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 testudinids are highly associated with terrestrial habitats and the few tortoises with high affinity to aquatic environments are found within the genus *Manouria*. *Manouria* belongs to a clade which forms a sister taxon to all remaining tortoises and is suitable as a model for studying evolutionary transitions within modern turtles. We analysed the feeding behaviour of *Manouria emys* and due to its phylogenetic position, we hypothesise that the species might have retained some ancestral features associated with an aquatic lifestyle. We tested whether *M. emys* is able to feed both in aquatic and terrestrial environments. In fact, *M. emys* repetitively tried to reach submerged food items in water, but always failed to grasp them - 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 generally accepted as 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 profiles 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 the tortoises.

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

2 **the dawn of modern land turtle evolution**

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

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INTRODUCTION

19   Comprising more than 180 species, the cryptodiran taxon Testudinoidea represents the most

20   diverse group of extant turtles (e.g., Fritz and Havaš, 2007; Thomson and Shaffer, 2010).

21   Traditionally, it contains three major extant groups, including the emydids, the geoemydids and

22   the testudinids (tortoises) (Fig. 1). All molecular phylogenetic studies (Iverson et al., 2007;

23   Shaffer, 2009; Thomson and Shaffer, 2010; Barley et al., 2010) confirm a sister group

24   relationship of the testudinids and the geoemydids (Fig. 1). The relationship of Platysternidae to

25   other turtle groups remains unresolved (Parham et al. 2006), but most molecular studies support

26   a closer relationship to emydids (e.g., Thomson and Shaffer, 2010; Crawford et al., 2015).

27   Palaeontological studies have shown that all testudinoids share aquatic ancestors, from which

28   terrestrial species evolved (Danilov, 1999; Sukhanov, 2000; Joyce and Gauthier, 2004).

29   The majority of the Triassic stem turtles were terrestrial as indicated by the design and

30   proportions of the limbs, which were adapted for terrestrial locomotion (for a comprehensive

31   discussion see Joyce, 2015). With the emergence of modern turtles (Testudines) during the

32   Jurassic period (e.g., Danilov and Parham, 2006; Sterli, 2010; Sterli and de la Fuente, 2011), a

33   general transition of turtles into an aquatic environment occurred (Willis et al., 2013). The

34   invasion of aquatic environments induced diversification into several subgroups (see Joyce,

35   2007; Thomson and Shaffer, 2010). Due to the different physical properties (drag, viscosity, etc.)

36   of air and water, the new environment required morphological and functional adaptations of the

37   locomotion- and feeding system to enable efficient swimming behaviour and aquatic food uptake

38   (i.e. suction feeding) (Schumacher, 1973; Lemell et al., 2002).

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

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

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

42 documented aquatic taxa such as †Haichemydidae and the †Sinochelyidae may perhaps also

43 belong to †Lindholmemydidae. This group was recorded from aquatic sediments of the late Early

44 Cretaceous and apparently had an amphibious lifestyle (Sukhanov, 2000). Among recent

45 cryptodirans, semi-aquatic to semi-terrestrial lifestyle is typical for most emydids (plus

46 platysternids) and geoemydids, as well as for some kinosternids (see Depecker et al., 2006, but

47 also Nakajima et al., 2014). The remaining extant species are entirely terrestrial (tortoises), or

48 predominantly aquatic (see Joyce and Gauthier, 2004; Rasmussen et al., 2011).

49   Among modern turtles, a transition from an aquatic to a semi-terrestrial or fully terrestrial

50 habitat and the capacity to exploit terrestrial food sources has evolved independently within all

51 three major testudinoid lineages (for overview see Summers et al., 1998; Natchev et al., 2009).

52 At least eight emydid species are able to feed on land as well as under water (see Bels et al.,

53 1997, 2008; Summers et al., 1998; Stayton, 2011). During terrestrial feeding, such amphibious

54 emydids use their jaws to grasp food items (jaw prehension). Similarly, all amphibious

55 geoemydids studied to date also use jaw prehension in terrestrial food uptake (see Heiss et al.,

56 2008; Natchev et al., 2009). In contrast, all testudinids studied so far use the tongue to touch the

57 food items, a behaviour referred to as "lingual prehension" (see Wochesländer et al., 1999; Bels

58 et al., 2008). According to Bels et al. (2008), lingual prehension is obligatory for all tortoises.

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

60 living and feeding medium (see Pritchard, 1979; Ernst and Barbour, 1989; Bonin et al., 2006). In

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

62 on terrestrial trophic ecologies. Some predominantly terrestrial geoemydids are able to complete

63 the whole feeding process on land and under water (Natchev et al., 2010). Similarly, testudinids

64 with tendencies towards an amphibious lifestyle might have retained the ancestral skill to feed

65 underwater. Hence, information on bimodal feeding mechanisms in tortoises is of great

66 importance to understand the evolution of terrestrial feeding mechanisms and subsequent

67 evolution of the predominantly terrestrial lifestyle in tortoises.

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

69 aquatic environments (Høybye-Mortensen, 2004; Stanford et al., 2015), constitutes a suitable

70 model to study the feeding mechanisms in testudinids. Its partially aquatic feeding habit

71 purported to be associated with the observed morphological extension of the palatines onto the

72 triturating surface of the upper jaw (character 30 sensu Gerlach, 2001), a diagnostic feature

73 common to geoemydid turtles. Another geoemydid-like feature is the unique existence of class II

74 mental glands (Winokur and Legler, 1975).

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

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

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

78 under both aquatic and terrestrial conditions as some geoemydids do (see Natchev et al., 2009,

79 2010).

80   Similar to all investigated testudinids, the Asian forest tortoise possesses a well developed

81 tongue. The hyoid complex is predominantly cartilaginous (Heiss et al., 2011). On the base of

82 the specific morphology of the feeding apparatus (elastic basis of the oropharynx and

83 voluminous lingual structures) we suggest a poor suction feeding performance in case *Manouria*

84 attempts to feed under water.

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

86 all testudinids involve two common features: an obligatory lingual prehension and the split of the

87   gape cycle in four main phases: slow open phase I (SOI); slow open phase II (SOII); fast open

88   phase (FO); fast close phase (FC). In our experiments we test whether these kinematical

89   elements are present in the feeding behaviour of *M. emys*. On the base of our findings, we fine-

90   tune the kinematical feeding models proposed for tortoises. The gained new data require a re-

91   evaluation of the concept on the function of the tongue in the food uptake in tortoises. Having in

92   mind the phylogenetical position of *M. emys* and the specifics of its feeding behaviour, we

93   propose hypothesis on the evolution of the terrestrial feeding among testudinoids in particular

94   and turtles in general. We discuss also on the interrelationship between the diet and the feeding

95   media in the course of turtle's evolution.

96

97   **MATERIALS AND METHODS**

98

99   Ecological background

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

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

102   distribution in Bangladesh, India (Assam, Meghalaya, Mizoram, Nagaland), Myanmar, Thailand,

103   Malaysia (East and West), and Indonesia (Kalimantan, Sumatra). The nominate subspecies, *M.*

104   *emys emys* - the subject of this study, inhabits the southern part of the species range (Fritz and

105   Havaš, 2007; TTWG, 2014, Stanford et al., 2015).

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

107   moisture. It is commonly found reposing in wet areas, buried in mud or under the leaf litter

108   where it may spend long periods of time. It is active even during rainy weather. Direct sun

109   exposure and basking are not required. Furthermore, this species has a mostly crepuscular and

110   nocturnal lifestyle (Ernst, Altenburg, and Barbour, 2000; Vetter and Daubner, 2000, Stanford et

111   al., 2015).

