 2004). In the Amboina box turtle, *Cuora amboinensis* (Daudin 1892), the tongue is used only for prey fixation underwater (Natchev et al. 2009). The gape cycle profile of aquatic transport in *C. amboinensis* lacks slow open phases prior to fast open (Natchev et al. 2009). The kinematical patterns in this species resemble those proposed by the model of Reilly and Lauder (1990). Still, the hyoid retraction – which is crucial for creating suction in aquatic tetrapods (Lauder and ShaVer 1985) including aquatic turtles (Aerts et al. 2001) – starts upon reaching peak gape and not at the beginning of fast opening. The occurrence of discrete slow open gape phases in *C. galbinifrons* when feeding on *Zophobas* larvae could be regarded as direct evidence supporting the existence of a "general intraoral transport cycles" in chelonians (Bels et al. 2008) and as indirect support for the validity of the general feeding cyclic model of Bramble and Wake (1985). Alternatively, the existence and separation of SO I and SO II in transport cycles in *C. galbinifrons* may be a consequence of newly evolved tongue involvement in the aquatic transport and do not represent an ancestral feeding trait. Additional information concerning the mechanism of the underwater food transport in other

terrestrial emydids and geoemydids able to feed in both media (e.g. *Terrapene carolina*, *Cyclemys sp.*, *Heosemys sp.*, but also *C. flavomarginata* (Gray 1863)) is required for verification of one of those hypotheses. As *C. flavomarginata* possesses a relatively large tongue (Natchev et al. 2009) and the hyoid apparatus is highly cartilagenous (Richter et al. 2007), IALT can be expected for this species. In conclusion, we propose that within the genus *Cuora*, aquatic tongue-based food transport represents a recently evolved and aberrant feeding modus, typical only for the most land-related box turtles – *C. galbinifrons* and possibly *C. flavomarginata*.

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Tables

Table 1. Mean ± standard deviation for eight kinematic variables in aquatic feeding transport of *Zophobas* larvae (38-49 mm) in *C. galbinifrons*.

	Individual 1		Individual 2		Individual 3		
Variable	Mean	Std.Dev.	Mean	Std.Dev.	Mean	Std.Dev.	p-value
TPG (s)	0,432	0,053	0,430	0,093	0,486	0,132	0,712
Duration „SO I“ (s)	0,079	0,019	0,101	0,012	0,100	0,015	0,036
Duration „SO II“ (s)	0,292	0,056	0,268	0,092	0,323	0,122	0,746
Duration „FO“ (s)	0,062	0,007	0,060	0,006	0,063	0,008	0,875
Duration MG (s)	0,040	0,008	0,043	0,005	0,044	0,007	0,448
Duration „FC“ (s)	0,059	0,011	0,057	0,012	0,061	0,011	0,758
Duration GC (s)	0,531	0,042	0,529	0,097	0,591	0,139	0,691
Duration HR (s)	0,074	0,011	0,069	0,009	0,080	0,012	0,158

N = 8 pro specimen; *TPG* Time to peak gape, *SO I* Slow open I, *SO II* Slow open II, *FO* Fast open, *MG* Maximal gape, *FC* Fast close, *GC* Gape cycle, *HR* Hyoid retraction; Time in s

Figures

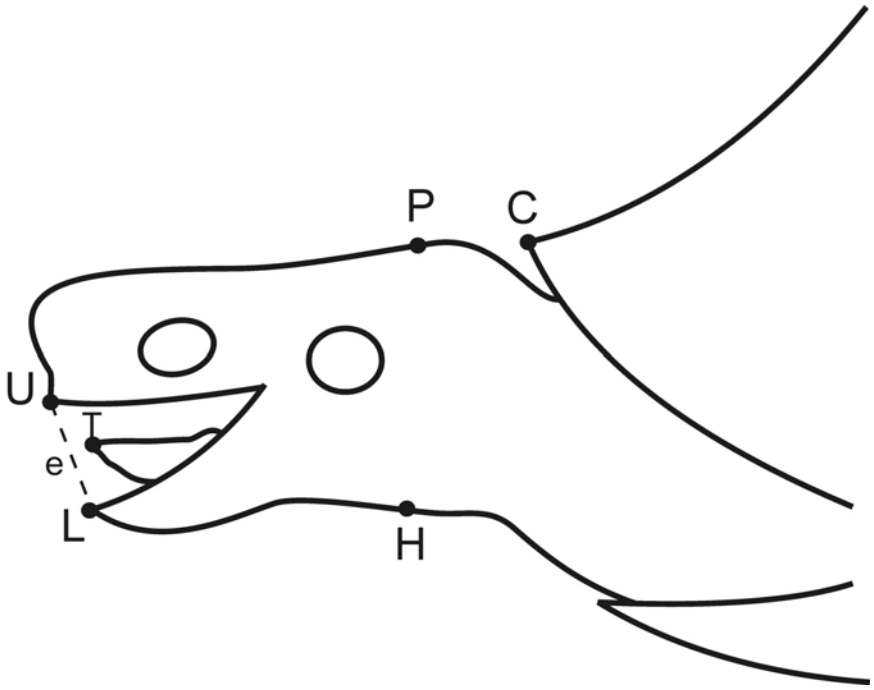

Fig. 1: Points used for kinematical analyses of feeding cycles of *Cuora galbinifrons*. *U* Anterior tip of upper jaw, *L* Anterior tip of lower jaw, *H* Hyoid, *P* Parietal bone, *C* Anterior tip of carapace, *T* Anterior tip of tongue; *e* Imaginary line connecting U and L; The distance between *U* and *L* is referred to as gape; the distance between H and P indicates the dorso-vertical movements of the hyobranchial apparatus; the distance between P and C shows the head extension rate

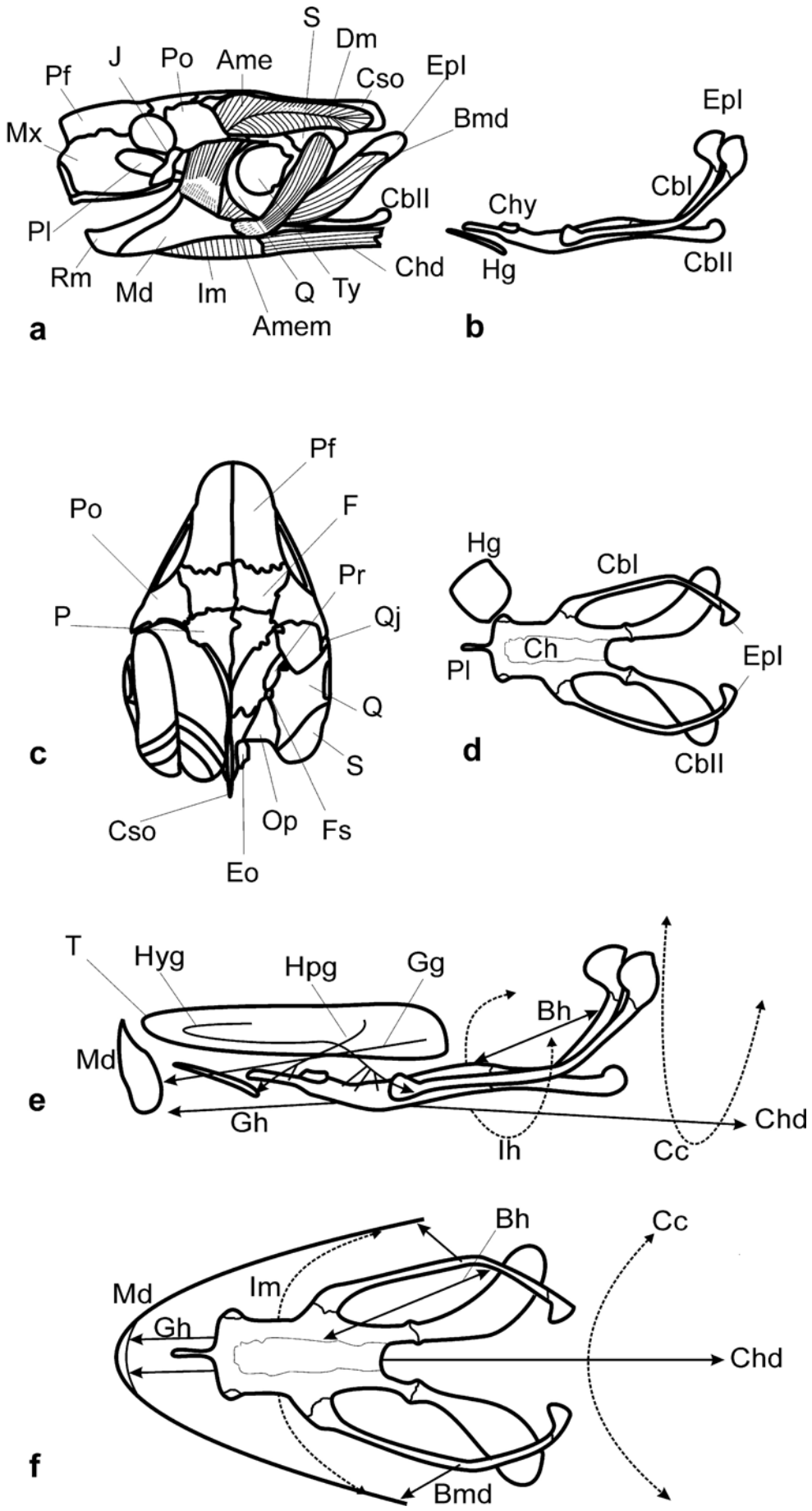
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Fig. 2: *C. galbinifrons*. Schematic illustration of head morphology. **a** Osteology and myology of the skull; **b** Hyoid and hypoglossum, lateral view; **c** Skull in dorsal view (right adductor complex removed; left jugal bar removed); **d** Hyoid and hypoglossum, dorsal view; **e** Direction of forces during contraction of the hyolingual muscles (*lateral view*); **f** Direction of forces during contraction of the hyolingual muscles (dorsal view). *Ame* M. add. mand. externus, *Amem* M. add. mand. externus pars media, *Bh* M. branchiohyoideus, *Bmd* M. branchiomandibularis, *CbI* Ceratobranchiale I, *CbII* Ceratobranchiale II, *Cc* M. constrictor colii, *Ch* Corpus hyoidei, *Chy* Cornu chyale, *Chd* M. coracohyoideus, *Cso* Crista supraoccipitalis, *Dm* M. depressor mandibulae, *Eo* Os exoccipitale, *EpI* Epibranchiale I, *F* Os frontale, *Fs* Foramen stapedius, *Gh* M. geniohyoideus, *Gg* M genioglossus, *Hg* Hypoglossum, *Hpg* M. hypoglossoglossus, *Hyg* M. hyoglossus, *Ih* M. interhyoideus, *Im* M. intermandibularis, *J* Os jugale, *Md* Mandibula, *Mx* Os maxillare, *Op* Os opistoticum, *P* Os parietale, *Pf* Os praefrontale, *Pl* Os palatinum, *Po* Os postorbitale, *Pr* Os prooticum, *Pl* Processus lingualis hyoidei, *Q* Os quadratum, *Qj* Os quadratojugale, *Rm* Ramphotheca, *S* Os squamosum, *T* Tongue, *Ty* Tympanum

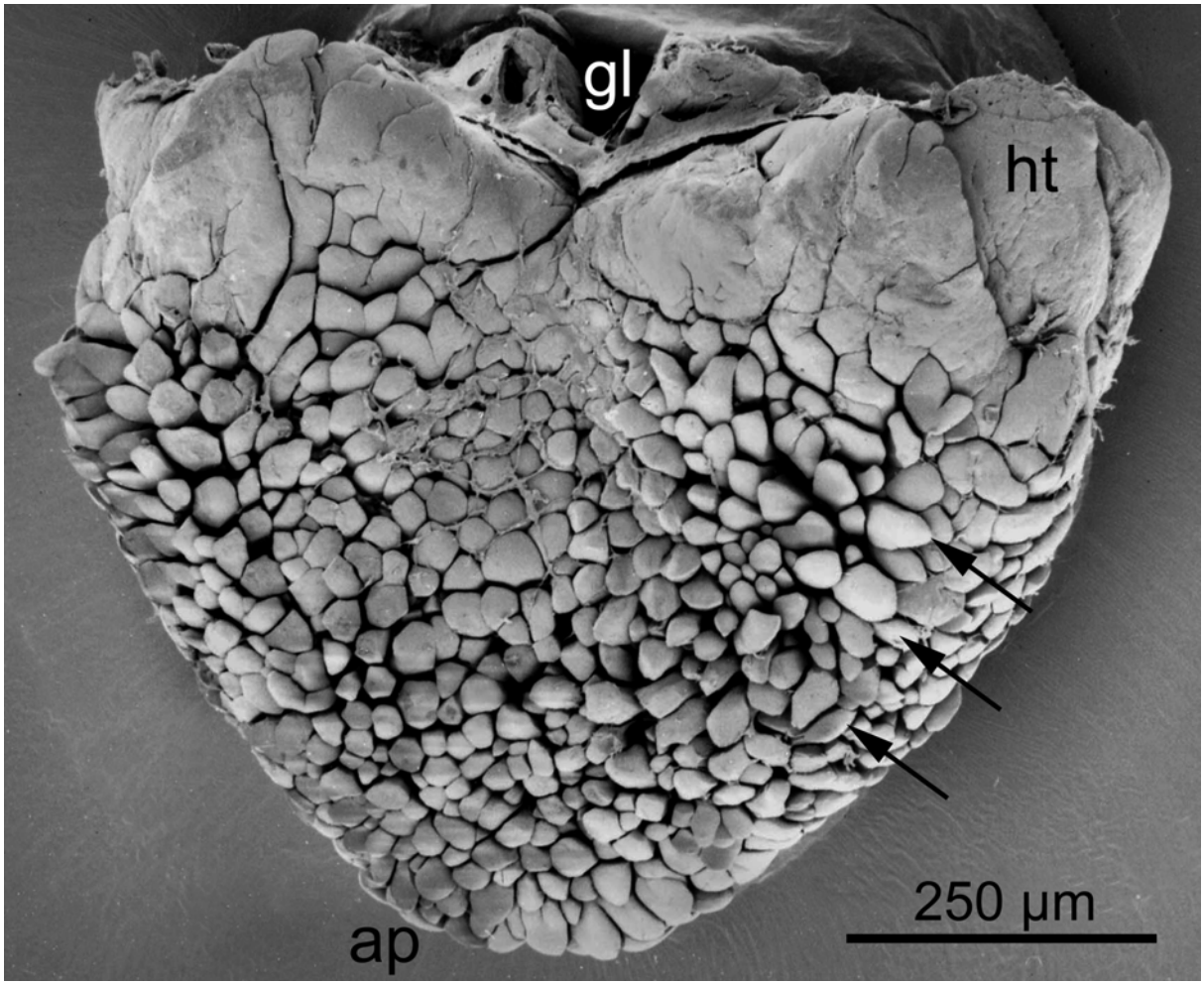

Fig. 3: Scanning electron micrograph showing total view of the tongue of *C. galbinifrons*. The high and slender lingual papillae (*arrows*) are distributed throughout the dorsal tongue surface, but are fused together in the area of the *ht* "hindtongue", *ap* Lingual apex, *gl* glottis

