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Feeding behaviour in a ‘basal’ tortoise provides insights on the transitional feeding mode at the dawn of modern land turtle evolution

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Almost all extant land turtles are highly associated with terrestrial habitats and the few tortoises with high affinity to aquatic environment are found within the genus *Manouria*. *Manouria* belongs to a clade which forms the sister taxon to all remaining tortoises and is suitable to be used as a model for studying evolutionary transitions from water to land within modern turtles. We analysed the feeding behaviour of *M. emys* and due to its phylogenetic position, we hypothesise that the species might have retained some ancestral characteristics associated to aquatic lifestyle. We tested whether *M. emys* is able to feed both in aquatic and terrestrial environments as mud turtles do. In fact, *M. emys* repetitively tried to reach submerged food items in water, but always failed to grasp them and no suction feeding mechanism was applied. When feeding on land, *M. emys* showed another peculiar behaviour; it grasped food items by its jaws – a behaviour typical for aquatic or semiaquatic turtles – and not by the tongue as in the typical feeding mode in all tortoises studied so far. In *M. emys*, the hyolingual complex remained retracted during all food uptake sequences, but the food transport was entirely lingual based. The kinematical profile significantly differed from those described for other tortoises and from those proposed from the general models on the function of the feeding systems in lower tetrapods. We conclude that the feeding behaviour of *M. emys* might reflect a remnant of the primordial condition expected in the aquatic ancestor of tortoises.

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Feeding behaviour in a 'basal' tortoise provides insights on the transitional feeding mode at the dawn of modern land turtle evolution

Abbreviated title: **Feeding ethology in *Manouria emys***

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INTRODUCTION

By comprising more than 180 species, the cryptodiran taxon Testudinoidea represents the most diverse group of extant turtles (e.g., Fritz and Havaš, 2007; Thomson and Shaffer, 2010; Turtle Taxonomy Working Group 2014). Traditionally, it contains three major extant groups, including the mud turtles Emydidae (marsh turtles), the Geoemydidae (pond, river and wood turtles), and the land turtles Testudinidae (tortoises) (Fig. 1). All molecular phylogenetic studies confirm a sister group relationship of Testudinidae and Geoemydidae (Iverson et al., 2007; Shaffer, 2009; Thomson and

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Shaffer, 2010; Barley et al., 2010) and Emydidae evolved independently from the former groups (Fig. 1). How Platysternidae are related to other turtle groups is uncertain (Fig. 1; reviewed by Parham et al. 2006), but most molecular studies provide evidence for a closer relationship to emydids (e.g., Shaffer and Thomson, 2010, Crawford et al., 2015). Paleontological studies revealed that all testudinoids had an aquatic ancestor and that terrestrial forms evolved secondarily (Danilov, 1999; Sukhanov, 2000; Joyce and Gauthier, 2004). The only pure terrestrial living turtle group are the tortoises.

Amphibious to terrestrial lifestyles and the capacity to exploit terrestrial food sources had evolved independently within all three major testudinoid lineages (for overview see Summers et al., 1998; Natchev et al., 2009). At least eight emydid species are able to feed on land as well as under water (see Bels et al., 1997, 2008; Summers et al., 1998; Stayton, 2011). During terrestrial feeding events, such amphibious emydids use their jaws to grasp food items (jaw prehension). Similarly, all amphibious geoemydids studied to date also use jaw prehension in terrestrial food uptake (see Heiss et al., 2008; Natchev et al., 2009). By contrast, all testudinids studied so far use their tongue to grasp food items - behaviour referred to as lingual prehension (see Wochesländer et al., 1999; Bels et al., 2008). According to Bels et al. (2008), lingual prehension is obligatory for all tortoises. Tortoises show a broad variety in their feeding ecology, with a clear tendency towards herbivory and emancipation from water as living and feeding media (see Pritchard, 1979; Ernst and Barbour, 1989; Bonin, Devaux and Dupre, 2006). In fact, testudinids seem to have lost their ancestral ability to feed under water and to exclusively rely on terrestrial trophic ecologies. However, some predominantly terrestrial geoemydids are able to fulfil the whole feeding process on land and under water (Natchev et al., 2010). Similarly, testudinids with tendencies towards an amphibious lifestyle might have retained the ancestral capacity to feed underwater. Accordingly, information on ambiguous feeding mechanisms in tortoises are important to understand the evolution of terrestrial feeding mechanisms and subsequently the evolution of the predominantly terrestrial lifestyle in tortoises. *Manouria*, as one of the ‘basal’-most extant tortoises with strong association to water (Høybye-Mortensen, 2004; Stanford et al., 2015), provides a promising candidate to study ambiguous feeding mechanisms in land turtles. Its partially aquatic feeding habit was supposed to be associated with the observed morphological extension of the palatine onto the triturating surface of the upper jaw (character 30 sensu Gerlach, 2001), a diagnostic feature common to geoemydid (=batagurid) turtles. Another geoemydid-like feature is the unique existence of class II mental glands (Winokur and Legler, 1975).

The present study was designed to provide a detailed analysis of the feeding behaviour in *Manouria*. *Manouria emys* is closely related to water; hence, we provide experiments to reveal whether this species is able to fulfil the whole feeding process in both media water and air as analogy to geoemydids. The kinematical data of the feeding mechanism are compared with data available from other tortoises. The development of the classical tetraphasic models of feeding kinematics proposed by Bramble and Wake (1985) in lower tetrapods was largely based on kinematics of the feeding apparatus of tortoises. According to Wochesländer et al. (1999, 2000) and Bels et al. (2008), the feeding kinematics in tortoises sticks exactly to the generalised cyclic model (GCM) which was suggested to be common for all lower tetrapods (Bramble and Wake, 1985). Accordingly, the second goal of this study is to test if the GCM can also be applied to one of the most 'basal' recent tortoises, i.e. *Manouria*. Based on the newly gained information, we formulate hypotheses concerning the evolution of the food prehension mode within turtles. Additionally, we test and fine tune the classical feeding models in tetrapods and discuss on their general validity.

MATERIALS AND METHODS

Ecological background

Both species of *Manouria*, the Asian forest tortoise *M. emys* and the impressed tortoise *M. impressa*, have a restricted distribution in Southeast Asia. *M. emys* has a narrow distribution in Bangladesh, India (Assam, Meghalaya, Mizoram, Nagaland), Myanmar, Thailand, Malaysia (East, West), and Indonesia (Kalimantan, Sumatra). The nominate subspecies, *M. emys emys* - the subject of this study, inhabits the southern part of the species range (Fritz and Havaš, 2007; TTWG, 2014, Stanford et al., 2015).

M. emys inhabits tropical evergreen monsoon forests and tolerates high soil moisture. It is commonly found reposing in wet areas, buried in mud or under the leaf litter where it may spend hours or days. It is active even at rainy weather and not depending of sun basking, the species is mostly crepuscular and nocturnal (Ernst, Altenburg, and Barbour, 2000; Vetter and Daubner, 2000, Stanford et al., 2015).

According to the available literature, the diet of *M. emys* includes plants (mostly aquatic), fungi, invertebrates, and frogs (Nutphand, 1979; Das, 1995; Lambert and Howes, 1994, Høybye-Mortensen, 2004). It has been reported to feed on plants in shallow mountain streams (Nutphand, 1979).

Experimental setup

The animals used in the present study were obtained commercially and kept at 12 h dark/light cycles in a large terrarium (150x100 cm ground area) with a permanently filtered water basin and spacious terrestrial area. The turtles were fed different fruits, vegetables, commercially obtained tortoise pellets, dead mice, as well as pieces of beef heart and liver, offered on the terrestrial part of the terrarium. The three subadult experimental animals had a carapace length between 109 and 135 mm and weighted between 234 and 265 g.For filming terrestrial feeding, the specimens were put in a dry glass aquarium (24x60x30 cm) and the food items were offered via forceps in front of the animals. As food items, we used small cattle heart pieces measuring approximately 4x4x6 mm. The turtles were filmed in left lateral view (with a reference grid 1x1 cm in the background) via the digital high-speed camera system Photron Fastcam-X 1024 PCI (Photron limited; Tokyo, Japan) at 500 fps with a highly light-sensitive objective AF Zoom - Nikkor 24-85 mm (f/2,8-4D IF). Two "Dedocool Coolh" tungsten light heads with 2 x 250 W (ELC), supplied by a "Dedocool COOLT3" transformer control unit (Dedo Weigert Film GmbH; München, Germany) were used for illumination. We filmed and analysed the food uptake and the food transport cycles in eight feeding sequences for each specimen.

