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A quantitative analysis of gilthead seabream, *Sparus aurata,* juvenile dentition as a tool to assess the effect of diet

A.M. de Azevedo[a,b], R. Fontanillas[c], M.A.G. Owen[c], S. Busti[d], L. Parma[d], A. Bonaldo[d], P.E. Witten[b], and A. Huysseune[b*]

[a]Department of Anatomy, Animal Production and Clinical Veterinary Sciences, Faculty of Veterinary Science, University of Santiago de Compostela. Campus Universitario S/N, 27002 Lugo, Spain.

[b]Evolutionary Developmental Biology Research Group, Biology Department, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium

[c]Skretting Aquaculture Research Centre, Sjøhagen 3, 4016 Stavanger, Norway

[d]Dipartimento di Scienze Mediche Veterinarie, Università di Bologna, Italy

[*]Corresponding author

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Abstract

Gilthead seabream, *Sparus aurata* Linnaeus, 1758 (Perciformes, Sparidae), is an important aquaculture species in the Mediterranean Sea basin. Yet, quantitative data on its dentition under standard farming conditions are currently lacking. It is furthermore unknown if the dentition can adapt to food of different sizes. Here, we describe the lower jaw dentition of juvenile *S. aurata* fed a standard pellet size (4 mm), and present a detailed analysis of eleven representative teeth. Overall, the number of teeth showed large individual variation, yet not significantly related to fish length. Considerable left-right differences were observed, without clear side dominance. We also assessed the influence of feeding *S. aurata* a smaller (2 mm) or larger (6 mm) pellet size. Four months of feeding with different pellet sizes did not cause detectable differences in total tooth number on the dentaries at the time of harvest, nor in size of the teeth assumed to be most relevant in food processing. If and how different pellet sizes may nevertheless affect digestion, and eventually fish health, is subject for further studies.

Keywords: Gilthead seabream (*Sparus aurata*); lower jaw; dentary; dentition, teeth; pellet size

Introduction

Gilthead seabream (*Sparus aurata* Linnaeus, 1758) (Perciformes, Sparidae) is a warm-temperate coastal teleost fish species in the Eastern Atlantic, and a species intensely produced in aquaculture in the Mediterranean Sea (FEAP 2017). As in many other species of teleost fish, teeth on the oral jaws are the primary structures handling food items during the initial phase of food processing. The first teeth in the oral dentition of *S. aurata* are observed in fish of 7.78 mm total length (TL) (Saka et al. 2008). Tooth patterns gradually change, adapting to the food type, from juveniles to adults (Cataldi et al. 1987; El Bakary 2012; Elgendy et al. 2016). The adult oral jaws possess a complex heterodont dentition, composed of caniniform and molariform teeth, as well as teeth of intermediate shape (Germain and Meunier 2020, and Figure 1a). Tooth shape and distribution resemble what is observed in Atlantic wolffish, *Anarhichas lupus* Linnaeus, 1758 (Bemis and Bemis 2015). Teeth are generally organised in rows (three rows on the dentary, four rows on the premaxilla, Cataldi et al. 1987; Elgendy et al. 2016). Caniniform teeth are located in the rostral region of the jaws, whereas molariform teeth with different sizes occur in the caudal region of the jaws (Castejón and Mitjans 1985; Cataldi et al. 1987; Elgendy et al. 2016; Germain and Meunier 2020). Beyond these qualitative descriptions, relatively little data exists on number, size and distribution of the oral teeth in *S. aurata*, neither in natural, nor in farming conditions.

In nature, *S. aurata* feed on hard- and soft-bodied prey such as bivalve molluscs, polychaetes and crustaceans, using the powerful oral jaws and molariform teeth to grind and crush the prey before swallowing (Castejón and Mitjans 1985; Cataldi et al. 1987; El Bakary 2012; Jobling et al. 2012). In *S. aurata*, molariform teeth are found exclusively on the oral jaws whereas the pharyngeal jaws bear numerous filiform teeth only. The adult oral dentition presents morphohistological specializations that can be linked to the durophagous diet of the fish

(Germain and Meunier 2020). The differentiation of simple nonramified folds of the dentine walls (simplexodont plicidentine, Meunier et al. 2015) in the base of caniniform and molariform teeth and the development of bony alveolae and bony shafts reinforces the jaws and the attachment of the teeth to the jaws to resist the strong mechanical forces derived from crushing hard-bodied preys (Germain and Meunier 2020).