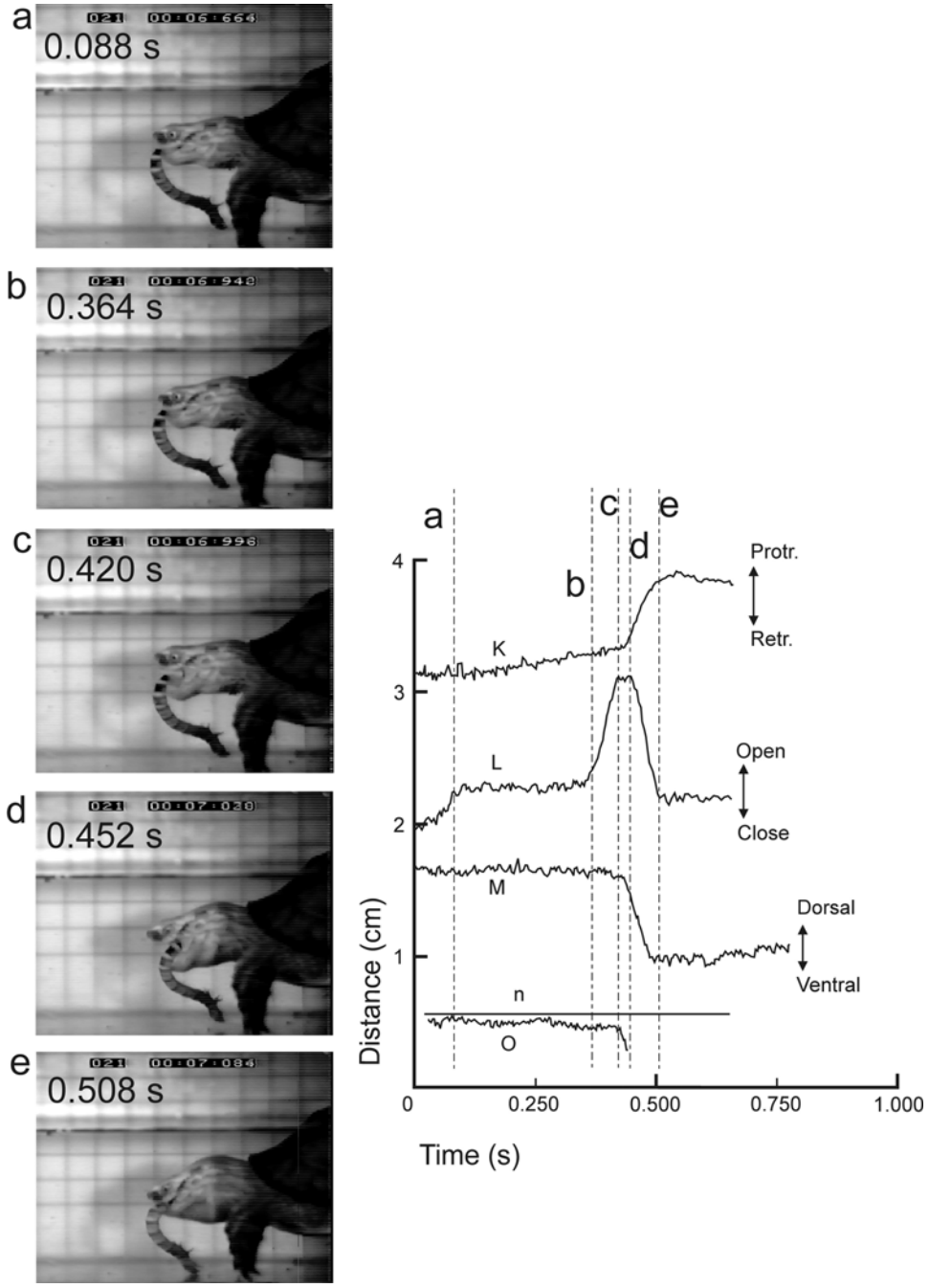

Fig. 4: *C. galbinifrons*: Aquatic food transport of a large prey item (*Zophoba* sp. larva): kinematical pattern diagram and frame sequence from a highspeed video (250 fr/s). *K* Kinematics of neck extension, *L* Gape, *M* Dorso-ventral hyoid movement, *O* Anterio-posterior movement of tongue, *n* Imaginary line connecting the jaw tips

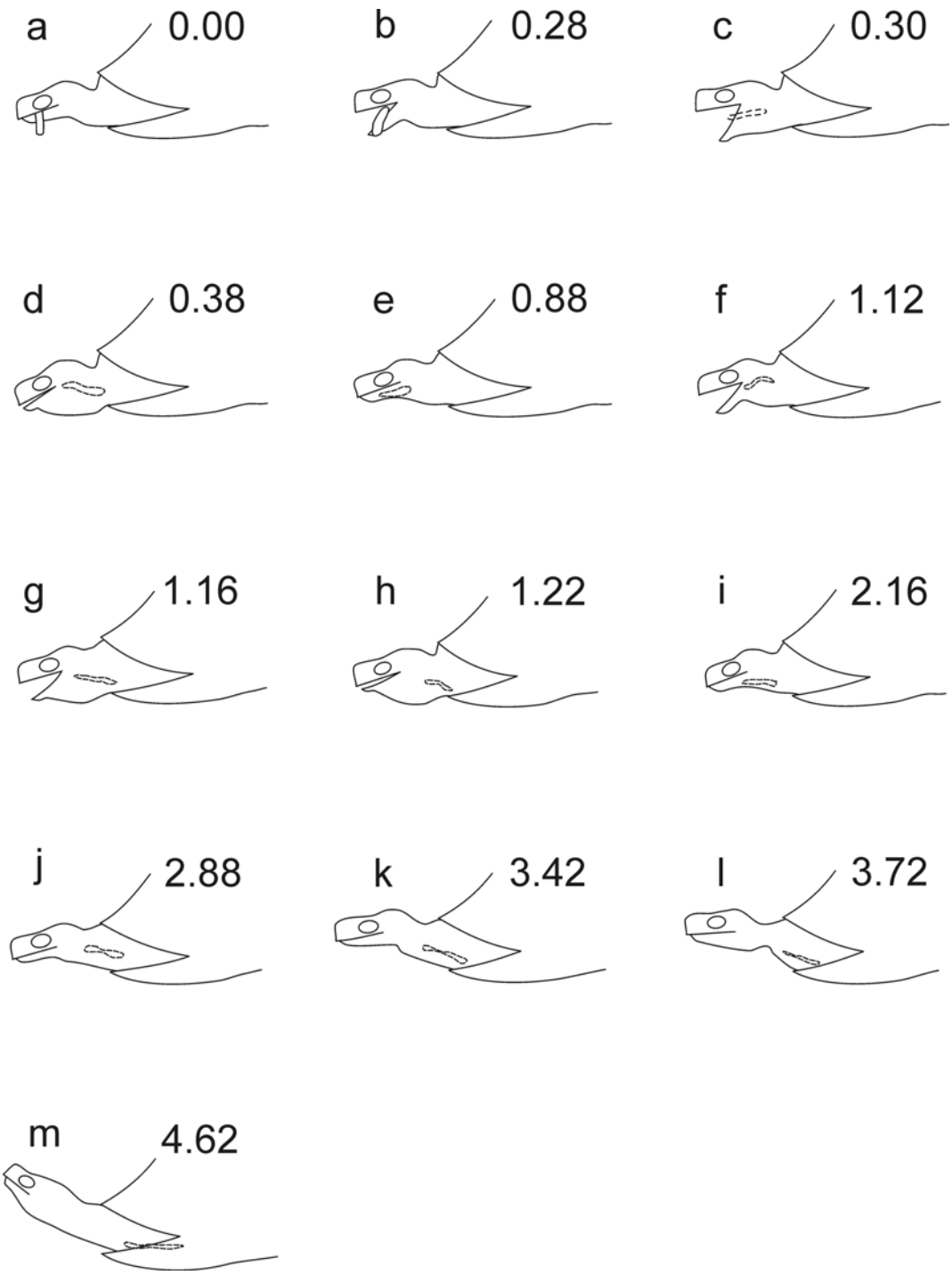

Fig. 5: *C. galbinifrons*: Aquatic food transport (**a**–**e**); pharyngeal packing (**f**–**i**) and swallowing (**j**–**m**) of a small prey item (mealworm); time in s; based on X-ray film sequence (50 fr/s)

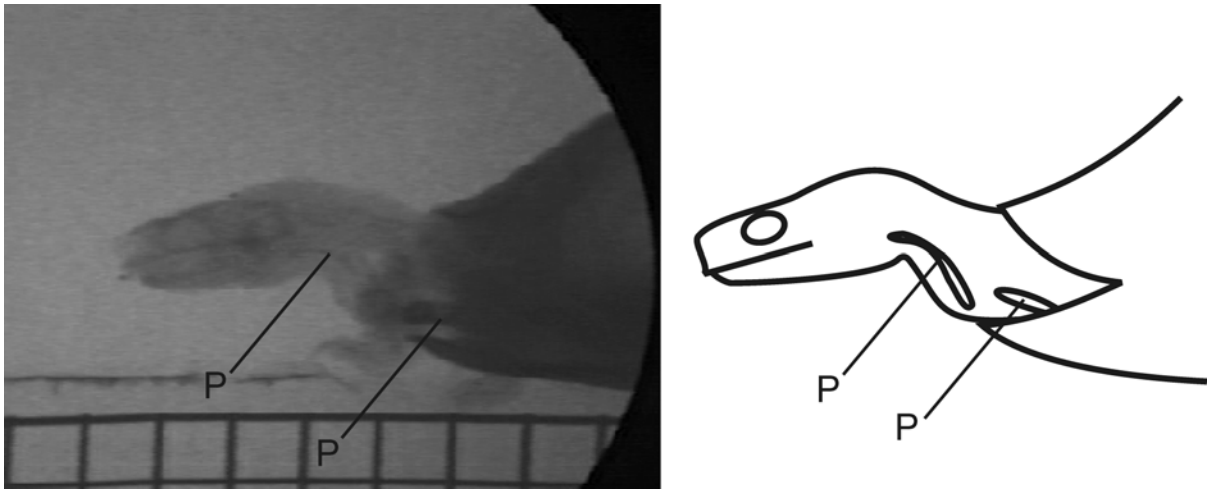

Fig. 6: “Oesophageal packing” event in *C. galbinifrons*; frame selected from x-ray film sequence (50 fr/s). P – prey.

2.6. Basal tortoise with primitive feeding biology? Structure and function of the oropharyngeal cavity in the Asian Forest Tortoise, *Manouria emys emys*

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Abstract

In turtles, the ability to take up food on land is based on certain morphological requirements of the feeding apparatus. The only exclusively terrestrial recent turtle family is represented by the tortoises, or testudinids. The ancestors of the testudinids were aquatic or semiaquatic forms, and *Manouria* is considered a very primitive, if not the most primitive member of this family. Ecological observations demonstrate *Manouria* sp. to show a semiaquatic lifestyle and therefore it was hypothesised that some primitive features could have been retained in form and function of the feeding apparatus in the species *M. emys emys*.

The present study tests this by providing new morpho-functional data on the oropharyngeal cavity of this primitive testudinid. The oropharyngeal cavity in *M. emys emys* is richly structured. The tongue is large, bearing a large number of slender, non-muscular papillae. The oral glands are numerous and highly complex structures, represented as tubular, polystomatic, or monostomatic entities. Keratinisation of the oral mucosa is extensive and found (apart from the rhamphothecae) on the anterior palate, as well as on the anterior half of the tongue. The taste buds are barrel- to onion-shaped and their regional distribution corresponds to the primitive food prehension mode used by this species. The data provided in the present study are congruent with the primitive status of *M. emys* within the testudinids. Even if clearly bearing strong characteristics of the "highest evolved turtle clade", it still shows primitive features which could reflect the aquatic to semiaquatic ancestor of testudinids.

