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.

2 **Feeding behaviour in a 'basal' tortoise provides insights on the transitional feeding mode at the**

3 **dawn of modern land turtle evolution**

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5 Abbreviated title: **Feeding behaviour in *Manouria emys***

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18

19 **ABSTRACT**

20

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

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39 **Key words:** turtle, food uptake, feeding kinematics, evolution, Tetrapoda, terrestrial transition

INTRODUCTION

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 emydid turtles, the geoemydid turtles, and the testudinid turtles (tortoises) (Fig. 1). All molecular phylogenetic studies confirm a sister group relationship of Testudinidae and Geoemydidae (Iverson et al., 2007; Shaffer, 2009; Thomson and Shaffer, 2010; Barley et al., 2010) and Emydidae evolved independently from the former groups (Fig. 1). The relationship of Platysternidae to other turtle groups remains unresolved (Fig. 1; reviewed by Parham et al. 2006), but most molecular studies support a closer relationship to emydids (e.g., Shaffer and Thomson, 2010, Crawford et al., 2015). Palaeontological studies have shown that all testudinoids share a common aquatic ancestor, from which terrestrial species evolved (Danilov, 1999; Sukhanov, 2000; Joyce and Gauthier, 2004).

The majority of the Triassic stem turtles were terrestrial as indicated by the design and proportions of the limbs, which were adapted for terrestrial locomotion (for a comprehensive discussion see Joyce, in press). With the emergence 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 occurred (Willis et al., 2013). The invasion of aquatic environments induced diversification into several subgroups (see Joyce, 2007; Thomson and Shaffer, 2010). Due to the different physical properties (drag, viscosity, etc.) 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).

Among modern turtles, a transition from an aquatic to a semi-terrestrial or fully terrestrial lifestyles and the capacity to exploit terrestrial food sources has evolved independently within all three major

65   testudinoid lineages (for overview see Summers et al., 1998; Natchev et al., 2009). At least eight

66   emydid species are able to feed on land as well as under water (see Bels et al., 1997, 2008; Summers et

67   al., 1998; Stayton, 2011). During terrestrial feeding, such amphibious emydids use their jaws to grasp

68   food items (jaw prehension). Similarly, all amphibious geoemydids studied to date also use jaw

69   prehension in terrestrial food uptake (see Heiss et al., 2008; Natchev et al., 2009). In contrast, all

70   testudinids studied so far use the tongue to touch the food items, a behaviour referred to as "lingual

71   prehension" (see Wochesländer et al., 1999; Bels et al., 2008). According to Bels et al. (2008), lingual

72   prehension is obligatory for all tortoises.

73   The tortoises show a clear tendency towards herbivory and emancipation from water as living and

74   feeding medium (see Pritchard, 1979; Ernst and Barbour, 1989; Bonin, Devaux and Dupre, 2006). In

75   fact, testudinids seem to have lost their ancestral ability to feed under water and exclusively rely on

76   terrestrial trophic ecologies. Some predominantly terrestrial geoemydids are able to complete the whole

77   feeding process on land and under water (Natchev et al., 2010). Similarly, testudinids with tendencies

78   towards an amphibious lifestyle might have retained the ancestral feature to feed underwater. Hence,

79   information on bimodal feeding mechanisms in tortoises is of great importance to understand the

80   evolution of terrestrial feeding mechanisms and subsequent evolution of the predominantly terrestrial

81   lifestyle in tortoises.

82   The genus *Manouria*, being of the most 'basal' extant tortoises, with a strong association to aquatic

83   environments (Høybye-Mortensen, 2004; Stanford et al., 2015) constitutes a suitable model to study

84   the feeding mechanisms in testudinids. Its partial aquatic feeding habit purported to be associated with

85   the observed morphological extension of the palatines onto the triturating surface of the upper jaw

86   (character 30 sensu Gerlach, 2001), a diagnostic feature common to geoemydid (=batagurid) turtles.

87   Another geoemydid-like feature is the unique existence of class II mental glands (Winokur and Legler,

88   1975).

89   The present study was conceived to provide a detailed analysis of the feeding behaviour in a

90   species of the genus *Manouria*. *Manouria emys* is found in close association with water. Hence, we

91   designed experiments to reveal whether this species is able to complete the entire feeding process

92   under both aquatic and terrestrial conditions as some geoemydids do.

93   Wochesländer et al. (1999, 2000) and Bels et al. (2008) stated that the feeding kinematics in all

94   testudinids involve two common features: an obligatory lingual prehension and the split of the gape

95   cycle in four main phases [slow open phase I (SOI); slow open phase II (SOII); fast open phase (FO);

96   fast close phase (FC)]. Accordingly, the second main goal of this study is to test whether these

97   elements are also typical for the feeding behaviour in the 'basal' modern tortoises, i.e. *Manouria*.

98   Based on the results of our experiments, we formulate hypotheses concerning the evolution of the food

99   prehension mode within different turtle groups. Additionally, we test and adjust the classical feeding

100   models in lower tetrapods and discuss their general validity.

101

102   **MATERIALS AND METHODS**

103

104   Ecological background

105   Both extant species of *Manouria*, the Asian forest tortoise *M. emys* and the impressed tortoise *M.*

106   *impressa*, have a restricted distribution in Southeast Asia. *M. emys* has a narrow distribution in

107   Bangladesh, India (Assam, Meghalaya, Mizoram, Nagaland), Myanmar, Thailand, Malaysia (East and

108   West), and Indonesia (Kalimantan, Sumatra). The nominate subspecies, *M. emys emys* - the subject of

109   this study, inhabits the southern part of the species range (Fritz and Havaš, 2007; TTWG, 2014,

110   Stanford et al., 2015).

111   *M. emys* inhabits tropical evergreen monsoon forests and exhibits high tolerance for soil moisture.

112   It is commonly found reposing in wet areas, buried in mud or under the leaf litter where it may spend

113   long periods of time. It is active even during rainy weather and direct sun exposure and basking are not

114   required. Furthermore, this species has a mostly crepuscular and nocturnal lifestyle (Ernst, Altenburg,

115   and Barbour, 2000; Vetter and Daubner, 2000, Stanford et al., 2015).

116   According to the available literature, the diet of *M. emys* includes plants, fungi, invertebrates, and

117   frogs (Nutphand, 1979; Das, 1995; Lambert and Howes, 1994, Høybye-Mortensen, 2004). It has been

118   reported to feed on plants in shallow mountain streams (Nutphand, 1979).

119

120   Experimental setting

121   Animal husbandry and experiments were in strict accordance with the Austrian Protection of

122   Animals Act. The animals used in the present study were obtained commercially and kept at 12 h

123   dark/light cycles in a large terrarium (150x100 cm ground area) with a permanently filtered water basin

124   and spacious terrestrial area. The turtles were fed different fruits, vegetables, commercially obtained

125   tortoise pellets, dead mice, as well as pieces of cattle heart and liver, offered on the terrestrial part of

126   the terrarium. Carapace lengths in the three subadult experimental animals ranged between 109-135

127   mm with body masses between 234-236 g. For filming terrestrial feeding, the specimens were put in a

128   dry glass cuvette (24x60x30 cm). When the food was offered on the floor of the cuvette, the tortoises

129   permanently twisted their necks and rotated their heads in attempt to grasp the food item. The side

130   movements made the filming of the animals in strict lateral view not possible and the landmarks were

131   not clearly visible during the sequence. By the use of forceps for food display we completely

132   eliminated these problems and were able to shoot perfect lateral plans of the feeding turtles. The food

133   in the feeding experiments was offered at a position which was similar to the position on which we

134   offered the food in the terrarium where the tortoises were housed. The animals did not extend their

135   necks extraordinary to reach the food items (see Suplements). The position of the offered food was

136 completely "natural" for the tortoises and they needed to stretch their necks forwards rather than

137 downwards, which did not impacted other kinematic patterns of the feeding cycles.

138   As food items we used small pieces of cattle heart measuring approximately 5x5x5 mm. The

139 turtles were filmed from lateral aspect (with a reference grid 1x1 cm in the background) via the digital

140 high-speed camera system Photron Fastcam-X 1024 PCI (Photron limited; Tokyo, Japan) at 500 fps

141 with a highly light-sensitive objective AF Zoom - Nikkor 24-85 mm (f/2,8-4D IF). Two "Dedocool

142 Coolh" tungsten light heads with 2 x 250 W (ELC), supplied by a "Dedocool COOLT3" transformer

143 control unit (Dedo Weigert Film GmbH; München, Germany) were used for illumination. We filmed

144 and analysed the food uptake and the food transport cycles in eight feeding sequences for each

145 specimen.

146   The setting for filming aquatic feeding of submerged food comprised the experimental aquarium

147 filled with water to a level of 3 cm and presentation of food items in front of the turtle's snout. In order

148 to reduce the light intensity and for optimisation of the digitising process, the frame rate was reduced to

149 250 fps. As the tortoises were unable to grasp the food item in a total of 36 trials, the kinematics of the

150 feeding apparatus were analysed (see below) in nine selected representative feeding trials.