For filming aquatic feeding on submerged food, we filled the experimental aquarium with a water level of 3 cm and offered food in front of the turtle's snout. For reducing the light intensity and for optimisation of the digitising process, the frame rate was reduced to 250 fr/s. As the tortoises were not able to grasp the food item in a total of 36 trials, the kinematics of the feeding apparatus were analysed (see below) in nine selected representative feeding trials. Horizontal (X-axis) and vertical (Y-axis) coordinates of relevant landmarks (see Fig. 2) were digitised frame by frame using "SIMI-MatchiX" (SIMI Reality Motion Systems; Unterschleißheim, Germany). On the base of the displacement of the markers, we were able to calculate the gape amplitude (distance between the tip of the upper and lower beak), head movement (distance between the anterior tip of the carapace and the point "P" on Figure 2), tongue movements (distance between the most ventral point on tympanum and the tip of the tongue when visible), and hyoid movements (distance between the point "P" on Figure 2 and the basis of the posterior ceratobranchial). These data were used to calculate the following kinematical variables: duration of Slow open phase (SO); duration of Slow open phase I and II (SOI and SOII) when present; duration of fast open (FO); duration of maximum gape phase (MG); duration of fast close (FC); time to peak gape (TPG); tatal cycle duration (TCD); duration of hyoid protraction (HDD); duration of hyoid retraction (HVD); duration of the total hyoid cycle (THC); hyoid retraction velocity (HRV); duration of head protraction (HP); duration of head retraction (HR); duration of tongue protraction (TP); tongue retraction velocity; delay of the start of hyoid retraction to the tongue retraction start; delay of reaching peak gape relative to start of the hyoid retraction; delay of reaching peak gape relative to tongue retraction start (see Table 1).

Statistics

Differences in food uptake and food transport cycles respectively with presence/absence of split in jaw opening in SO and FO phases; split of SO phase in SOI and SOII phases; occurrence of MG phase were tested with Yates corrected Chi-square test. Datasets for the studied variables were tested with Shapiro-Wilk test for normal distribution. When the p-value was less than the chosen alpha level ($p<0.05$), the null hypothesis was rejected and data were excluded from further analyses. In addition, variables were tested with Levene’s and Brown-Forsythe tests and then processed with Welch’s ANOVA for heteroscedastic data. Tukey’s honest significant difference test (HSD) was performed for post-hoc analyses when applicable. Standard descriptive statistics including mean, range, and standard deviation (SD) were performed. Canonical discriminant analysis was also performed, in order to observe the individual differentiation of all measured variables in the transport phase.

RESULTS

When fed on land, the Asian forest tortoises always grasped their food by the jaws. After food uptake, one to four transport cycles followed prior to oesophageal packing (see Schwenk, 2000). The tip of the tongue was barely seen during food uptake (see Fig 3b-c) indicating that the tongue was not protracted. By contrast, during transport cycles, the tongue was well visible as it was rhythmically pro- and retracted to transport the food item towards the oesophagus (Fig. 5).

When trying to feed under water (Fig. 4), *M. emys* submerged its head under the water level, and by protruding the head, the animals were trying to position the gaping mouth around the food item. The gape cycle was newer split in slow and fast jaw open phases. The tongue tip was not visible in lateral view and the hyolingual complex did not protract prior reaching peak gape. No retraction of the hyoid complex was detected prior jaw closure. The gape cycle duration exceeded for one and a half seconds and was 1.94 ± 0.36 s (mean ± SD) in all digitised sequences. Despite the lack of success, the turtles had repeatedly tried to catch the submerged food. In several events, we were able to detect that the food item was carried away by the bow wave induced by jaw closing.

The variables of the kinematical profiles are summarised in Table 1. In the statistic tests, we found highly significant differences in sequences with and without both slow open phase I (SO I) and slow open phase II (SO II) (see Bramble and Wake, 1985) when food uptake and transport phases were compared ($\chi^2_{(1,\ N=98)}=25.05$, $p<0.001$). Similarly significant differences were observed when comparing food uptake and transport cycles in respect to sequences with and without slow jaw open phases as well as with and without maintaining jaw maximum gape (MG phase) ($\chi^2_{(1,\ N=98)}=6.10$, p=0.02; $\chi^2_{(1,\ N=98)}=6.52$, p=0.01).

When testing the variables of the feeding kinematics (Table 1), nine variables, which describe the food uptake process, were detected to show significant differences between individuals for time to peak gape duration (TPG; $F_{Welch(2,21)}=5.03$, p=0.024), total gape cycle duration (TCD; $F_{Welch\ (2,21)}=12.68$, p=0.001), fast closing duration (FC, $F_{Welch(2,21)}=10.34$, p=0.002) and head retraction duration (HR; $F_{Welch\ (2,21)}=13.86$, p=0.001). In transport cycles, six out of 18 variables differed significantly amongst individuals: hyoid dorsal displacement duration (HDD; $F_{Welch\ (2,62)}=6.32$, p=0.005); total hyoid cycle duration (THC, $F_{Welch\ (2,62)}=7.46$, p=0.002); hyoid retraction velocity (HRV, $F_{Welch\ (2,66)}=4.66$, p=0.016); head protraction duration (HPR; $F_{Welch\ (2,53)}=11.47$, p=0.001); the delay in the start of hyoid ventral displacement (HVD) relative to the start of tongue retraction (TR) ($F_{Welch\ (2,55)}=6.38$, p=0.005); the delay of time to peak gape (TPG) relative to the start of hyoid ventral displacement (HVD) ($F_{Welch\ (2,66)}=4.74$, p=0.014). When testing for differences between grasping and transport cycles, seven out of nine variables differed significantly: fast jaw open duration (FO; $F_{Welch\ (1,43)}=15.17$, $p=0.011$); maximum gape dutaion (MG; $F_{Welch\ (1,26)}=15.89$, $p=0.001$); fast closure (FC; $F_{Welch\ (1,26)}=7.86$, $p=0.010$); time to peak gape (TPG, $F_{Welch\ (1,72)}=46.78$, $p<0.001$); total gape cycle duration (TCD; $F_{Welch\ (1,72)}=52.50$, $p<0.001$); head protraction duration (HP; $F_{Welch\ (1,67)}=52.23$, $p<0.001$); and head retraction duration (HR; $F_{Welch\ (1,47)}=12.57$, $p=0.002$).

When comparing three further parameters among the transport cycles in all three specimens (delay of HVD start relative to TR start; delays of TPG relative to HVD; delay of TPG relative to TR starts), statistically significant differences were found among all compared pairs ($F_{Welch(2,105)}=41.58$, $p<0.001$).

In order to visualize the kinematical variability of transport cycles amongst individuals a canonical discriminant analysis (CDA) was performed (Fig. 6). . In total, both axes explained 100% of the total variance among individuals (70% explained by the first axis). Seven out of 18 variables loaded positively to the first canonical axis (SO II, FC, TPG, TGC, HDD, HRV, HP - durations and delay of TPG relative to TR). Respectively, six variables loaded positively to the second canonical axis (FC, TPG, THR, THC, HR, and TR). Differences between hyoid retraction velocity (HRV) and tongue retraction velocity (TRV) was highly statistically significant ($F_{(2,60)}=5.39$, $p<0.001$, $V_{HRV}=1{,}79\pm1.21$ vs. $V_{TRV}=6.85\pm2.80$).

DISCUSSION

The present contribution shows that *M. emys* repetitively tried to feed on dispersed food items under water, which was a surprising and unknown behaviour among tortoises. However, the species always failed to uptake the submerged food. On land, *M. emys* grasped food by the jaws, just like all known aquatic turtles do in terrestrial situations, but not with the tongue as formerly predicted for all tortoises. On the base of our results we discuss several important evolutionary, behavioural, and functional aspects.

Evolution of food uptake among turtles

All, or almost all stem turtles of the Triassic were terrestrial as indicated by the anatomy and proportions of the limbs, which were adapted for terrestrial locomotion (for a comprehensive discussion see Joyce, in press). With the rise of modern turtles (Testudines) during the Jurassic period (e.g., Danilov and Parham, 2006; Sterli, 2010; Sterli and de la Fuente, 2011), a general transition of turtles into an aquatic environment took place (Willis et al. 2013). The invasion of aquatic environments induced an immense radiation and diversification into several subgroups related to the great evolutionary success of turtles (Joyce, 2007; Thomson and Shaffer, 2010). Due to the different physical properties of air and water, the new environment required morphological and functional adaptations of the locomotion- and feeding system to enable efficient swimming behaviour and aquatic food uptake (i.e. suction feeding) (Schumacher, 1973; Lemell et al., 2002).