In natural populations of some teleost fish species, the pharyngeal dentition can adapt in response to differences in food hardness, showing plasticity in the number and size of the teeth (Huysseune 2000). Grigorakis et al. (2002) noted some differences in the external morphology of the teeth between wild and cultured *S. aurata*. However, it is unknown whether the dentition in *S. aurata* can adapt to, for example, the size of the feed offered. Under farming conditions, *S. aurata* process extruded pellets by opening and closing the mouth repeatedly - "chewing" (Andrew et al. 2003; Ballester-Moltó et al. 2016). A similar "chewing" behaviour was also observed in *Diplodus sargus* Linnaeus, 1758, another species with molariform teeth, when fed natural preys (Vandewalle et al. 1995). Usually, the fish crushes the pellets before swallowing or swallows them as a whole, depending on size and appetite of the fish (Ballester-Moltó et al. 2016). Sometimes whole pellets or fragments are ejected, and can be re-ingested by the same or other individuals, or can be lost as waste feed (Andrew et al. 2003; Andrew et al. 2004*a*; Andrew et al. 2004*b*; Ballester-Moltó et al. 2016*)*. Pellet size could play an important role in fish performance and feed conversion ratio (Zakes et al. 2013; Mattila and Koskela 2018), as well as in the control of feed waste through chewing, thus influencing feeding efficiency (Andrew et al. 2004*b*; Andrew et al. 2004*a*; Ballester-Moltó et al. 2016; Aguado-Giménez 2020). For *S. aurata*, the optimal feed size has been established for larvae only (Fernández-Díaz et al. 1994; Ballester-Moltó et al. 2016). However, for other rearing stages, it was probably estimated by comparison with other species or simply by subjective observations (Ballester-Moltó et al. 2016).

Knowing if and to what extent food particle size can affect the dentition in *S. aurata* can be useful for the aquaculture industry. While farming conditions for *S. aurata* have been well established (Teles et al. 2011), optimizing feed efficiency may indeed result in less production costs (Basurco et al. 2011), as in other intensive aquaculture. Apart from the descriptions in the studies of Castejón and Mitjans (1985), Cataldi et al. (1987), El Bakary (2012), Elgendy et al. (2016) and Germain and Meunier (2020), the size of some molariform teeth was reported in Sisma-Ventura et al. (2015). Yet, baseline information on tooth number, size and distribution in the oral dentition of *S. aurata* in farmed juveniles is currently lacking.

The aim of the present study was threefold: (1) to provide quantitative data on the dentition of farmed *S. aurata* at the on-growing stage, focusing on the lower jaw (left and right dentary), (2) to define a region of the dentition that can be used for comparative purposes, and (3) to assess the influence of pellet size on total number of teeth and on the different tooth variables (size of the teeth) for the selected region.

Material and Methods

Diets, fish and rearing conditions

Sparus aurata (initial weight: 216.1 ± 0.9 g; *n* = 120) were fed a diet (44% protein; 23% lipid) with a pellet size of 4 mm (named hereafter as standard diet), which is a commonly used pellet size under farming conditions at this rearing stage (Andrew et al. 2003; Ballester-Moltó et al. 2016; Aguado-Giménez 2020; Parma et al. 2020). At the same time, two additional diets with the same formulation of the standard diet but different pellet size (2mm and 6mm) were provided to two additional fish groups (*n*=120 fish/diet; initial weight: 216.4 ± 3.3 g and 215.1 ± 0.8 g for 2 mm and 6 mm, respectively). Diets were formulated with practical ingredients currently used for *S. aurata* in aquafeed (Parma et al. 2016) and were manufactured by Skretting Aquaculture

Research Centre (Stavanger, Norway) via extrusion technology. The rearing trial was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna (Cesenatico, Italy). Triplicate groups of 40 fish $tank^{-1}$ were fed ad libitum twice a day (8:30, 16:30) for six days a week via automatic feeders using the overfeeding approach according to Parma et al. (2016). Nine, 800L square conical bottom tanks were provided with natural seawater (23.0 ± 1.0°C) and connected to a closed recirculation system (Parma et al. 2020). The rearing trial lasted 122 days, i.e., until the fish had reached a commercial size (which can vary from 250 g to more than 2.0 kg, APROMAR 2020). All experimental procedures were evaluated and approved by the Ethical-scientific Committee for Animal Experimentation of the University of Bologna, in accordance with the European directive 2010/63/UE on the protection of animals used for scientific purposes.