151   For both terrestrial and "aquatic feeding" sequences, horizontal (X-axis) and vertical (Y-axis)

152 coordinates of relevant landmarks (see Fig. 2) were digitised frame by frame using "SIMI-MatchiX"

153 (SIMI Reality Motion Systems; Unterschleißheim, Germany). Based on the displacement of the

154 markers, we were able to calculate the gape amplitude (distance between the tip of the upper and lower

155 beak), head movement (distance between the anterior tip of the carapace and the point "P" on Fig. 2),

156 tongue movements (distance between the most ventral point on tympanum and the tip of the tongue

157 when visible), and hyoid movements (distance between the point "P" on Fig. 2 and the basis of the

158 posterior ceratobranchial). To compare the kinematic feeding pattern of *M. emys* to those of other

159 studied turtles and to understand the coordination between the elements of the feeding apparatus, these

160   data were used for calculation of the following kinematical variables: duration of Slow open phase

161   (SO); duration of Slow open phases I and II (SOI and SOII) when present; duration of fast open (FO);

162   duration of maximum gape phase (MG); duration of fast close (FC); time to peak gape (TPG); total

163   cycle duration (TCD); duration of hyoid protraction (HyDD); duration of hyoid retraction (HyVD);

164   duration of the total hyoid cycle (THC); hyoid retraction velocity (HyRV); duration of head protraction

165   (HP); duration of head retraction (HR); duration of tongue protraction (TP); tongue retraction velocity;

166   delay of the start of hyoid retraction relative to the tongue retraction start; delay of reaching peak gape

167   relative to start of the hyoid retraction; delay of reaching peak gape relative to tongue retraction start

168   (see Table 1).

169

170   Statistics

171   We tested whether there are any differences among the frequency of occurrences of defined

172   patterns both in food uptake (FU) and food transport (T), i.e. sequences with: missing split of the jaw

173   opening in SO and FO; without detectable split of discrete SOI and SOII slow gape phase; lacking MG

174   phase. In order to provide the comparisons, Chi-square test with Yates' correction was performed.

175   Then we tested for possible existence of differentiation in kinematical variables in both feeding stages.

176   All variables were tested with the Shapiro-Wilk test for normal distribution. When the p-value was less

177   than the chosen alpha level ($p<0.05$), the null hypothesis was rejected and data were excluded from

178   further analyses. In addition, all variables included in Table 1 were tested with Levene's and Brown-

179   Forsythe tests and then processed with Welch's ANOVA for heteroscedastic data. Tukey's honest

180   significant difference test (HSD) was performed for post-hoc analyses when applicable.

181   Furthermore, in order to express the degree of individual differentiation among the studied specimens,

182   a Canonical discriminant analysis (CDA) was performed. Standard descriptive statistics including

183   mean, range, standard deviation [(SD) and confidential interval at 95% CI] were presented.

184

RESULTS

185

186

187   When feeding on land, the Asian forest tortoises always grasped food by the jaws. After food

188   uptake, one to four transport cycles followed prior to oesophageal packing (see Schwenk, 2000). The

189   tip of the tongue was barely visible during food uptake (see Fig. 3b-c) indicating that the tongue was

190   not protracted. By contrast, during transport cycles, the cyclic movements of the tongue were well

191   visible as it was rhythmically pro- and retracted to transport the food item towards the oesophagus (Fig.

192   5).

193   When trying to feed under water (Fig. 4), *M. emys* submerged its head under the water level, and

194   by protruding the head, the animals were trying to position the gaping mouth around the food item. The

195   gape cycle was newer split in slow and fast jaw open phases. The tongue tip was not visible from the

196   lateral aspect and the hyolingual complex did not protract prior reaching peak gape. No retraction of

197   the hyoid complex was detected prior jaw closure. The gape cycle duration exceeded for one and a half

198   seconds and was 1.94 ± 0.36 s (mean $\pm$ SD). Despite the unsuccessful attempts, the turtles repeatedly

199   tried to catch the submerged food. In several events, we were able to detect that the food item was

200   carried away by the bow wave induced by jaw closing.

201   The variables of the kinematical profiles are summarised in Table 1. In the statistic tests, we found

202   highly significant differences in sequences with and without both SO I and SO II when food uptake and

203   transport stages were compared ($\chi^2_{(1,\ N=98)}=25.05$, p <0.001). Similarly significant differences were

204   observed when comparing food uptake and transport cycles in respect to sequences with and without

205   slow jaw open phases as well as with and without maintaining jaw maximum gape - MG phase ($\chi^2_{(1,}$

206    $_{N=98)}=6.10$, p=0.02; $\chi^2_{(1, N=98)}=6.52$, p=0.01).

207   When testing the variables of the feeding kinematics (Table 1), nine variables, which describe the

208   food uptake process, were detected to show significant differences between individuals. In transport

209   cycles, six out of 18 variables differed significantly amongst individuals (see Table 1). When testing

210   for differences between grasping and transport cycles, seven out of nine variables differed

211   significantly: fast jaw open duration (FO; $F_{Welch\ (1,43)}$=15.17, p=0.011); maximum gape dutaion (MG;

212    $F_{Welch\ (1,26)}$=15.89, p=0.001); fast closing (FC; $F_{Welch\ (1,26)}$=7.86, p=0.010); time to peak gape (TPG;

213    $F_{Welch\ (1,72)}$=46.78, p<0.001); total gape cycle duration (TCD; $F_{Welch\ (1,72)}$=52.50, p<0.001); head

214   protraction duration (HP; $F_{Welch\ (1,67)}$=52.23, p<0.001); and head retraction duration (HR; F_{Welch}

215    $_{(1,47)}$=12.57, p=0.002).

216   When comparing three further parameters among the transport cycles in all three specimens (delay

217   of HyVD start relative to TR start; delays of TPG relative to HyVD; delay of TPG relative to TR

218   starts), statistically significant differences were found among all compared pairs ($F_{Welch(2,105)}$=41.58,

219   p<0.001).

220   The performed canonical discriminant analysis (CDA) revealed the existence of substantial

221   degree of individualism among the studied specimens (Fig. 7). However, only the first axis eigenvalue

222   exceeded the level of acceptance, i.e. 1. First axis explained 70% of the total variance. Among the 18

223   studied variables only two (THC and HPR) showed higher correlation scores than 0.75. On the base of

224   the CDA and the detected degree of individualism mentioned above, we can conclude that the patterns

225   displayed by the studied specimens can be regarded as similar but not as uniform.

226

227   **DISCUSSION**

228

229   *M. emys* repetitively tried to feed on dispersed food items under water, which was an unexpected

230   and hitherto unknown behaviour among tortoises. However, *M. emys* always failed to consume the

231   submerged food. On land, *M. emys* grasped food with the jaws, just like all known aquatic or

232   semiterrestrial turtles do, but not with the tongue as formerly predicted for all tortoises. On the basis of

233   our results we discuss several important evolutionary, behavioural, and functional aspects.

234

235   **Evolution of food uptake among turtles**

236   In general, most aquatic turtles combine a fast acceleration of the head towards the food or prey

237   item and a suction feeding mechanism induced by fast oropharyngeal volume expansion. In some

238   extant turtles, a strong suction flow can be generated and prey is directly sucked into the oropharynx

239   without contact with the jaws [e.g., *Chelus fimbriatus* (Lemell et al., 2002), *Apalone spinifera*

240   (Anderson, 2009), *Pelodiscus sinensis* (N.N. and I.W. unpublished data)]. However, most extant turtles

241   cannot generate such strong suction flows and only compensate ("gulp") the bow wave that otherwise

242   would push small to moderately sized food items away from the fast approaching head.   Such species

243   finally fix and grasp prey with the jaws (see Lauder and Prendergrast, 1992; Lemell et al., 2000; Aerts

244   et al., 2001, Natchev et al., 2009, 2011). We consider the latter as plesiomorphic behaviour for extant

245   turtles.

246   Among extant turtles, the ability to complete the whole feeding process (including food uptake,

247   food manipulation and transport, esophagial packing, and swallowing) on land has been tested and

248   documented for only six species so far. All of them were members of Testudinoidea (Fig. 1; see also

249   Summers et al., 1998; Bels et al., 2008; Natchev et al., 2009). The terrestrial mode of food uptake

250   differs dramatically among and within the three testudinoid subgroups (see Bels et al., 1997, 2008;

251   Summers et al., 1998; Wochesländer et al., 1999, 2000; Natchev et al., 2009, present study).

252   Correspondingly, it appears as if terrestrial feeding re-evolved several times independently amongst

253   turtles. Unfortunately, only limited experimental data are available on feeding mechanisms in emydids

254 and geoemydids. Further functional and palaeontological investigations may help to sort out the issues

255 on the evolution of the feeding behaviour and the morphology of the feeding apparatus in testudinoids.