In general, most aquatic turtles combine a fast acceleration of the head towards the food or prey item and a suction feeding mechanism induced by fast oropharyngeal volume expansion. In some extant turtles, a strong suction flow can be generated and prey is directly sucked into the oropharynx without contact with the jaws [e.g., *Chelus fimbriatus* (Lemell et al., 2002), *Apalone spinifera* (Anderson, 2009), *Pelodiscus sinensis* (N.N. and I.W. unpublished data)]. However, most extant turtles cannot generate such strong suction flows and only compensate ("gulp") the bow wave that otherwise would push small to moderately sized food items away from the fast approaching head. Such species finally fix and grasp prey with the jaws (see Lauder and Prendergrast, 1992; Lemmel et al., 2000; Aerts et al., 2001, Natchev et al., 2009, 2011). We consider the latter as plesiomorphic behaviour for extant turtles.

Among extant turtles, the ability to fulfil the whole feeding process (including food uptake, food manipulation and transport, oesophageal packing, and swallowing) on land was tested and documented for only six species so far. All of them were members of the taxon Testudinoidea (Fig. 1; see also Summers et al., 1998; Bels et al., 2008; Natchev et al., 2009). The terrestrial mode of food uptake differs dramatically among and within the three testudinoid subgroups (see Bels et al., 1997, 2008; Summers et al., 1998; Wochesländer et al., 1999, 2000; Natchev et al., 2009, present study). Accordingly, it seems that terrestrial feeding re-evolved several times independently amongst turtles. Unfortunately, only limited experimental data are available on feeding mechanisms in emydid and geoemydid species and accordingly, palaeontological evidence and functional indications can help to address such questions. The sister group to all remaining Testudinoidea is the diverse and possibly paraphyletic extinct taxon †Lindholmemydidae (Fig. 1; Lourenço et al., 2012), which contains genera such as †*Mongolemys* and †*Lindholmemys* (Danilov, 1999; Joyce and Gauthier, 2004). Some poorly known aquatic taxa such as †Haichemydidae and the †Sinochelyidae may perhaps also belong to †Lindholmemydidae. This group is first known from aquatic sediments of the late Early Cretaceous and apparently had an amphibious lifestyle (Sukhanov, 2000). Among cryptodirans, amphibious lifestyle is retained in most living emydids (plus platysternids) and geoemydids, as well as in kinosternids (see Depecker et al., 2006, but also Nakajima et al., 2014). All other taxa are fully terrestrial (tortoise), freshwater inhabitants or marine turtles (see Joyce and Gauthier, 2004; Rasmussen et al., 2011).

Very limited information is available on feeding mechanisms employed by amphibious non-testudinoid turtles that occasionally exploit terrestrial food sources. Weisgram (1985a) documented a non-testudinoid turtle (*Claudius angustatus*) that caught prey on land and dragged it into water for transport and swallowing. Natchev et al. (2008) documented another non-testudinoid (*Sternotherus odoratus*) catching food on land, but failing to transport it through the oropharynx. Among extant turtles, successful food transport on land seems to be restricted to testudinoids, because they have evolved enlarged and muscular tongues (von Bayern, 1884; Werneburg, 2011,).

Natchev et al. (2009) described and summarised three categories of terrestrial food uptake modes among Testudinoidae, based on experimental data: (A) Jaw prehension with retracted hyolingual complex: the tongue is not protracted towards the food, as observed in the geoemydid genus *Cuora* (Natchev et al., 2009). (B) Jaw prehension with slightly protracted hyolingual complex: a protraction of the tongue is detectable during food uptake but the tongue does not touch the food, as observed in emydids (Bels et al., 1997; Stayton, 2011). (C) Lingual prehension: The tongue touches the food item prior to food uptake and possibly carries the food item into the mouth, as documented in all tortoises studied so far (Wocheslánder et al., 1999, Bels et al., 2008). The food uptake mode of *M. emys*, however, differs substantially from that of all remaining tortoises (category C). In fact, the hyolingual complex in *M. emys* remained fully retracted during the food prehension on land, and the first contact with the food item was by the jaws. Accordingly, the feeding mechanism of *M. emys* should be assigned to category A, next to semiaquatic geoemydids. In contrast to semiaquatic geoemydids, *M. emys* was unable to grasp submerged food. We consider that the adaptations in the morphology of the feeding apparatus (see Heiss et al., 2011) and the feeding motorics of this species are adapted for terrestrial feeding .However, unlike other tortoises, *M. emys* is documented to actually feed in water (Nutphand, 1979) but only on rigid water plants (equivalent to feeding plants on land) and not on loose submerged food items as tested in our study.

The closest extant sister taxon to *Manouria* is that of *Gopherus sp* (Thompson and Shaffer, 2010) - the desert tortoises. Like all other tortoises, the desert tortoises posses a fleshy tongue (Winokur, 1988). There is no published data on the feeding kinematics of *Gopherus*, but personal observations have shown that *Gopherus* grasps food by the jaws (N.N. pers. obs.). Accordingly, preliminary observations imply that the terrestrial food uptake mechanism in *Gopherus* is similar to the feeding modes from categories A or B (see above). Therefore, both *Gopherus* (preliminary observations) and *Manouria* (this study) seem to share the plesiomorphic behaviour of jaw prehension with all amphibious turtles studied so far.

Analyzing the feeding behaviour in extant turtles, we now aim to reconstruct a theoretical scenario on the evolution of terrestrial feeding mechanisms in turtles. The ancestors of all living turtles were aquatic animals. The functional evolution from aquatic to terrestrial feeding mechanisms could theoretically have involved four stages, starting from exclusively aquatic feeding ancestor. In different lineages, turtles may have left the water for example in search for food. The species that retained predominantly aquatic life style grasped food by the jaws and brought it to water for further intraoral (hydrodynamic based) transport. Such behaviour is still found in the kinosternids *C. angustatus* (Weisgram, 1985a; Weisgram, 1985b) and *S. odoratus* (Heiss et al., 2010; Natchev et al., 2011), as well as in the emydid *Trachemys scripta* (Weisgram, 1985b; Weisgram et al., 1989) and other emydids (see Stayton, 2011). Turtles of the second theoretical evolutionary stage grasped food by the jaws and the tongue was used for intraoral food transport on land. Such species had not lost the ability to feed underwater by using hydrodynamic mechanisms. When grasping food on land, the tongue remained retracted or was protracted without however touching the food item. Among extant turtles, such feeding mode is found in the geoemydid genus *Cuora* (Heiss et al., 2008; Natchev et al., 2009,, 2010) and in some emydids (Bels et al., 1997; Summers et al., 1998; Stayton, 2011). In the next theoretical evolutionary step (stage three), behavioural and morphological specializations for terrestrial feeding advanced further, increasing the efficiency of terrestrial food transport at the cost of the capability to use effective hydrodynamic mechanisms in water. Such forms still grasped food items with their jaws on land (as typical for aquatic or semiaquatic turtles), but were no longer able to take up dispersed food if submerged. Such a situation is still present in *M. emys*. Most importantly, stage three could have marked the “point of no return” in the evolution of terrestrial feeding in turtles. Finally, in a fourth stage, turtles became fully terrestrial and their tongue was obligatorily involved in food uptake as documented in the tortoises *Testudo* (*Eurotestudo*) *hermanni boettgeri* (Weisgram, 1985b; Wocheslánder et al., 1999; 2000), *Kinixis belliana*, *Geochelone elephantopus* and *G. radiata* (Bels et al., 2008).

According to Bels et al. (2008), tortoises exhibit very stereotypical kinematical patterns in food ingestion and transport. These authors predicted obligatory lingual prehension for initial food uptake, a split of the gape cycle in slow open (SO) and fast open (FO) phases and start of the tongue retraction at the beginning of the fast open phase (see also Bramble and Wake, 1985). Specifically, the authors demonstrated these elements of the feeding behaviour in the tortoises *G. radiata*, *G. elephantopus*, and *K. belliana*. In these species both in food uptake and in food transport, the gape cycle starts with a slow opening of the jaw to approximately one third of the maximum gape angle (slow open phase I; SOI). The slow open phase I is followed by a prolonged slow open phase II (SOII), where the gape angle remains similar as at the end of slow open phase I. During both slow open phases, the hyolingual complex protracts and reaches its rostral most position, with the tongue protruding. At the end of slow open phase II starts the fast opening of the jaws (fast open phase; FO) and the initiation of the hyoid retraction. Immediately after the peak gape is reached, the jaws start to close fast (FC phase) and during this gape phase the hyoid retraction is finished. Wocheslánder et al. (1999, 2000) reported similar patterns in the feeding kinematics of *T. hermanni boettgeri.* These four species were the only tortoises in which feeding behaviour was studied on the base of high-speed sequence analyses - still, the generalized gape cycle model (GCM) of Bramble and Wake (1985) was considered to apply to all tortoises (Bels et al., 2008).