Introduction

Feeding plays a critical role in survival. The contribution of feeding systems to fitness is therefore likely to be substantial (Schwenk and Wagner, 2001). Studies on aquatic and terrestrial turtles showed a correlation between phylogeny, feeding mode, and oropharyngeal morphology (Winokur, 1973; Weisgram et al., 1989; Iwasaki et al., 1996a, 1996b; Lemell et al., 2000, 2002; Beisser et al., 2004; Richter et al., 2007; Heiss et al., 2008; Natchev et al., 2009). In other words, the oropharyngeal anatomy both enables and constrains the food uptake potential. Purely aquatic (non-marine) turtles are known to have a smoothened oral surface with a flat palate (Winokur, 1988; Weisgram et al., 1989) and a tiny tongue (Bramble and Wake, 1985; Weisgram et al., 1989; Beisser et al., 1995, 1998, 2001; Lemell and Weisgram, 1997; Lemell et al., 2000, 2002, 2010). Such a design seems to be advantageous for suction feeding, the preferred feeding mode used by aquatic turtles. An oral cavity rich in structures would interfere with hydrodynamic mechanisms, possibly preventing an efficient water flow into the oral cavity. Furthermore, the oral glands are poorly developed in these turtles, being present only as single, mucus-producing goblet cells (Iwasaki, 1992, 1996b; Beisser et al., 1995, 1998, 2001). Mucus as a lubricating substance in the oral cavity clearly plays a subordinate role in aquatic turtles. By contrast, fully terrestrial turtles ("tortoises": family Testudinidae) show an oral cavity rich in structures (e.g. Winokur, 1988; Weisgram et al., 1989), with epithelial flaps and ridges around the choanae on the palate and a large tongue ventrally (Wochesländer et al., 1999, 2000). The tongue in the so-far-studied testudinids is well developed with numerous slender and densely packed, movable lingual papillae (see Winokur, 1988 for overview). These turtles use their tongue for food prehension and intraoral transport (Weisgram et al., 1989; Wochesländer et al., 1999; 2000; Bels et al., 2008). The lingual papillae are needed for surface amplification to increase the friction force between tongue and food item (Bramble and Wake, 1985; Wochesländer et al., 1999). Additionally, the well-developed oral mucous glands in testudinids thoroughly lubricate the tongue to promote wet adhesion between tongue and food (Wochesländer et al., 1999). Paleoecological studies imply that all existing turtles had an aquatic ancestor (Joyce and Gauthier, 2004; Li et al., 2008; Anquetin et al, 2009). This means that terrestrial feeding in turtles – along with all the morphological requirements – is derived rather than ancestral. The genus *Manouria* is suggested to represent the most primitive (according to Gaffney and Meylan, 1988; Meylan and Sterrer, 2000; Takahashi et al., 2003; Spinks et al., 2004) – or at least one of the most primitive (according to Le et al., 2006; Fritz and Bininda-Emonds, 2007) – recent members of the unique, truly terrestrial turtle-clade, the testudinids or tortoises. Additionally, *Manouria*

emy emys is reported to live a somewhat amphibious life in the wild (Ernst and Barbour, 1989; Obst, 1986; Høybye-Mortensen, 2004). The oropharyngeal design of this species therefore could retain certain primitive features possibly reflecting an aquatic or semiaquatic ancestor. The present study tests this by providing gross- and micro-anatomical data on the oropharyngeal cavity in *M. emys emys*, a largely uninvestigated species. The results will be compared with information available from other vertebrates, focusing on aquatic, semiaquatic, and terrestrial turtles. Furthermore, we provide data from a preliminary functional study and discuss our morphological findings in the context of the feeding behaviour of the species.

Material and Methods

Manouria emys emys (Schlegel and Müller, 1844), the Asian Forest Tortoise, ranges from southern Thailand through Malaysia, Sumatra, and Borneo, where it preferably inhabits tropical evergreen woodlands (Ernst and Barbour, 1989). *Manouria emys emys* prefers moist situations (Ernst and Barbour, 1989; Bonin et al., 2006) and is described as having a strong association to water (Høybye-Mortensen, 2004) or even as exhibiting a partially aquatic lifestyle (Obst, 1986). The diet of these tortoises consists of land- and water plants, mushrooms, insects, but also amphibians and dead animals (Bonin et al., 2006).

The six captive-bred juvenile animals used in the present study were obtained commercially and ranged in size (straight carapace length) between 109 and 135 mm, and in weight between 234 and 265 g. They were kept in a terrarium with 150 x 100 cm ground area at a 12 h dark, 12 h light cycle. The terrarium contained bark mulch (5 cm high) as substrate, some bigger cork bark pieces as hiding facilities, and a low basin (40 x 100 x 7 cm) filled with water which was permanently filtered by an external aquarium filter. The animals were fed with a variety of vegetables (salads, carrots, cucumbers, bananas, apples, tomatoes, and mushrooms), tortoise pellets from the pet trade, and pieces of fresh cattle heart and dead mice.

For morphological investigations, 4 animals were anesthetised by intraperitoneal injection of sodium pentobarbital and, after deep narcosis, decapitated. The heads were immersed immediately in fixation solution. For scanning electron microscopy (SEM), two heads were immersed for 24 h at room temperature in modified Karnovsky solution (2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer; Karnovsky, 1965). After rinsing in 0.1 M cacodylate buffer, the lower jaw with the tongue was removed from the head in order to better view both the ventral and dorsal surfaces of the oropharyngeal cavity. Then, samples were postfixed in buffered 1% osmium tetroxide for 2 h at 37 °C, washed in distilled water, and treated with 25% HCl at 40 °C for 30 min to remove the mucus from the surface. After repeated washing in distilled water, the samples were dehydrated in a graded ethanol and acetone series and immersed in HMDS (hexamethyldisilazane) for seven days. Then, the HMDS was evaporated and the samples were mounted on aluminium stubs for SEM and coated with gold in an AGAR B7340 Sputtercoater (Agar Scientific Ltd, Stansted, UK). Observations and digital photographic documentations were made by using a Philips XL-20 scanning electron microscope (Philips, Eindhoven, NL) and a Philips XL-30 environmental scanning electron microscope (Philips, Eindhoven, NL).

For paraffin-based histology, two animals were used. The heads were immersed in Bouin's fixative (Romeis, 1989) for 30 days, changing the solution twice a week. After complete

fixation and decalcification, the upper jaw was removed from the rest of the head and the cornified rhamphothecae were cut off. Then, the samples were dehydrated in a graded ethanol-isopropanol series and embedded in paraffin. After polymerisation, 7 µm serial-sections were made on a Reichert-Jung 2030 rotation microtome (Reichert-Jung, Bensheim, GER). These series included about 2000 sections of the upper and 2000 sections of the lower jaw per animal. The sections were mounted on glass slides and, after removing the paraffin, standard stained with Haematoxylin (H), Eosin (E), periodic acid Schiff (PAS), Alcian blue (AB), and Coomassie Brilliant blue (after Romeis, 1989; Kiernan, 2003). The preparations were documented by digital photography under a Nikon Eclipse 800 light microscope (Nikon, Tokyo, JP).

For filming the feeding events of all six animals, food items were offered in front of the animals by forceps (to avoid head rotation during terrestrial feeding) or on the bottom of a glass aquarium (24 x 60 x 30 cm) without water or with 4 cm of water level, respectively. To film animals, all turtles were fed with small cattle heart pieces, measuring approximately 5 x 5 x 7 mm. The turtles were filmed in lateral view with the digital high-speed camera Photron Fastcam-X 1024 PCI (Photron, Tokyo, JP) at 250 fr/s, with a reference grid (1 x 1 cm) as a background. Kinematical analyses were made but are not shown in the present study as they are subject of a separate paper.

Results

Morphology

Scanning electron microscopy

The oral cavity of *M. emys emys* was rich in structures. The ventral part was largely occupied by the elongated and well-developed tongue. The tongue was somewhat triangular with blunt apex and rounded posterolateral ends (Fig. 1 and Fig. 2A). In between the two posterolateral lingual ends was the glottis (Fig. 1 and Fig. 2A). Two bulging elevations were present on the floor of the mouth, one on each side of the tongue; these elevations contained slot-shaped openings of glands (Fig. 2A). The tongue itself bore numerous tall papillae (Fig. 2A). These lingual papillae were not homogeneously shaped. They were slender, sharply pointed with a rough surface in the dorsalmost, anterior and middle portion of the tongue, but tall with a blunt apex and a smooth surface in the lateral and posteriormost parts (Fig. 2A, B, C). Higher magnification revealed that the rough surface of the slender papillae was due to numerous keratinocytes there; such keratinocytes were lacking in the blunt-tipped papillae (compare Figs. 2B and 2C). The keratinised, sharply pointed papillae tended to point posteriorly, an orientation that became gradually stronger toward the median line of the tongue (Fig. 2A).

The surface of the palate was also richly structured and bore the two large openings of the inner choanae (see schema in Fig. 1). Higher magnification revealed that the whole anterior palatal epithelium was keratinised (shown schematically in Fig. 1 and in detail in Fig. 3A). Keratinised epithelial cells exhibited maze-like shaped microplicae on their surface (Fig. 3F), whereas non-keratinised cells were studded with microvilli (Fig. 3E).

Taste buds were detected on the oropharyngeal surface based on their typical crater-like shape consisting of somewhat concentrically arranged, small epithelial cells (Fig. 3D). The taste pore, with its characteristic field of microvilli, lay in the centre of the taste bud swirl (Fig. 3D). These microvilli were larger and more numerous than those of other epithelial cells. Taste buds were not homogeneously distributed throughout the oropharyngeal cavity. Most taste buds occured in the anteriormost floor of the mouth, but were also found on a small lateral band, as well as adjacent to the tongue and behind it (Fig. 1). On the palate, taste buds were present posteriorly and on a small strip lateral to the choanae, in the very posterior interchoanal and postchoanal regions of the palate (Fig. 1). No taste buds were found in the anteriormost, praechoanal palate and on the anterior interchoanal part.

Light microscopy

General structure of the mucosa

The oral mucosa in *M. emys emys* consisted of an epithelial layer and the underlying lamina propria (Fig. 4A). Both epithelium and lamina propria varied in thickness and shape, depending on the regional specialisation of the mucosa. The lamina propria largely impacted the relief of the oral cavity by building up structures such as palatal ridges (Fig. 4A) and the tall and slender lingual papillae, which never contained muscle fibres (Fig. 5C). The epithelium was stratified and squamous, cuboidal or columnar. It consisted of three cell layers: the basal layer, the intermediate layer, and the superficial layer. The cells of the basal layer were large, rounded to cuboidal, and with a large round to oval nucleus in the centre. They directly overlay the basement membrane, which connected the epithelium to the lamina propria. The cells in the intermediate layer increased in size and passed into the superficial layer. Cells from the superficial layer were highly specialised and varied in their form according to their function. They were flattened (Fig. 4C, D), cuboidal (Fig. 4B) or columnar (mucus-producing goblet cells).

Secretory cells and glands

The oral glands were well developed in *M. emys emys*. Secretory cells were mostly organised

into glandular entities and single goblet cells were rare, but present. Glandular entities were found on the floor of the mouth ("sublingual glands", Fig. 5A, B, G), on the dorsal and lateral tongue ("lingual glands", Fig. 5B, C, G), around and above the glottis ("gular glands"), on the palate posterior to the choanae ("palatine glands", Fig. 5D, F, H). A giant glandular entity was present beneath the eye and embedded in adductor musculature; this entity emptied into the oropharyngeal cavity through a single duct ("*glandula anguli oris*", Fig. 5D, E). Generally, the above-described glands consisted of multiple, densely adjoining epithelial goblet cells, folded into the lamina propria just beneath the epithelial lining. They opened to the oropharyngeal cavity through more than one duct (except the *glandula anguli oris*) (Fig. 5A, B, F, G, H). The lingual glands were glandular krypts build up by numerous goblet cells and arranged between the tall lingual papillae (Fig. 5B, C). They were relatively small on the middle tongue part and increased in size and complexity laterally (Fig. 5B).

In contrast, the *glandula anguli oris* lay deeply sunken between jaw musculature, bony palate and beneath the eye (Fig. 5D). This very well-developed gland opened into the oral cavity near the corner of the mouth by a single but large duct with the pore between the posterior end of the choanae and the rhamphothecae. Histochemically, the oral glands of *M. emys emys* showed a positive reaction to Alcian blue (for acid mucosubstances), PAS (for neutral mucosubstances), or both Alcian blue and PAS (see Fig. 5A, B, D, E, F), but a negative reaction to Coomassie brilliant blue (for proteins, Fig. 5G, H). When stained with AB and PAS, all oral glands showed a certain polarity, with both AB- and PAS-positive goblet cells proximally and AB-positive cells distally (Fig. 5A, B, E, F).

Taste buds

We detected only one taste bud type in *M. emys emys*: the barrel- or onion- to flask-shaped one (Fig. 6A, B). Taste buds extended over the whole thickness of the oral epithelium and consisted of specialised epithelial cells. They were clearly distinguishable from the surrounding cells by their vertically clustered, slender construction (Fig. 6A, B). The narrow cytoplasmatic processes of the apical region reached freely with their microvilli into the taste pore (Fig. 6B). Taste buds were found in un- and slightly keratinised epithelia, but never in heavily keratinised areas. Sometimes they were found in small but dense aggregations close to glands (Fig. 6A, B). The heterogeneous distribution of these taste organs is illustrated in Fig. 1 and described in the SEM results above.

Keratinised epithelium

Beside the heavily keratinised rhamphothecae and adjoining regions, keratinisation in the oral cavity was present on the anterior floor of the mouth, the anterior lingual papillae, and the anterior part of the palate (see schema in Fig. 1). The keratinised cells belong to the superficial layer of the epithelium and were flattened and eosinophilic (Fig. 4A, C). During the final stages of maturation, the keratinocytes lost their nucleus and became pyknotic (Fig. 4C, D). Such matured keratinocytes appeared single layered or they were organised into keratinocyte-stacks. The thickness of these keratinocyte-stacks could vary from very few (Fig. 4C) to numerous keratinocytes (Fig. 4D). The epithelium in heavily keratinised areas was connected to the underlying lamina propria by periplasmatic protrusions (Fig. 4E), which were lacking in lightly or non-keratinised epithelia.

Feeding behaviour

The *M. emys emys* used in the present study spent approximately one third of their active phase within the water-filled basin in their terrarium. When food was offered on land, all animals grasped the food item with their jaws (Fig. 7A-C). During food ingestion, the tongue was always retracted (Fig. 1A, B) and lingual food prehension was never observed. When food was offered underwater, all animals sighted the food item and repeatedly tried to grasp it (Fig. 7A'-C'). As on land, the animals retracted their tongue and tried to seize the food with their jaws, but failed in all cases (as exemplarily shown in Fig. 7A'-C').

