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The incidence of different pellet size on growth, gut evacuation, feed digestibility and feed waste in gilthead sea bream (*Sparus aurata*)

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1 **The incidence of different pellet size on growth, gut evacuation, feed digestibility**  
2 **and feed waste in gilthead sea bream (*Sparus aurata*)**

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17

18 **Abstract**

19 The feeding behaviour of gilthead sea bream (*Sparus aurata*) consists in cracking and  
20 chewing feed. In farming condition, this results in crushing feed pellets with an occasional  
21 loss of some fragments which can vary in response to pellet dimension, thus affecting  
22 feed waste at the on-growing stage. However, few studies have addressed this issue and  
23 even less information on the further effect of different pellet size on growth, gut  
24 evacuation and feed efficiency are available on this species. Thus, a 122-day study was

25 undertaken to assess the effects of three pellet size (2 mm, S; 4 mm, M and 6 mm, L) on  
26 growth, gut evacuation, feed waste and feed digestibility during the on-growing of  
27 gilthead sea bream (initial weight:  $215.9 \pm 1.8$  g). No significant effects of pellet size on  
28 growth (final body weight and SGR) were observed. Pellets diameters had no effects on  
29 feed digestibility (protein and dry matter) and feed efficiency parameters (FCR, PER,  
30 GPE, GLE) even if differences in the gastric evacuation rate were detected at different  
31 pellet size. At this regard, the shape-rate model developed to estimate the gastrointestinal  
32 evacuation pattern, evidenced a slower gastric evacuation rate in the 6 mm diet, while no  
33 differences in foregut evacuation rate were observed. Data on feed waste, highlighted  
34 how feed losses by chewing was practically absent in the S (2mm) diet while in the M  
35 (4mm) and L (6mm) diets 24.3 and 17.3 % of the entire meal was losses by chewing  
36 activity, respectively. The study reinforces previous observation that feeding pellets size  
37 of 4 and 6 mm in gilthead sea bream within 200-450g could induced an excess of feed  
38 waste by chewing activity with economic and environmental implication. Despite the  
39 reduced feed intake observed, pellets size of 2 mm did not lead to any feed losses by  
40 chewing and was able to guarantee similar growth compared to the other diets. Further  
41 studies considering intermediate pellets size (3 mm) may be useful in order to further  
42 optimize the pellet size choice during the on-growing phase of this species.

43 **Keywords:** gilthead sea bream, pellet size, growth, feed waste, gut evacuation.

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## 49 **1. Introduction**

50 In nature, gilthead sea bream (*Sparus aurata*) feeds on molluscs, crustaceans,  
51 polychaetes, echinoderms and small fish (Nikolopoulou et al., 2011) and its normal  
52 feeding behaviour includes cracking preys. This food processing, observed also in other  
53 sparids species (Vandewalle et al., 1995), consists in opening and closing mouth in a  
54 series of movements (chewing) in which food items can be ejected from the mouth and  
55 on some occasions re-ingested or seized (Andrew et al., 2003). Sea bream showed the  
56 same feeding behaviour also in captivity, chewing and crushing pellets, and occasionally  
57 ejecting parts of feed before ingesting but also losing some fragments which can be  
58 consumed by other individuals or be lost, thus affecting feed utilization (Andrew et al.,  
59 2003, 2004a). Previous studies showed that in teleost fish, feeding mechanisms may be  
60 hardly variable on the basis of prey type (Andrew et al., 2004b; Wainwright and Friel,  
61 2000). It has been observed that the *Sparids* white bream, *Diplodus sargus*, can modulate  
62 the mouth movements speeding up or slowing down chewing, depending on whether the  
63 prey is soft or hard-textured (Vandewalle et al., 1995). Andrew et al. (2003), found that  
64 also gilthead sea bream has a similar feeding behaviour and hypothesized that chewing  
65 could vary not only for the nature of the prey regarding its hardness but also in response  
66 to pellet dimensions.