112   According to the available literature, the diet of *M. emys* includes plants, fungi,

113   invertebrates, and frogs (Nutphand, 1979; Das, 1995; Lambert and Howes, 1994, Høybye-

114   Mortensen, 2004). It has been reported to feed on plants in shallow mountain streams (Nutphand,

115   1979).

116

117   Experimental setting

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

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

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

121   water basin and spacious terrestrial area. The turtles were fed different fruits, vegetables,

122   commercially obtained tortoise pellets, dead mice, as well as pieces of cattle heart and liver,

123   offered on the terrestrial part of the terrarium. Carapace lengths in the three subadult

124   experimental animals ranged between 109-135 mm with body masses between 234-236 g. For

125   filming terrestrial feeding, the specimens were put in a dry glass cuvette (24x60x30 cm). When

126   the food was offered on the floor of the cuvette, the tortoises often twisted their necks and rotated

127   their heads in attempt to grasp the food item. The side movements made the filming of the

128   animals in strict lateral view very difficult and the landmarks were not clearly visible during the

129   sequence. By the use of forceps for food display we completely eliminated these problems and

130   were able to shoot perfect lateral plans of the feeding turtles. The food in the feeding

131   experiments was offered at a position which was similar to the position on which we offered the

132   food in the terrarium where the tortoises were housed. The animals did not extend vastly their

133   necks to reach the food items (see Supplements). The position of the offered food was

134   completely "natural". The tortoises needed to stretch their necks forwards rather than

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

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

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

138   the digital high-speed camera system Photron Fastcam-X 1024 PCI (Photron limited; Tokyo,

139   Japan) at 500 fps with a highly light-sensitive objective AF Zoom - Nikkor 24-85 mm (f/2,8-4D

140   IF). Two "Dedocool Coolh" tungsten light heads with 2 x 250 W (ELC), supplied by a

141   "Dedocool COOLT3" transformer control unit (Dedo Weigert Film GmbH; München, Germany)

142   were used for illumination. We filmed and analysed the food uptake and the food transport

143   cycles in eight feeding sequences for each specimen.

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

145   aquarium filled with water to a level of 3 cm and presentation of food items in front of the

146   turtle's snout. In order to reduce the light intensity and for optimisation of the digitising process,

147   the frame rate was reduced to 250 fps. As the tortoises were unable to grasp the food item in a

148   total of 36 trials, the kinematics of the feeding apparatus had been analysed (see below) in nine

149   selected representative feeding trials.

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

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

152   MatchiX" (SIMI Reality Motion Systems; Unterschleißheim, Germany). Based on the

153   displacement of the markers, we were able to calculate the gape amplitude (distance between the

154   tip of the upper and lower beak), head movement (distance between the anterior tip of the

155   carapace and the point "P" on Fig. 2), tongue movements (distance between the most ventral

156   point on tympanum and the tip of the tongue when visible), and hyoid movements (distance

157   between the point "P" on Fig. 2 and the basis of the posterior ceratobranchial). To compare the

158   kinematic feeding pattern of *M. emys* to those of other studied turtles and to understand the

159   coordination between the elements of the feeding apparatus, these data were used for calculation

160   of the following kinematical variables: duration of Slow open phase (SO); duration of Slow open

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

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

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

164   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

166   velocity; delay of the start of hyoid retraction relative to the tongue retraction start; delay of

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

168   to tongue retraction start (see Table 1).

169

170   Statistics

171   We tested for any differences among the frequency of occurrences of defined patterns both in

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

173   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

175   performed. Then we tested for possible existence of differentiation in kinematical variables in

176   both feeding stages (FU and T). All variables were tested with the Shapiro-Wilk test for normal

177   distribution. When the p-value was less than the chosen alpha level ($p<0.05$), the null hypothesis

178   was rejected and data were excluded from further analyses. In addition, all variables included in

179 Table 1 were tested with Levene's and Brown-Forsythe tests and then processed with Welch's

180 ANOVA for heteroscedastic data. Tukey's honest significant difference test (HSD) was

181 performed for post-hoc analyses when applicable.

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

183 specimens, a Canonical discriminant analysis (CDA) was performed. Standard descriptive

184 statistics including mean, range, standard deviation [(SD) and confidential interval at 95% CI]

185 were presented.

186

187 **RESULTS**

188

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

190 food uptake, one to four transport cycles followed prior to oesophageal packing (see Schwenk,

191 2000). The tip of the tongue was barely visible during food uptake (see Fig. 3b-c) indicating that

192 the tongue was not protracted. By contrast, during transport cycles, the cyclic movements of the

193 tongue were well visible as it was rhythmically pro- and retracted to transport the food item

194 towards the oesophagus (Fig. 5).

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

196 and protruded the gaping jaws toward the food item. The gape cycle was newer split in slow and

197 fast jaw open phases. The tongue tip was not visible from the lateral aspect and the hyolingual

198 complex did not protract prior reaching peak gape. No retraction of the hyoid complex was

199 detected prior jaw closure. The gape cycle duration exceeded one and a half seconds and was

200 1.94 ± 0.36 s (mean $\pm$ SD). Despite the unsuccessful attempts, the turtles repeatedly tried to catch

201   the submerged food. In several events, we were able to detect that the food item was carried

202   away by the bow wave induced by jaw closing.

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

204   found highly significant differences in sequences with and without both SO I and SO II when

205   food uptake and transport stages were compared ($\chi^2_{(1,\ N=98)}$=25.05, p <0.001). Similarly

206   significant differences were observed when comparing food uptake and transport cycles in

207   respect to sequences with and without slow jaw open phases as well as with and without

208   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).

209   Nine of the variables which describe the food uptake process were detected to show

210   significant differences between individuals (Table 1). In transport cycles, six out of 18 variables

211   differed significantly amongst individuals (see Table 1)., Seven out of nine variables differed

212   significantly when testing for differences between grasping and transport cycles: fast jaw open

213   duration (FO; $F_{Welch\ (1,43)}$=15.17, p=0.011); maximum gape (MG; $F_{Welch\ (1,26)}$=15.89, p=0.001);

214   fast closing (FC; $F_{Welch\ (1,26)}$=7.86, p=0.010); time to peak gape (TPG; $F_{Welch\ (1,72)}$=46.78,

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

216   (HP; $F_{Welch\ (1,67)}$=52.23, p<0.001); and head retraction duration (HR; $F_{Welch\ (1,47)}$=12.57, p=0.002).

217   When comparing three further parameters among the transport cycles in all three specimens

218   (delay of HyVD start relative to TR start; delays of TPG relative to HyVD; delay of TPG relative

219   to TR starts), statistically significant differences were found among all compared pairs

220   ($F_{Welch(2,105)}$=41.58, p<0.001).

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

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

223   eigenvalue exceeded the level of acceptance, i.e. 1. First axis explained 70% of the total

224 variance. Among the 18 studied variables only two (THC and HPR) showed higher correlation

225 scores than 0.75. On the base of the CDA and the detected degree of individualism mentioned

226 above, we can conclude that the patterns displayed by the studied specimens can be regarded as

227 similar but not as uniform.

228

229 **DISCUSSION**

230

231 The Asian forest tortoise repetitively tried to feed on dispersed food items under water,

232 which was an unexpected and hitherto unknown behaviour among tortoises. However, *M. emys*

233 always failed to consume the submerged food. On land, *M. emys* grasped food with the jaws, just

234 like all known aquatic or semiterrestrial turtles do, but not with the tongue as formerly predicted

235 for all tortoises. On the basis of our results we discuss several important evolutionary,

236 behavioural, and functional aspects.