Discussion

The oropharyngeal cavity in tetrapods is involved in a variety of functions, comprising thermoregulation, oropharyngeal respiration, defense, mating, and perhaps most importantly, feeding (Druzisky and Brainerd, 2001). As an integral part of the oral cavity, the oral mucosa shows a variety of specialisations which are – alone or in combination – crucial for the adaptational success of a species to its environment. Turtles show a great diversity regarding their ecology – they span the range from fully aquatic to fully terrestrial with a full range of intermediate variations (Schwenk, 2000). This diversity is congruent with their feeding biology and is reflected in structural specialisations of the oropharyngeal mucosa (Winokur, 1988; Weisgram et al., 1989; Iwasaki, 2002; Iwasaki et al., 1996a, 1996b; Beisser et al., 1995, 1998, 2001, 2004; Heiss et al., 2008). The oropharyngeal mucosa in *M. emys emys*, consisting of lamina propria and stratified, mostly triple-layered epithelium, does not differ notably from the general structure described in other turtles (see Beisser et al., 2004 for overview). However, differences were found in the complexity, extension and topography of specialised epithelial tissue such as secretory entities, taste organs, and keratinisation.

Secretory cells and oral glands are found in all major vertebrate groups, from cyclostomes (see Gibbs, 1956; Cook et al., 1990; Xiao et al., 2007; Renaud et al., 2009) to mammals (e.g. Fahrenholz, 1937; Denny et al., 1997). Kochva (1978) listed 9 major oral gland types in reptiles. Most of these glands produce and contain mucosubstances to lubricate the oral surface. Others are used to secrete various bioactive substances (see Fry et al., 2006; 2009 for overview). Squamates (lizards and snakes) typically exhibit the greatest variety of oral glands within reptiles (e.g. Fahrenholz, 1937; Dornesco and Andrei, 1966; Kochva, 1978; Fry et al., 2006). While squamate oral glands have been studied in great detail (Fahrenholz, 1937; Dornesco and Andrei, 1966; Kochva, 1978; Kardong, 1982; Gopalakrishnakone and Kochva, 1990; Young et al., 2000; Fry et al., 2009), our knowledge with regard to turtles is limited. The occurrence, distribution, and complexity of oral glands in turtles have been suggested to correlate with feeding ecology (water vs. air and carnivory vs. herbivory) (Fahrenholz, 1937; Kochva, 1978; Winokur, 1988; Iwasaki, 2002; Beisser et al., 2004; Heiss et al., 2008; Natchev et al., 2009). Aquatic turtles (which are usually carnivorous to omnivorous) show simple single-celled glands in their oral cavity (Nalavade and Varute, 1976; Winokur, 1988; Weisgram et al., 1989; Iwasaki, 1992; Iwasaki et al., 1996b; Beisser et al., 1995; 1998; 2001). Terrestrial turtles (typically herbivorous to opportunistic) have prominent oral glands (Fahrenholz, 1937; Kochva, 1978; Winokur, 1988; Weisgram et al., 1989; Wochesländer et al., 1999). These glands were reported from the floor of the mouth (sublingual glands), the

palate (palatal glands), the corner of the mouth (*glandula anguli oris*), the area around the glottis (gular glands), and from the dorsal and lateral tongue between the lingual papillae (lingual glands).

Manouria is suggested to be basal to all other recent tortoises (according to Gaffney and Meylan, 1988; Meylan and Sterrer, 2000; Takahashi et al., 2003; Spinks et al., 2004) – or to share this basalmost position with the sister genus *Gopherus* (Le et al., 2006; Fritz and Bininda-Emonds, 2007). *Manouria* however, and especially the subspecies *M. emys emys*, shows a strong affinity toward aquatic environments (Obst, 1986; Ernst and Barbour, 1989; Hoybye-Mortensen, 2004). As all testudinids had aquatic ancestors (Joyce and Gauthier, 2004; Li et al., 2008; Anquetin et al, 2009), and aquatic turtles show scarcely developed oral glands, *M. emys emys* was expected to show moderately developed glandular tissue in its mouth, as has been observed in semiaquatic geoemydids (Heiss et al., 2008; Natchev et al., 2009). Contrary to these predictions, its oral glands are very well developed. Compared with the data from more highly evolved testudinids, *M. emys emys* shows a similar amount and complexity of glandular oral tissue. Even if single secretory goblet cells are still present, most glandular tissue is organised into large entities: tubular, monostomatic, and polystomatic glands. The latter are often organised into glandular fields (e.g. on the postchoanal palate). The columnar secretory cells forming the glandular entities contain neutral (PAS-positive) to acidic (AB-positive) mucosubstances and no detectable amounts of proteins (Coomassie Brilliant blue-negative). The main function of a mucus film covering the oral epithelium is probably to lubricate the oral epithelium to avoid dehydration and to improve food ingestion, oropharyngeal transport, and swallowing (Bramble and Wake, 1985; Schwenk, 2000). The abundant mucus in the oral cavity of *M. emys emys* may be especially advantageous when feeding on hard plant material in a terrestrial environment.

The taste organs in *M. emys emys* are represented by a single type of taste buds: the barrel- or onion- to flask-shaped one. This type apparently represents the typical reptile taste organ, as found in a variety of species: Lepidosauria (Squamata and *Sphenodon*) (Kroll, 1973; Schwenk, 1985, 1986, 2000; Uchida, 1980; Berkhoudt et al., 2001), crocodiles (Bath, 1905; Puterill and Soley, 2003, 2004), and turtles (Korte, 1980; Uchida, 1980; Beisser et al., 1998; Heiss et al., 2008). In contrast, birds show three morphologically distinguishable types (Berkhoudt, 1985). Taste buds are used as a chemosensory organ at close distance to discriminate between potential food items when food is held between the jaws. Berkhoudt (1985) was the first to observe that taste buds are sometimes aggregated around mucus glands in birds. Such a correlation between taste buds and compound oral glands is also found in *M.*

emys emys but in no other turtle studied so far (see Weisgram et al., 1989; Beisser et al., 1998; Iwasaki, 2002; Heiss et al., 2008 for overview). Navalade and Varute (1976) suggested that mucus may have an intricate functional significance in taste reception.

Taste buds are not distributed randomly within the oral cavity in reptiles. Schwenk (1985), for example, reported the highest taste bud density on the tongue tip of iguanid lizards. They use lingual food prehension (Delheusy et al., 1994; Herrel et al., 1995; Schwenk, 2000; Schwenk and Rubega, 2005; Schaerlaeken et al., 2007) and the tongue tip therefore contacts the food first. The semiaquatic turtle *Cuora amboinensis*, however, showed the significantly highest taste bud density on the small palatal area anterior to the choanae (Heiss et al., 2008). That species practices jaw prehension to grasp food items (Heiss et al., 2008; Natchev et al., 2009) and the very anterior palate is therefore the first to contact food. In *M. emys emys*, most taste buds occur in the anteriormost floor of the mouth, but were also found on a small lateral band, as well as adjacent to the tongue and behind it. Dorsally, taste buds were present laterally to the choanae and on the more posterior part of the palate (see Fig. 1). The highest taste bud density on the anteriormost floor of the mouth is congruent with preliminary studies performed on the feeding mechanism of this species. They showed tongue retraction and jaw prehension when grasping food. The first food contact therefore involves the anterior floor of the mouth and palate. While the anteriormost palate is strongly keratinised and lacks taste buds, the taste bud aggregation on the anteriormost floor of the mouth allows a fast feedback response to the just grasped food item. A fast chemical test of the food before further processing is crucial to allow a fast decision to avoid harmful items. Taste buds located more posteriorly in the oral cavity indicate that a continuous examination of the food during oral processing is also important. Analogous correlations of taste bud aggregations and feeding mechanisms were also reported for some birds (see Berkhoudt, 1977, 1985; Zweers et al., 1977).

The most efficient mechanical protection of the oral mucosa against noxious influences is probably keratinisation. Such keratinisation is quite common in tetrapods (Zweers et al., 1977; Schwenk, 1985; Toubeau et al., 1994; Iwasaki et al., 1996a; 1996c; 1997; Schwenk, 2000; Putterill and Soley, 2003, 2004; Squier and Finkelstein, 2003) but is especially evolved in turtles and birds. The cornified beaks, or rhamphothecae, replace the teeth and play an important role in a variety of functions (see Heiss et al., 2008). In *M. emys emys*, the most heavily keratinised areas are, apart from the rhamphothecae, the anterior region of the palate and the anterior half of the dorsal lingual surface (Fig. 1). These areas are most exposed during jaw prehension, when injuries are most likely. To withstand the mechanical stress, the

basal cells of heavily keratinised epithelia are interconnected to the basal membrane by basal cytoplasmatic protrusions. This increases the adhesion to the lamina propria (Heiss et al., 2008) and ensures mechanical stability. The amply keratinised oral surface in *M. emys emys* represents an adaptation to the terrestrial habitat. Such keratinisation, together with further adaptational changes during evolution, is thought to be essential during the transition of vertebrates from freshwater to land (Iwasaki, 2002).

The preliminary functional study showed that *Manouria* is clearly a terrestrial feeder, as expected for all testudinids (see Bels et al., 2008 for overview). Nonetheless, *M. emys emys* uses jaw prehension when grasping food and the tongue plays a subordinate role. Even if large and beefy, the tongue shows a primitive character not shared with other testudinids studied so far: The intrinsic tongue muscle fibers do not extend into the lingual papillae. Primitive non-muscular lingual papillae are known only in aquatic and semiaquatic ("non testudinid") turtles. Similarly, jaw prehension is not known for any other testudinid, but is obligatory for semiaquatic and aquatic turtles (Lauder and Prendergast, 1992; Bels et al., 1997, 2008; Lemell and Weisgram, 1997; Summers et al., 1998; Heiss et al., 2008; 2010; Natchev et al., 2009; 2010). The high affinity of *M. emys emys* to water is reflected by its habit of spending a lot of time in the water. It repeatedly submerges the whole body when trying to ingest food items from the bottom of the aquarium, although our animals never succeeded in feeding there.

In summary, the primitive testudinid *Manouria emys emys* shows oral anatomical features believed to be characteristic for purely terrestrial turtles. These include the large tongue that bears abundant tall and slender lingual papillae, the well-developed oral glands, and the extensive keratinisation of the oral mucosa. On the other hand, some primitive features in this species are characteristic for aquatic or semiaquatic turtles. Accordingly, the tongue lacks muscular papillae and seems to play a subordinate role in food prehension. While all purely terrestrial turtles use obligatory lingual prehension, *Manouria* retracts the tongue and food is grasped by the jaws. Jaw prehension is typically used by aquatic and semiaquatic turtles. Like semiaquatic turtles, *Manouria* shows a high affinity to water but, contrary to them, is unable to ingest food underwater. This turtle therefore could have retained some primitive features – a heritage from the aquatic (or semiaquatic) testudinid ancestors and could represent a link between semiaquatic and terrestrial turtles.

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Figures

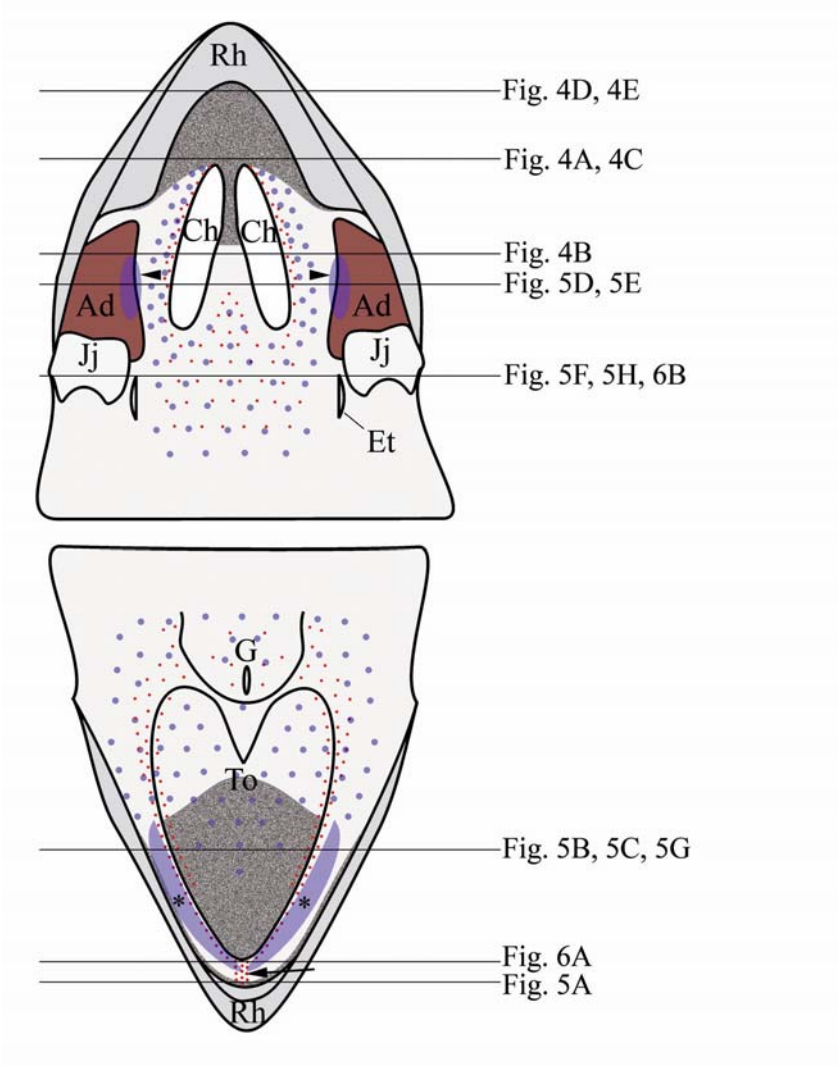

Fig. 1. Schematic drawing showing the oral topography in *M. emys emys*. Top: palate, bottom: floor of mouth. Strongly keratinised regions of anterior palate and anterior half of tongue are visualised grey-speckled. Oral glands are tagged in blue. Sublingual gland (asterisks) and *glandula anguli oris* (arrowheads) are the largest oral secretory entities in *M. emys emys*. Smaller gland distribution indicated by blue dots. Taste buds (small red dots) are not homogeneously distributed but restricted to certain regions. Note taste bud aggregation in anteriormost floor of mouth (arrow). Horizontal lines indicate where histological sections were taken (Fig. 4, 5, 6). Ad, adductor mandibulae; Ch, chonae; Et, eustachian tube; G, glottis; Jj, jaw joint; Rh, rhamphothecae (horny beak); To, tongue.