Sampling

At the end of the four month period, three fish per tank (nine fish per diet) were sampled for the current study; the remaining fish were used for purposes outside this study. Specimens were weighed (total weight in g) and TL (mm) was measured. The heads were dissected and frozen at -20°C until further processing. Samples were defrosted and the lower oral jaws (left and right dentaries) were dissected and photographed with a Canon PowerShot A640 digital camera. The jaws were next fixed in 4% buffered paraformaldehyde (PFA) for at least 48h and stored in 70% ethanol.

Stereomicroscopic analysis

Whole-mount staining with alizarin red S was performed as modified from Witten et al. (2019). Modifications to the protocol consisted of the omission of acetone steps and the use of a higher concentration of KOH clearing solution (10%). The dentition on the dentaries was examined and photographed using a Zeiss Axio Zoom V16 Stereo Zoom Microscope equipped with a 5MP CCD digital camera. The photographs and the stained dentaries were evaluated in a blind analysis. Due to the loss of several teeth in certain specimens after staining (one fish in each diet group), only eight fish per diet could be evaluated. For each fish, the number of teeth on the right and left dentaries, (NRD and NLD respectively) was counted, their sum representing the total number of dentary teeth on the lower jaw (TN). Teeth that were not emerged from their alveolus were not considered. Next, a subset of 11 teeth on each dentary was chosen for a detailed analysis, based on the assumption that their location and typology reflect the functionally most relevant part of the dentition for crushing food pellets (Huysseune 1995; Jobling et al. 2012). These teeth were distributed in the caudalmost region of the three tooth rows: five teeth in the labial row, three teeth in the middle row, and three teeth in the lingual row (Figure 1b). The area of the occlusal surface of this set of selected teeth was measured with the help of the image processing program ImageJ 1.52a (Schneider et al. 2012), using the perimeter of the occlusal surface (Figure 1c). The areas were summed, both on the right and left dentary (selected occlusal area on right dentary, ARD, and selected occlusal area on left dentary, ALD, respectively). The sum of ARD and ALD was calculated (total selected occlusal area, TA). From this subset of 11 teeth, the total area of the three largest teeth was calculated (A3RD, A3LD, TA3; for right and left dentary, and both dentaries, respectively), as well as the area of the largest tooth (A1RD, A1LD, TA1; for right and left dentary, and both dentaries, respectively) (see Table 1 for a list of terminology and abbreviations). Altogether, 528 teeth were measured.

Statistics

Spearman's rho correlation coefficient (and correlation test) was calculated to analyze the association between TL of the fish, and either the total number of teeth (TN) or the number of teeth in the right and left dentaries (NRD and NLD, respectively). To adjust for fish size, the variables related to number and occlusal area of teeth (as in Table 1A) were divided by TL of the fish (in mm) (Table 1B). Basic descriptive statistics (mean, standard deviation (Stdev), median, minimum and maximum) were used for measurements and tooth variables. A Kruskal-Wallis non-parametric test was used to assess the differences between the diet groups in the calculated variables. In case of significant differences ($P < 0.05$), pairwise Mann-Whitney comparison tests (with Bonferroni corrections) were performed. Data of initial fish weight were analysed by One-way analysis of variance (ANOVA) with Tukey's post hoc test. Statistical analysis was performed using SPSS (Version 26, IBM, Armonk, NY, USA) and using a significance level of 5%.

Results

Sparus aurata juveniles fed the standard pellet size (4 mm) showed an average number of 74 (ranging from 56 to 92) teeth on their dentaries (Table 2). These were arranged in three rows on each dentary, as described by Cataldi et al. (1987) and Elgendy et al. (2016), although some teeth were difficult to assign due to an intermediate position (Figure 1a). Teeth of the labial row were usually medium-sized and conical with at least two caniniform teeth in the most rostral region of the dentary. Teeth of the middle row were mostly small and conical in the rostral region of the dentary. Towards the caudal region of the dentary, the teeth showed a flatter occlusal surface in all three rows, with prominently large caudal teeth in the middle row (Figure 1d-g).

The number of teeth showed large individual variation as well as considerable left-right differences within individual fish (Figure 1e-g, Table 2). The association between the number of teeth and TL was weak for total number (TN) and NRD (Spearman's rho coefficient, $|rho|<0.3$, $P>0.05$), and moderate for NLD ($rho=0.440$, $P=0.276$), although non-significant for the three variables (Figure 2a) (see Table 1 for abbreviations).