256   The sister group of all remaining Testudinoidea is the diverse and possibly paraphyletic extinct

257 taxon †Lindholmemydidae (Fig. 1; Lourenço et al., 2012), which contains genera such as

258 †*Mongolemys* and †*Lindholmemys* (Danilov, 1999; Joyce and Gauthier, 2004). Some poorly

259 documented aquatic taxa such as †Haichemydidae and the †Sinochelyidae may perhaps also belong to

260 †Lindholmemydidae. This group was recorded from aquatic sediments of the late Early Cretaceous and

261 apparently had an amphibious lifestyle (Sukhanov, 2000). Among recent cryptodirans, semi-aquatic to

262 semi-terrestrial lifestyle is typical for most emydids (plus platysternids) and geoemydids, as well as for

263 some kinosternids (see Depecker et al., 2006, but also Nakajima et al., 2014). The remaining extant

264 species are entirely terrestrial (tortoises), or predominantly aquatic (see Joyce and Gauthier, 2004;

265 Rasmussen et al., 2011).

266   Very limited information is available on feeding mechanisms employed by amphibious non-

267 testudinoid turtles that occasionally exploit terrestrial food sources. Weisgram (1985) documented a

268 kinosternid (*Claudius angustatus*) that caught prey on land and dragged it into water for transport and

269 swallowing. Natchev et al. (2008) documented another kinosternid (*Sternotherus odoratus*) catching

270 food on land, but failing to transport it through the oropharynx. Among extant turtles, successful food

271 transport on land seems to be restricted to testudinoids and the development of enlarged and muscular

272 tongues within this group (von Bayern, 1884; Werneburg, 2011) can be considered as adaptation to

273 improved terrestrial food manipulation.

274   Based on experimental data, Natchev et al. (2009) described and summarised three categories of

275 terrestrial food uptake modes among Testudinoidae: (A) Jaw prehension with retracted hyolingual

276 complex, as observed in the geoemydid genus *Cuora* (Natchev et al., 2009); (B) Jaw prehension with

277 slightly protracted hyolingual complex, as observed in emydids (Bels et al., 1997; Stayton, 2011); (C)

278 Lingual prehension: The tongue touches the food item prior to food uptake and possibly carries the

279 food item into the mouth, as documented in all tortoises studied so far (Wochesländer et al. 1999, Bels

280 et al. 2008). The food uptake mode of *M. emys*, however, differs substantially from that of all

281 remaining tortoises (category C). In fact, the hyolingual complex in *M. emys* remained fully retracted

282 during the food prehension on land, and the first contact with the food item was by the jaws.

283 Accordingly, the feeding mechanism of *M. emys* should be assigned to category A, along with that of

284 semi-aquatic geoemydids.

285   We now aim to construct a theoretical scenario on the evolution of terrestrial feeding mechanisms

286 in turtles. Given the aquatic origin of all living turtles, the functional transition from aquatic to

287 terrestrial feeding mechanisms could hypothetically have involved four stages, beginning with an

288 exclusively aquatic feeding ancestor. In different lineages and stages, turtles may have left their aquatic

289 environments for various reasons e.g. for exploiting new food niches. The species that retained

290 predominantly aquatic life styles may grasp food by the jaws on land, but have to drag it into the water

291 for further intraoral (hydrodynamic based) transport. In recent turtles, such behaviour was documented

292 in the kinosternids *C. angustatus* (Weisgram, 1985a,b) and *Sternotherus odoratus* (Heiss et al., 2010;

293 Natchev et al., 2011), as well as in the emydid *Trachemys scripta* (Weisgram, 1985b; Weisgram et al.,

294 1989) and other emydids (see Stayton, 2011). Turtles of the second hypothetical evolutionary stage

295 grasped food by the jaws and the tongue was used for intraoral food transport on land. Such species

296 would still have retained their underwater feeding ability by using hydrodynamic mechanisms. When

297 grasping food on land, the tongue remained retracted or was protracted without however touching the

298 food item. Among extant turtles, such a feeding mode is found in the geoemydid genus *Cuora* (Heiss et

299 al., 2008; Natchev et al., 2009, Natchev et al., 2010) and in some emydids (Bels et al., 1997; Summers

300 et al., 1998, Stayton, 2011). In the next theoretical evolutionary step (stage three), behavioural and

301 morphological adaptations for terrestrial feeding were further advanced, increasing the efficiency of

302   terrestrial food transport at the expense of the ability to use effective hydrodynamic mechanisms in

303   water. Such species still grasped food items with their jaws on land (as typical for aquatic or

304   semiaquatic turtles), but were no longer able to take up dispersed food if submerged, which features

305   prominently in the present case of *M. emys*. Most importantly, stage three could have marked the

306   "point of no return" in the evolution of terrestrial feeding in extant tortoises. Finally, in a fourth stage,

307   turtles became fully terrestrial and their tongue was obligatorily involved in food uptake as

308   documented in the tortoises *Testudo* (*Eurotestudo*) *hermanni boettgeri* (Weisgram, 1985b;

309   Wochesländer et al., 1999; 2000), *Kinixis belliana*, *Geochelone elephantopus* and *G. radiata* (Bels et

310   al., 2008).

311   According to Bels et al. (2008), tortoises exhibit very stereotypical kinematical patterns in food

312   ingestion and transport. These authors predicted: obligatory lingual prehension for initial food uptake:

313   split of the gape cycle in slow open (SO) and fast open (FO) phases: start of the tongue retraction at the

314   beginning of the fast jaw open phase. Specifically, the authors demonstrated these elements of the

315   feeding behaviour in the tortoises *G. radiata*, *G. elephantopus*, and *K. belliana*, and Wochesländer et

316   al. (1999, 2000) in *T. hermanni boettgeri*.

317   In contrast, our investigations demonstrate that the 'basal' tortoise *M. emys* does not contact food

318   with the tongue prior to jaw prehension on land. This shows that tongue to food contact is

319   characteristic of advanced tortoises only. We consider the terrestrial feeding behaviour of *M. emys* as

320   plesiomorphic and potentially inherited from its semiaquatic ancestors. On that base, *M. emys* can be

321   considered a transitional turtle in regard to secondary terrestriality.

322   We propose that hyolingual protrusion evolved in the linage forming to advanced tortoises (Fig.

323   1). *Manouria emys* has a large tongue with massive intrinsic and extrinsic musculature (see Heiss et al.,

324   2011). The advanced and complex lingual musculo-skeletal architecture permits the turtle to protrude

325   the tongue outside the margins of the rhamphothecae (see Fig. 2). However, *M. emys* does not use

326 lingual food prehension as typical for all other tortoises studied so far. In fact, it seems that the

327 *Manouria* [and perhaps *Gopherus* (N.N. personal observations)] "linage" has retained the jaw

328 prehension mechanism inherited from earlier aquatic ancestors. It seems that the tortoises in general

329 evolved fleshy tongues which improve the food transport performance and advanced tortoises only

330 refined the behaviour of food uptake on land via lingual food contact prior to jaw closure (see

331 Wochesländer et al., 1999; Bels et al., 2008).

332

Function of the protruded tongue in the testudinid's food uptake

334   What would the potential advantage of the obligatory lingual protrusion, found in the more derived

335 tortoises, be? One possible explanation is that the tongue is used as a prehensile organ for food

336 ingestion analogous to that found in other tetrapod groups (for overview see Schwenk, 2000; Schwenk

337 and Wagner, 2001). However, for tortoises such anlagues explanation might be put into question. By

338 examining all published data available (Wochesländer et al., 1999, Bels et al., 2008), we were not able

339 to find any convincing evidence that tortoises collect food with their tongues - they just touch it. In all

340 published feeding sequences, the contact between the food and the tongue is clearly demonstrated - yet,

341 in all cases, after the initial contact of the tongue with the food, the head moves forward and the food

342 item is not dragged into the mouth by tongue retraction, but is grasped by the jaws during the fast jaw

343 closing (FC gape phase). Hence, in extant tortoises, the tongue may not be the main organ that is

344 catching (collecting) the food, but these are in fact the jaws. Initial food ingestion in tortoises might not

345 be considered "lingual prehension" (see Schwenk, 2000; Bels et al., 2008) in the strict sense, but

346 should be regarded as "jaw prehension following lingual contact". This is imperative for differentiating

347 and prompts the question: why is an obligatory contact of the tongue to the food present in extant

348 tortoises (except *Manouria* and also belike *Gopherus*) during food uptake? In other words: why do

349 tortoises apply a more complex and presumably more energetically expensive food uptake mechanism

350 by including movements of the hyolingual complex in addition to the movements of the neck and jaws

351 alone?

352   We propose that the lingual contact provides tactile information on the position of the food item

353 and helps the advanced tortoises to compensate the "information gap" which occurs when the food is

354 approached to a distance where it located out of sight. The eyes of tortoises are positioned laterally on

355 the head (Pritchard, 1979) and the turtles are not able to permanently observe the position of the food

356 item when the neck is protracted and the gape is positioned around it. The prolonged maximum gape

357 (MG) phase found in most ingestion cycles of *M. emys* (see Table 1, Figure 3 and Figure 6) might be

358 the result of lack of lingual contact with the food surface. In all published sequences and kinematical

359 profiles on food uptake in tortoises, there is a clear tendency toward a split of the gape cycle into slow

360 open (SO) and fast open (FO) gape phases (see Wochesländer, 1999; Bels et al., 2008). The lack of

361 tongue protrusion might explain the lack of slow open (SO) and fast open (FO) split in the gape cycle

362 of food uptake in the geoemydid *Cuora* (see Natchev et al., 2009). In most food uptakes analysed in *M.*

363 *emys,* SO phases are not present and the gape increases gradually (see Table 1 and Figure 6). Similar as

364 in *Cuora*, the lack of SO phases might be explained by the lack of tongue protrusion in food uptake

365 (see Natchev et al., 2009).