237

238 **Evolution of food uptake among turtles**

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

240 prey item and a suction feeding mechanism is induced by fast oropharyngeal volume expansion.

241 In some extant turtles, a strong suction flow can be generated and prey is directly sucked into the

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

243 *spinifera* (Anderson, 2009), *Pelodiscus sinensis* (N.N. and I.W. unpublished data)]. However,

244 most extant turtles cannot generate such strong suction flows and only compensate ("gulp") the

245 bow wave that otherwise would push small to moderately sized food items away from the fast

246 approaching head. Such species finally fix and grasp prey with the jaws (see Lauder and

247 Prendergrast, 1992; Lemell et al., 2000; Aerts et al., 2001, Natchev et al., 2009, 2011). We

248 consider the latter as plesiomorphic behaviour for extant turtles.

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

250 uptake, food manipulation and transport, esophageal packing, and swallowing) on land has been

251 tested and documented for only six species so far. All of them were members of Testudinoidea

252 (Fig. 1; see also Summers et al., 1998; Bels et al., 2008; Natchev et al., 2009). The terrestrial

253 mode of food uptake differs dramatically among and within the three testudinoid subgroups (see

254 Bels et al., 1997, 2008; Summers et al., 1998; Wochesländer et al., 1999; Natchev et al., 2009,

255 present study). Correspondingly, it appears as if terrestrial feeding re-evolved several times

256 independently amongst turtles. Unfortunately, only limited experimental data are available on

257 feeding mechanisms in emydids and geoemydids. Further functional and palaeontological

258 investigations may help to sort out the issues on the evolution of the feeding behaviour and the

259 morphology of the feeding apparatus in testudinoids.

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

261 testudinoid turtles that occasionally exploit terrestrial food sources. Weisgram (1985 a,b)

262 documented a kinosternid (*Claudius angustatus*) that caught prey on land and dragged it into

263 water for transport and swallowing. Natchev et al. (2008) documented another kinosternid

264 (*Sternotherus odoratus*) catching food on land, but failing to transport it through the oropharynx.

265 Among extant turtles, successful food transport on land seems to be restricted to testudinoids.

266 The development of enlarged and muscular tongues within this group (von Bayern, 1884;

267 Werneburg, 2011) represents adaptation to improved terrestrial food manipulation.

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

269 categories of terrestrial food uptake modes among Testudinoidae: (A) Jaw prehension with

270   retracted hyolingual complex, as observed in the geoemydid genus *Cuora* (Natchev et al., 2009);

271   (B) Jaw prehension with slightly protracted hyolingual complex, as observed in emydids (Bels et

272   al., 1997; Stayton, 2011); (C) Lingual prehension - the tongue touches the food item prior to food

273   uptake, as documented in all tortoises studied so far (Wochesländer et al. 1999, Bels et al. 2008).

274   The food uptake mode of *M. emys*, however, differs substantially from that of all remaining

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

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

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

278   that of semi-aquatic geoemydids.

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

280   mechanisms in turtles. Given the aquatic origin of all living turtles, the functional transition from

281   aquatic to terrestrial feeding mechanisms could hypothetically have involved four stages,

282   beginning with an exclusively aquatic feeding ancestor. In different lineages and stages, turtles

283   may have left their aquatic environments for various reasons e.g. for exploiting new food niches.

284   The species that retained predominantly aquatic life styles may grasp food by the jaws on land,

285   but have to drag it into the water for further intraoral (hydrodynamic based) transport. In recent

286   turtles, such behaviour was documented in the kinosternids *C. angustatus* (Weisgram, 1985 a, b)

287   and *Sternotherus odoratus* (Natchev et al., 2011), as well as in the emydid *Trachemys scripta*

288   (Weisgram, 1985 b; Weisgram et al., 1989) and other emydids (see Stayton, 2011). Turtles of the

289   second hypothetical evolutionary stage grasped food by the jaws, while the tongue was used for

290   intraoral food transport on land. Such species would still have retained their underwater feeding

291   ability by using hydrodynamic mechanisms. When grasping food on land, the tongue remained

292   retracted or was protracted without however touching the food item. Among extant turtles, such a

293   feeding mode is found in the geoemydid genus *Cuora* (Heiss et al., 2008; Natchev et al., 2009,

294   Natchev et al., 2010) and in some emydids (Bels et al., 1997; Summers et al., 1998, Stayton,

295   2011). In the next theoretical evolutionary step (stage three), behavioural and morphological

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

297   food transport at the expense of the ability to use effective hydrodynamic mechanisms in water.

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

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

300   features prominently in the present case of *M. emys*. Finally, in a fourth stage, turtles became

301   fully terrestrial and their tongue was obligatorily involved in food uptake as documented in the

302   tortoises *Testudo* (*Eurotestudo*) *hermanni boettgeri* (Weisgram, 1985 b; Wochesländer et al.,

303   1999), *Kinixis belliana*, *Geochelone elephantopus* and *G. radiata* (Bels et al., 2008).

304   Our investigations demonstrate that the 'basal' tortoise *M. emys* does not contact food with

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

306   characteristic of advanced tortoises only. We consider the terrestrial feeding behaviour of *M.*

307   *emys* as plesiomorphic and potentially inherited from its semiaquatic ancestors. On that base, *M.*

308   *emys* can be considered a transitional turtle in regard to secondary terrestriality.

309   We propose that hyolingual protrusion evolved in the lineage forming to advanced tortoises

310   (Fig. 1). *Manouria emys* has a large tongue with massive intrinsic and extrinsic musculature (see

311   Heiss et al., 2011). The advanced and complex lingual musculo-skeletal architecture allows the

312   turtle to protrude the tongue outside the margins of the rhamphothecae (see Fig. 2). However, *M.*

313   *emys* does not use lingual food prehension as typical for all other tortoises studied so far. In fact,

314   it seems that the *Manouria* [and perhaps *Gopherus* (N.N. personal observations)] "lineage" has

315   retained the jaw prehension mechanism inherited from earlier aquatic ancestors. It seems that the

316   tortoises, in general, evolved fleshy tongues which improve the food transport performance. The

317   advanced tortoises only refined the behaviour of food uptake on land via lingual food contact

318   prior to jaw closure (see Wochesländer et al., 1999; Bels et al., 2008).

319

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

321   What would be the potential advantage of the obligatory lingual protrusion, found in the

322   more derived tortoises? One possible explanation is that the tongue is used as a prehensile organ

323   for food ingestion analogous to that found in other tetrapod groups (for overview see Schwenk,

324   2000; Schwenk and Wagner, 2001). However, for tortoises such interpretation might be put into

325   question. By examining all published data available (Wochesländer et al., 1999; Bels et al.,

326   2008), we were not able to find any convincing evidence that tortoises collect food with their

327   tongues - they just touch it. In all published feeding sequences, the contact between the food and

328   the tongue is clearly demonstrated - yet, in all cases, after the initial contact of the tongue with

329   the food, the head moves forward and the food item is not dragged into the mouth by tongue

330   retraction, but is grasped by the jaws during the fast jaw closing (FC gape phase). Initial food

331   ingestion in tortoises might not be considered "lingual prehension" (see Schwenk, 2000; Bels et

332   al., 2008) in the strict sense, but should be regarded as "jaw prehension following lingual

333   contact". This prompts the question: why is an obligatory contact of the tongue to the food

334   present in extant tortoises (except in *Manouria* and also conceivably in *Gopherus*) during food

335   uptake? In other words: why do tortoises apply a more complex and presumably more

336   energetically expensive food uptake mechanism by including movements of the hyolingual

337   complex in addition to the movements of the neck and jaws alone?