The area of each of the selected teeth (11 on each dentary) was determined as a proxy of tooth size. The largest teeth were the ones situated in the caudal region of the middle row, particularly, teeth in positions 7 and 8, on both dentaries (Figure 2b). The area of the largest tooth ranged from 7.5 to 18.2 mm^2. The mean and median total area of the 11 selected teeth was larger for the left dentary (ALD) compared with the right side (ARD) (Table 3). Similar results were found for the area of the three largest teeth (A3LD *VS* A3RD) and the area of the largest tooth (A1LD *VS* A1RD) (Table 3). However, within individuals, 50% of the specimens showed a largest tooth on the right dentary.

Next, we compared the dentition of fish fed a standard pellet size, with fish fed a smaller (2 mm) and a larger (6 mm) pellet size. The weight of the fish at the start of the experiment was similar for the three diet groups (Table 4). Likewise, total weight and TL of the fish sampled at the end of the four month experiment did not statistically differ between the diet groups ($P > 0.05$) (Table 4).

Comparison of the three diet groups revealed very subtle differences for all the tooth variables analysed. Fish fed smaller pellets showed a slightly higher mean for the number of teeth on the left dentary (NLD/TL) compared with the other diets. On the right dentary (NRD/TL), both the standard and the smaller pellet size diet showed more teeth than the larger pellet size diet (Table 5, Figure 3a). Likewise, fish of the standard and the smaller pellet size diet displayed a slightly higher total number of teeth (TN/TL) than the larger pellet size diet (Table 5). These slight differences in the number of teeth (NRD/TL, NLD/TL, TN/TL) were, however, not statistically significant with Kruskal-Wallis tests (P>0.05). The number of teeth on the right dentary (NRD/TL) was slightly higher than on the left dentary (NLD/TL) especially for the 4 mm pellet size (Table 5, Figure 3a). The association between the number of teeth (TN, NRD, NLD) and TL was weak and non-significant for both smaller and larger pellet size ($|rho|<0.3$, P>0.05). The correlation coefficient was slightly higher than 0.3 in NRD for the diet with larger pellets (rho=-0.357, P=0.385) but also non-significant.

Finally, for the two experimental diets, we selected 11 teeth in the same positions as those selected in the standard diet and compared their area. The mean and median of the total area of these 11 teeth (ARD/TL, ALD/TL and TA/TL) was higher in fish fed the standard diet compared to the two other diets, which showed similar values (Table 6, Figure 3b). A similar trend was observed when considering only the three largest teeth, or the single largest tooth. Thus, with two exceptions, fish fed the standard diet showed the highest mean and median for the area of the three largest teeth (A3RD/TL, A3LD/TL, TA3/TL) as well as of the largest tooth (A1RD/TL, A1LD/TL, TA1/TL) (Table 6, Figure 3c,d). However, for both A3RD/TL and A1LD/TL, the highest median was also displayed by fish fed the larger pellet size (Table 6). The mean area of the largest tooth (A1RD/TL, A1LD/TL and TA1/TL) was consistently lower in fish fed the smaller pellet size (Table 6, Figure 3d). However, none of the observed slight differences between the diets was statistically significant (P>0.05).

Discussion

This study presents quantitative data on tooth number, distribution and size of the teeth on the dentaries of farmed *S. aurata* fed a standard (4 mm) pellet size. These data were next used for comparative purposes in an experimental trial using diets with different pellet sizes. The results complement previous, qualitative, descriptions for the lower jaw dentition in *S. aurata* juveniles and adults (Elgendy et al. 2016; Germain and Meunier 2020).

Fish fed the standard pellet size showed a large individual variation in tooth number, as well as between left and right dentaries. This contrasts with certain other species of teleosts, such as piranhas and other characins, which maintain the same number of teeth throughout life (Berkovitz and Shellis 2017). The variation in *S. aurata* juveniles could not be attributed to fish length, as the relationship between total fish length and the number of teeth on the dentaries was non-significant, not just for *S. aurata* fed the standard pellet size, but also for the two experimental diets (smaller and larger pellet size). A relevant comparison can be made to *Astatoreochromis alluaudi* Pellegrin, 1904, a cichlid species showing considerable phenotypic plasticity. In this species, pharyngeal tooth number increased significantly with fish standard length in soft-food, but not in hard food-fed fish (Huysseune 1995). The absence of a correlation between fish size and tooth number both in *S. aurata* and hard-food fed cichlids could be related to the possession of large molariform teeth in the two cases. Such teeth take up a large surface of the tooth-bearing bone, at the expense of many smaller teeth, thus concealing a potential increase in tooth number with fish size.