In contrast, our investigations demonstrate that the ‘basal’ tortoise *M. emys* does not contact food with the tongue prior to jaw prehension on land. This shows that tongue to food contact is characteristic for advanced tortoises only. We consider the terrestrial feeding behaviour of *M. emys* as plesiomorphic and potentially inherited from its semiaquatic ancestors. On that base, *M. emys* can be considered a transitional turtle in regard to secondary terrestriality.

Almost all tortoises are predominantly terrestrial animals. They feed exclusively on land and protrude their tongues toward the food for initial contact during food uptake. We propose that hyolingual protrusion evolved in the linage leading to advanced tortoises (Fig. 1). *Manouria emys* has a large tongue with massive intrinsic and extrinsic musculature (see Heiss et al., 2011). The advanced and complex lingual musculo-skeletal architecture permits the turtle to protrude the tongue outside the margins of the rhamphothecae (see Fig. 2). The food transport in *M. emys* is totally lingual based, as cyclical tongue movements bring the food to the posterior pharynx. However, *M. emys* does not use lingual food prehension as typical for all other tortoises studied so far. In fact, it seems that the *Manouria* (and *Gopherus*, see above) “linage” has retained the jaw prehension mechanism inherited from its aquatic ancestors. Tortoises in general evolved fleshy tongues which improve the food transport performance and advanced tortoises only refined the behaviour of food uptake on land via lingual food contact (see Wocheländer et al., 1999; Bels et al., 2008) prior to jaw closure.

Function of the protruded tongue in testudinid food uptake

What could be the potential advantage of the obligatory lingual protrusion during food uptake found in the more derived tortoises? One possible explanation is that the tongue is used as a prehensile organ for food ingestion as found in other tetrapod groups (for overview see Schwenk, 2000; Schwenk and Wagner, 2001; Heiss et al., 2014). However, this historical explanation might be put into question. By examining all published data available (Wocheländer et al., 1999, Bels et al., 2008), we were not able to find any hard evidences that tortoises collect food with their tongues - they just touch it. In all feeding sequences, the contact between the food and the tongue is clearly demonstrated - still, in all cases, after the initial contact of the tongue to the food, the head moves forward and the food item is not dragged into the mouth by tongue (or hyolingual) retraction, but is grasped in the jaws during fast jaw closing (FC gape phase). Hence, in extant tortoises, the tongue is not the main organ that is catching (collecting) the food, but these are in fact the jaws. Accordingly, initial food ingestion in tortoises might not be considered "lingual prehension" (see Schwenk, 2000; Bels et al., 2008) in the strict sense, but rather as "jaw prehension following lingual contact". This is a crucial difference, which sets the question: why do extant tortoises except *M. emys* (and *Gopherus*) obligatory protrude their tongues during food uptake? In other words: why do tortoises apply a more complex and presumably more energetically expensive food uptake mechanism by including movements of the hyolingual complex besides the movements of the neck and jaws alone?

We propose that the lingual contact provides tactile information on the position of the food item and helps the advanced tortoises to compensate the "information gap" which occurs when the food is approached to a distance where it is lost by the sight. The eyes of tortoises, similar to those of most other turtles, are positioned laterally on the head (Pritchard, 1979) and the turtles are not able to observe permanently the position of the food item when the neck is protracted and the gape is positioned around it. The prolonged maximum gape (MG) phase found in most ingestion cycles of *M. emys* (see Tble 1, Figure 3 and Figure 6) might be the result of lack of lingual contact to the food surface. In all published sequences and kinematical profiles on food uptake in tortoises, there is a clear tendency toward a split of the gape cycle into slow open (SO) and fast open (FO) gape phases (see Wocheländer, 1999; Bels et al., 2008). The split of the gape cycle in slow and fast phases is associated with the movements of the tongue (for overview see Bramble and Wake, 1985). The lack of tongue protrusion might explain the lack of slow open (SO) and fast open (FO) split in the gape cycle of food uptake in the geoemydid *Cuora* (see Natchev et al., 2009). In most food uptakes analysed in *M. emys,* SO phases are not present and the gape increases gradually (see Table 1 and Figure 6). This fact may be conditioned by the same factors as in the investigated geoemydids, namely a lack of tongue protrusion in food uptake (Natchev et al., 2009).

Intraoral food transport on land

The intraoral transport of *M. emys* is entirely lingual based. The cycle duration is much shorter than that of food uptake (see Table 1 and results), despite the involvement of the hyoid and the tongue in the complex rhythmic motoric of transport mechanism. The execution of the transport cycles demand complicated coordination of the activities of the contractile elements such as the jaw opening and closing muscles, head protracting and retracting muscles, intrinsic and extrinsic lingual muscles, as well as muscles that protract and retract the hyolingual complex as a whole unit (Jones et al., 2012; Werneburg, 2011, 2013).

In opposite to this complicated choreography, the mode of food prehension in *M. emys* suggests less complex neuromotoric coordination only between neck and jaw movements. The execution of the transport cycles is at least more than 100 % shorter in duration (see Table 1). A possible explanation for the longer duration of food uptake cycles relative to transport cycles might be that during transport the coordination centres of the muscle activities execution are permanently supplied by feed-back information concerning the position of the food item and the proper movements can be executed precisely in a shorter time.

Alternative interpretation of our results might be that the food uptake cycle duration is longer, as the movements have to be permanently fine-tuned according to the position of the food. The transport cycles might be shorter, because of pre-programmed neuromotor control of rhythmic movement patterns. Wocheslănder et al. (1999, 2000) and Bels et al. (2008) hypothesised that the feeding kinematical patterns in tortoises adhere exactly to those of the generalized cycle model (GCM) proposed by Bramble and Wake (1985). In his work on the feeding mechanisms in domestic animals, Bels (2006) established that the pre-programmed GCM is universally valid along different groups of tetrapods, including amphibians, sauropsids, and mammals. On the base of our results, the feeding kinematics of the 'basal' extant Asian forest tortoise differs significantly from those of the GCM. The kinematics of the feeding system in *M. emys* seems to be though pre-programmed, under permanent feed-back control. The values of the gape and hyoid/hyolingual cycle patterns in the three specimens studied here show high degrees of variation, both concerning food uptake and food transport (see Table 1 and Figures 5-7). The slow open phases (SO) are obligatory neither for food uptake, nor for food transport gape cycles. The gape cycle often includes a phase of retaining maximum gape (see Table 1, Figure 3, 5). In turtles, the maximum gape phase (MG) was described for the gape cycle in *Cuora sp.* (Natchev et al., 2009, 2010) and was confirmed for other turtles (Natchev et al., 2011). The presence of a prolonged maximum gape phase (MG) can be easily overseen when the frame rate of the film sequence is not high enough (i.e., step between successive frames over 10 milliseconds) In this case the durations of the time to peak gape, or the fast close phase may be miscalculated.

. The GCM presupposes that the start of hyoid retraction coincidence with the start of fast open phase (FO). However, our calculations (see Table 1 and Figure 5) demonstrate that in *M. emys* the hyoid retraction in the food transport cycle starts shortly prior reaching peak gape. The same pattern was detected by the investigation of aquatic, semi-aquatic, but also predominantly terrestrial cryptodirans (Natchev et al., 2008, 2009, 2010, 2011).

We observed a clear delay in the offset of depression of the oropharyngeal basis (which is a good indicator for the start of hyoid retraction in lower vertebrates (Schwenk, 2000) relative to the initial retraction of the tip of the tongue (see Table 1). The tongue tip retraction velocity is much higher (see Table 1) than the velocity of the mouth base depression. This fact indicates that there is an initial caudal retraction of the tongue tip (probably by contraction of the intrinsic lingual muscles) prior to the movement of the hyolingual complex as a whole unit. Another explanation might be that, the hyolingual complex is retracted caudally and the depression of the orpharyngeal base is a result of the repositioning of the voluminous tongue.