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

339   item and helps the advanced tortoises to compensate the "information gap" which occurs when

340   the food is approached to a distance where it is out of sight. The eyes of tortoises are positioned

341   laterally on the head (Pritchard, 1979) and the turtles are not able to permanently observe the

342   position of the food item when the neck is protracted and the gape is positioned around it. The

343   prolonged maximum gape (MG) phase found in most ingestion cycles of *M. emys* (see Table 1,

344   Figure 3 and Figure 6) might be the result of lack of lingual contact with the food surface. In all

345   published sequences and kinematical profiles on food uptake in tortoises, there is a clear

346   tendency toward a split of the gape cycle into slow open (SO) and fast open (FO) gape phases

347   (see Wochesländer, 1999; Bels et al., 2008). The lack of tongue protrusion might explain the lack

348   of slow open (SO) and fast open (FO) split in the gape cycle of food uptake in the geoemydid

349   *Cuora* (see Natchev et al., 2009). In most food uptakes analysed in *M. emys,* SO phases are not

350   present and the gape increases gradually (see Table 1 and Figure 6). Similar as in *Cuora*, the lack

351   of SO phases might be explained by the lack of tongue protrusion in food uptake (see Natchev et

352   al., 2009).

353

354   **Intraoral food transport on land**

355   The execution of the transport cycles require coordination of the activities of contractile

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

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

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

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

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

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

362 longer duration of food uptake cycles relative to transport cycles might be that during transport,

363 the coordination centres of the muscle activities execution are permanently supplied with

364 information concerning the position of the food item within the oropharynx and the proper

365 movements can be executed precisely in a shorter time.

366   In his work on the feeding mechanisms in domestic animals, Bels (2006) established that a

367 pre-programmed Generalise Cyclic Model (GCM) – very similar to those proposed from

368 Bramble and Wake (1985) – is universally valid among the different groups of tetrapods,. The

369 feeding kinematics of the Asian forest tortoise differs in some aspects from those proposed by

370 the GCM. The kinematics of the feeding system in *M. emys* seems to be pre-programmed, but

371 under permanent feed-back control. The values of the gape and hyoid/hyolingual cycle patterns

372 in the three specimens studied here show high degrees of variation, both concerning food uptake

373 and food transport (see Table 1 and Figures 5-7). The slow open phases (SO) are not obligatory.

374 The gape cycle often includes a phase of retaining maximum gape (see Table 1, Figure 3, 5). In

375 turtles, the maximum gape phase (MG) was described for the gape cycle in *Cuora sp.* (Natchev

376 et al., 2009, 2010) and was confirmed for kinostrnids (Natchev et al., 2011). The presence of a

377 prolonged maximum gape phase (MG) can be easily overlooked when the frame rate of the film

378 sequence is not high enough (i.e., step between successive frames over 10 milliseconds). Thus, it

379 may be present in other turtles but was not taken into account by the calculations of the

380 kinematical profile (see Stayton, 2011; Nischizawa et al., 2014).

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

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

383 the hyoid retraction in the food transport cycle starts shortly prior reaching peak gape. The same

384   pattern was detected by the investigation of aquatic, semi-aquatic, but also predominantly

385   terrestrial cryptodirans (Natchev et al., 2008, 2009, 2010, 2011).

386

387   **Relations between the habitat preferences and the diet in turtles**

388   In the evolution of the testudinids there is a clear shift not only in the habitat preferences

389   (from aquatic to terrestrial), but corresponding shifts are also seen in dietary preferences. In that

390   taxon it manifests in a tendency toward herbivory. Most of the recent tortoises rely on diets

391   mainly composed of plant material (for overview see Pritchard, 1979; Ernst et al., 2000; Bonin et

392   al., 2006). The partly carnivorous lifestyle of *Manouria sp.* (Bonin et al., 2006) may be a relic of

393   the carnivorous diet of the ancestor of the tortoises and supports the transitional status of the

394   genus.

395   Apparently the feeding media (air vs. water) and the dietary shift had a large influence on

396   the overall feeding behaviour of the testudinids (see Bels et al., 2008). The suction mechanism

397   was lost and replaced by a jaw food prehension system (this study) or by "lingual prehension"

398   (Wochesländer et al., 1999; Bels et al., 2008). Probably, the switch to herbivory determined the

399   reorganisations in the morphology of the jaw muscle system and the proportions of the skull in

400   tortoises (see Werneburg 2011, 2012, 2013). By feeding predominantly on plants (immobile

401   items), the tortoises may prolong the duration of the food uptake cycles and have more time to

402   adjust their prehension kinematics to every single feeding situation.

403   We propose that the habitat preferences and the diet change in turtles are firmly correlated

404   (e.g. Bels et al., 2008; Werneburg, 2014). The overall rigid design of the turtle 'body plan'

405   hinder the animals to actively hunt for agile prey in terrestrial environments (King, 1996). We

406   hypothesise that this statement is also valid for the terrestrial stem turtles and that these animals

407   were predominantly herbivorous (sensu King, 1996). In aquatic turtles, in contrast, the buoyancy

408   of the water overrides the shell-caused restrictions of mobility and also suction feeding can be

409   applied for carnivorous feeding. Those advantages might have been the key factor for the turtles

410   to become aquatic (and carnivorous) in the Jurassic. There may be several reasons for the

411   secondary terrestriality in tortoises and presumably one of the main was the inter- and

412   intraspecific concurrence for food resources.

413

414   **CONCLUSIONS**

415

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

417   land. During the shift from aquatic to terrestrial lifestyle, including a shift from aquatic to

418   terrestrial feeding biology, the *Manouria* (and most likely also *Gopherus*) "lineage" had retained

419   pure jaw prehension in food uptake. The hyolingual complex in that lineage exhibits the typical

420   morphological features of tortoises that feed exclusively on land (see Bramble, 1973; Winokur,

421   1988; Heiss et al., 2011), such as an enlarged fleshy tongue with abundant papillae, a complex

422   tongue musculature, a relatively small and mainly cartilaginous hyoid and hypoglossum. The

423   evolutionary shift in the morphology of the hyolingual complex was apparently primed by the

424   optimisation of the food transport behaviour and not for food uptake. We suggest that the

425   involvement of the tongue during food uptake found in the derived extant tortoises serves as a

426   tactile sensory tool for the localisation of the food item prior to jaw prehension. Thus, the tongue

427   is not used as the main food collecting organ in modern tortoises and the food uptake mode

428   represents a derived jaw prehension system.