Much of the outcome of this study is based on measurements on a selected number of teeth, positioned largely in the caudal area of the dentaries. A similar approach was used by Huysseune (1995) to compare cichlids fed a soft or a hard diet. Using this approach, we compared *S. aurata* fed the standard pellet size, with two experimental diets, consisting of a smaller (2 mm) or larger (6 mm) pellet size.

Left and right dentary frequently presented only very subtle differences with respect to various variables. Overall, the left dentary showed a higher mean total area of the teeth selected, the three largest teeth and the largest tooth, compared with the right dentary, and this for all three pellet sizes. However, the left-right differences at individual level did not show a clear side dominance. In cichlids of the Eretmodini tribe, the oral dentition showed left-right differences in the number of tooth groups, which could be explained by a specific tooth replacement pattern (Huysseune et al. 1999). These fish also displayed side asymmetries in the shape of their oral teeth, which could indicate side feeding preferences (Wautier 2005). Left-right asymmetry in the number of micro-teeth at the tip of the bill was also observed in hunting sailfish, *Istiophorus platypterus* Shaw, 1792, suggesting unequal tooth abrasion and providing support for attack lateralization in this species (Kurvers et al. 2017). Whether the observed left-right differences in *S. aurata* are related to a possible side preference during feeding is worth of further exploration. Asynchrony in the replacement of the teeth on the two dentaries could also be a base for left-right differences in the number and area of the teeth. Different arguments hint at a possible asynchrony of tooth replacement. First, in the experiments on *S. aurata* conducted by Pucéat et al. (2010), teeth labelled by calceine were randomly distributed, suggesting asynchronous replacement. Second, data on double labelled *S. aurata* that are currently being analysed appear to support this suggestion (unpublished results). As mentioned before, the dentition in *S. aurata* bears striking similarities to that of Atlantic wolffish, where replacement is nevertheless synchronous. In the latter, however, the molariform teeth are tightly packed, and their outline reflects the shape of the adjacent molariform teeth (Bemis and Bemis 2015), suggesting that mutual (perhaps mechanical) interactions take place during a synchronous phase of growth (replacement) of the teeth. In contrast, *S. aurata* teeth are more widely spaced and there is no evidence for a synchronised phase of growth.

The differences between the diets in terms of fish size, total tooth number and area of selected teeth, were very subtle for all the variables considered. Larger pellets tended to produce fish with the lowest total number of teeth on the dentary. Specimens fed the smallest pellet size presented an overall smaller median of the area (hence, smaller size) for the selected teeth, the three largest teeth and the lowest mean for the largest tooth. Differences were however not statistically significant, and during this specific experimental period, the pellet size seemed to produce no effect on the number of teeth and size of the selected teeth. The subtle differences between the three diet groups, both in terms of tooth number and tooth size, are intriguing. In the cichlid *A. alluaudi,* hard-food fed specimens presented fewer but larger teeth on their pharyngeal jaws than soft-food fed specimens (Huysseune 1995; Gunter et al. 2013). In a feeding experiment where papilliform morphs of the cichlid fish *Cichlasoma citrinellum* Günther, 1864 (i.e., morphs with slender and pointed teeth) were fed hard-snail diets, the fish changed their tooth morphology to an intermediate or molariform type (Meyer 1990). Following this line of thought, it could be hypothesised that the use of smaller feed sizes (a size that could be swallowed directly) could favour a higher number of smaller teeth in *S. aurata.* That such a result is not observed may be due to the length of the replacement cycle of the teeth. In *S. aurata*, as in most teleosts, tooth replacement is a continuous, lifelong process. Over the course of ontogeny, tooth number can change by the addition of teeth in new positions, by replacement (e.g. by replacing smaller teeth by fewer, but larger teeth, resulting in a decrease of tooth number), or by arrest of replacement. At present, data on the length of the replacement cycle of individual teeth in *S. aurata* are not available. However, *S. aurata* injected with calceine still showed labelled teeth after a four to five month period, suggesting a replacement cycle longer than four months, at least for some teeth (Pucéat et al. 2010).