67 Currently, many different pellet sizes are used during the husbandry of sea bream at  
68 the on growing stage but detecting feed loss is still a challenge. Sea bream producers  
69 estimated an average general waste of 50-100 g per Kg<sup>-1</sup> of feed administered under  
70 offshore conditions (Piedecausa et al., 2009). However, the waste can change widely  
71 depending on several variables related to feed composition, fish feeding behaviour and  
72 feeding management (Ballester-Moltó et al., 2017; Cho and Bureau, 2001; Zhou et al.,

2018). Few studies tried to calculate feed loss in sea bream caused by chewing. Among these, Ballester-Moltó et al. (2016) quantified the loss rate by mean of using mesh screen and taking into account full pellets disaggregation and leaching. The author found that feed loss increases with the increase not only of feed size but also of fish size. They hypothesized that although several studies stated that the appropriate pellet size should represent from 25% to 50% of the fish mouth amplitude (Linnér and Brännäs, 1994; Smith et al., 1995), alternative feeding regimes in which even large fish are fed with small- size feeds (e.g. 2 mm) could help to reduce feed waste. Pellet size could also play a significant role in gut evacuation potentially affecting feed efficiency and growth performance (Aguado-Giménez, 2020; Andrew et al., 2004a; Ballester-Moltó et al., 2016; Mazumder et al., 2020). Ballotini beads (inert metal powders incorporated in feed) have been used as a feed marker to estimate feed intake (FI) and to study the trophic and behavioural dynamics of fish (Andrew et al., 2004b; Talbot and Higgins, 1983). Moreover, it could become a valid tool for obtaining a precise quantification of feed waste relative to different pellet sizes. The aims of this work are i. to study the effects of 3 different pellet sizes 2mm, 4mm, and 6mm, on growth, feed efficiency, and gut evacuation during the on-growing stage of gilthead sea bream; ii. to quantify the proportion of feed waste using ballotini beads.

91

## 92 **2. Materials and methods**

93

### 94 *2.1 Experimental Diet*

95 Three experimental diets with the same formulation were produced by Skretting  
96 Aquaculture Research Centre, Stavanger, Norway, in 3 different pellet sizes named S

97 (small size, 2mm), M (medium size, 4mm) and L (large size, 6mm) (ingredients and  
98 proximate composition in Table 1). All feed were extruded with a double screw extruder  
99 at 85°C for 5 minutes. Oil was coated using a vacuum coater at 200 mb of pressure for 90  
100 seconds. Pellets were dried at 60°C during 10 minutes in an horizontal dryer. Extra  
101 amount of feed for each size was also produced to contain 0.2 % of ballotini glass beads  
102 to be used to evaluate the chewing estimation.

103 All feeds were analysed for bulk density, durability, oil leaking, water stability,  
104 floating rate, and water absorption index according to Irungu et al., 2018, Aas et al., 2011,  
105 Sørensen et al., 2011, Khater et al., 2014, Alcaraz et al., 2021, Rosentrater et al., 2009.  
106 Physical pellet quality characteristics are shown in Table 2

107

## 108 *2.2 Feed Calibration straight lines*

109 In order to estimate the quantity of feed lost by chewing, calibration straight lines were  
110 calculated. Known quantities of pellets (1.0, 3.0, 7.0, 9.0, 11.0, 13.0, 15.0 g) containing  
111 ballotini beads for each pellet size were x-rayed (Talbot & Higgins, 1983). A protocol for  
112 automatic detection of the ballotines in x-ray images was developed using the Visiopharm  
113 software with app author module (version 2020.09). The ballotines were detected using  
114 the k-means clustering classification method, segmenting the image into 6 different  
115 classes defined by the pixel values. Two of these classes represented the range of pixel  
116 values in the ballotines. False positives, (artefacts with same ballotines' pixel value), were  
117 removed using post processing steps based on size and shape. Also, ballotines lying close  
118 together were separated using the post processing step separate objects. Afterwards the  
119 software automatically counted the number of ballotines per image and for each known  
120 pellet quantity a correlation between the number of beads and feed weight was built up