429

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441

443

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Figure 1

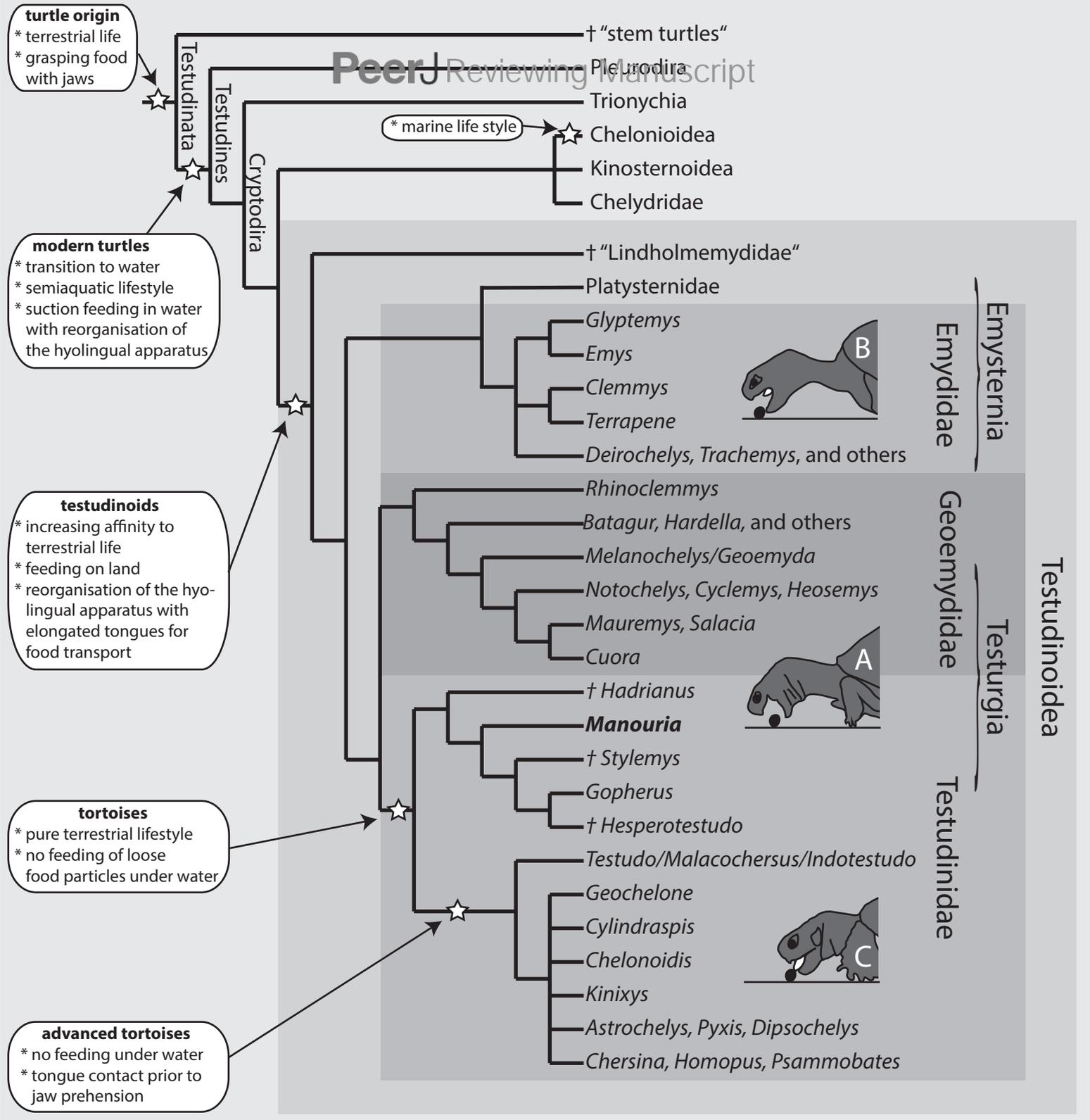
Phylogeny of turtle clades with a focus on Testudinoidea. Interrelationship following Thomson and Shaffer (2010). Major evolutionary changes are listed; for details see text. Three modes of terrestrial food uptake are illustrated. A) Jaw prehension; the tongue is not protruded and is only used for food 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).

2

Figure 2

Selected frame from a high-speed video sequence (500 frs) of food transport in *Manouria emys*, showing the landmarks used for kinematic analyses: C, rostral tip of sagital line of the carapace, Hy, hyoid at the basis of ceratbranchial I; LJ, tip of the lower jaw; P, posterior most 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. Abbreviations in Appendix 1.

3

Figure 3

Selected frames and graphics (based on a high-speed video with 500 frs) 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; arrows indicate the position of the food item; arrowheads represent the position of the tip of the tongue; grid 10x10 mm. Abbreviations in Appendix 1.

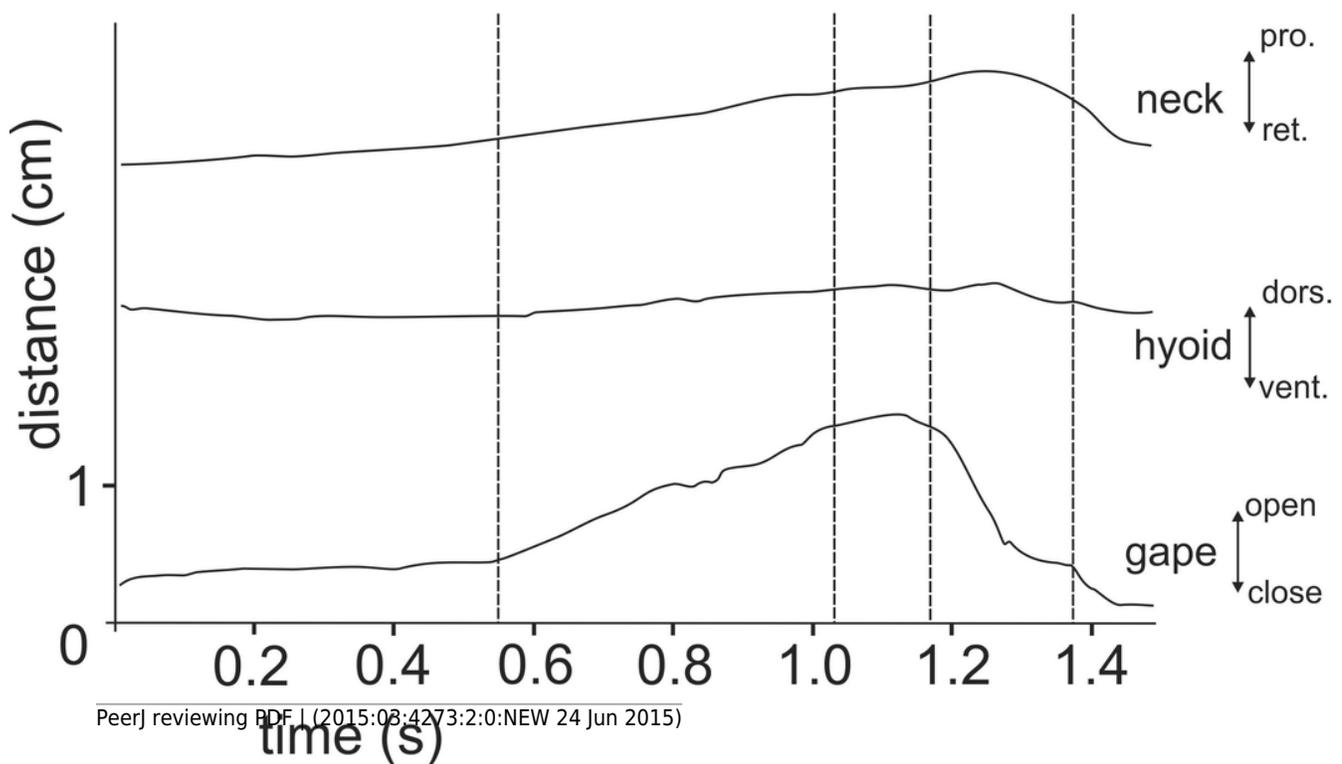
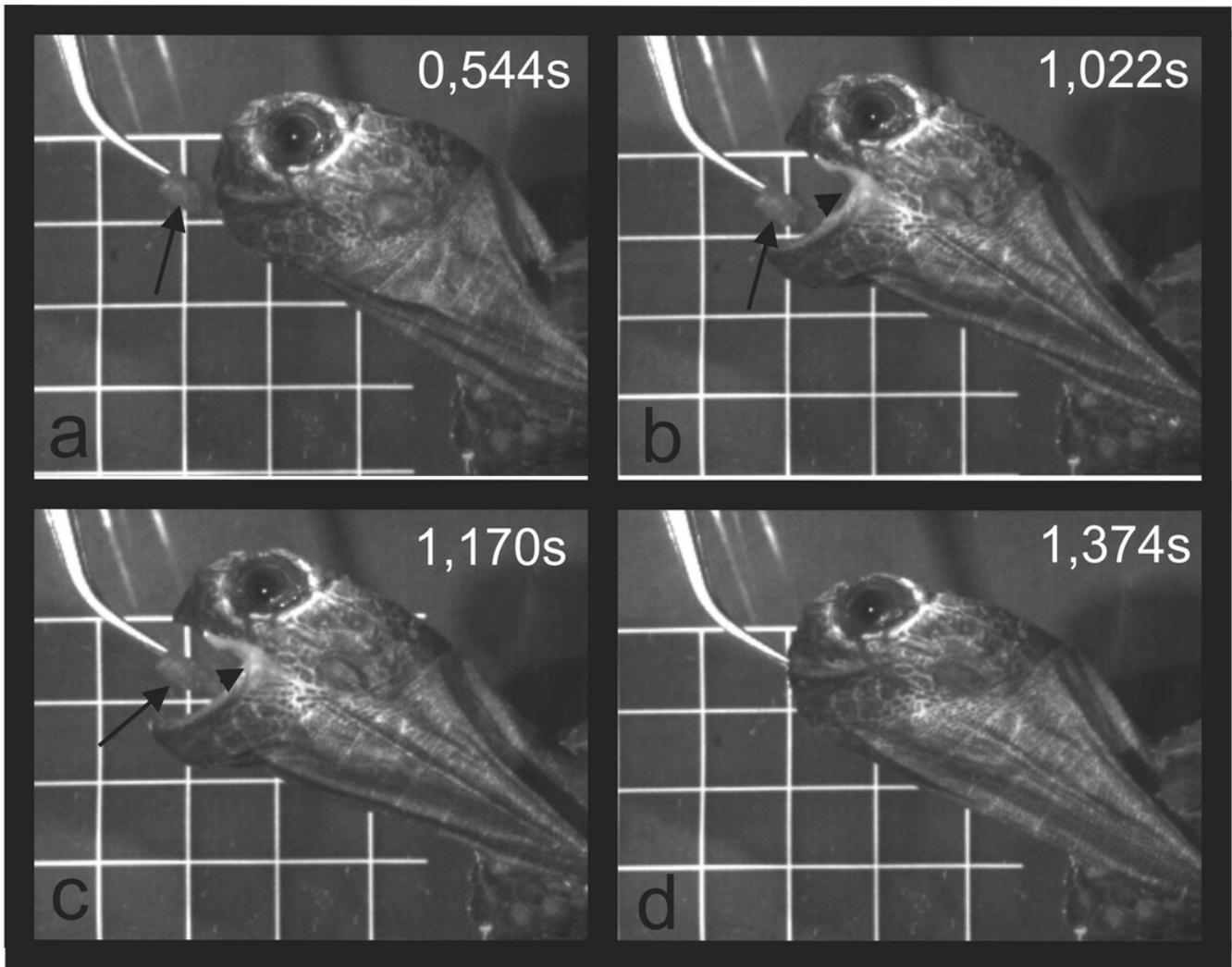