In conclusion, farmed *S. aurata* at the on-growing stage show a large variation in number and size of dentary teeth. Four months of feeding with different pellet sizes does not cause detectable differences in total tooth number on the dentaries at harvest, nor in size of the teeth assumed to be most relevant in food processing. If and how digestion may nevertheless be affected is worth considering, but not part of this study. Further experiments are required to assess long term effects of pellet size on the dentition, especially if fish are harvested at larger size. In this case, the length of the tooth replacement cycle – suspected to be over four months

– will need to be taken into account. Experiments in which *S. aurata* of different rearing stages were double labelled with fluorochromes are currently being analysed to collect such data.

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Data availability statement

Underlying study data are available from the corresponding author upon request.

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Figure legends

Figure 1*:* Dentary of *Sparus aurata* Linnaeus, 1758 fed the standard pellet size (4 mm). a. Left and right dentary of a specimen of 286 mm TL. Teeth are mostly arranged in three rows on each dentary, although some teeth are present in intermediate positions between these rows (arrowheads). b. Teeth selected from the three rows for area measurements: labial (yellow), middle (pink) and lingual tooth row (blue). c. Perimeter of the occlusal surface used for area measurements (black line); left dentary. d. Left dentary of a specimen of 319 mm TL. Note the canine shape of the rostralmost teeth in the labial row (black arrowheads). Small conical teeth (black arrows) are predominant in the rostral area of the dentary whereas teeth become larger and with a flatter occlusal surface in the caudal region of the dentary, especially in the middle row (asterisks). Note an erupting tooth (white arrow) and a shedding tooth (white arrowhead) in the labial row. Inset: lingual view of caudal molariform teeth showing flatter occlusal surface. Rostral to the left of the image. e-g. Variation of tooth number on the right dentary of two similar-sized fish (TL = 293 mm) (NRD = 48 and 39, e and f resp.), and substantially less teeth (NRD = 29) in a slightly smaller specimen (TL = 275 mm; g). Alizarin red S staining. Scale bars = 1 mm.

Figure 2: *Sparus aurata* Linnaeus, 1758 fed the standard pellet size (4 mm). a. Number of teeth on the left and right dentaries and sum for both dentaries, compared with TL of the fish (mm). b. Distribution of the area of selected teeth (mm^2), on the left and right dentaries, respectively. The largest teeth were situated in positions 7 and 8 in most fish. Scale bars = 1 mm.

Figure 3: Comparison of *Sparus aurata* Linnaeus, 1758 fed three different pellet sizes (mean + standard deviation; *n*=8 fish per diet). a. Number of teeth in the three diet groups. Tooth number on the left and right dentaries, NRD/TL and NLD/TL, respectively. b. Sum of the area of all 11 selected teeth on the right and left dentaries, ARD/TL and ALD/TL, respectively (mm). c. Area of the three largest teeth on the right and left dentaries, A3RD/TL and A3LD/TL, respectively (mm). d. Area of the largest tooth on the right and left dentaries, A1RD/TL and A1LD/TL, respectively (mm). LD: left dentary; RD: right dentary.