CONCLUSIONS

We propose that the ancestral food uptake mode in tortoises, when feeding on land, was jaw based. During the shift from aquatic to terrestrial lifestyle and feeding, the "*Manouria* (and most likely also *Gopherus*) linage" had retained pure jaw prehension in food uptake. The hyolingual complex in that lineage exhibit the typical morphological features of tortoises that feed exclusively on land (see Bramble, 1973; Winokur, 1988; Heiss et al., 2011), such as an enlarged fleshy tongue with abundant papillae, a complex tongue musculature, a relatively small and mainly cartilaginous hyoid and hypoglossum. The evolutionary shift in the morphology of the hyolingual complex was conditioned by the optimisation of the food transport behaviour and not for the food uptake. We suggest that the involvement of the tongue during food uptake found in the derived extant tortoises serves a tactile sensory tool for the localisation of the food item position prior to jaw prehension. The tongue is not used as the main apprehensive organ in modern tortoises and the food uptake mode represents a deriven jaw prehension system.

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FIGURE CAPTIONS

Figure 1. Phylogeny of turtle clades with a focus on Testudinoidea. Interrelationship following Shaffer and Thomson (2011). Those authors assign Platysternidae as sister taxon to Emydidae; here we show different hypothesis for the position of this species. Major evolutionary changes are listed; for details see text. Three modes of terrestrial food uptake are illustrated. **A)** Jaw prehension; the tongue is not protruded and is only used for food transport (Geoemydida, *Manouria*). **B)** Jaw prehension; the elongated tongue is protruded during prehension but does not contact the food and is only used for food transport (Emydidae). **C)** Prior to jaw prehension, the tongue contacts the food (advanced tortoises).

Figure 2. Selected frame from a high-speed video sequence (500 fr/s) of food transport in *Manouria emys*, showing the landmarks used for kinematic analyses: **C**, rostral tip of sagital carapace, **Hy**, hyoid at the basis of ceratbranchial I; **LJ**, tip of the lower jaw; **P**, posteriormost point of crista supraoccipitale; **TT**, tip of the tongue; **Tv**, ventral most point of the tympanum at the position of the jaw joint; **UJ**, tip of the upper jaw; grid 10x10 mm.

Figure 3. Selected frames and graphics (based on a high-speed video with 500 fr/s) represent the movements of jaws, hyoid and t head during terrestrial food uptake in *Manouria emys* when feeding on pieces of beef heart; **a**, slow open phase end (lacking discrete SOI and SOII); **b**, fast open end; **c**, fast close start; **d**, fast close end; arrow indicates the position of the food item; arrowheads represent the position of the tip of the tongue; grid 10x10 mm.

Figure 4. Selected frames and graphics (based on high-speed video with 250 fr/s showing the movements of jaws, hyoid, and head during attempts of aquatic food uptake in *Manouria emys*; **a**, start of the gape cycle; **b**, end of jaw opening; **c**, maximum gape end; **d**, fast closure end; note the lack of movement of the hyoid complex during the whole cycle; grid 10x10 mm. dors., – dorsal; vent. – ventral; pro – protraction; ret – retraction.

Figure 5. Selected graphics (based on a high-speed video with 500 fr/s) showing the movement patterns of jaws, hyoid, tongue and head during terrestrial food transport in *M. emys*; note the delay in hyoid ventral displacement relative to the start the retraction of the tongue tip, as well as the delay of both the tongue retraction and hyoid retraction relative to the start of the FO phase. Abbreviations: **FC**, fast closure; **GCM**, generalised cyclic model; **HDD**, hyoid dorsal displacement; **HPR**, head protraction; HR, neck retraction; **HRV**, hyoid retraction velocity; **HVD**, hyoid ventral displacement; **MG**, maximum gape; **PG**, peak gape; **SO**, slow open phase lacking discrete SOI and SOII; **SO I**, slow open phase I; **SO II**, slow open phase II; **T**, transport; **TCD**, total cycle duration; **THC**, total hyoid cycle; **TP**, tongue plateau; **TPG**, time to peak gape; **TPR**, tongue protraction; **TR**, tongue retraction.

Figure 6. Frequencies of occurrence of sequences with absence of selected variables in food uptake (FU) (light grey bars) and food transport (T) (black bars) phases, expressed as: percentages missing any possible split in slow and fast open gape phases - SO and FO (A); cycles in which SO phase was detectable, but the split of discrete slow gape phase in SOI and SOII is missing (B); lack of MG phase (C).

Figure 7. Canonical centroid plots of three *M. emys* specimens (T1–T3), centroid scores for each individual and measurement repetition in food transport phase.

TABLES AND TABLE LEGENDS

Table. 1: Variables describing the feeding process in *Manouria emys*, present as means ± SD; n, sample size; *, significant differences ($\alpha=0.05$) among individuals in the ingestion phase (P1), in the transport phase (P2), and between both mode (P3); n.c., p value not calculated.

	Food uptake (FU)				Transport (T)				I vs. T
Variable	individual 1	individual 2	individual 3	p1	individual 1	individual 2	individual 3	p2	p3
	(n=8)	(n=8)	(n=8)		(n=33)	(n=20)	(n=21)		
SOI duration [s]	0.168±0.060	0.618±0.231	0.562	n.c.	0.146±0.016	0.126±0.014	0.115±0.015	0.378	0.068
	n=2	n=3	n=1		n=18	n=14	n=11		
SOII duration [s]	0.738±0.508	0.453±0.294	1.024	n.c.	0.147±0.014	0.187±0.027	0.190±0.021	0.187	0.072
	n=2	n=3	n=1		n=18	n=13	n=11		
FO duration [s]	0.450±0.060	0.379±0.150	0.694	n.c.	0.122±0.009	0.126±0.012	0.102±0.006	0.111	0.011*
	n=2	n=3	n=1		n=25	n=15	n=19		
MG duration [s]	0.079±0.017	0.095±0.031	0.166±0.044	0.271	0.025±0.003	0.033±0.004	0.042±0.019	0.318	0.001*
	n=4	n=4	n=6		n=6	n=10	n=4		
FC duration [s]	0.157±0.079	0.105±0.036	0.158±0.030	0.024*	0.089±0.020	0.186±0.040	0.119±0.088	0.155	0.010*
	n=8	n=8	n=8		n=33	n=20	n=21		
TPG [s]	0.943±0.144	0.989±0.177	1.784±0.137	0.002*	0.408±0.021	0.439±0.038	0.403±0.028	0.187	<0.001*
	n=8	n=8	n=8		n=33	n=20	n=21		
TCD duration [s]	1.139±0.148	0.128±0.169	2.073±0.144	0.001*	0.499±0.020	0.655±0.098	0.510±0.030	0.311	<0.001*
	n=8	n=8	n=8		n=33	n=20	n=21		
HDD duration [s]					0.281±0.025	0.216±0.035	0.169±0.019	0.005*	
					n=30	n=14	n=21		
HVD duration [s]					0.176±0.011	0.167±0.014	0.149±0.009	0.162	
					n=31	n=17	n=21		
THC duration [s]					0.456±0.028	0.384±0.041	0.317±0.022	0.002*	
					n=30	n=14	n=21		
HRV velocity [cm/s]					0.718±0.059	0.938±0.107	0.551±0.071	0.016*	
					n=31	n=17	n=21		
HP duration [s]	1.345±0.159	1.204±0.246	2.494±0.177	0.001*	0.220±0.049	0.864±0.132	0.464±0.089	0.001*	<0.001*
	n=8	n=8	n=8		n=32	n=14	n=10		
HR duration [s]	0.296±0.041	0.487±0.086	0.704±0.174	0.052	0.236±0.027	0.211±0.025	0.316±0.065	0.333	0.002*
	n=8	n=7	n=8		n=14	n=13	n=10		
TP duration [s]					0.165±0.008	0.160±0.017	0.133±0.025	0.483	
					n=30	n=19	n=13		
TR velocity [cm/s]					7.459±0.550	5.798±0.547	6.562±0.595	0.121	
					n=31	n=20	n=13		
delay of HVD start relative to TR start [s]					-0.2011±0.026	-0.039±0.231	-0.082±0.025	0.005*	
					n=30	n=15	n=14		
delay of TPG relative to HVD start [s]					-0.007±0.007	-0.016±0.008	-0.032±0.005	0.014*	
					n=31	n=17	n=21		
delay of TPG relative to TR start [s]					-0.062±0.007	-0.045±0.013	-0.055±0.008	0.521	
					n=31	n=20	n=14		

APPENDIX

List of die abbreviations in the text in alphabetic order:

C – Carapax rostral tip;

cbI – Ceratobranchiale I;

ch – Corpus of the hyoid;

chII – Ceratohyale II;

cm – Centimetre;

crh – Cornu hyale;

dors. – Dorsal;

ep I – Epibranchiale I;

FC – Fast Close;

GCM – Generalised Cyclic Model;

hg – Hypoglossum;

HDD – Hyoid dorsal displacement;

HP – Head protraction;

HPR – Head protraction;