Figure 4

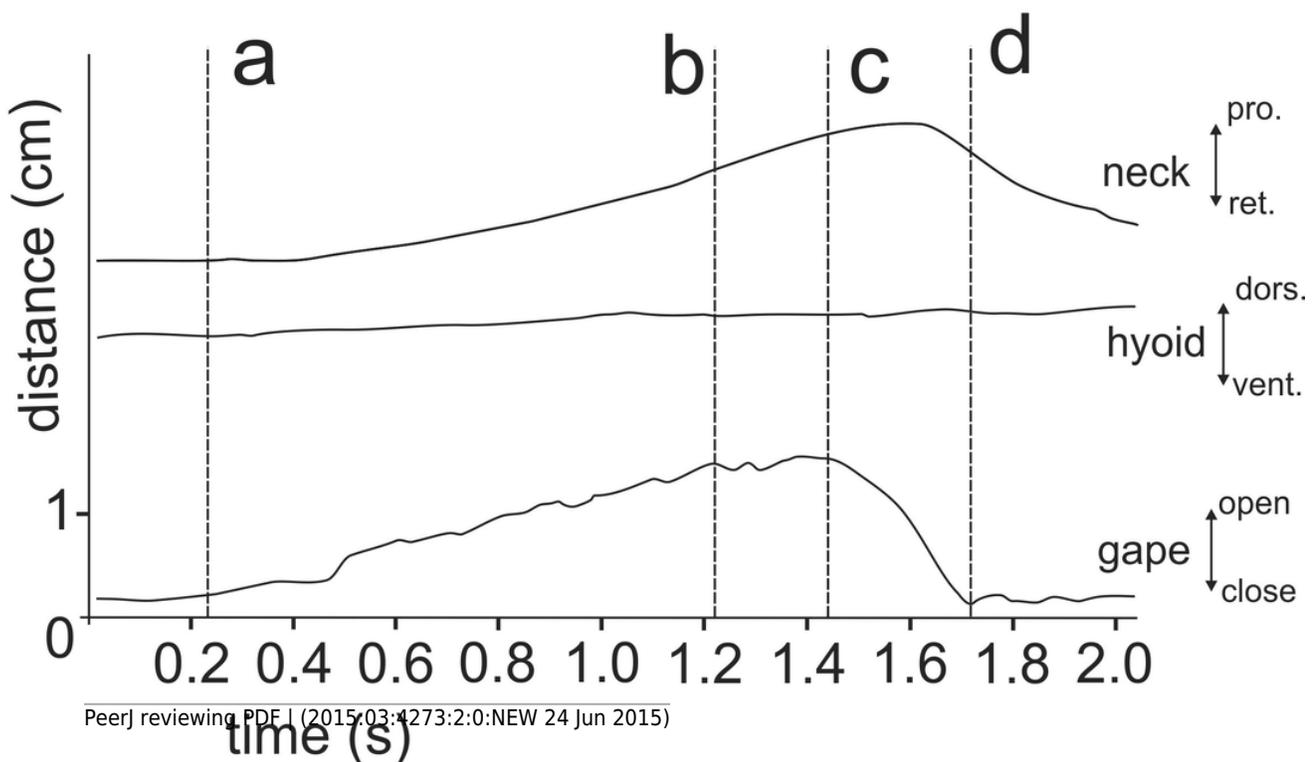
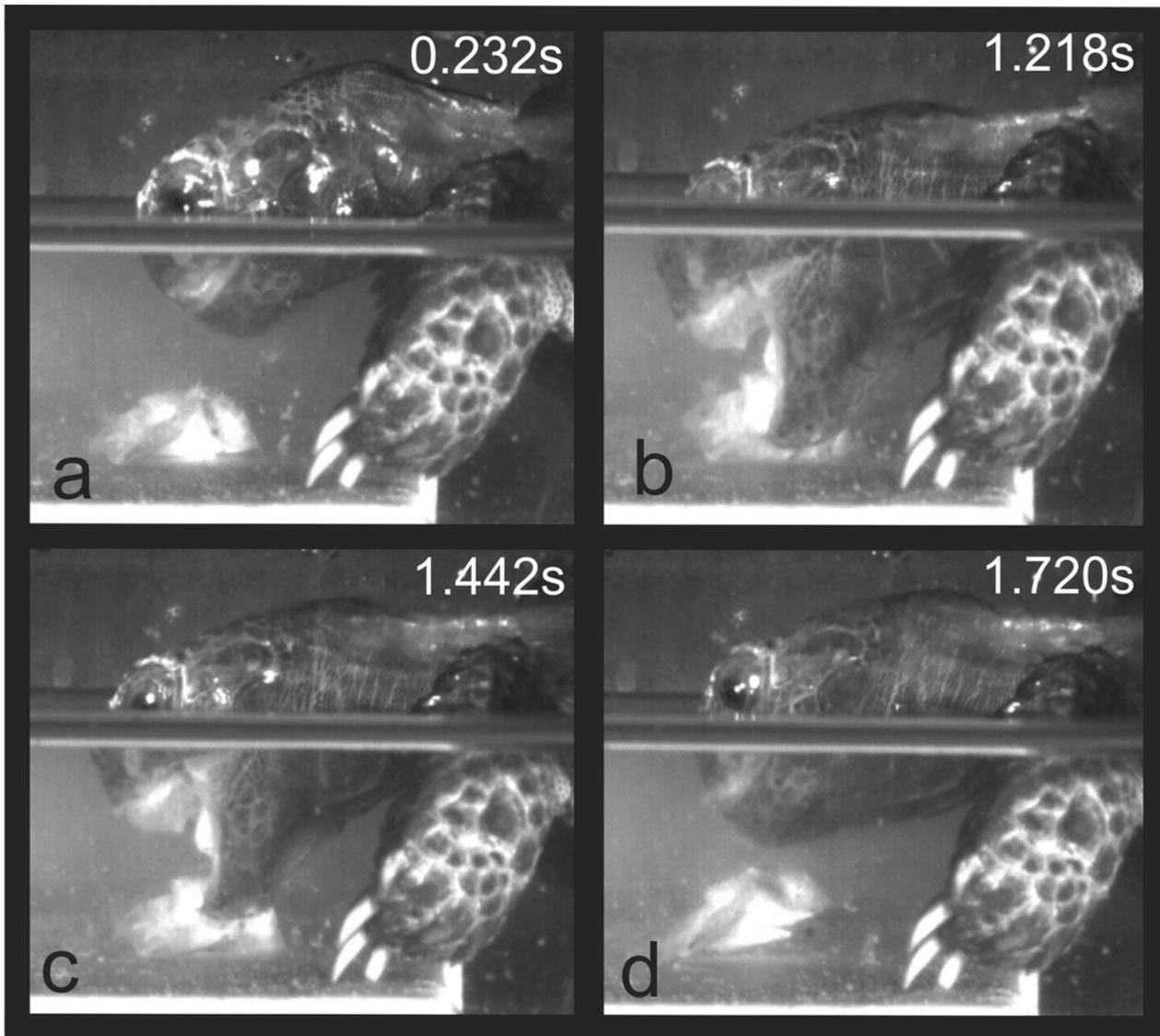
Selected frames and graphics (based on high-speed video with 250 frs) showing the movements of jaws, hyoid, and head during attempts of aquatic food uptake in *Manouria emys*; a, start of the gape cycle; b, end of jaw opening; c, maximum gape end; d, fast closure end; note the lack of movement of the hyoid complex during the whole cycle; grid 10x10 mm. Abbreviations in Appendix 1.