366

367 **Intraoral food transport on land**

368   The execution of the transport cycles demand complicated coordination of the activities of the

369 contractile elements such as the jaw opening and closing muscles, head protracting and retracting

370 muscles, intrinsic and extrinsic lingual muscles, as well as muscles that protract and retract the

371 hyolingual complex as a whole unit (Jones et al., 2012; Werneburg, 2011; 2013). In contrast to this

372 complicated choreography, the mode of food prehension in *M. emys* suggests less complex

373 neuromotoric coordination between neck and jaw movements. Yet, the execution of the transport

374 cycles is often more than twice shorter in duration (see Table 1). A possible explanation for the longer

375 duration of food uptake cycles relative to transport cycles might be that during transport the

376 coordination centres of the muscle activities execution are permanently supplied with information

377 concerning the position of the food item within the oropharynx and the proper movements can be

378 executed precisely in a shorter time.

379 Wochesländer et al. (1999, 2000) and Bels et al. (2008) hypothesised that the feeding kinematical

380 patterns in tortoises adhere exactly to those of the generalized cycle model (GCM) proposed by

381 Bramble and Wake (1985). In his work on the feeding mechanisms in domestic animals, Bels (2006)

382 established that the pre-programmed GCM is universally valid among different groups of tetrapods,

383 including anamniotes, sauropsids, and mammals. The feeding kinematics of the 'basal' extant Asian

384 forest tortoise differs in some aspects from those proposed by the GCM. The kinematics of the feeding

385 system in *M. emys* seems to be pre-programmed, but under permanent feed-back control. The values of

386 the gape and hyoid/hyolingual cycle patterns in the three specimens studied here show high degrees of

387 variation, both concerning food uptake and food transport (see Table 1 and Figures 5-7). The slow

388 open phases (SO) are not obligatory. The gape cycle often includes a phase of retaining maximum gape

389 (see Table 1, Figure 3, 5). In turtles, the maximum gape phase (MG) was described for the gape cycle

390 in *Cuora sp.* (Natchev et al., 2009, 2010) and was confirmed for other turtles (Natchev et al., 2011).

391 The presence of a prolonged maximum gape phase (MG) can be easily overlooked when the frame rate

392 of the film sequence is not high enough (i.e., step between successive frames over 10 milliseconds).

393 The GCM presupposes that the start of hyoid retraction coincides with the start of fast open phase

394 (FO). However, our calculations (see Table 1 and Figure 5) demonstrate that in *M. emys* the hyoid

395 retraction in the food transport cycle starts shortly prior reaching peak gape. The same pattern was

396 detected by the investigation of aquatic, semi-aquatic, but also predominantly terrestrial cryptodirans

397 (Natchev et al., 2008, 2009, 2010, 2011).

398

Relations between the habitat preferences and the diet in turtles

In the evolution of the testudinids there is a clear shift not only in the habitat preferences (from aquatic to terrestrial), but corresponding shifts are also seen in dietary preferences. In the particular taxon it manifests in a tendency toward herbivory. Most of the recent tortoises rely on diets mainly composed of plant material (for overview see Pritchard, 1987; Ernst et al., 2000; Bonin et al., 2006). The partly carnivorous lifestyle of *Manouria sp.* (Bonin et al., 2006) may be a relic of the carnivorous diet of the ancestor of the tortoises and supports the transitional status of the genus.

Apparently the feeding media (air vs. water) and the dietary shift had a large influence on the overall feeding behaviour of the testudinids (see Bels et al., 2008). The suction mechanism was lost and replaced by a jaw food prehension system (this study) or by "lingual prehensuion" (Wochesländer et al., 1999; Bels et al., 2008). Probably, the switch to herbivory determined the reorganisations in the morphology of the jaw muscle system and the proportions of the skull in tortoises (see Werneburg 2011, 2012, 2013). By feeding predominantly on plants (immobile items), the tortoises may prolong the duration of the food uptake cycles and have more time to adjust their prehension kinematics to every single feeding situation.

We propose that the habitat preferences and the diet change in turtles are firmly correlated (e.g. Bels et al., 2008; Werneburg, 2014). The overall rigid design of the turtle 'body plan' prohibits the animals to actively hunt for agile prey in terrestrial environments (King, 1996). We hypothesise that this statement is also valid for the terrestrial stem turtles and that these animals were predominantly herbivorous (sensu King, 1996). In aquatic turtles, in contrast, the buoyancy of the water overrides the shell-caused restrictions of mobility and also suction feeding can be applied for carnivorous feeding. Those advantages might have been the key factor turtles to become aquatic (and carnivorous) in the

421   Jurassic (as Testudines). There may be several reasons for the secondary terriallity in tortoises and

422   presumably one of the main was the inter- and intraspecific concurrence for food resources.

423

424   **CONCLUSIONS**

425

426   We propose that the ancestral food uptake mode in tortoises was jaw based when feeding on land.

427   During the shift from aquatic to terrestrial lifestyle, including a shift from aquatic to terrestrial feeding

428   biology, the "*Manouria* (and most likely also *Gopherus*) linage" had retained pure jaw prehension in

429   food uptake. The hyolingual complex in that lineage exhibits the typical morphological features of

430   tortoises that feed exclusively on land (see Bramble, 1973; Winokur, 1988; Heiss et al., 2011), such as

431   an enlarged fleshy tongue with abundant papillae, a complex tongue musculature, a relatively small

432   and mainly cartilaginous hyoid and hypoglossum. The evolutionary shift in the morphology of the

433   hyolingual complex was apparently primed by the optimisation of the food transport behaviour and not

434   for food uptake. We suggest that the involvement of the tongue during food uptake found in the

435   derived extant tortoises serves as a tactile sensory tool for the localisation of the food item position

436   prior to jaw prehension. Thus, the tongue is not used as the main apprehensive organ in modern

437   tortoises and the food uptake mode represents a derived jaw prehension system.

438

451

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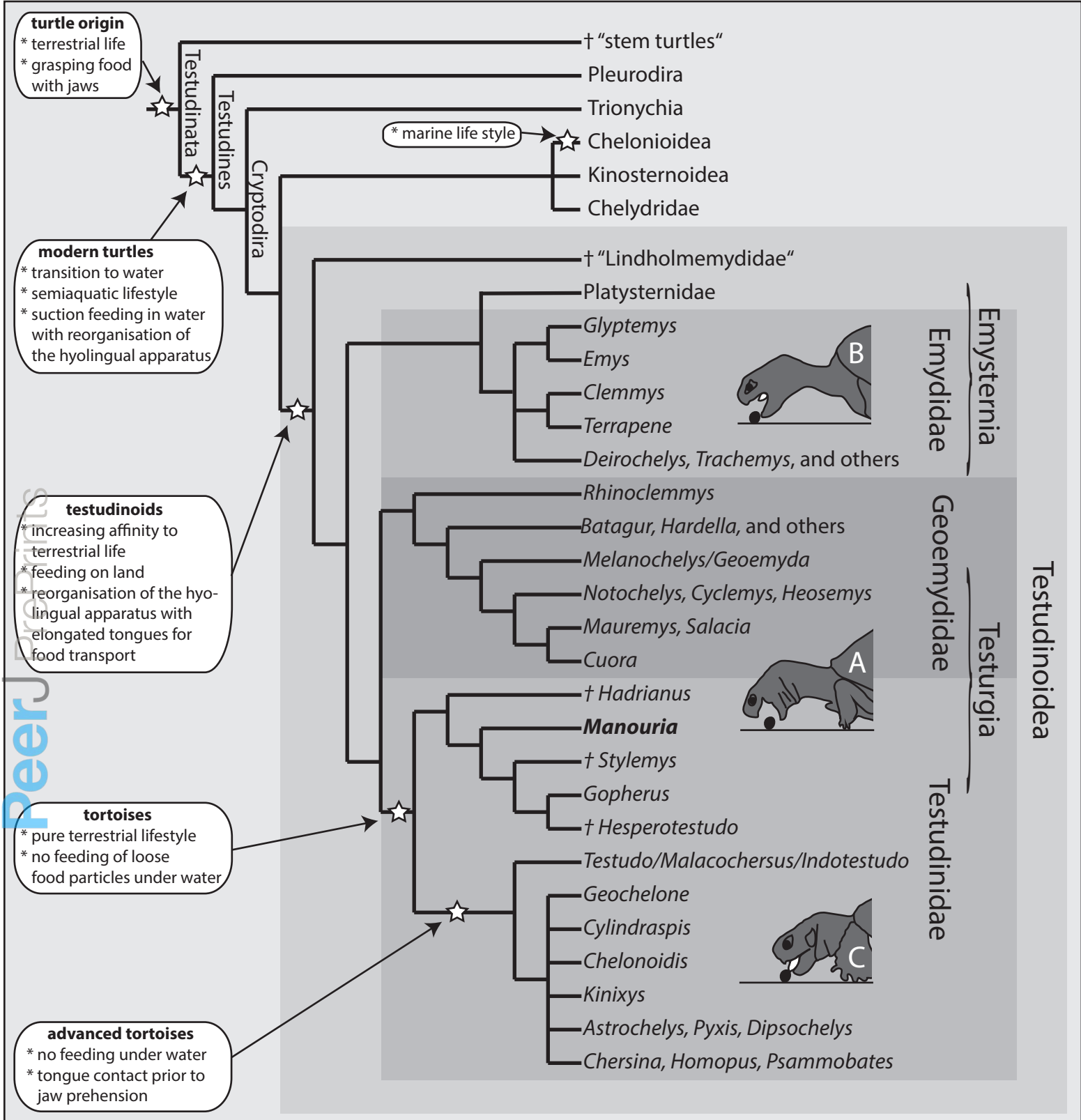
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595