Figure 1

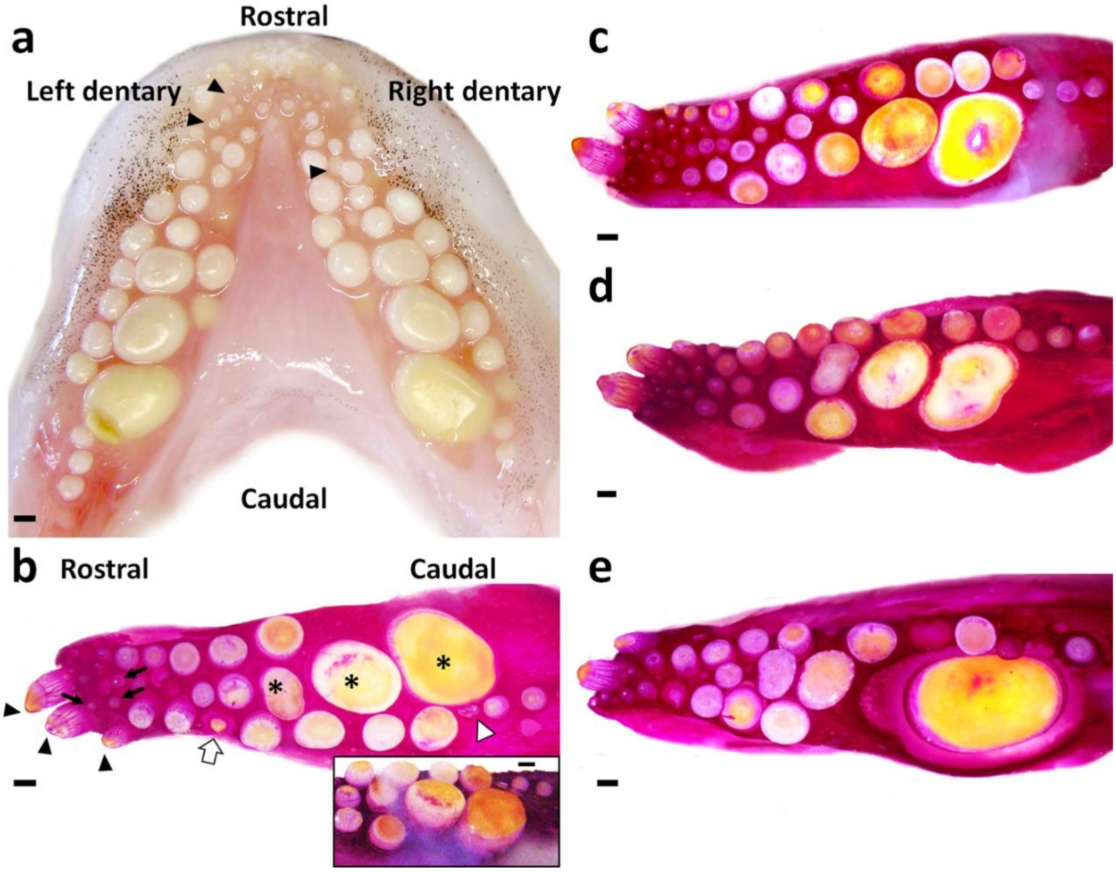

a
Rostral
Left dentary
Right dentary
Caudal
b
Rostral
Caudal
c
d
e

Figure 2

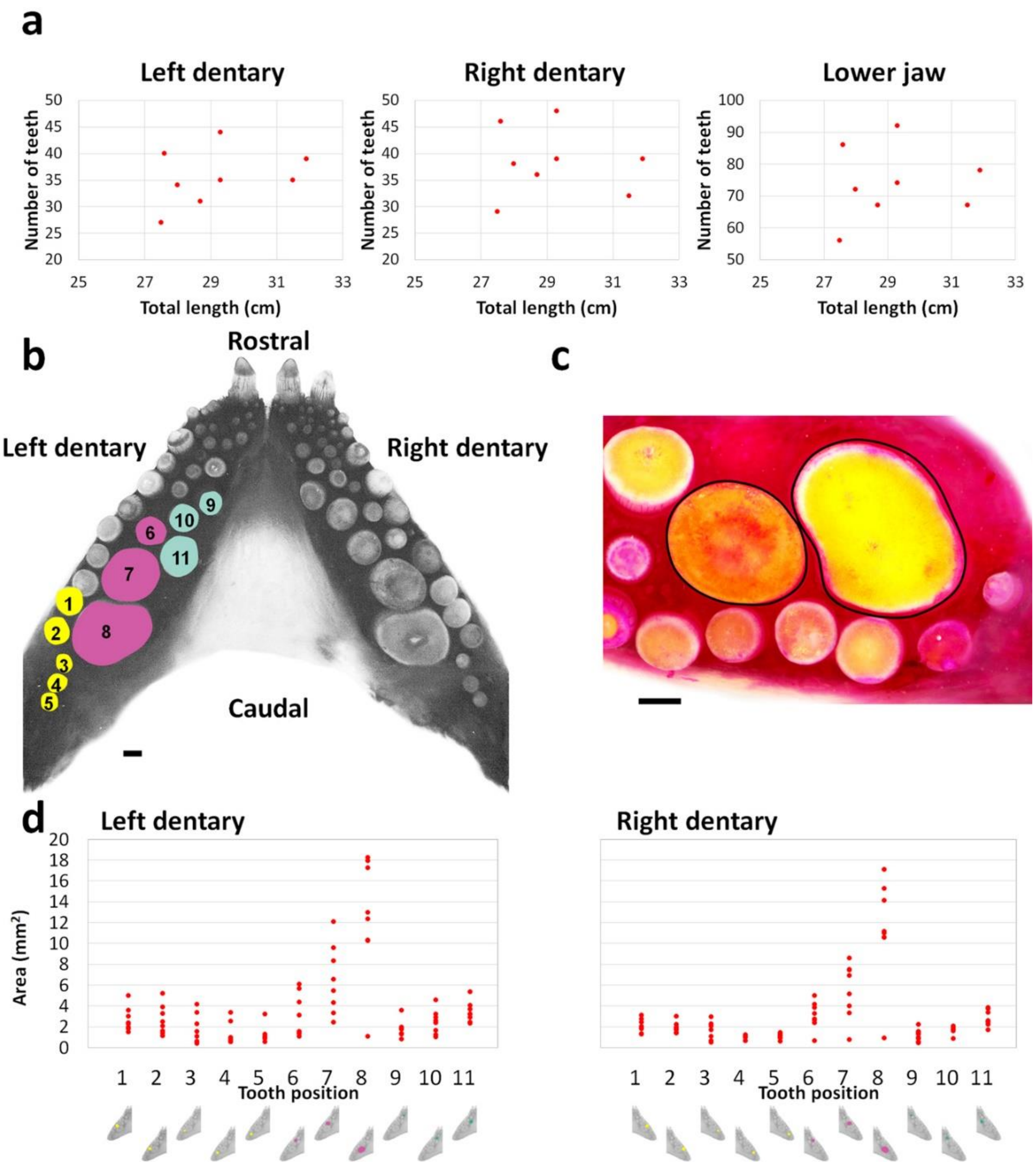
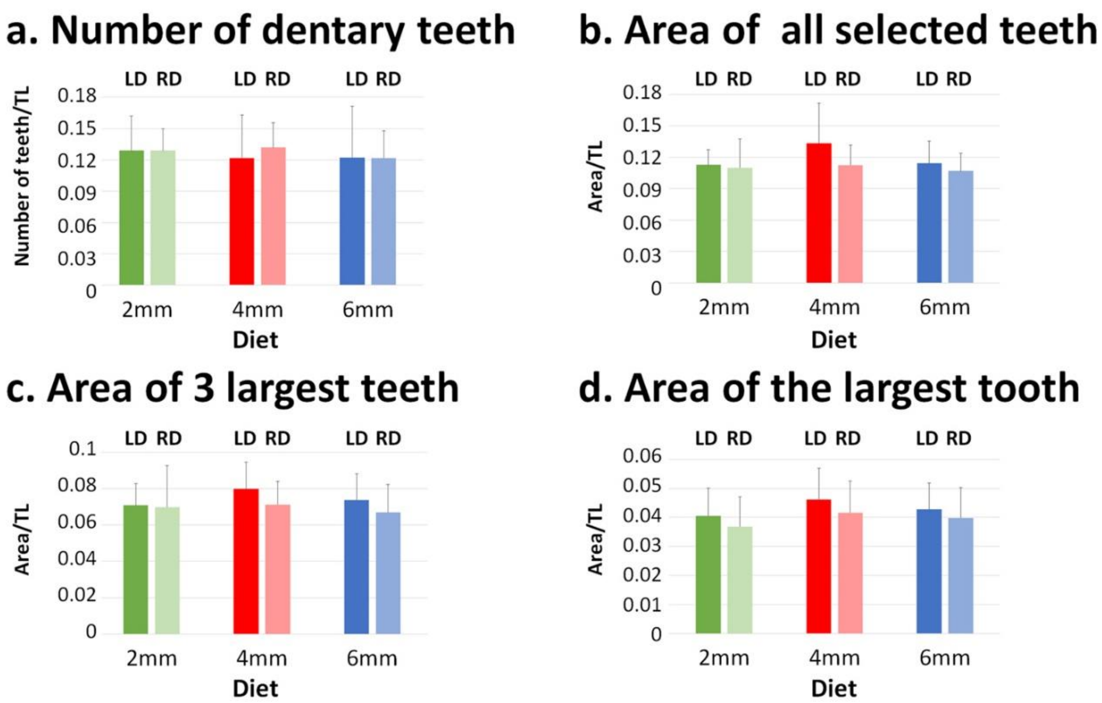

a
Left dentary
Right dentary
Lower jaw
Number of teeth
Total length (cm)
b
Rostral
Left dentary
Right dentary
Caudal
c
d
Left dentary
Right dentary
Area (mm²)
Tooth position

Figure 3


a. Number of dentary teeth
Number of teeth/TL
LD RD
0.18
0.15
0.12
0.09
0.06
0.03
0
2mm
4mm
6mm
Diet
b. Area of all selected teeth
Area/TL
c. Area of 3 largest teeth
Area/TL
0.1
0.08
0.06
0.04
0.02
d. Area of the largest tooth
Area/TL
0.06
0.05
0.04
0.03
0.02
0.01