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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 *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 in Appendix 1.
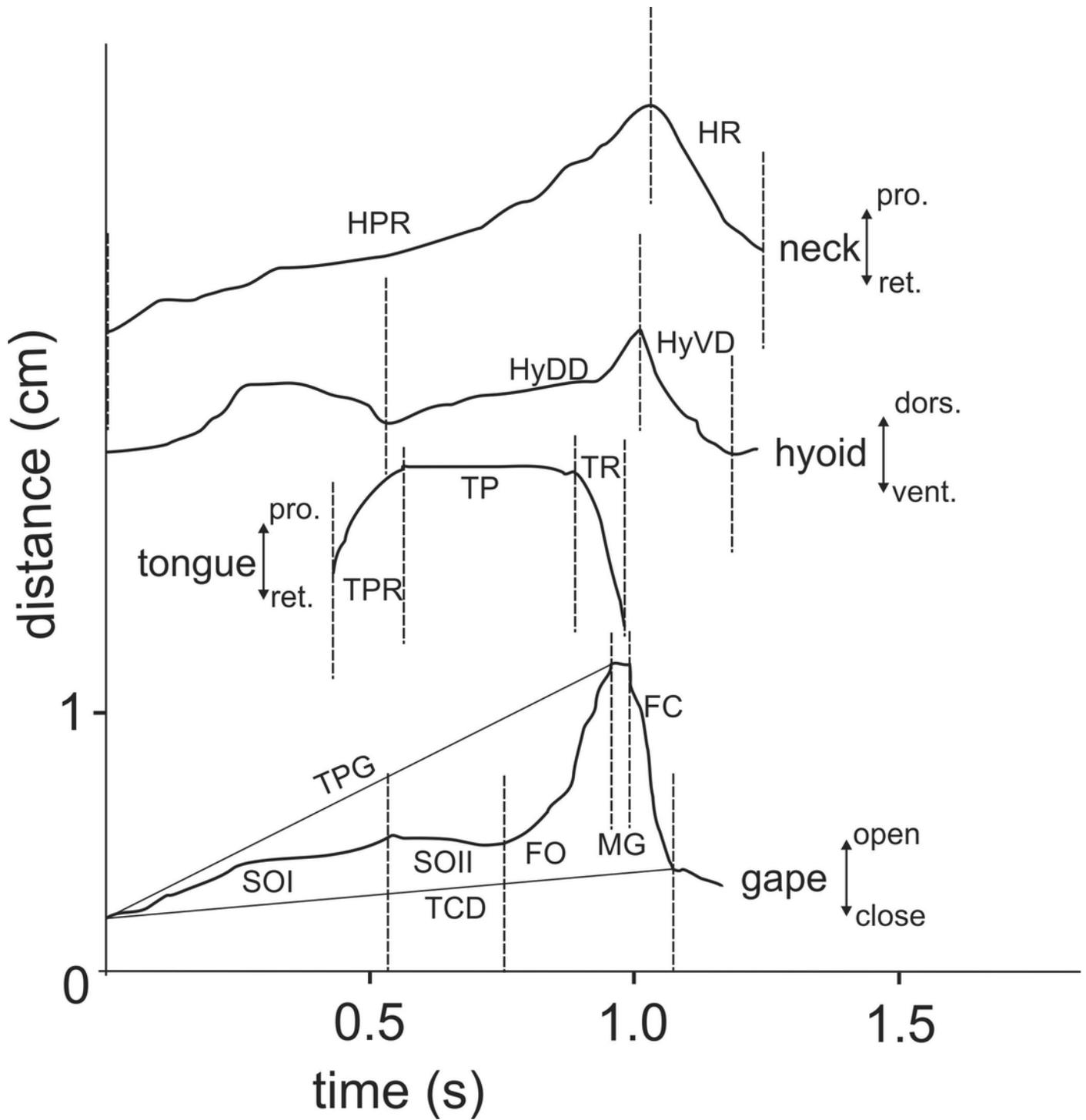
Figure 6