596

Figure 1(on next page)

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).

turtle origin
* terrestrial life
* grasping food with jaws

modern turtles
* transition to water
* semiaquatic lifestyle
* suction feeding in water with reorganisation of the hyolingual apparatus

testudinoids
* increasing affinity to terrestrial life
* feeding on land
* reorganisation of the hyolingual apparatus with elongated tongues for food transport

tortoises
* pure terrestrial lifestyle
* no feeding of loose food particles under water

advanced tortoises
* no feeding under water
* tongue contact prior to jaw prehension

* marine life style

† "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/Malacochersus/Indotestudo
Geochelone
Cylindraspis
Chelonoidis
Kinixys
Astrochelys, Pyxis, Dipsochelys
Chersina, Homopus, Psammobates

Testudinata
Testudines
Cryptodira
Emysternia
Emydidae
Geoemydidae
Testurgia
Testudinidae
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.
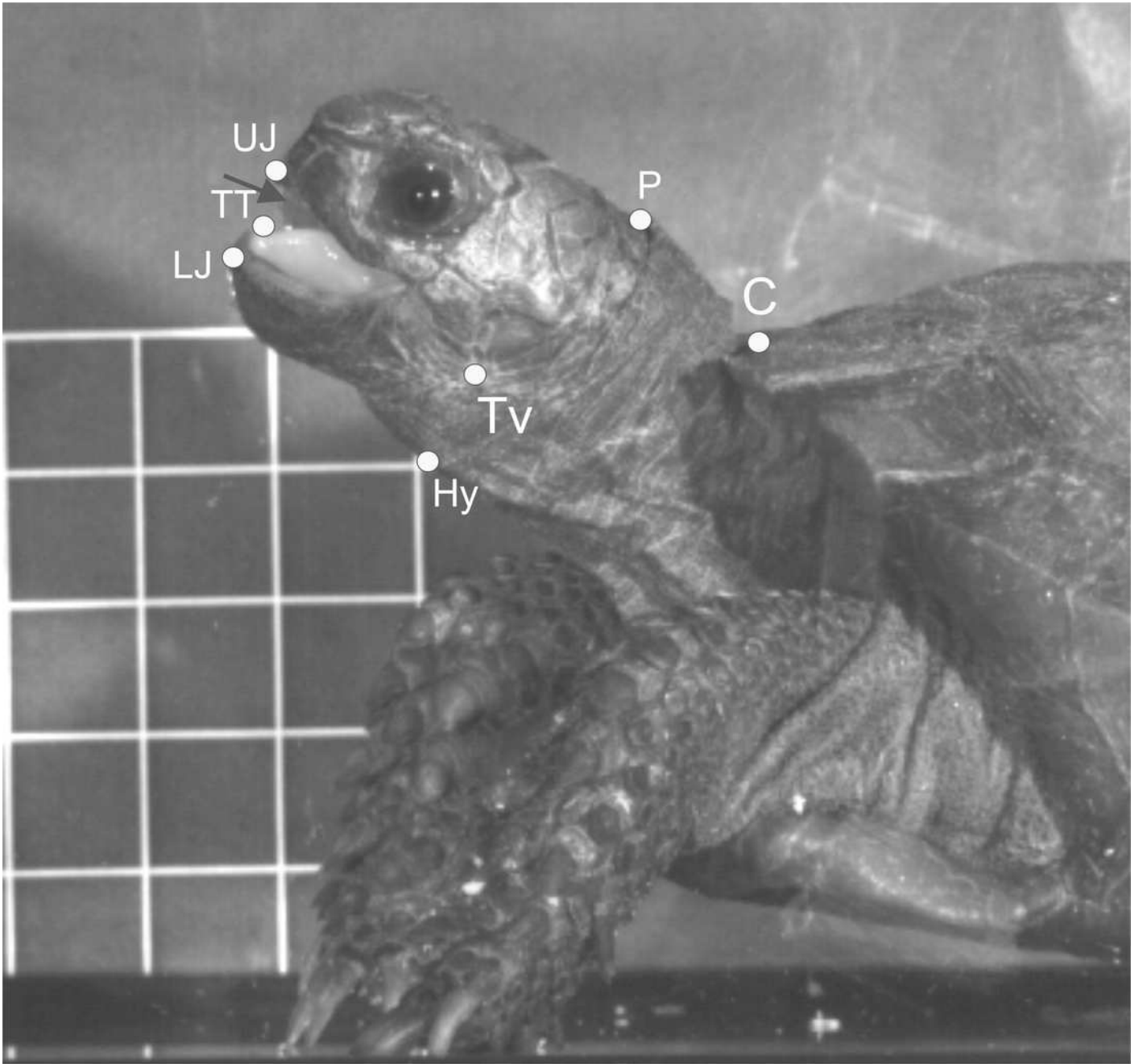
3