HR – Head retraction;

HRV – Hyoid retraction velocity;

Hy – Hyoid at the basis of ceratbranchial I;

HVD – Hyoid ventral displacement;

I – Ingestion (food uptake);

LJ – Lower jaw tip;

MG – Maximum gape;

Mm – Millimetre;

oss – Ossification islands on cornu branchiale II;

P – Parietale;

PG – Peak gape;

pro – Protraction;

pl – Processus lingualis;

ret – Retraction;

s – Second;

SO – Slow open phase lacking discrete SOI and SOII;

SO I – Slow Open I;

SO II – Slow Open II;

T – Transport;

TAG – Terrestrial aquatic gradient;

TCD – Total cycle duration;

THS – Total hyoid cycle;

TP – Tongue plateau;

TPG – Time to Peak Gape;

TPR – Tongue protraction;

TR – Tongue retraction;

TT – Tip of the tongue;

Tv – Tympanim venral most point;

UJ – Upper jaw tip;

vent. – Ventral;

1

Figure 1

Phylogeny of turtle clades with a focus on Testudinoidea. Interrelationship following Shaffer and Thomson (2011). Those authors assign Platysternidae as sister taxon to Emydidae; here we show different hypothesis for the position of this species. Major evolutionary changes are listed; for details see text. Three modes of terrestrial food uptake are illustrated. A) Jaw prehension; the tongue is not protruded and is only used for food transport (Geoemydida, *Manouria*). B) Jaw prehension; the elongated tongue is protruded during prehension but does not contact the food and is only used for food transport (Emydidae). C) Prior to jaw prehension, the tongue contacts the food (advanced tortoises).

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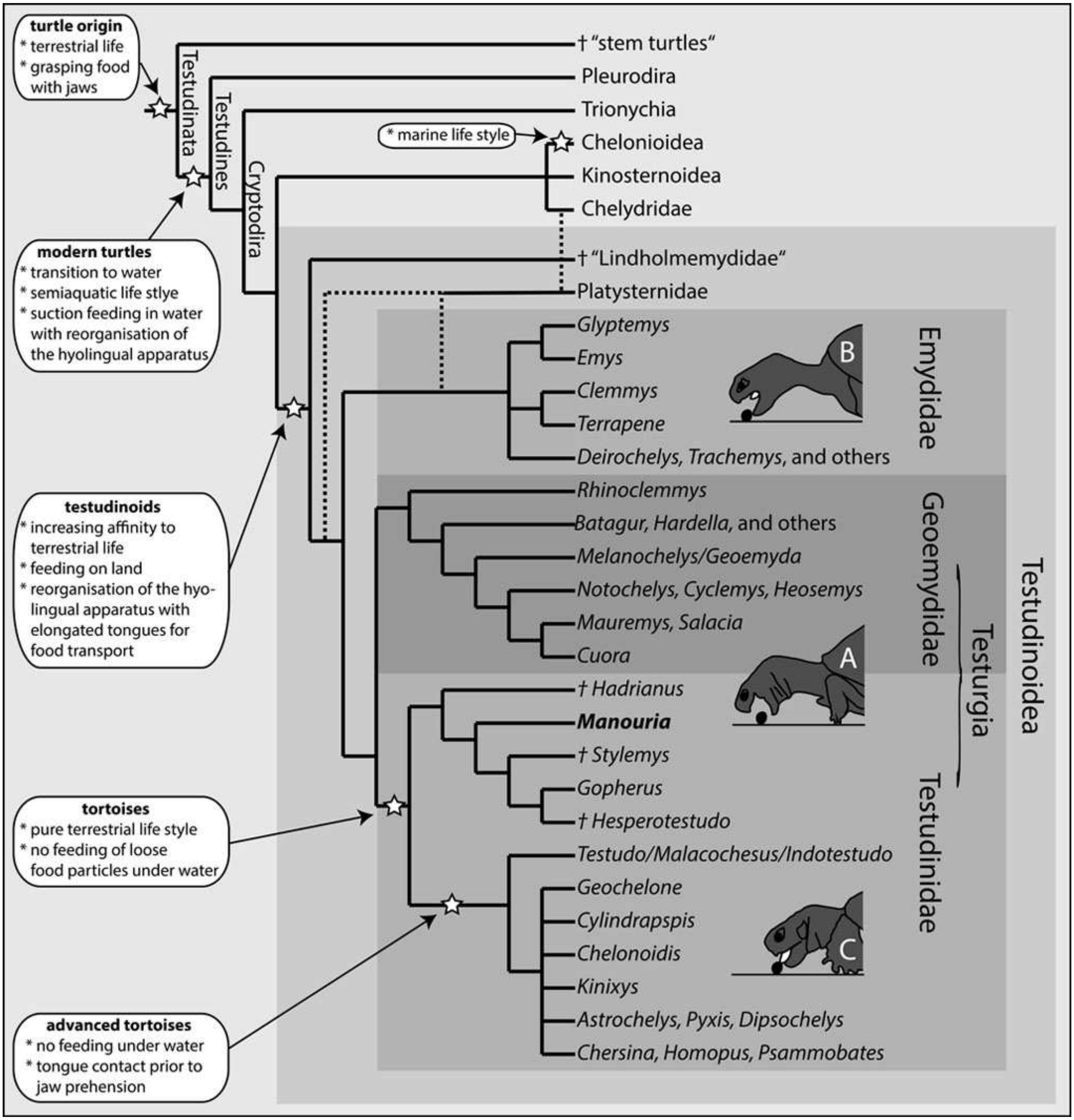
turtle origin
* terrestrial life
* grasping food with jaws
modern turtles
* transition to water
* semiaquatic life stlye
* suction feeding in water with reorganisation of the hyolingual apparatus
testudinoids
* increasing affinity to terrestrial life
* feeding on land
* reorganisation of the hyo-lingual apparatus with elongated tongues for food transport
tortoises
* pure terrestrial life style
* no feeding of loose food particles under water
advanced tortoises
* no feeding under water
* tongue contact prior to jaw prehension
* marine life style
Testudinata
Testudines
Cryptodira
† "stem turtles"
Pleurodira
Trionychia
Chelonioidea
Kinosternoidea
Chelydridae
† "Lindholmemydidae"
Platysternidae
Glyptemys
Emys
Clemmys
Terrapene
Deirochelys, Trachemys, and others
Rhinoclemmys
Batagur, Hardella, and others
Melanochelys/Geoemyda
Notochelys, Cyclemys, Heosemys
Mauremys, Salacia
Cuora
† Hadrianus
Manouria
† Stylemys
Gopherus
† Hesperotestudo
Testudo/Malacochesus/Indotestudo
Geochelone
Cylindrapspis
Chelonoidis
Kinixys
Astrochelys, Pyxis, Dipsochelys
Chersina, Homopus, Psammobates
B
A
C
Emydidae
Geoemydidae
Testudinidae
Testurgia
Testudinoidea

2

Figure 2

Selected frame from a high-speed video sequence (500 fr/s) of food transport in *Manouria emys*, showing the landmarks used for kinematic analyses: C, rostral tip of sagital carapace, Hy, hyoid at the basis of ceratbranchial I; LJ, tip of the lower jaw; P, posteriormost point of crista supraoccipitale; TT, tip of the tongue; Tv, ventral most point of the tympanum at the position of the jaw joint; UJ, tip of the upper jaw; grid 10x10 mm.

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Figure 3

Selected frames and graphics (based on a high-speed video with 500 fr/s) represent the movements of jaws, hyoid and t head during terrestrial food uptake in *Manouria emys* when feeding on pieces of beef heart; a, slow open phase end (lacking discrete SOI and SOII); b, fast open end; c, fast close start; d, fast close end; arrow indicates the position of the food item; arrowheads represent the position of the tip of the tongue; grid 10x10 mm.