Graphical representation of three selected variables in food uptake (FU) and food transport (T) phases. Bars are denoted by their mean values and whiskers present the 95% CI; A, sequences with discrete SO and FO phases; B, sequences with no detectable split of discrete slow gape phase (SOI and SOII were inseparable); C - sequences with lacking MG phase. Abbreviations in Appendix 1.
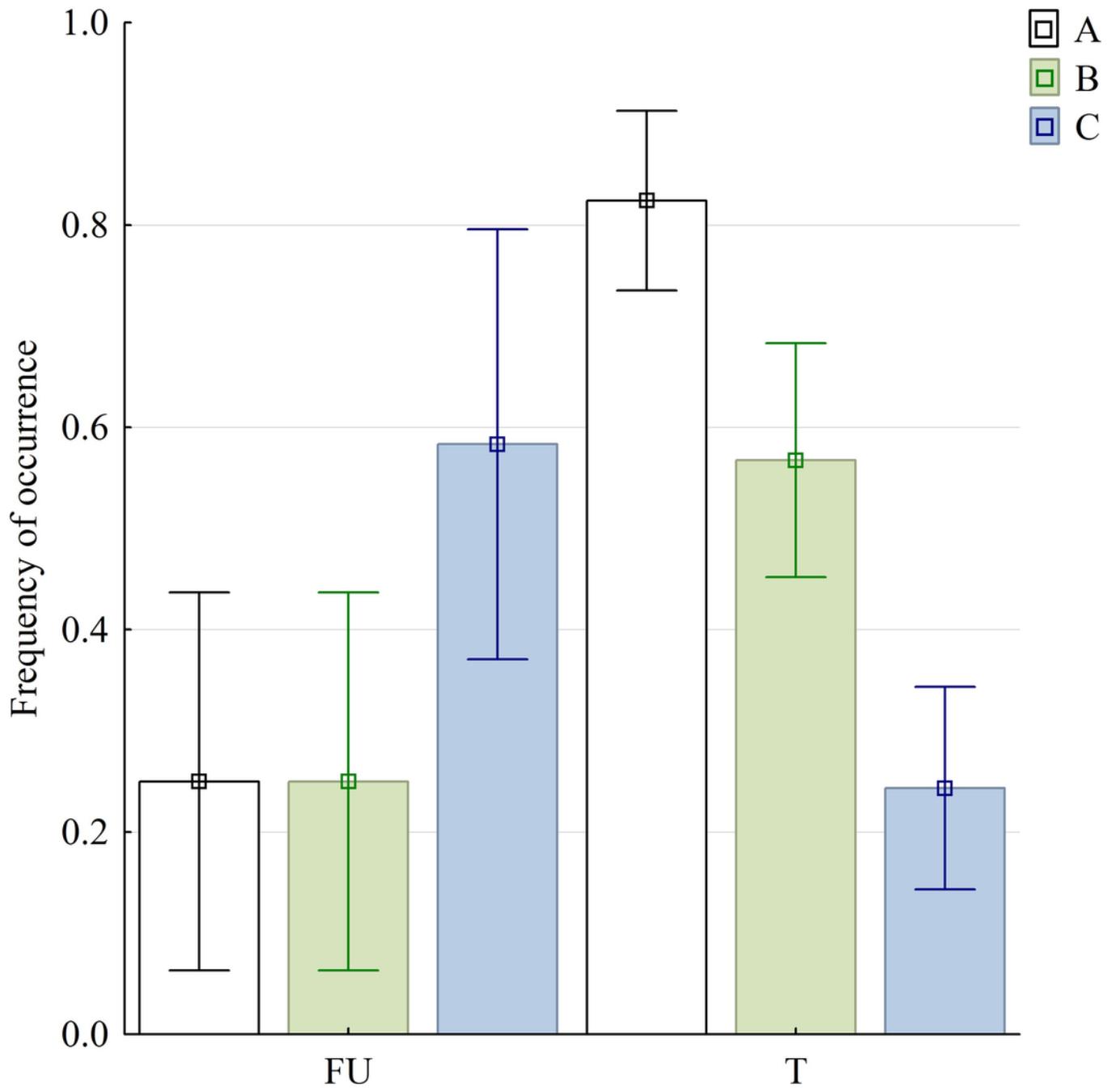
Figure 7

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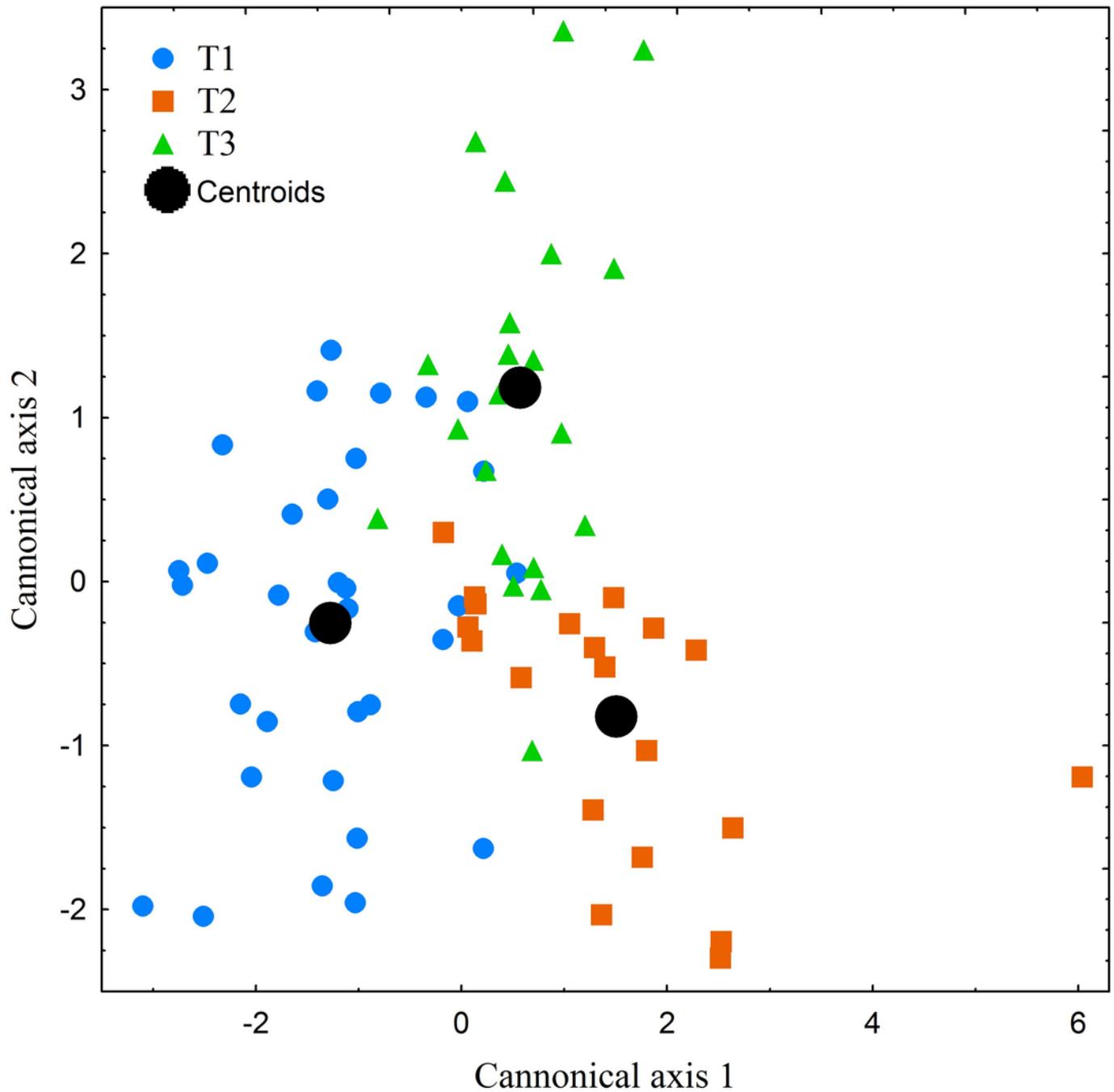
Table 1

Variables describing the feeding process in Manouria emys, present as means ± SD; n, sample size; *, significant differences (α=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. Abbreviations in appendix 1.

	Food uptake (FU)				Transport (T)				I vs. T
Variable	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	