121 (Figure 1 a-c). The equations deriving from the calibration lines for each pellet size are  
122 the following:

123 Diet S (2 mm pellet)  $y=0.059x+0.2401$

124 Diet M (4 mm pellet)  $y=0.0604x+0.028$

125 Diet L (6 mm pellet)  $y=0.0704x+0.0474$

126 where y indicates the feed weight (g), and x indicates the ballotini beads number.

127

### 128 *2.3 Fish and feeding trial*

129 The experiment was carried out at the Laboratory of Aquaculture, Department of  
130 Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. Sea bream  
131 specimens were obtained from an Italian hatchery. At the beginning of the trial, 40 fish  
132 (initial average weight:  $215.9 \pm 1.8$  g) per tank were randomly distributed into nine 800  
133 L square tanks with a conical base. Each diet was administered to triplicate groups,  
134 assigned in a completely random manner, over 122 days. Tanks were provided with  
135 natural seawater and connected to a closed recirculation system (RAS) (overall water  
136 volume:  $15 \text{ m}^3$ , RAS utilized and water flow rate according to Busti et al. (2020). The  
137 oxygen level was maintained at  $8.0 \pm 1.0 \text{ mg L}^{-1}$  through a liquid oxygen system  
138 connected to a software controller (B&G Sinergia snc, Chioggia, Italy); temperature was  
139 kept at  $24 \pm 1.0 \text{ }^\circ\text{C}$  during the entire trial, salinity ( $25 \text{ g L}^{-1}$ ) was measured by a salt  
140 refractometer (106 ATC), photoperiod was held constant at 12 h day through artificial  
141 light, ammonia (total ammonia nitrogen  $\leq 0.1 \text{ mg L}^{-1}$ ) and nitrite ( $\text{NO}_2^- \leq 0.2 \text{ mg L}^{-1}$ )  
142 were spectrophotometrically monitored once a day (Spectroquant Nova 60, Merck, Lab  
143 business, Darmstadt, Germany), and sodium bicarbonate was added on a daily basis to  
144 keep pH at 7.8–8.0.



145 Feed was provided to satiation by oversupplying feed via automatic feeders by  
146 approximately 10% of the daily ingested ration, twice a day: the first 60% of the daily  
147 ration was administered at 8.30 and the last 40% at 16.00 for six days a week, while on  
148 Sundays fish fasted. Each meal lasted 1 hour, after which the uneaten pellets, including  
149 chewed pellet, of each tank were collected thanks to the use of strainers with a mesh of 1  
150 mm. The uneaten pellets were then gathered, dried overnight at 105°C, and weighted for  
151 overall calculation.

152

#### 153 *2.4 Sampling for growth parameters*

154 At the beginning and at the end of the experiment, all the fish in each tank were  
155 anaesthetised by tricaine methane sulfonate (MS-222) at 100 mg L<sup>-1</sup> and individually  
156 weighed. Specific growth rate (SGR), feed intake (FI) and feed conversion rate (FCR)  
157 were calculated. The proximate composition of the carcasses was determined at the  
158 beginning of the trial on a pooled sample of 10 fish and on a pooled sample of 5 fish per  
159 tank at the end of the trial. Protein efficiency rate (PER), gross protein efficiency (GPE)  
160 and gross lipid efficiency (GLE) were calculated.