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.
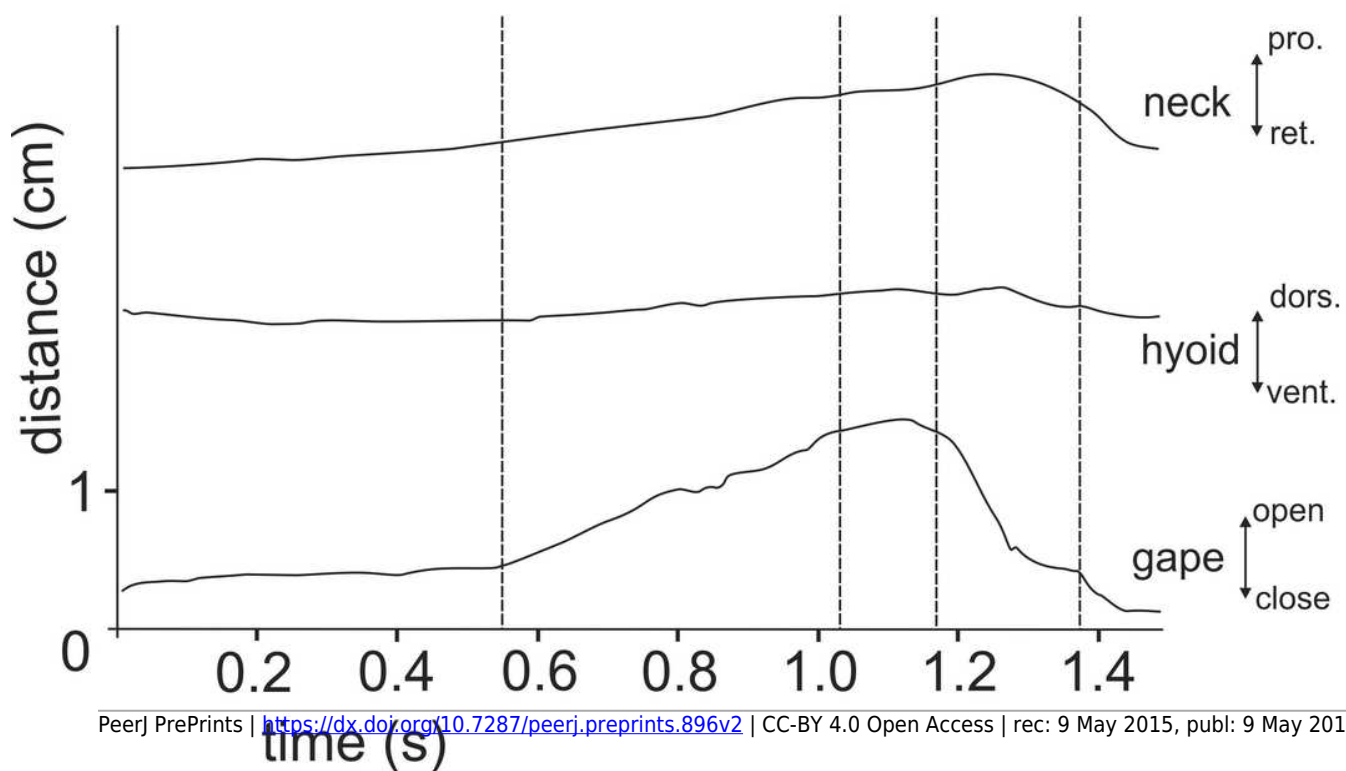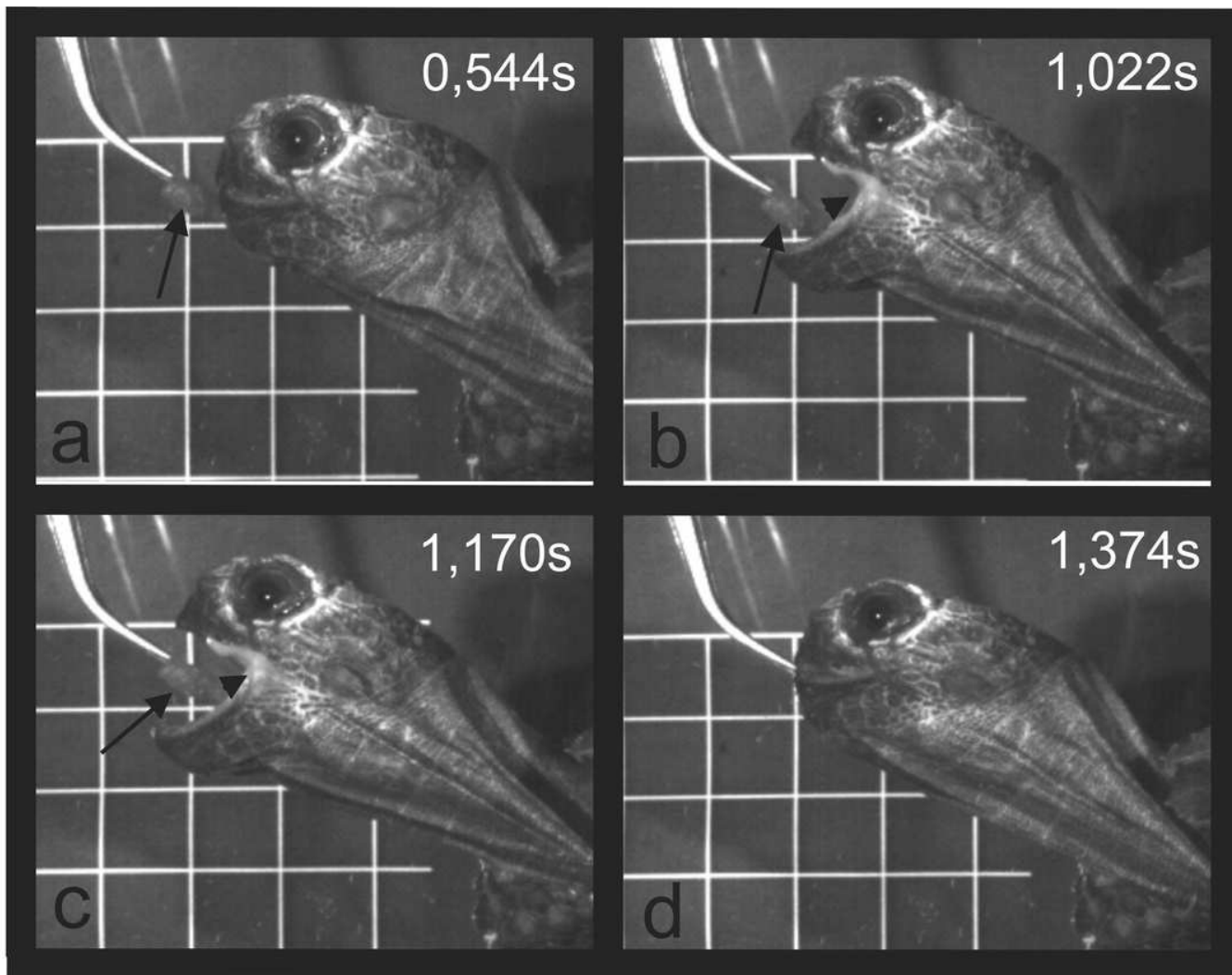
4

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.
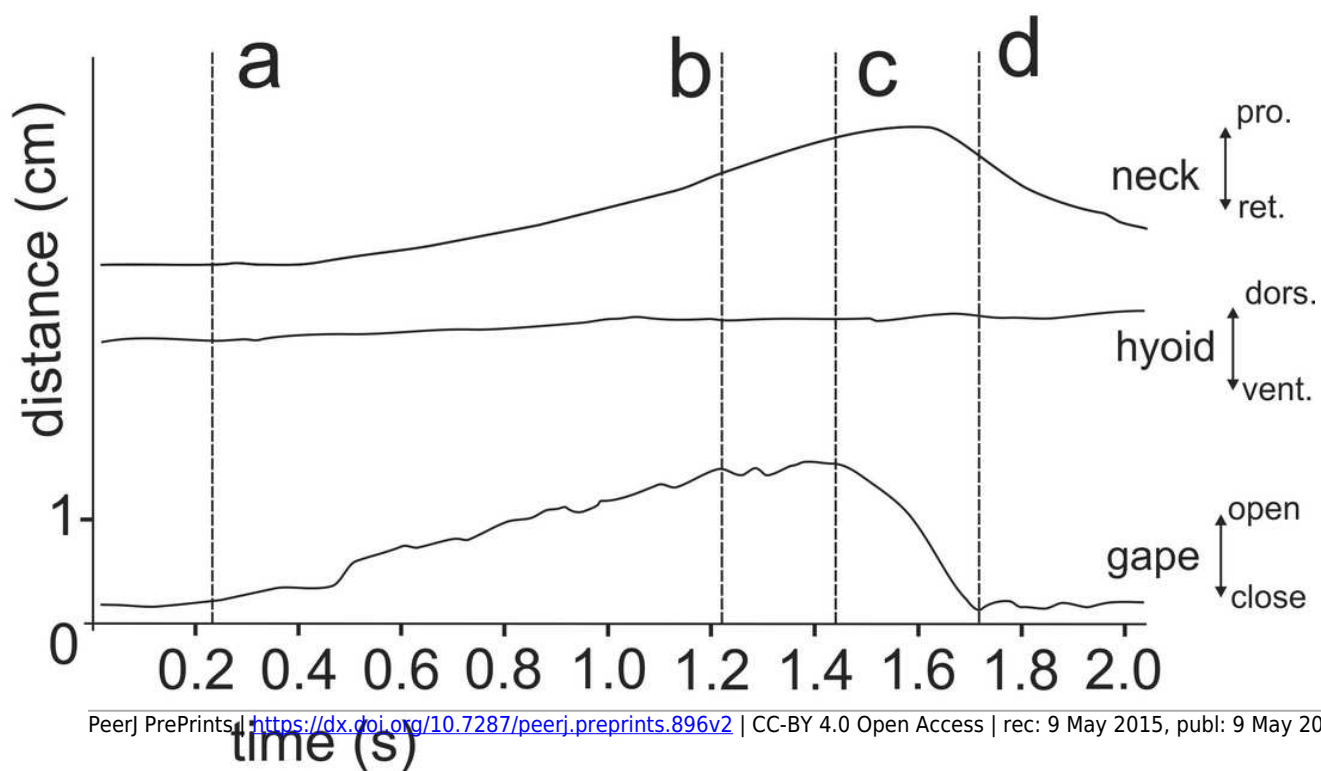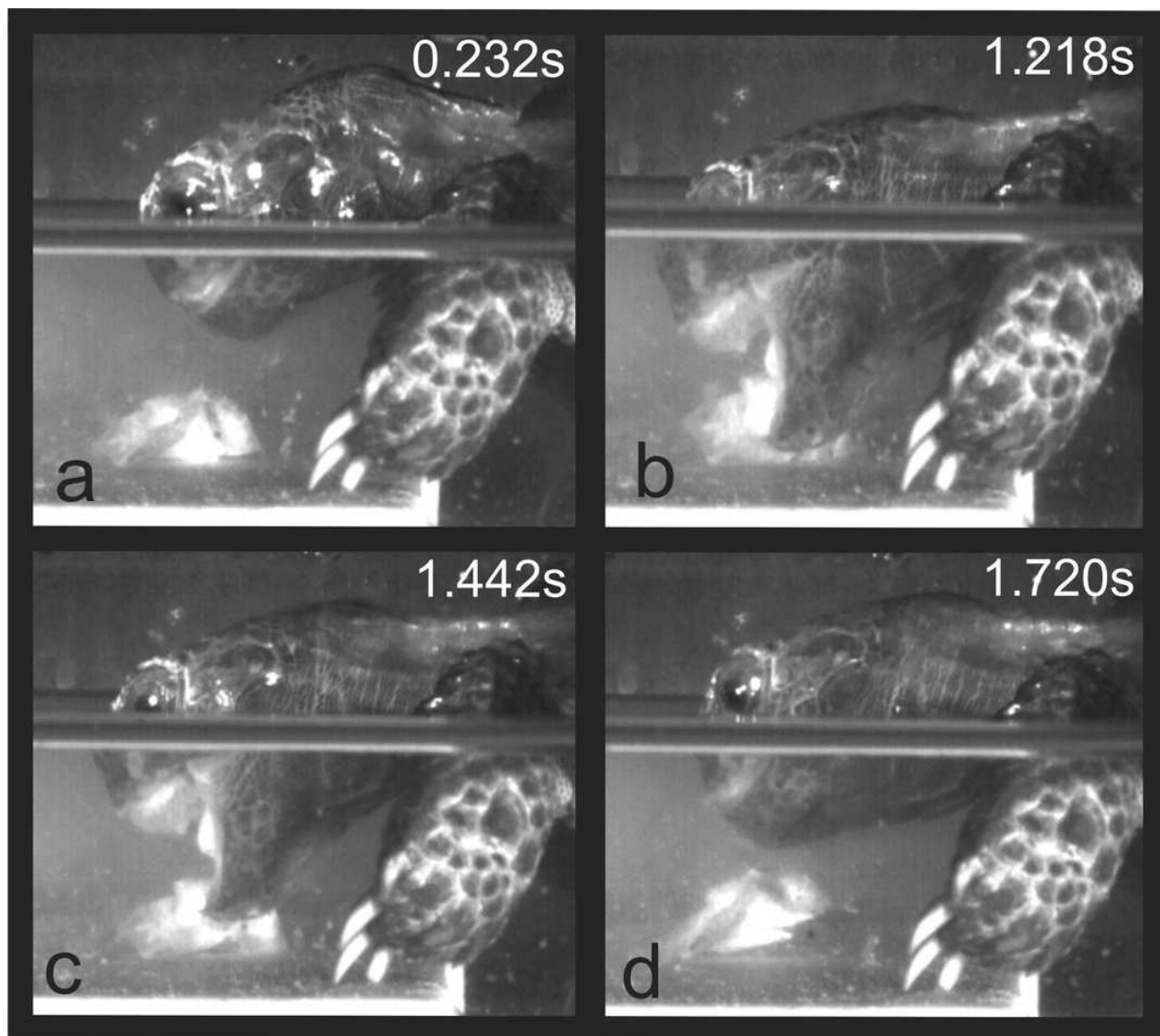
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Figure 5