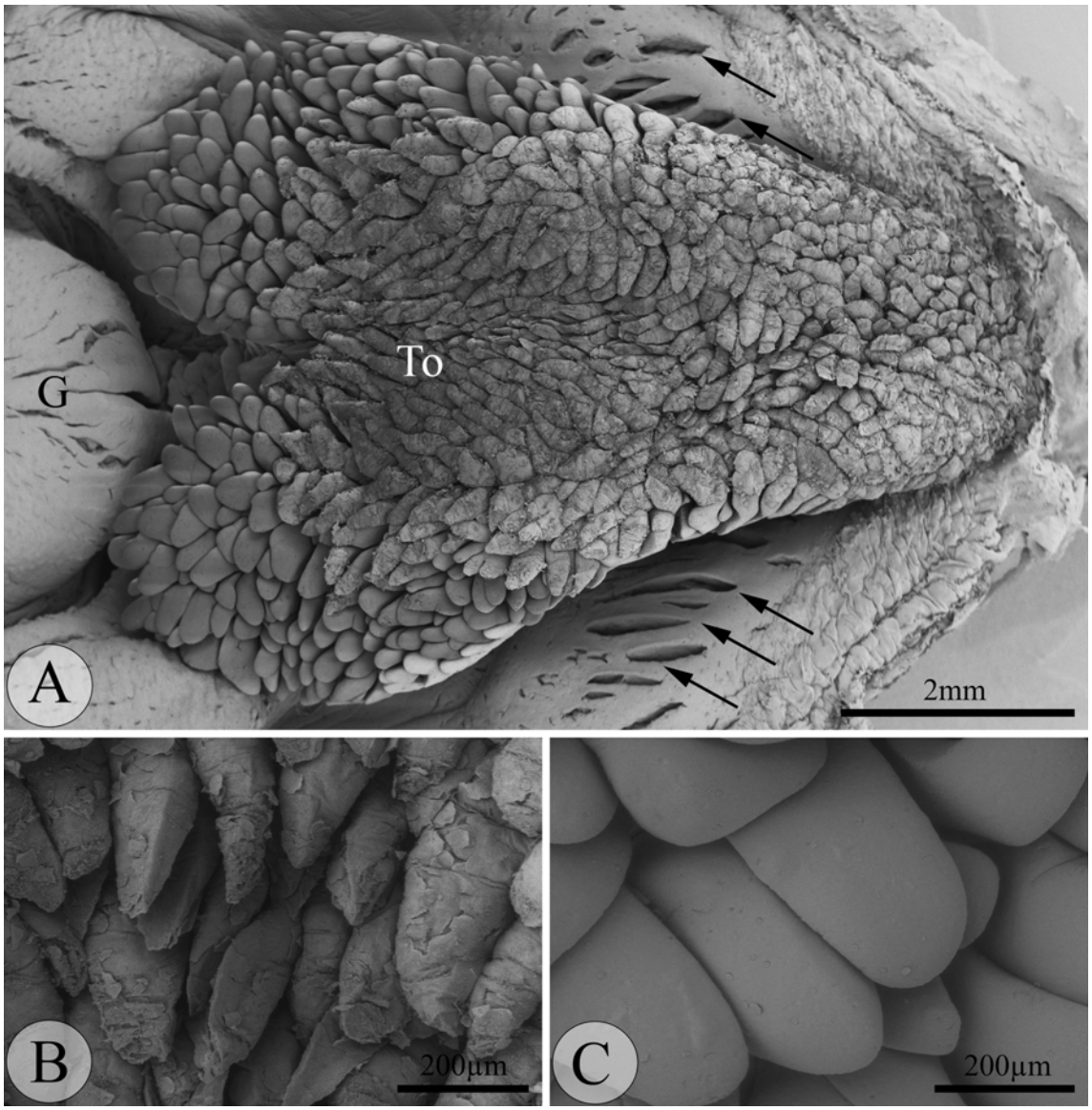

Fig. 2. Scanning electron micrographs of floor of mouth. A. overview showing large tonge (To), glottis (G), and bulge lateral to tongue bearing openings of sublingual gland (arrows). B. Detail of strongly keratinised lingual papillae. Note rough surface and pointed apex. C. Detail of non-keratinized lingual papillae showing smooth surface and blunt apex.

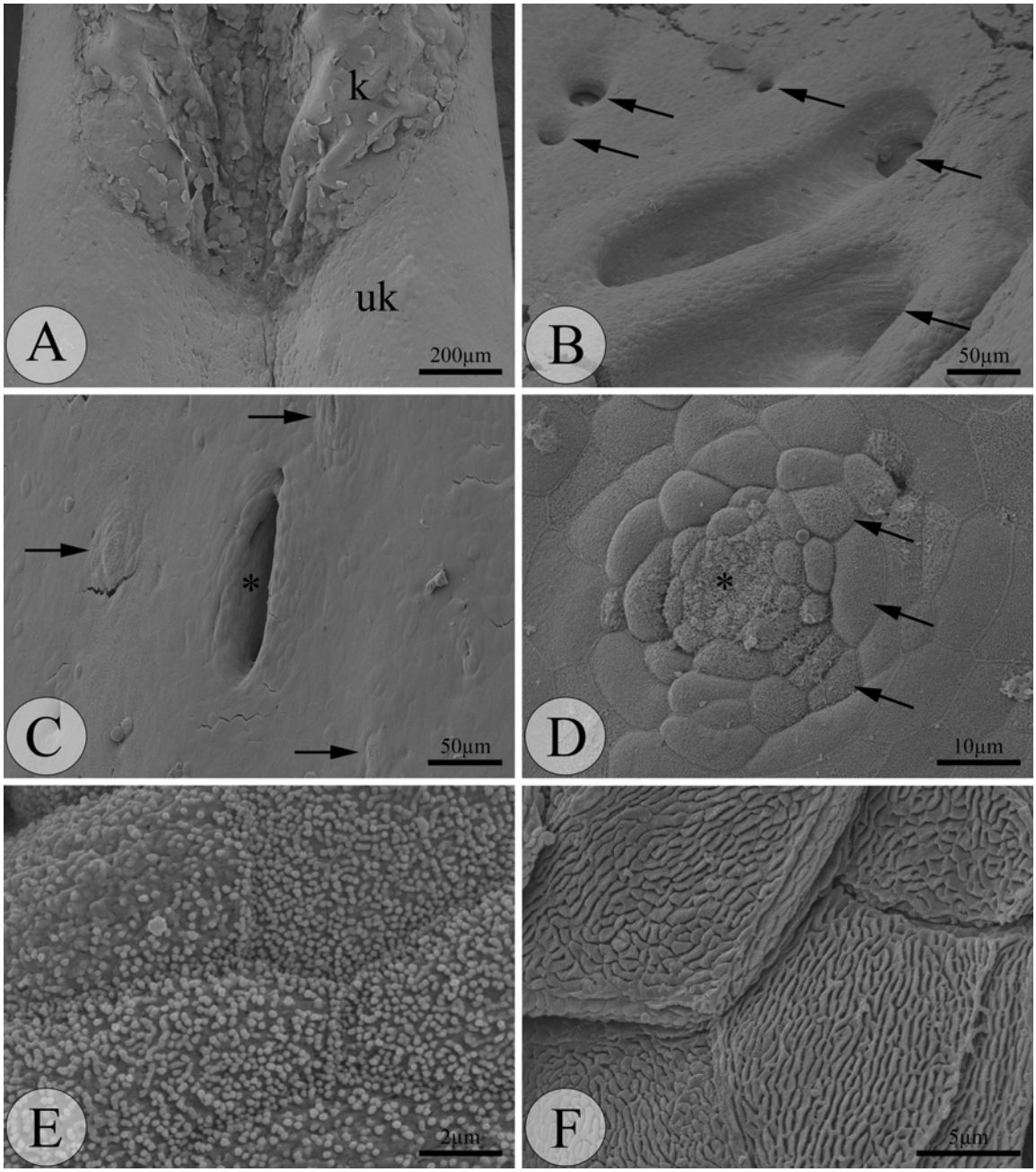

Fig. 3. Scanning electron micrographs of palate. A. Keratinised mucosa (k) shows a much rougher surface than the unkeratinised one (uk) and the border line is easy to follow. B. gland pores (indicated by arrows) that open into oral cavity. C. Taste buds (arrows) next to a gland opening (asterisk). D. Surface structure of taste bud characterised by small cells (arrows) surrounding taste pore, which lies in the centre (asterisk). E. non-keratinised epithelial cells are studded with microvilli. F. Keratinocytes show a maze-like relief on the surface, the so-called microplicae.

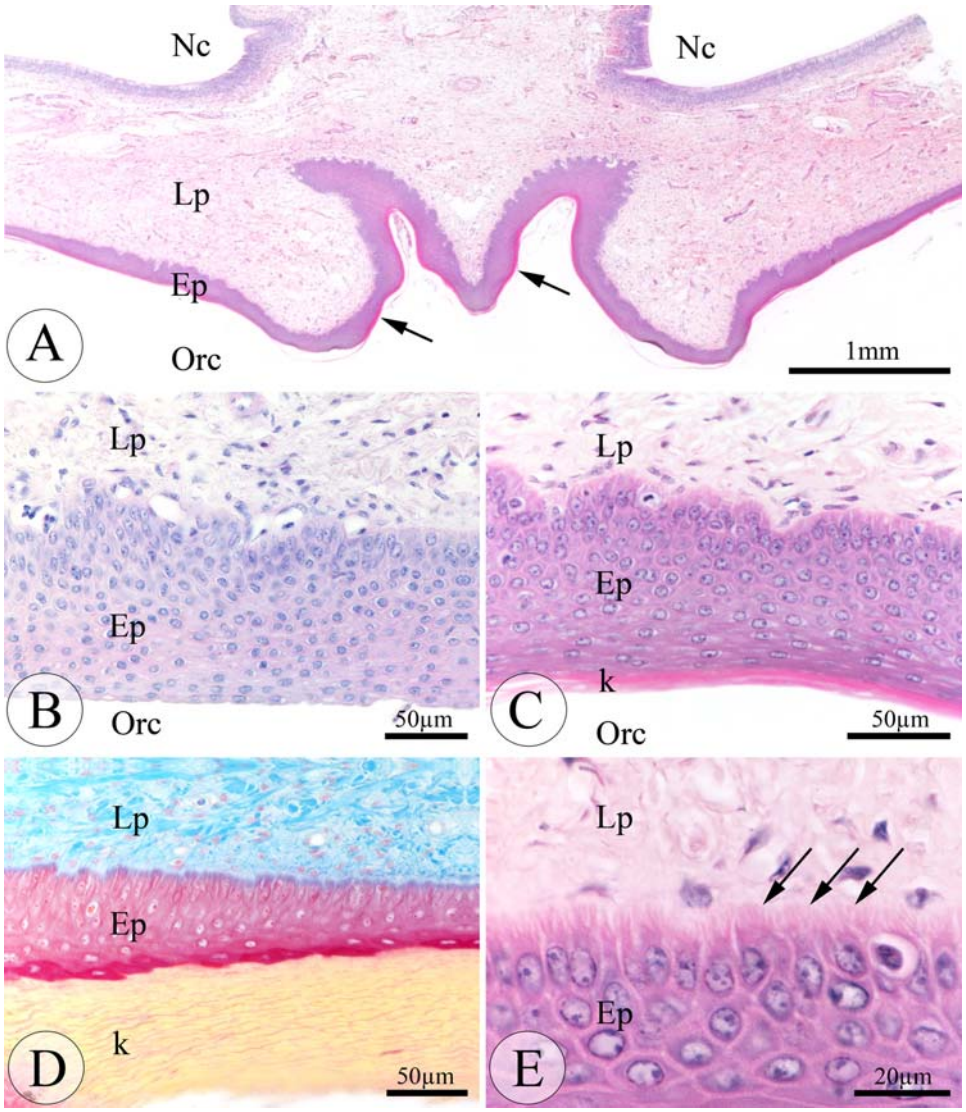

Fig. 4. Light micrographs of histological cross sections from palate. For better orientation, the black horizontal lines in the schematic drawing of Fig. 1 indicate where the sections were taken. A. overview of praechoanal region. In general, the mucosa consists of a connective tissue sheath, the lamina propria (Lp), and the overlying epithelium (Ep). Note eosinophilic (pink) keratinocytes (arrows) and lack of glandular tissue here. Epithelium of oral mucosa can be non-keratinised (shown in B), lightly keratinised (shown in C), or heavily keratinised (shown in D). Note lack of nuclei in the flat mature keratinocytes in C and D. E. In heavily keratinised regions, epithelium is connected basally to lamina propria through numerous periplasmatic protrusions (arrows). Staining: A, B, C & D: H-E, E: Azan. Abbreviations: Ep, epithelium; k, keratin layer; Lp, lamina propria; Nc, nasal cavity; Orc, oral cavity.