161

#### 162 *2.5 Digestibility experiment*

163 At the end of the growth trial, 14 fish per tank were sampled to determine the apparent  
164 digestibility coefficient (ADC) of dry matter and protein by the indirect method with diets  
165 containing yttrium oxide. Eight hours after the meal fish were euthanised by overdose of  
166 anaesthetic and dissected. Then, the distal intestine (5 cm portion) was stripped on a  
167 previously sterilized surface. Faeces were collected for each tank (pooled in one falcon

168 per tank) and immediately kept at  $-20^{\circ}\text{C}$  until analysis (Busti et al., 2020). ADC was  
169 calculated as follows:

170 
$$\text{ADC} = 100 * (1 - (\text{dietary Y2O2 level} / \text{faecal Y2O2 level})) * ((\text{faecal nutrient or energy}$$
  
171 
$$\text{level} / \text{dietary nutrient or energy level})).$$

172 All experimental procedures were evaluated and approved by the Ethical-Scientific  
173 Committee for Animal Experimentation of the University of Bologna, in accordance with  
174 European directive 2010/63/UE concerning the protection of animals used for scientific  
175 purposes.

176

#### 177 *2.6 Gastrointestinal evacuation experiment*

178 At the end of the growth trial, to estimate the gastric evacuation time fish were sampled  
179 according to the following protocol: fish were hand-fed up to visual satiation, being  
180 careful not to lose any feed. In case of loss, pellets were collected from the outlet pipe of  
181 the tank and deducted from administered feeds. At 30 minutes, 4, 8, 12, 16 and 24 hours  
182 postprandial fish were euthanised by MS-222 at  $300 \text{ mg L}^{-1}$ . The abdominal cavity was  
183 opened, and the digestive tract carefully removed and ligated at the pylorus and anus. The  
184 gut was also ligated (approximately 4 centimetres from the pyloric ligature) to separate  
185 stomach, foregut, and hindgut. Compartments of the gastrointestinal tract were bound  
186 using a Teflon robe to prevent flow of content from one compartment to another. Each  
187 gut was identified with fish number and tank number, and frozen immediately. After  
188 being frozen at  $-20^{\circ}\text{C}$  guts were x-rayed to count the number of ballotini beads for gut  
189 evacuation calculations.

190

#### 191 *2.7 Estimation of feed loss by chewing*

192 In order to perform the estimation of feed loss by chewing based on ballottini beads,  
193 in the middle of the growth trial five fish per tank were moved to other tanks in triplicate  
194 condition and fed with the same diets for a few days. Fish were then fasted for 36 hours  
195 and then each tank received the same feed size provided during the growth trial but  
196 containing ballottini beads. Fish were hand-fed up to visual satiation, being careful not to  
197 lose any feeds. In case of loss, feed left (excluding chewed) was collected from the outlet  
198 pipe of the tank and deducted from administered feeds. Thirty minutes after feeding all  
199 fish were euthanized by MS-222 at 300 mg L<sup>-1</sup>. Each fish sampled was weighed, then the  
200 abdominal cavity was opened, and the digestive tract carefully removed and ligated at the  
201 pylorus and anus. The gut was also ligated (approximately 4 centimetres from the pyloric  
202 ligature) to separate stomach, foregut, and hindgut. Compartments of the gastrointestinal  
203 tract were bound using a Teflon robe to prevent flow of content from one compartment  
204 to another. Each gut was identified with fish number and tank number, and frozen  
205 immediately. After being, frozen at -20°C guts were x-rayed to count the number of  
206 ballottini beads. The number of ballottini beads was used in the equations of calibration  
207 straight lines to quantify the amount of feed lost by chewing via the formula:

208  $\% \text{ loss by chewing on feed eaten} = \text{feed chewed, g} / \text{feed eaten, g} \%$

209 where feed chewed (g) is calculated as: (feed administered, g – feed left, g - feed ingested  
210 estimated from ballottini beads calculation, g); and Feed eaten (g) is: (feed administered,  
211 g – feed left, g).