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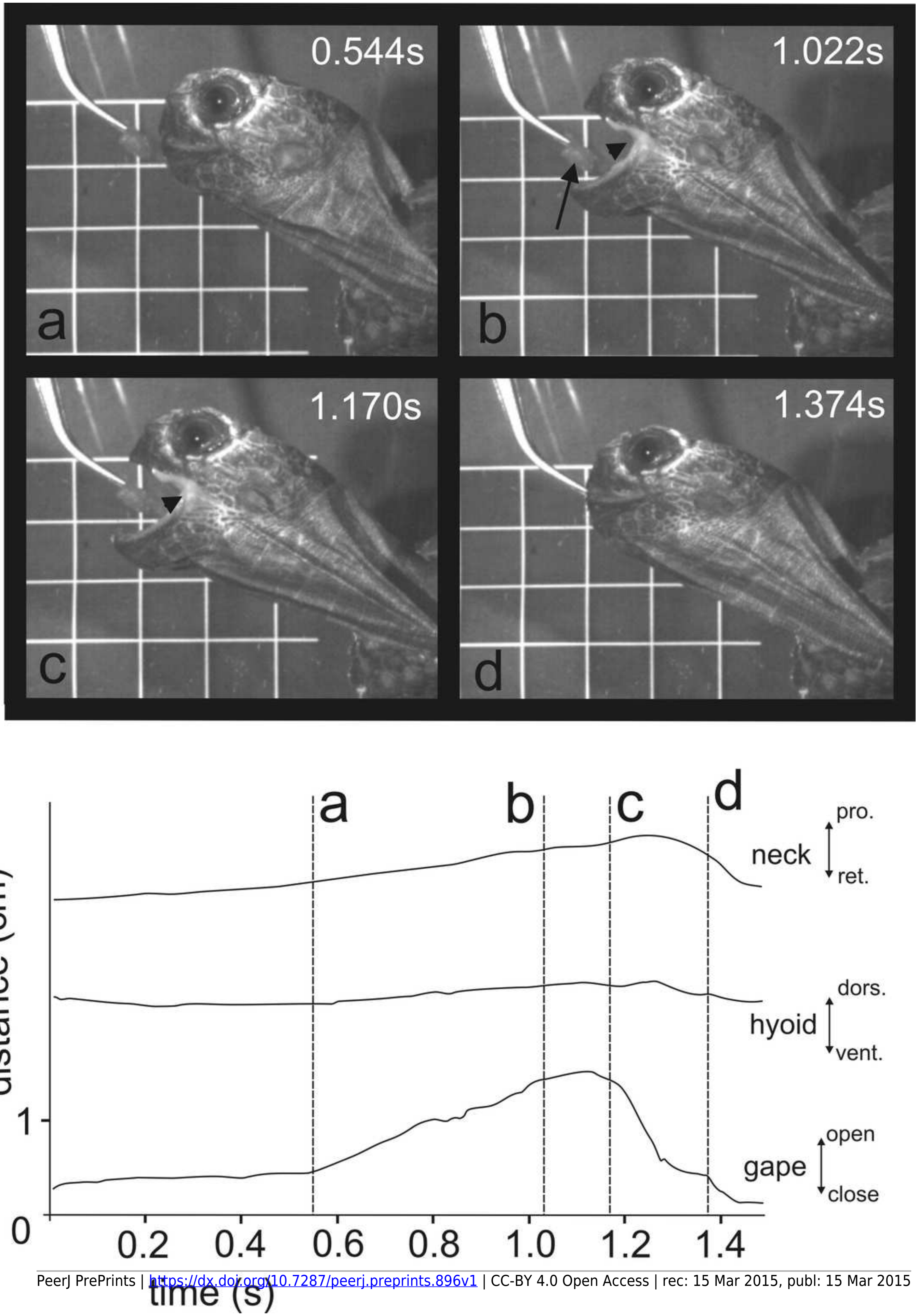
0.544s
a
1.022s
b
1.170s
c
1.374s
d
a
b
c
d
neck
pro.
ret.
hyoid
dors.
vent.
gape
open
close
distance (cm)
1
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
time (s)

4

Figure 4

Selected frames and graphics (based on high-speed video with 250 fr/s showing the movements of jaws, hyoid, and head during attempts of aquatic food uptake in *Manouria emys*; a, start of the gape cycle; b, end of jaw opening; c, maximum gape end; d, fast closure end; note the lack of movement of the hyoid complex during the whole cycle; grid 10x10 mm. dors., – dorsal; vent. – ventral; pro – protraction; ret – retraction.

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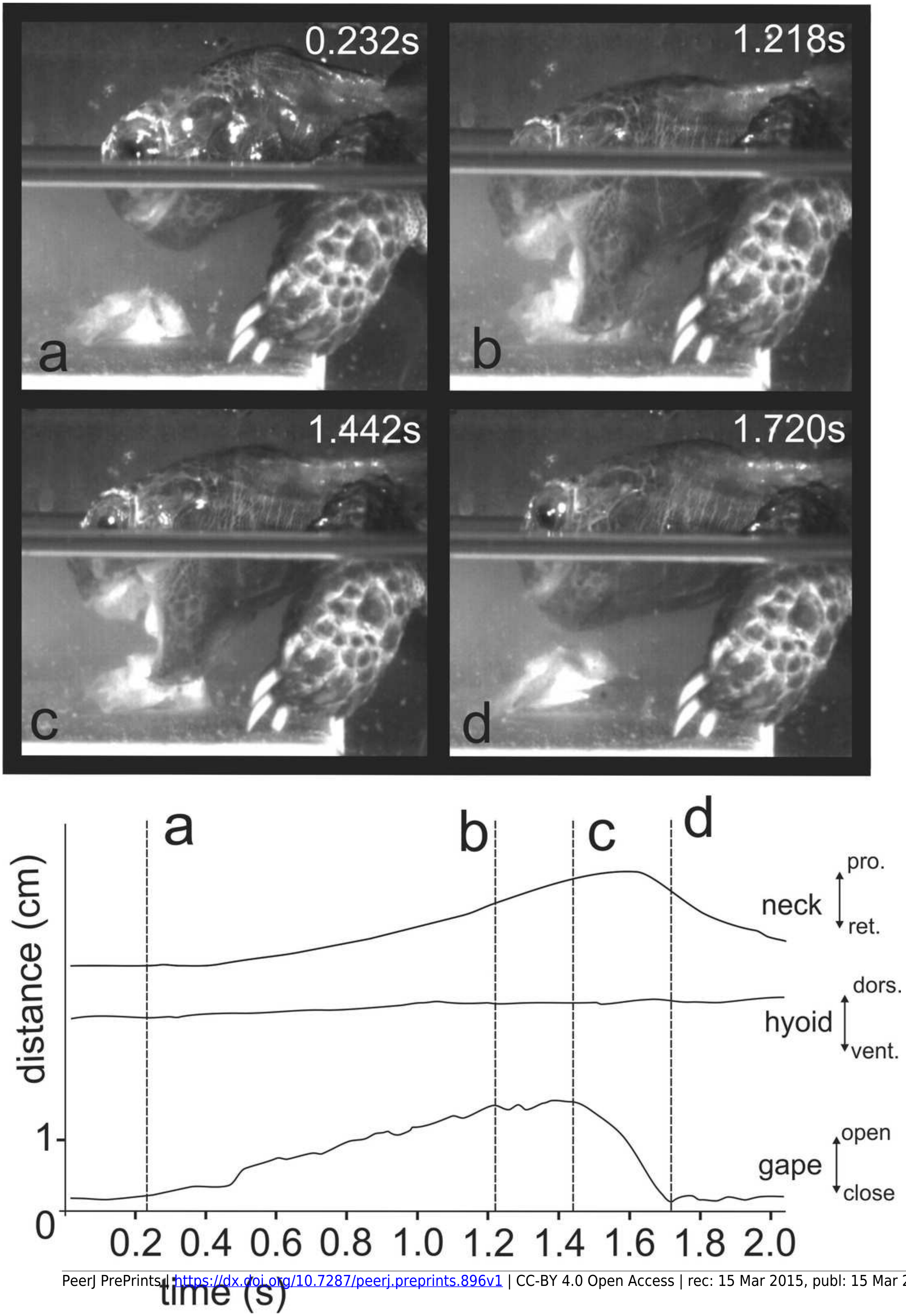
0.232s
a
1.218s
b
1.442s
c
1.720s
d
a
b
c
d
distance (cm)
neck
pro.
ret.
hyoid
dors.
vent.
gape
open
close
1
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
time (s)

5

Figure 5

Selected graphics (based on a high-speed video with 500 fr/s) showing the movement patterns of jaws, hyoid, tongue and head during terrestrial food transport in *Manouria emys*; note the delay in hyoid ventral displacement relative to the start the retraction of the tongue tip, as well as the delay of both the tongue retraction and hyoid retraction relative to the start of the FO phase. Abbreviations: FC, fast closure; GCM, generalised cyclic model; HDD, hyoid dorsal displacement; HPR, head protraction; HR, neck retraction; HRV, hyoid retraction velocity; HVD, hyoid ventral displacement; MG, maximum gape; PG, peak gape; SO, slow open phase lacking discrete SOI and SOII; SO I, slow open phase I; SO II, slow open phase II; T, transport; TCD, total cycle duration; THC, total hyoid cycle; TP, tongue plateau; TPG, time to peak gape; TPR, tongue protraction; TR, tongue retraction.