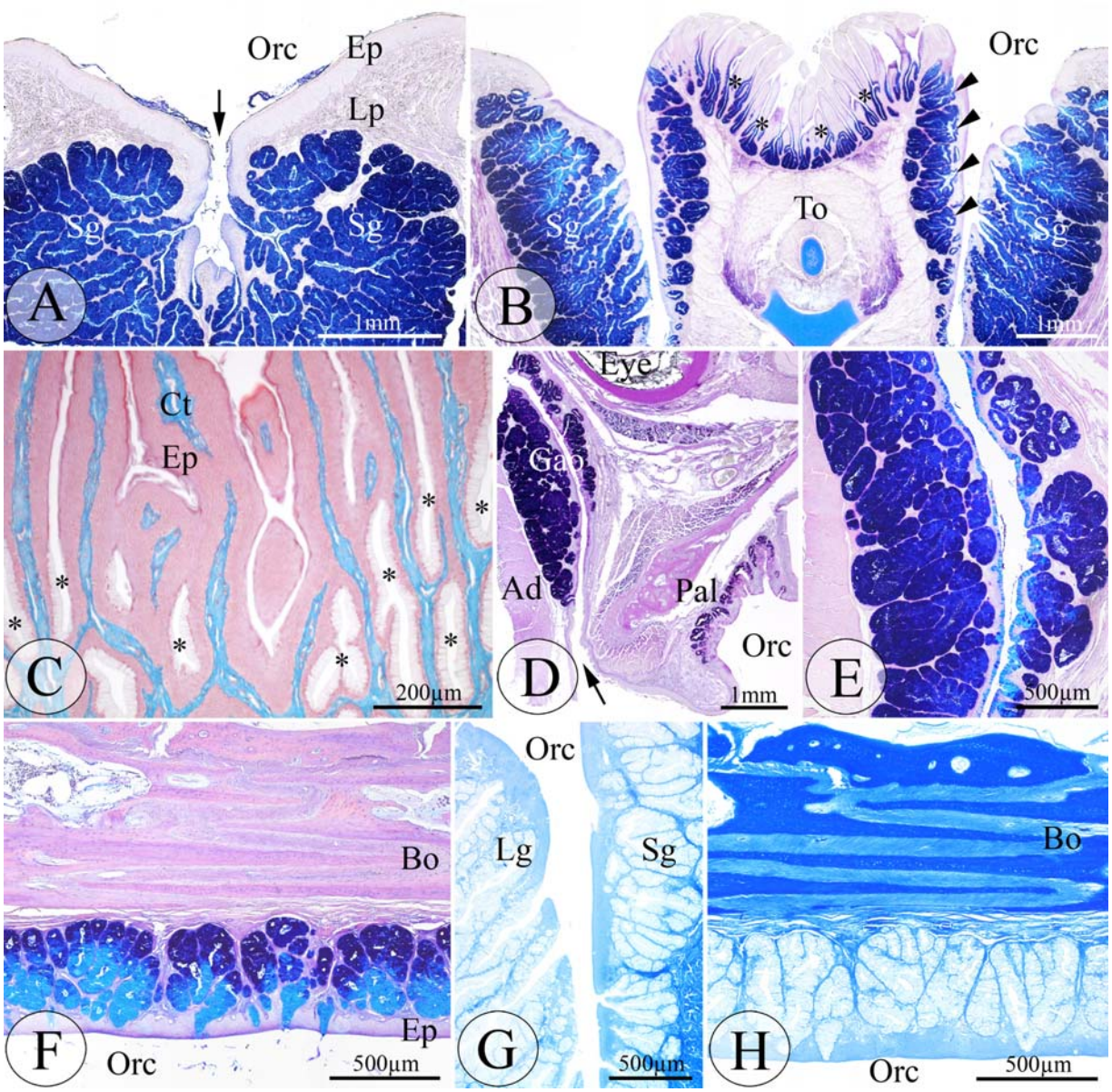

Fig. 5. Light micrographs of histological cross sections. For better orientation, the black horizontal lines in the schematic drawing of Fig. 1 indicate where the sections were taken. A. Anteriormost part of floor of mouth. The very well-developed left and right sublingual glands (Sg) fuse together in this region. Note median groove in which they empty their secretion (arrow). B. More posteriorly, sublingual glands (Sg) shift to the lateral wall and the median groove is replaced by the well-developed beefy tongue (To). Note tube-like glands between lingual papillae in apical region of tongue (asterisks) and branched glands in lateral tongue area (arrowheads). C. Detail of apical tongue area showing that lingual papillae are not infiltrated by intrinsic musculature. Papillae are surrounded by epithelium (Ep, red stained) and contain connective tissue (Ct, blue stained). Asterisks mark the apical, tube-shaped lingual glands. D. Overview and E detail of *glandula anguli oris* (Gao). Note that this gland entity opens through a single common duct to the oral cavity (arrow in D). F. Posterior to

glandula anguli oris, the palatal glands are also well developed and fill most of the space within the lamina propria between the bony roof of the mouth (Bo) and the palatal epithelium (Ep). No oral glands in *Manouria* showed positive reaction to Coomassie brilliant blue (shown in G for sublingual and lingual glands and in H for palatal glands). This indicates insignificant amount of proteins there. Staining: A, B, E, F: PAS-AB-H; D: PAS-H; G & H: Coomassie brilliant blue. Abbreviations: Ad, adductor musculature; Bo, bone; Ct, connective tissue; Ep, epithelium; Eye, eye; Gao, *glandula anguli oris*; Lg, lingual gland; Lp, lamina propria; Orc, oral cavity; Pal, palate; Sg, sublingual gland; To, tongue.

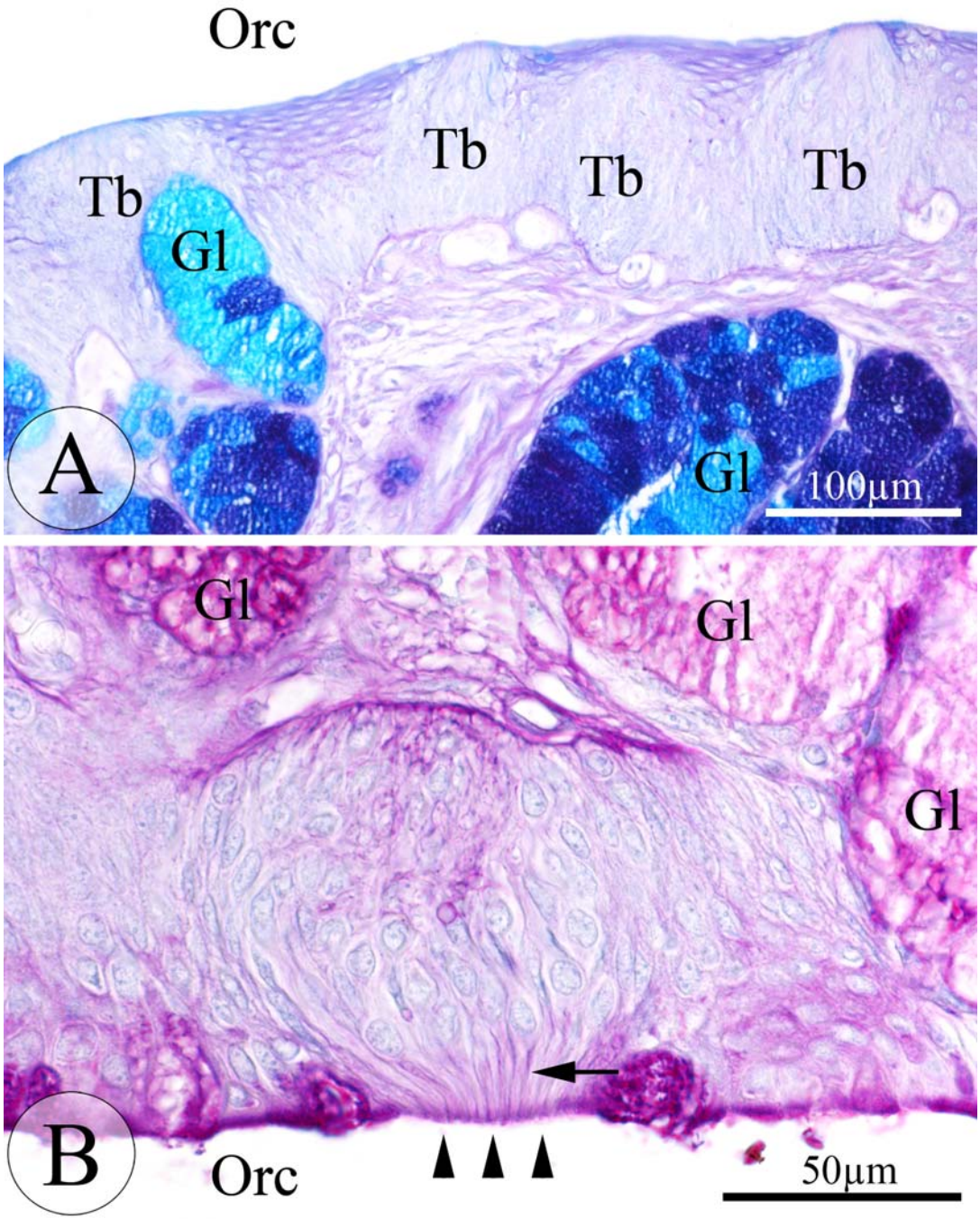

Fig. 6. Light micrographs showing taste buds in *M. emys emys*. A. Overview with four taste buds (Tb) next to glands (Gl). B. Detail of a typically barrel- to onion-shaped taste bud. The nuclei are located in the basal three fourths and the slender cytoplasmatic processes (arrow) in the superficial fourth. Arrowheads indicate taste pore. Staining: A: AB-PAS; B: PAS-H. Abbreviations: Gl, gland; Orc, oral cavity; Tb, taste bud.

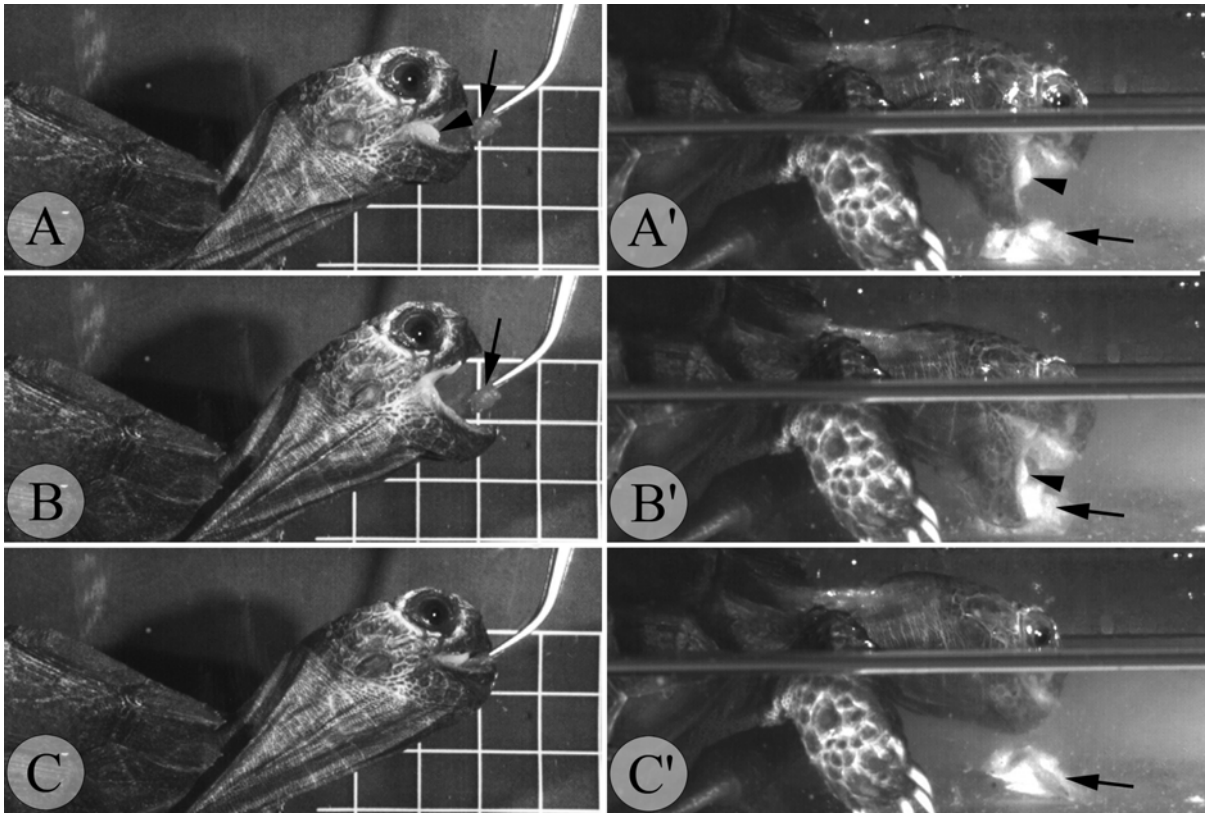

Fig. 7. Selected video frames showing *M. emys emys* grasping a small piece of meat (arrow) offered by forceps on land (A-C) and on the bottom of an aquarium with 4 cm water level (A'-C'). A. Head approaches food item and mouth begins to open. Tongue indicated by arrowhead. B. Mouth opening continues until peak gape is reached, the tongue is retracted. C. Tongue remains retracted and as mouth is closed; jaws hold food item. When food was offered underwater, animals sighted it (A') and approached with opened mouth (B') while the tongue (arrowhead) remained retracted. *Manouria emys emys* never successfully ingested food underwater (C').