212

## 213 2.8 Calculations

### 214 2.8.1 Gastrointestinal evacuation pattern calculation

215 The Elliott regression is one of the most widely used models to describe the stomach  
 216 evacuation pattern after feeding (Elliot, 1972; Nikolopoulou et al., 2011). Consider for  
 217 each sea bream the stomach ballotini content is divided by the sea bream weight and  
 218 denote by  $W_t$  the mean of the normalized stomach ballotini contents of all fishes in all  
 219 tanks at time  $t$ , with  $t=(0.5, 4, 8, 12, 16, 24)$ . The Elliot regression model is an exponential  
 220 curve describing the stomach ballotini content as a function of the time,  $W_t = A e^{-rt}$  or  
 221  $\ln W_t = \ln A - rt$  where  $A=W_0$  is a constant representing the ballotini in a standard meal at  
 222 time 0, and the parameter  $r$  represents the gastric evacuation rate (GER). An interesting  
 223 aspect of this model is that it makes it possible to estimate the gastric evacuation time  
 224 (GET) as a function of  $r$ . More precisely, since  $\ln W_0 - \ln W_t = rt$ , the GET can be  
 225 estimated by  $GET_{p\%} = [ \ln 100 - \ln (100-p) ]/r$ .

226 Despite these interesting properties, the evacuation pattern of different gastrointestinal  
 227 tracts, such as foregut or hindgut, cannot successfully be described by an exponential  
 228 curve, since the typical shape is first increasing (filling) and then decreasing (evacuation)  
 229 during time. To this aim, Bonvini et al. (2018) applied a quadratic regression model, with  
 230 interesting results. In this work, a more flexible solution is presented, which describes the  
 231 different evacuation patterns of the gastrointestinal tracts in a unique formulation since it  
 232 includes the Elliott model as a special case. The proposed rate-shape model extends the  
 233 Elliot model, by adding an additional part depending on a shape parameter  $s$ :

$$234 \quad W_t = A e^{-rt} t^s \quad \text{or} \quad \ln W_t = \ln A - rt + s \ln t$$

235 When  $s=0$  the model coincides with the Elliott exponential curve; for  $s>0$  the curve can  
 236 take different shapes, as shown in Figure 2. This shape-rate curve is essentially equivalent  
 237 to fitting a Gamma probabilistic model on the normalized ballotini content as a function  
 238 of time. Since the mode of the Gamma distribution is  $s/r$ , the quantity

239

$$W_0 = A e^{-s} (s/r)^s$$

240 represents the ballotini content. In other term, the time of maximum ballottini content is

241  $t=s/r$ . Since  $\ln W_0 - \ln W_t = rt - s - s \ln (s/rt)$ , the GET of this model can be estimated by

242 solving the nonlinear equation as function of  $t$ :

243

$$[ \ln 100 - \ln (100-p) ] = rt - s - s \ln (s/rt).$$

244 In order to check the effect of the diet on evacuation time, the model has been applied to

245 each gut segment separately on the weights of the sea breams distinguished by diets. Let's

246  $W_{ti}$  the weight means of all fishes at time  $t$  and for diet  $i$ , with  $i=1,2,3$ , corresponding to

247 the three diets S (2mm), M (4mm) and L (6mm) and for a specific gut segment. The

248 model with rate-varying parameter is  $\ln W_{ti} = \ln A - r_i t + s \ln t$ .

249

### 250 2.8.2 Performance parameters calculation

251 The formulae employed were as follows:

252 Specific growth rate (SGR) (% day<sup>-1</sup>) = 100 \* (ln FBW - ln IBW) / days (where FBW and

253 IBW represent the final and the initial body weights). Feed intake (FI) (% ABW<sup>-1</sup> day<sup>-1</sup>)

254 = ((100 \* total ingestion)/(ABW))/days)) (where average body weight, ABW = (IBW +

255 FBW)/2; Feed conversion ratio (FCR) = feed intake / weight gain. Protein efficiency rate

256 (PER) = (FBW - IBW) / protein intake. Gross protein efficiency (GPE) (%) = 100 \* [(%

257 final body protein \* FBW) - (% initial body protein \* IBW)] / total protein intake fish.