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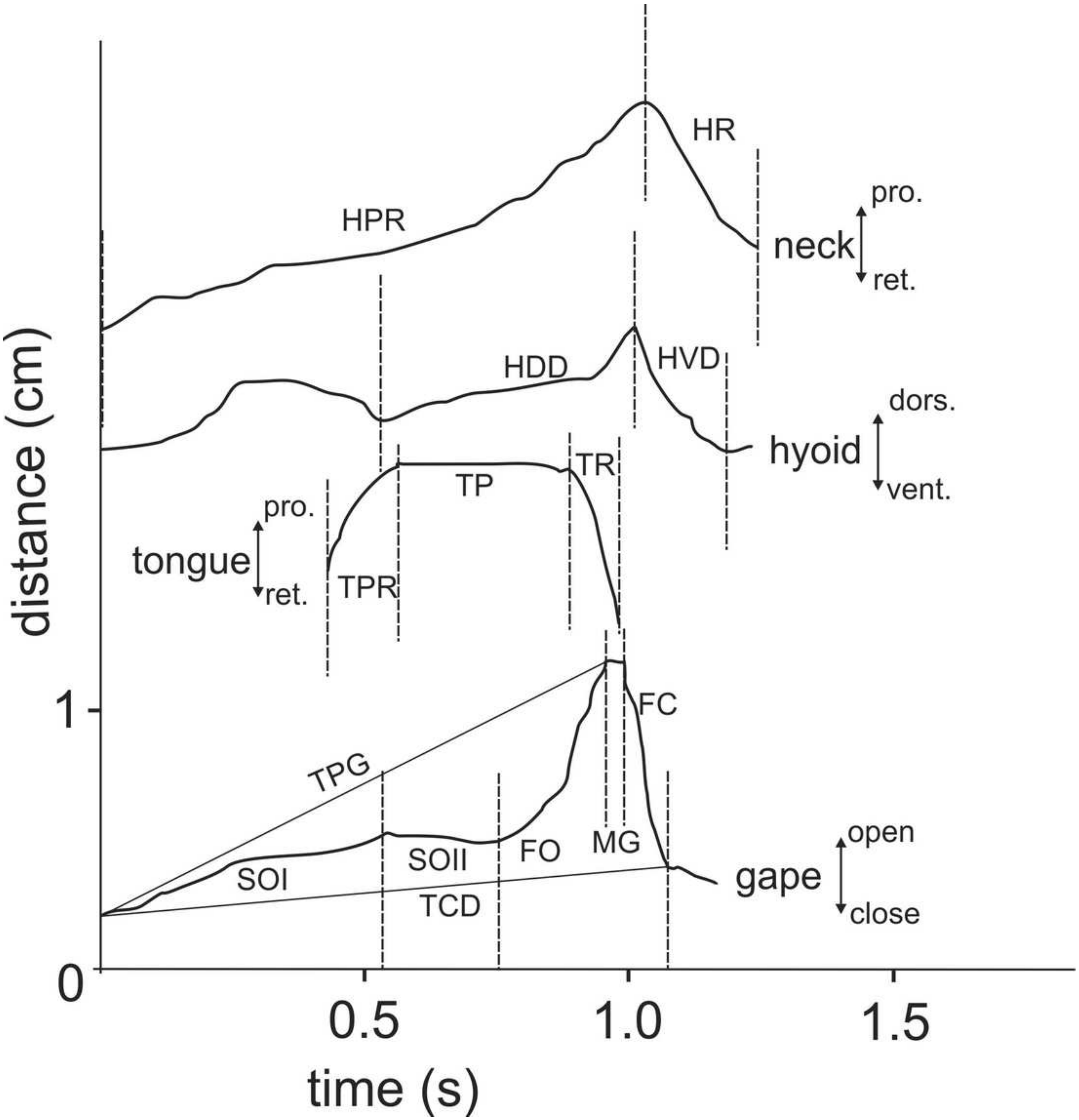
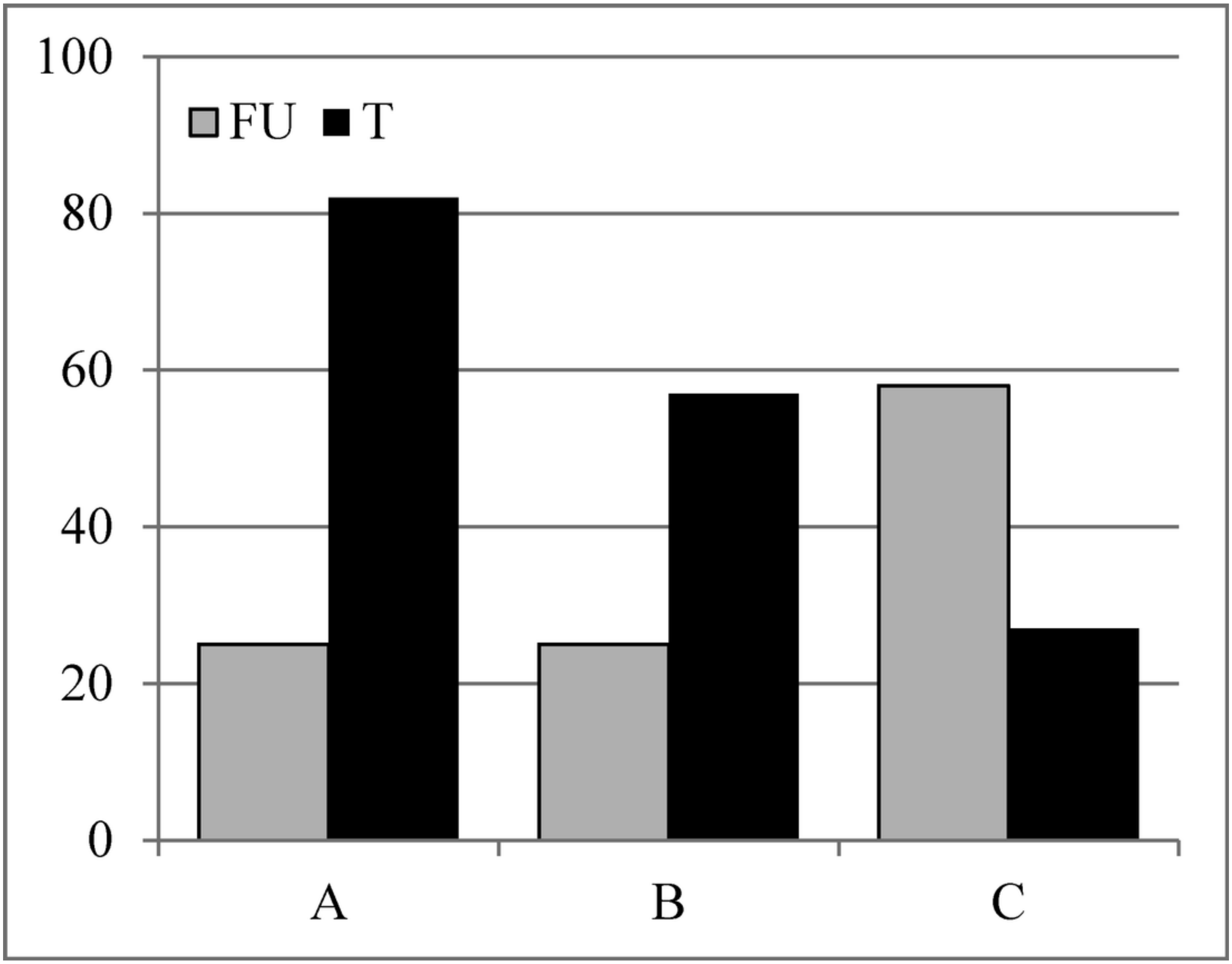

distance (cm)
time (s)
0
1
0.5
1.0
1.5
HPR
HR
neck
pro.
ret.
HDD
HVD
hyoid
dors.
vent.
TP
TR
TPR
tongue
pro.
ret.
TPG
SOI
SOII
TCD
FO
MG
FC
gape
open
close

Figure 6

Frequencies of occurrence of sequences with absence of selected variables in food uptake (FU) (light grey bars) and food transport (T) (black bars) phases, expressed as: percentages missing any possible split in slow and fast open gape phases - SO and FO (A); cycles in which SO phase was detectable, but the split of discrete slow gape phase in SOI and SOII is missing (B); lack of MG phase (C).



7

Figure 7

Canonical centroid plots of three *Manouria emys* specimens (T1–T3), centroid scores for each individual and measurement repetition in food transport phase.