3. Conclusions

3.1. The oropharyngeal mucosa: always the same?

The general structure of the oropharyngeal mucosa in the examined species does not differ notably from those described in other turtles (Beisser et al., 2004), or amniotes in general (Iwasaki, 2002). However, direct comparisons of *Sternotherus odoratus*, *Cuora* spp. and *Manouria emys* revealed differences in the complexity, extension and topography of specialised mucosal tissue such as secretory entities, taste organs and keratinisation. Secretory cells and oral glands are present in all major vertebrate groups, but seem to have gained special importance in tetrapods. The increase in number and complexity of compound oral glands was probably connected with the emergence of amphibians onto land: a lubricating substance was necessary in the oral cavity to avoid dehydration and help catch and swallow prey in the new environment (Fahrenholz, 1937). The first oral glands in tetrapods were probably mucosubstances-producing structures to help maintain homeostasis. Amphibian oral glands show the full range from simple (single-celled glands) to highly complex structures (Fahrenholz, 1937). As anamniotes, however, most amphibians are still highly associated with water (especially for reproduction). Reptiles were therefore the first amniotes to invade a truly terrestrial environment and were faced there with the need to subsist on dry food. In turtles, the occurrence, distribution and complexity of oral glands have been suggested to correlate with feeding ecology (Fahrenholz, 1937; Kochva, 1978; Winokur, 1988; Iwasaki, 2002; Beisser et al., 2001a; 2004; Heiss et al., 2008, 2010; Natchev et al., 2009a). Consequently, the oropharyngeal glands in the aquatic species *S. odoratus* are single goblet cells embedded in the superficial layer of the epithelium (Heiss et al., 2010). No multicellular glands were found in *S. odoratus* – a situation typical for highly aquatic turtles (Beisser et al., 1995, 1998, 2001b, Iwasaki et al., 1996; Lemell et al., 2010). By contrast, the *Cuora* material examined showed fields of goblet cells, partly folded into shallow intraepithelial crypts on the palate and deeper ones on the tongue (Heiss et al., 2008; Natchev et al., 2009a). This situation probably represents the next step in the evolution of glandular complexity in turtles, culminating in the highest-evolved family: the Testudinidae. Consequently, the testudinid *M. emys* showed enlarged glandular entities on palate, tongue and floor of the mouth (Heiss et al., to be submitted). These results clearly confirm the subordinate role of oropharyngeal glands in aquatic turtles and the need to develop larger glands when switching to a terrestrial feeding ecology.

Another important innovation in the context of turtle transition from water to land is the development of keratinisation in the oral mucosa (see Iwasaki, 2002 for overview). As mentioned above, cells of the mucosal epithelium migrate and mature from the basal to the superficial layer. In keratinocytes, this process includes the cellular production of keratin fibres. The cell becomes increasingly flattend on its way to the surface and, finally, the nucleus becomes pycnotic (Squier and Finkelstein, 2003). Such dead mature keratinocytes adhere to the epithelium and can build up layers of varying thickness. Keratinisation in the oral epithelium occurs as a mechanical protection for the soft epithelium, but plays also an important role in maintaining homeostasis in terrestrial amniotes (Iwasaki, 2002). Accordingly, one expectation was that the amount of keratinisation may also reflect the ecological framework of the turtles examined in the present study. In fact, the aquatic *S. odoratus* showed only the typically flattened keratinocytes on the ramphothecae and regions very close to them, while the semiaquatic *C. amboinensis* had additional keratinised tissue in the anterior (praechoanal) palate. In *M. emys*, large-scale keratinisation was detected on the prae- and interchoanal region and on the anterior two-thirds of the tongue, which largely covers the floor of the mouth (Heiss et al., to be submitted).

Taste buds in the oral cavity are described for most tetrapods and are used as chemosensory organs at close distance. While various taste bud types are described for birds (Berkhoudt, 1985) and mammals (Adler et al., 2000), only one morphologically distinct taste bud type could be detected in reptiles. Taste bud structure did not differ notably in the species examined here. They span the whole thickness of the epithelium, are barrel- to flask-shaped, and open with their microvilli into the oral cavity through the taste pore. Even if general taste bud morphology was similar amongst species, their distribution patterns varied considerably and apparently depended on the feeding biology. While *C. amboinensis* and *S. odoratus* showed the highest taste bud concentration on the anteriormost, praechoanal palate, *M. emys* lacked them completely there, but showed a striking accumulation of taste buds on the anteriormost floor of the mouth. This tendency toward taste bud concentration in the anterior mouth could be explained as a functional adaptation to jaw prehension, the food prehension mode used by all turtles examined in this study. When capturing prey or grasping food items by the jaws, the anteriormost palate and floor of the mouth are the first parts to have contact with the potential food. This enables a fast chemosensory feedback response and a rapid decision on the potential food: “eat it or leave it.” Rapid rejection is especially important to avoid further contact with harmful items. At the same time, a continuous examination of food

during intraoral processing and transport is also possible because taste buds were spread throughout the oropharyngeal cavity and even behind the glottis.

Apart from feeding, the oropharynx also contributes to a variety of other functions (Druzisky and Brainerd, 2001); the design of the oropharyngeal cavity meets the challenge to find compromises between structural design and function. The kinosternid *S. odoratus*, for example, relies on hydrodynamic feeding mechanisms. To induce an effective water stream into the mouth, a smooth oropharyngeal surface would be advantageous (Beisser et al., 1995, 2001b; Lemell et al., 2000, 2002, 2010). *S. odoratus*, however, was shown to practise oropharyngeal gas exchange underwater, and the higher the degree of mucosal surface amplification, the more effective oropharyngeal gas exchange is (according to Feder and Burggren, 1985). The surface-amplifying respiratory oropharyngeal papillae in *S. odoratus* are slender and tall, lobe-like and oriented longitudinally to the body axis. Such a design and orientation opposes only the smallest papillar surface to instreaming water, probably minimising any negative impacts on the smooth water flow in hydrodynamic feeding mechanisms. Due to that compromise, *S. odoratus* still can effectively feed underwater and has the advantage of being able to remain submerged for very long periods (Ultsch, 1985, 1988; Ultsch and Wasser, 1990; Ultsch and Cochran, 1994; Ultsch and Jackson, 1995; Heiss et al., 2010).

3.2. The tongue

As feeding ecology largely correlates with tongue morphology (see also Introduction section), morphological differences were expected in the examined species – and the differences were huge. The aquatic *S. odoratus* had a tiny tongue with minimally developed musculature and few but large, flattened and lobe-like papillae. The lingual mucosa contained single goblet cells and no keratinocytes were observed. The *Cuora* species had a much larger tongue with well-developed musculature and tall and slender papillae. In between these papillae were larger tubular mucous glands, consisting of densely packed goblet cells. As in *S. odoratus*, *Cuora* spp. showed no detectable keratinisation in the lingual mucosa. The tongue in *M. emys* was considerably larger, and especially longer than those of the other turtles. The lingual papillae of this beefy tongue were slender and high; large tubular glands were present on the bases of these papillae. The papillae of the anterior two-thirds had a pointed apex and were covered by keratinised epithelium. In contrast, those from the posterior third were blunt and unkeratinised. These differences in tongue morphology confirm the four working hypotheses: 1. during the transition from water to air, the tongue needs an increase in size and mobility; 2.

the increased requirement of mucus on land (wet adhesion, see Introduction) is reflected by considerable enlarged glands; 3. the lingual papillae become more numerous and tall (to promote the "interlocking effect", see Introduction); and 4. the lingual epithelium becomes gradually keratinised (e.g. to maintain homeostasis, see section above).

3.3. Feeding behaviour and kinematics

Many studies on the kinematics of feeding in tetrapods are based on the assumption that movements of jaws, hyoid and tongue are highly stereotypical amongst tetrapods when feeding (e.g. Bramble and Wake, 1985; Lauder and Shaffer, 1988; Lauder and Reilly, 1988; Bels and Goose, 1990; Reilly and Lauder, 1990, 1992; Bels and Delheusy, 1992; Lauder and Reilly, 1994; Reilly, 1996; Bels et al., 1997, 1998; McBrayer and Reilly, 2002; Bels et al., 2008). When feeding underwater, most tetrapods use hydrodynamic mechanisms to get the food item into the mouth (see chapter 1.4 for references). In most tetrapods, the approach to the food is followed (or combined in different grades) by a fast jaw opening and hyoid depression to compensate the bow wave and/or to suck the food item in (see also chapter 1.4). This was also true for the aquatic species *S. odoratus* and the semiaquatic *Cuora* spp. The terrestrial *M. emys* has lost the ability to create suction forces under water, resulting in an incapability to ingest food there. To transport the food from the jaws to the oesophagus, *Cuora* spp. and *S. odoratus* predominantly rely on hydrodynamic mechanisms. Interestingly, the most terrestrial *Cuora* species studied so far, *C. galbinifrons*, switches from hydrodynamic intraoral food transport to lingual transport when feeding on larger objects, pointing to a high degree of behavioural plasticity. On land, all turtles examined here used their jaws to grasp food. This was expected for *S. odoratus* and *Cuora* spp., but surprising for *M. emys*. While all aquatic to semiaquatic turtles studied so far use obligatory jaw prehension (see Bels et al., 2008 for overview), all exclusively terrestrial turtles (i.e. the family Testudinidae) were reported to rely on lingual food prehension (see Wochesländer et al., 1999; Bels et al., 2008 for overview). The intraoral food transport on land was lingual based in the species examined, except in *S. odoratus*, which was incapable of terrestrial transport due to missing structural prerequisites (Heiss et al., 2010; Natchev et al., 2010b).

Concerning intraoral food transport, a four-phase jaw cycle was proposed as the generalised feeding cycle – slow opening, fast opening, fast closing and slow closing – together with phase-specific movements of hyoid and tongue, (see also chapter 1.4). Consequently, the "neuro motor patterns" that should control these movements were suggested to be conserved during the origin, either in ontogeny or phylogeny, of behavioural novelties (Lauder and

Shaffer, 1988). The generalised feeding cycle model proposed by Bramble and Wake (1985) could not be supported by the kinematic analysis in the present study. Even if rhythmic movements of the jaws and hyoid apparatus were evident, a strict and objective distinction in the four jaw-movement-phases (see also section 1.4) was not possible. The turtle species examined showed a high variability in their kinematic profiles, pointing to a certain plasticity and modulatory potential of their feeding behaviour. As *Cuora* spp. and *S. odoratus* are opportunistic feeders rather than specialists (Pritchard, 1979; Obst, 1986; Bonin et al., 2006), a continuous sensorimotor feedback probably allows these modulations, because a certain modulatory capacity seems to be essential when relying on varying food types. The impact of different food types on feeding kinematics was clearly demonstrated in the pleurodiran *Pelusios castaneus* (Lemell and Weisgram, 1997). By contrast, species with a specialised feeding niche show certain similarities to the Bramble and Wake (1985) model. These so far studied specialists are the marine *Dermochelys coriacea* (Bels and Renous, 1992; Bels et al., 2008), presumably feeding on jellyfish, the piscivorous *Chelus fimbriatus* (Lemell et al., 2002), and the herbivorous *Testudo hermanni* (Wochesländer et al., 2000). The only exception is represented by the semiaquatic *Terrapene carolina*: an opportunistic feeder fitting into the generalised feeding cycle model (Bels et al., 1997).

When comparing the literature data, however, it is important to consider that the precision of the methods used to study feeding kinematics has increased dramatically in the past few years (e.g. digital cinematography allowing very high frame rates coupled with semiautomatic analysis software; Natchev et al., 2009b). Recent studies on the feeding kinematics of other tetrapods showed that feeding behaviour is much more complex than previously thought, requiring caution when formulating general theories that are based on a very small percentage of species (according to Alfaro and Herrel, 2001; Herrel et al., 2001; Ross et al, 2007; Vincent et al., 2007; Schaerlaeken et al., 2007, 2008; Montuelle et al., 2009). Another weakness of the Bramble and Wake model is the lack of evidences for homology of the movements of the feeding apparatus across tetrapods – though many morphological structures responsible for similar movements are not homologous amongst tetrapod classes (see Dullemeijer, 1994 and Smith, 1994 for overview).

In their comparative study on feeding performance in the urodele *Ambystoma tigrinum* during ontogeny, Lauder and Shaffer (1988) showed similar motor patterns in similar behaviours (i.e. aquatic feeding in pre- and post-metamorphosed animals). This behaviour, however, changed significantly when metamorphosed individuals fed on land, again weakening the hypothesis of motor pattern conservatism (Smith, 1994). Wainwright et al. (1989), comparing five highly

divergent species, showed that even if general features of the motor pattern were the same in all species, highly significant differences were found among the species in the average muscle activity pattern. This indicates that any tendency toward conservation within genera and families breaks down in a broader phylogenetic context (see also Smith, 1994).

In conclusion, a neuronal central pattern generator (CPG) which produces rhythmic jaw opening and closing may exist in tetrapods, but the oscillations of the CPG can be modified to produce a variety of movements, rhythms, and combinations of muscle activity patterns. When behavioural novelties appear (e.g. terrestrial feeding), motor patterns also change (Smith, 1994). According to Alfaro and Herrel (2001), "neuronal circuitry might be expected to show even greater evolutionary plasticity than other levels of design in order to accommodate the striking changes in morphology and function present during ontogeny and evolution."

3.4. Plasticity in feeding biology and evolutionary implications

As described in the previous chapter, feeding biology in tetrapods apparently involves much more variation than previously thought. Consequently, a high degree of plasticity rather than a stereotypical feeding biology seems to be valid in turtles. Plasticity is found in the interspecific morphology of the feeding apparatus not only in a broader phylogenetic context, but also within families and genera. While distantly related turtles such as *S. odoratus* and *M. emys* show a completely diverging design of their feeding apparatus, clear differences were also found within three species of the genus *Cuora*. All three *Cuora* species examined are semiaquatic and can feed both on land and underwater, but show varying tendencies toward one or the other medium. Whereas *C. amboinensis* prefers aquatic environments and *C. galbinifrons* terrestrial ones, *C. flavomarginata* assumes an intermediate position. The skull, hyoid, tongue, and associated muscles in *C. amboinensis* show similarities to aquatic feeding turtles, whereas the feeding apparatus of *C. flavomarginata* (and perhaps more so that of *C. galbinifrons*) more closely resemble those of terrestrial forms. Consequently, when feeding underwater, the mean capture cycle duration in *C. amboinensis* is about five times shorter than that in *C. galbinifrons.* In contrast, the better-developed tongue with numerous tall lingual papillae in *C. flavomarginata* and *C. galbinifrons* makes the lingual-based transport on land much more effective. *C galbinifrons* even uses lingual transport when feeding on larger prey underwater. This is atypical for turtles and again shows their modulatory behavioural capacity. The food transport in the investigated species (i.e. *Cuora* spp. and *S. odoratus*) furthermore seems to be permanently modulated, fine tuned, and optimised by sensory

feedback. A special example of plasticity in feeding behaviour was observed in *S. odoratus* and *M. emys*. Due to their ecomorphological specialisations, both species are restricted to one medium to take up food (*S. odoratus* to water, *M. emys* to land). Regardless of these constraints, both attempt to feed in the other medium (*S. odoratus* on land and *M. emys* under water). In this study they never succeeded despite repeated efforts. *S. odoratus*, however, was able at least to grasp food items on land and to ingest them when brought to water, and *M. emys* finally succeeded to feed on food floating on the water surface (data not shown in this thesis). This implies that a certain potential to plasticity in the cognitive capacity of turtles could strongly impact their ecological potential (see also Wilkinson et al., 2007, 2009). In other words, cognitive plasticity could open fresh opportunities, resulting in a broader feeding ecology or even in an ecological shift.