258 Gross lipid efficiency (GLE) (%) = 100 \* [(% final body lipid \* FBW) - (% initial body

259 lipid \* IBW)] / total lipid intake fish.

260

### 261 2.9 Analytical methods

262 *2.9.1 X-ray analyses*

263 Radiographic images of pellets and guts were acquired using a high-frequency X-Ray  
264 unit (Raffaello HF/40, ACEM s.p.a, Italy) assembled with the CR system (Carestream  
265 Vita Flex, Carestream Health, Milano, Italy). In order to obtain an adequate display and  
266 radiographic contrast of the ballotini beads, the exposure parameters were set at 45 kV  
267 and 2.5 mAs and 45kV and 4 mAs for feed and guts respectively. The focal distance was  
268 maintained constant (100 cm). Each type of feed, put into a Petri dish, was placed on the  
269 radiographic plate avoiding overlapping of the pellets. Three guts of fish fed the same  
270 feed were then placed on the radiographic plate. Radiographic images were recorded in  
271 DICOM format and transferred to a computer for the ballotini beads count using the  
272 Visiopharm software with app author module (version 2020.09).  
273

274 *2.9.2 Proximate composition*

275 Diets and whole body were analysed for proximate composition. Moisture content was  
276 obtained by weight loss after drying samples in a stove at 105 °C until a constant weight  
277 was achieved. Crude protein was determined as total nitrogen (N) by using the Kjeldahl  
278 method and multiplying N by 6.25. Total lipids were determined according to Bligh and  
279 Dyer's (1959) extraction method. Ash content was estimated by incineration to a constant  
280 weight in a muffle oven at 450 °C.

281

282 *2.10 Statistical analysis*

283 All data are presented as mean  $\pm$  standard deviation (SD). Tank was used as the  
284 experimental unit for analysing growth, digestibility and chewing loss. A pool of five  
285 sampled fish was considered the experimental unit for analysing carcass composition.  
286 Data of growth performance, nutritional indices and digestibility and chewing loss were

287 analysed by a one-way ANOVA. The differences among treatments were considered  
288 significant at  $P \leq 0.05$ , and in this case, Tukey's post hoc test was performed.  
289 Gastrointestinal evacuation data analyses were performed using the function *lm* in R  
290 (version 4.0) for the parameter estimation of the three models (corresponding to the three  
291 gut segments) and the function *uniroot* for computing the corresponding evacuation  
292 times.

293

### 294 **3. Results**

295

#### 296 *3.1 Growth and physical pellet quality*

297 Data on growth performances (final body weight and SGR), FI, FCR at the end of the  
298 trial, are summarised in Table 3. No significant differences were observed in FBW, SGR,  
299 FCR during the overall period ( $P > 0.05$ ) while FI showed a significant difference with  
300 higher values in diets M (4mm) and L (6mm) with respect to diet S (2mm) (Table 3).

301 Data on ballotini beads and feed loss by chewing are shown in Table 3. The feed loss  
302 by chewing was considerably lower in diet S (2mm) compared with diets M (4mm) and  
303 L (6 mm).

304 Also, data on nutritional indices (PER, GPE, GLE) are presented in Table 3. No  
305 significant pellet size effect was observed ( $P > 0.05$ ), however values referred to diet L  
306 (6 mm) are lower compared to values of diets S (2mm) and M (4mm) in the three  
307 nutritional indices examined.

308 Concerning the physical characteristics of feed, water stability and water absorption  
309 index were similar between the three diets. Durability and floating rate displayed the

310 highest values in diet L (6mm) while, in the same diet, oil leaking was the lowest. Bulk  
311 density tended to decrease at the increase of pellet size (Table 2).

312

### 313 *3.2 Digestibility*

314 Data on ADC analysis are shown in Table 4. No significant differences are present in  
315 ADC dry matter and ADC protein calculated ( $P > 0.05$ ). However, the latter showed a  
316 trend of values which decrease from diet S (2mm) to diet L (6mm).