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.
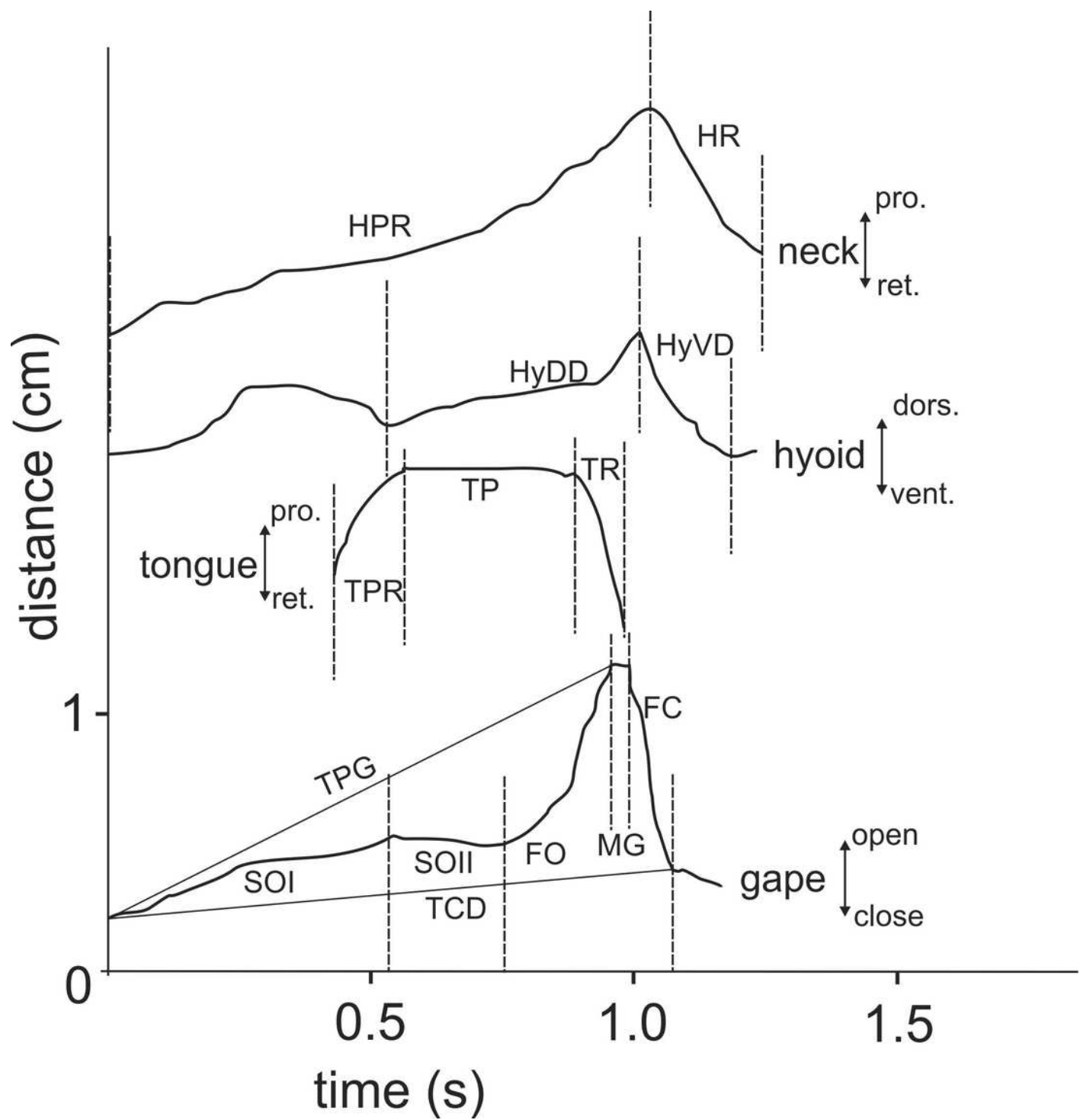
6

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).
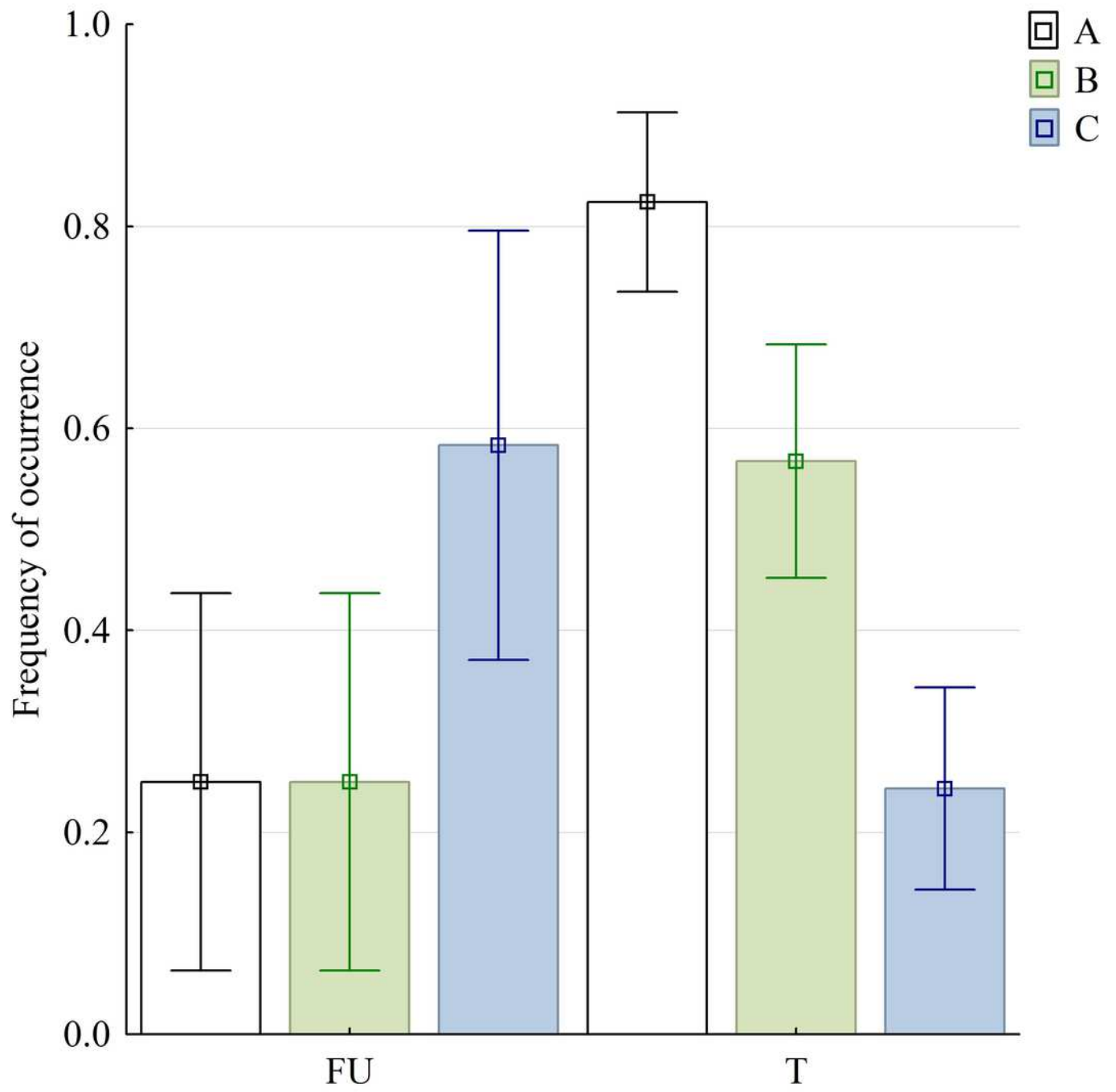
Figure 7