As stated in the Introduction, the ancestors of all living turtles were aquatic forms, and aquatic feeding was therefore the primitive food uptake mode in modern turtles. Terrestrial feeding developed much later and is found today in only one superfamily: the monophyletic Testudinoidea, which includes the three families Emydidae, Geoemydidae, and Testudinidae. While Emydidae and Geoemydidae are aquatic to semiaquatic, the Testudinidae exclusively contain terrestrial forms. The present study, together with literature data (e.g. Summers et al., 1998), imply that terrestrial feeding developed (and continues to develop) independently in these groups. The functional steps during the transition from water to air could theoretically involve 5 steps, starting from exclusively aquatic forms. First, animals could have left their home waters in search for food. When food was found, it could have been grasped by the jaws and brought to water for further intraoral (hydrodynamic) transport. Such a situation is still found in the kinosternids *Claudius angustatus* (Weisgram, 1985b) and *S. odoratus* (Heiss et al., 2010; Natchev et al., 2010b) as well as in the emydids *Trachemys scripta* (Weisgram, 1985a; Weisgram et al., 1989) and *Emys orbicularis* (Kummer, 2009; Singer, 2009). In a second step, if behavioural and morphological requirements were met, an animal could have grasped food by its jaws and used its tongue for intraoral transport on land, without losing the ability to feed underwater by hydrodynamic modes. Such a step is still represented by the semiaquatic emydid *Terrapene carolina* (Bels et al., 1997; Summers et al., 1998) and the geoemydid *C. amboinensis* (Heiss et al., 2008; Natchev et al., 2009a). In the next theoretical step, behavioural and morphological specialisations for terrestrial feeding continued, increasing the efficiency of terrestrial food uptake – at the cost of the capability to use highly effective hydrodynamic mechanisms. This is still found in *C. flavomarginata* and *C. galbinifrons* (Natchev et al., 2009, 2010). Further on in terrestrial specialisation, in a fourth

step, animals completely lost their ability to use hydrodynamic mechanisms. They still grasped food items with their jaws, typical for aquatic or semiaquatic turtles, but were no longer able to take up food if submerged. Such a situation is still present in one of the most primitive testudinids, *M. emys* (Heiss et al., to be submitted). This step could mark a "point of no return" in the evolution of terrestrial feeding in turtles. Consequently, animals that have reached the last step are highly specialised on terrestrial food uptake and have left their aquatic origin behind. These turtles no longer used their jaws to take up food, but have developed the lingual prehension mode (analogous to some terrestrial amphibians, lizards, and mammals; see Schwenk, 2000 for overview), associated with major changes in behaviour and morphology. This evolutionary step is characteristic for higher-evolved testudinids such as *Testudo hermanni* (Weisgram, 1985a; Wochesländer et al., 1999; 2000), *Kinixys belliana* (Bels et al., 2008), and *Geochelone radiata* (Bels et al., 2008). The ecological advantages of gaining a semiaquatic or terrestrial lifestyle with associated semiaquatic or terrestrial feeding modes becomes evident when we consider that the superfamily that exclusively developed semiaquatic or terrestrial forms comprises more species than all other ten families of recent turtles combined (according to Bonin et al., 2006).

3.5. References Conclusion

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4. Summary

Feeding plays a critical role in survival, making the contribution of the feeding system to fitness substantial. As turtles show a great diversity in their ecology – they span the range from fully aquatic to fully terrestrial with all possible variations in between – they are especially interesting for studying morphological, kinematic, and behavioural changes that have been taken place during the transition from water to land in tetrapod evolution. Previous studies on aquatic and terrestrial turtles showed a correlation between phylogeny, feeding mode, and oropharyngeal morphology. This means that the oropharyngeal anatomy both enables and constrains the food uptake potential. A purely aquatic turtle is therefore unable to feed on land, and a terrestrial turtle cannot take up food underwater. It is generally accepted that the ancestor of all living turtles was aquatic. Terrestrial feeding biology in extant turtles therefore is derived rather than ancestral. This study compares the oropharyngel morphology, feeding behaviour, and kinematic profiles of five extant turtle species. The five species represent one aquatic (*Sternotherus odoratus*), three amphibious (*Cuora amboinensis, C. flavomarginata*, *C. galbinifrons)* and one terrestrial (*Manouria emys*) form. Anatomical methods including dissection, histology, scanning electron microscopy (SEM), and computed tomography (CT) revealed that the design of the feeding apparatus differs considerably not only between distantly related species (e.g. *S. odoratus* vs. *M. emys*) but also within species of the same genus (*Cuora* sp.). This interspecific plasticity of the feeding apparatus reflects adaptations to various trophic niches. This also allows us to interpret the micro- and macro-anatomical patterns that have been taken place during the transition from an aquatic to a terrestrial lifestyle. Analogous to the differences in morphology, differences were also found in feeding behaviour and kinematics by analysing high-speed recordings. While the aquatic kinosternid *S. odoratus* can grasp food items on land but is unable to finalise feeding events out of the water, the geoemydid *Cuora* spp. are trophically well adapted to both environments, even if they show varying tendencies to one or the other medium. The testudinid *M. emys* by contrast is terrestrial and has lost the ability to take up food underwater, despite all efforts. The results of this study imply that even minor changes in the morphology of the feeding apparatus can lead to significant changes in its function, potentially resulting in ecological shifts. Consequently, a shift from aquatic to terrestrial or partly terrestrial lifestyle has developed at least three times independently amongst turtles. The intermediate character of the species selected for this study allow a theoretical step-by-step reconstruction model of the evolution of terrestrial lifestyle in turtles, based on the evolution of terrestrial feeding biology.

5. Zusammenfassung

Ohne Zweifel spielt die Effizienz der Nahrungsaufnahme eine zentrale Rolle im Überlebenspotential einer Tierart. Schildkröten zeichnen sich nicht nur durch ein stammesgeschichtlich extrem hohes Alter, sondern auch durch eine bemerkenswerte ökologische Heterogenität aus. Sie beinhalten von rein wasserlebenden Formen bis hin zu Wüstenbewohnern alle erdenklichen Zwischenstufen. Diese Tatsache macht sie für die Erforschung der Evolution terrestrischer Lebensweisen in Tetrapoden besonders interessant, va. morphologische und kinematische Veränderungen in der Biologie der Nahrungsaufnahme betreffend. Frühere Studien an Tetrapoden – und speziell an Schildkröten – konnten einen Zusammenhang zwischen Phylogenie, Art der Nahrungsaufnahme und oropharyngealer Morphologie aufzeigen. Morphologische Gegebenheiten erlauben bestimmte Arten der Nahrungsaufnahme, bzw. limitieren andere. Eine rein aquatische Schildkröte z.B. kann keine Nahrung an Land aufnehmen, genauso wie eine rein terrestrische Schildkröte nicht mehr unter Wasser fressen kann. Da alle heute lebenden Schildkröten von aquatischen Formen abstammen, muss die terrestrische Lebensweise dieser Ordnung sekundär entstanden sein. In diesem Zusammenhang werden in der vorliegenden Studie die Morphologie des oropharyngealen Systems, genauso wie das Fressverhalten und daraus resultierende kinematische Profile fünf selektierter Schildkrötenarten vergleichend untersucht. Bei den ausgesuchten Arten handelt es sich um die rein aquatische *Sternotherus odoratus*, drei amphibisch lebenden Cuora-Arten (*Cuora amboinensis*, *C. flavomarginata*, *C. galbinifrons*) und die terrestrische Art *Manouria emys*. Mit Hilfe mikro- und makroanatomische Verfahren wie Sektion, Histologie, Rasterelektronenmikroskopie (SEM) und Computertomographie konnten detaillierte morphologische Beschreibungen, genauso wie funktionelle Vergleiche erstellt werden. Dabei konnte gezeigt werden, dass der Bauplan des Nahrungsaufnahme-Apparates sich nicht nur bei weit entfernt verwandten Arten stark voneinander unterscheidet (z.B. *S. odoratus* vs. *M. emys*), sondern große Unterschiede auch innerhalb nur einer Gattung vorkommen (*Cuora* sp.) können. Diese interspezifische Plastizität im Design des Nahrungsaufnahme-Apparates spiegelt die Anpassung der Arten an unterschiedliche trophische Nischen wieder und erlaubt auch ein Erkennen von mikro- und makroanatomischen Mustern, welche im Laufe der Evolution der terrestrischen Lebensweise aus funktioneller Notwendigkeit entstanden sind. Analog zu den morphologischen Unterschieden konnten durch die Analyse von high-speed-Filmaufnahmen auch klare Abweichungen im Verhalten und kinematischen Profil zwischen den Arten bei der Nahrungsaufnahme gezeigt werden. So kann die Schildkröte *S. odoratus* (Familie

Kinosternidae) zwar an Land Futterstücke mit den Kiefern ergreifen, um den gesamten Fressvorgang vollenden zu können ist diese Art aber auf ein aquatisches Milieu angewiesen. Die amphibisch lebenden *Cuora* Arten (Familie Geoemydidae) hingegen sind sehr gut sowohl an ein aquatisches wie auch terrestrisches Milieu angepasst und sind befähigt in beiden Medien Nahrung aufzunehmen, auch wenn innerhalb des Cuora Arten-Komplexes verschiedene Habitatpräferenzen festzustellen sind. Im Gegensatz dazu hat die terrestrische Schildkrötenart *M. emys* im Laufe der Evolution ihre Fähigkeit verloren unter Wasser zu fressen, auch wenn ihre Lebensweise noch stark amphibischen Charakter aufweißt. Die in dieser Studie erzielten Ergebnisse deuten darauf hin, dass selbst kleine Veränderung in der Morphologie des Nahrungsaufnahmeapparates große Veränderungen in seiner Funktion mit sich bringen können, was u. U. zu ökologischen Verschiebungen („ecological shifts“) führen kann. Folglich wird gezeigt dass eine Verschiebung von primär aquatischer- hin zu primär terrestrischer Lebensweise in modernen Schildkröten mindestens dreimal unabhängig voneinander entstanden ist. Schlussendlich erlaubt der intermediäre Charakter der untersuchten Schildkrötenarten eine stufenweise Rekonstruktion evolutionärer Vorgänge, die im Laufe der Veränderung von aquatischen bis hin zu terrestrischen Schildkröten stattgefunden haben – basierend auf der Evolution einer terrestrischen Nahrungsaufnahme.

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6. Curriculum Vitae

Egon Heiss
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Italian nationality

education

•February 2010 introductory course in Laboratory Animal Science at the University of Veterinary Medicine Vienna, AUT
•September 2009 Cell Imaging Techniques course at the Oxford Brooks University, UK
•since November 2007 phD Student at the University of Vienna, AUT
•August 2006 TBA (Tropical Biology Associaton) - course in the Kibale Forest National Park, Uganda as a BES (British Ecology Society) - scholarship holder
•June 2006 Master's degree in zoology at the Unversity of Vienna
•2002-2006 specialisation in zoology and ecology at the University of Vienna
•1999-2002 study of biology at the Leopold Franzens University in Innsbruck, AUT
•1999 Matura: school leaving exam in Bozen, I

professions

•2008-present: lectorship in general zoology and vertebrate morphology at the University of Vienna
•2008-present: co-supervisor of master-students
•2007-present: research assistant at the Department of Theoretical Biology, University of Vienna
•2005-2008: tutor in general zoology and vertebrate morphology at the University of Vienna
•2004- 2007: lab assistant at the Department of Oral Implantology, Medical University of Vienna

memberships

•since October 2009 RMS: Royal Microscopical Society
•since July 2008 SEB: Society for Experimental Biology
•since August 2007 ISVM: International Society of Vertebrate Morphology
•since January 2006 ÖGH: Austrian Herpetological Society
•since August 2006 TBA: Tropical Biology Association

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publications

(i) journal articles

•Heiss E., Natchev N., Beisser C., Lemell P., Weisgram J. (to be subm.). Basal tortoise with primitive feeding biology? Structure and function of the oropharyngeal cavity in the Asian Forest Tortoise, *Manouria emys emys.*

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•Heiss E., Plenk H.Jr., Weisgram J. (2008). Microanatomy of the palatal mucosa of the Malayan Box Turtle, *Cuora amboinensis* (Daudin, 1802) with functional implications. Anat. Rec. 291:876-885.

(ii) published congress abstracts/papers

•Heiss E., Weisgram J., Natchev N. (2009). Feeding performance in two chelonian frontier-runners: *Manouria emys* and *Sternotherus odoratus*. Comp Biochem Phys A. 153(2):126. (SEB Annual Main Meeting; Glasgow, UK, 28/6-1/7/2009).

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(iii) scientific contributions

•Lechner, W., Wysocki L.E., Ladich, F. (2010). Ontogenetic development of auditory sensitivity and sound production in the squeaker catfish *Synodontis schoutedeni*. BMC Biol. 8:10.

(iv) attended congresses & meetings

•European Forum Alpbach 2009: Technology Forum; Alpbach, AUT. 27-29/8/2009
•SEB Annual Main Meeting 2009; Glasgow, UK. 28/6-1/7/2009
•VII Natural Sciences' 2008 Scientific Conference; Varna, BG., 28-30/9/2008
•SEB Annual Main Meeting 2008; Marseille, F. 28/6-1/72008
•VI Natural Sciences' 2007 Scientific Conference; Varna, BG. 27-30/9/2007
•8th ICVM; Paris, F. 16-21/8/2007

Vienna, April 2010 Egon Heiss