Canonical centroid plots of three *Manouria emys* specimens (T1–T3), centroid scores for each individual and measurement repetition in food transport phase.
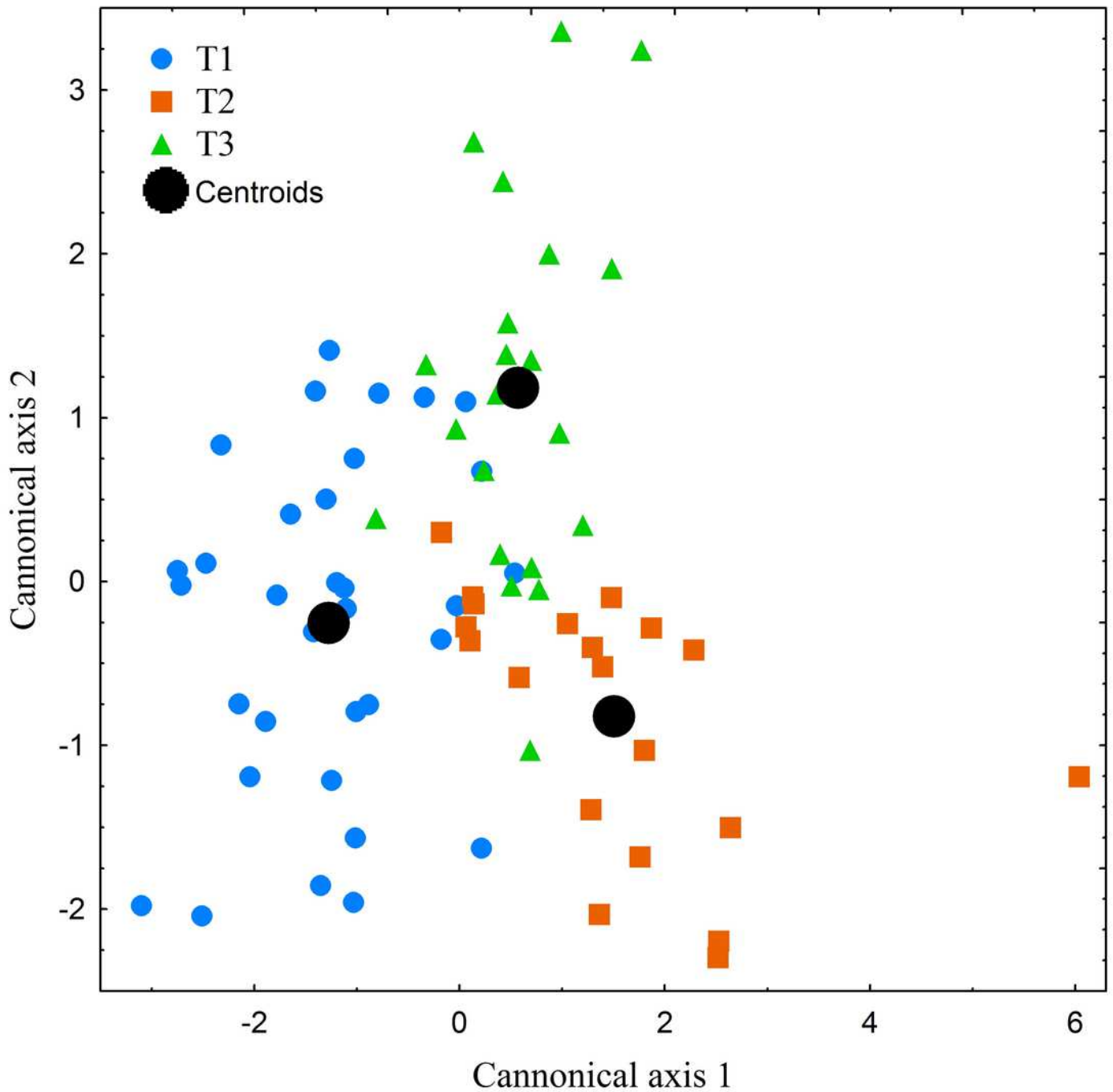
Table 1(on next page)

Table 1

2 Table and table legend

3 Table. 1: Variables describing the feeding process in *Manouria emys*, present as means ± SD; n,
4 sample size; *, significant differences (α=0.05) among individuals in the ingestion phase (P1), in the
5 transport phase (P2), and between both mode (P3); n.c., p value not calculated.
6

Variable	Food uptake (FU)				Transport (T)				I vs. T
	individual 1 (n=8)	individual 2 (n=8)	individual 3 (n=8)	p1	individual 1 (n=33)	individual 2 (n=20)	individual 3 (n=21)	p2	p3
SOI duration [s]	0.168±0.060 n=2	0.618±0.231 n=3	0,562 n=1	n.c.	0.146±0.016 n=18	0.126±0.014 n=14	0.115±0.015 n=11	0,378	0,068
SOII duration [s]	0.738±0.508 n=2	0.453±0.294 n=3	1,024 n=1	n.c.	0.147±0.014 n=18	0.187±0.027 n=13	0.190±0.021 n=11	0,187	0,072
FO duration [s]	0.450±0.060 n=2	0.379±0.150 n=3	0,694 n=1	n.c.	0.122±0.009 n=25	0.126±0.012 n=15	0.102±0.006 n=19	0,111	0.011*
MG duration [s]	0.079±0.017 n=4	0.095±0.031 n=4	0.166±0.044 n=6	0,271	0.025±0.003 n=6	0.033±0.004 n=10	0.042±0.019 n=4	0,318	0.001*
FC duration [s]	0.157±0.079 n=8	0.105±0.036 n=8	0.158±0.030 n=8	0.024*	0.089±0.020 n=33	0.186±0.040 n=20	0.119±0.088 n=21	0,155	0.010*
TPG [s]	0.943±0.144 n=8	0.989±0.177 n=8	1.784±0.137 n=8	0.002*	0.408±0.021 n=33	0.439±0.038 n=20	0.403±0.028 n=21	0,187	<0.001*
TCD duration [s]	1.139±0.148 n=8	1.128±0.169 n=8	2.073±0.144 n=8	0.001*	0.499±0.020 n=33	0.655±0.098 n=20	0.510±0.030 n=21	0,311	<0.001*
HDD duration [s]					0.281±0.025 n=30	0.216±0.035 n=14	0.169±0.019 n=21	0.005*	
HVD duration [s]					0.176±0.011 n=31	0.167±0.014 n=17	0.149±0.009 n=21	0,162	
THC duration [s]					0.456±0.028 n=30	0.384±0.041 n=14	0.317±0.022 n=21	0.002*	
HRV velocity [cm/s]					0.718±0.059 n=31	0.938±0.107 n=17	0.551±0.071 n=21	0.016*	
HP duration [s]	1.345±0.159 n=8	1.204±0.246 n=8	2.494±0.177 n=8	0.001*	0.220±0.049 n=32	0.864±0.132 n=14	0.464±0.089 n=10	0.001*	<0.001*
HR duration [s]	0.296±0.041 n=8	0.487±0.086 n=7	0.704±0.174 n=8	0,052	0.236±0.027 n=14	0.211±0.025 n=13	0.316±0.065 n=10	0,333	0.002*
TP duration [s]					0.165±0.008 n=30	0.160±0.017 n=19	0.133±0.025 n=13	0,483	
TR velocity [cm/s]					7.459±0.550 n=31	5.798±0.547 n=20	6.562±0.595 n=13	0,121	
delay of HVD start relative to TR start [s]					-0.2011±0.026 n=30	-0.039±0.231 n=15	-0.082±0.025 n=14	0.005*	
delay of TPG relative to HVD start [s]					-0.007±0.007 n=31	-0.016±0.008 n=17	-0.032±0.005 n=21	0.014*	
delay of TPG relative to TR start [s]					-0.062±0.007 n=31	-0.045±0.013 n=20	-0.055±0.008 n=14	0,521	

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