317

### 318 *3.3 Gastrointestinal evacuation rate and time*

319 Table 5 reports the estimated parameters of the shape-rate models. In order to check  
320 the effect of the diet on evacuation time we have considered the rate-varying model where  
321 the rate parameter changes according to the diet. More precisely, Diet M (4mm) is taken  
322 as the reference, and we measure the additional effect on the evacuation rate of Diet L  
323 (6mm) and Diet S (2mm) with respect to Diet M (4mm). The high R squares indicate the  
324 goodness of fit of the shape-rate model to the data. For the stomach tract the estimated  
325 shape parameter (GES) is approximately 0, meaning that the classic exponential Elliott  
326 curve fits the data well. For the other two tracts the patterns have a parabola shape. For  
327 the stomach, the effect of Diet L (6mm) is significantly different from the effect of Diet  
328 M (4mm) taken as reference: -0.029 is an additional effect with respect to M (4mm) and  
329 the GER of L (6mm) can be computed as  $0.148 - 0.029 = 0.119$ . For the foregut, both diet  
330 L (6mm) and S (2mm) are significant, and they cause a slowdown of evacuation time. In  
331 the hindgut there is no significant difference among the three diets, as also confirmed by  
332 the similar estimated evacuation times at 50%, 75% and 90%.

333



#### 334 4. Discussion

335 Feeding sea bream with diets of different pellet size, did not show significant  
336 differences ( $P > 0.05$ ) in growth performance, this indicates that animals between 200 and  
337 450 g can feed properly with pellet sizes ranging from 2 to 6 mm, which is in accordance  
338 to pellet granulometries recommended by the feed industry producers for this fish range  
339 size (Ballester-Moltó et al., 2016). However, it is worth noticing that fish fed with diet S  
340 (2mm) showed a final weight 5.8% lower compared with fish fed on diet M (4mm),  
341 indicating that probably small pellet diameters negatively affected feeding activities  
342 within the feed time administration adopted. It is known that the feed size is important  
343 for influencing its attractiveness, ease of capture and probability of ingestion once  
344 captured (Davis, 2015). The pellet sizes that apparently attract fish more, are not the size  
345 that they ingest most readily once grasped, and it seems to be confirmed by FI values  
346 which are significantly lower for the smallest pellet size diet S (2mm). Probably, this is  
347 because by choosing a larger pellet the fish minimizes predation energy consumption.  
348 Specifically, when smaller pellets are supplied fish need to increase predation activity  
349 (detection, predation, and ingestion) to obtain the same feed ration as when larger pellets  
350 are delivered (Robb and Crampton, 2013; Smith et al., 1995).

351 Gut evacuation has already been studied in Mediterranean fish species, and it is known  
352 it could be affected by several factors, including plant-based dietary ingredients (Bonvini  
353 et al., 2018 ; Adamidou et al., 2009; Zhou et al., 2004); difference in ingredient processing  
354 (Venou et al., 2003); high lipid levels; high starch content (Fountoulaki et al., 2005;  
355 García-Meilán et al., 2014); food type (pellet or natural prey) (Pedro Andrade et al.,  
356 1996); physiological and species-specific factors (stomach physiological properties,  
357 digesta moisture content), (Nikolopoulou et al., 2011 ; Hughes and Barrows, 1990);

358 temperature (Mazumder et al., 2020) ; feeding time and frequency (Gilannejad et al.,  
359 2019). Few studies have focused on the influence that feed size exerts on gut evacuation.  
360 Hossain et al. (2000), stated that small feed particles are evacuated more rapidly than  
361 larger particles, increasing the speed of gut evacuation. This could probably lead to a  
362 reduction of the time needed for the action of digestive enzymes and nutrient absorption,  
363 and consequently a low assimilation efficiency (Azaza et al., 2010). In the present study,  
364 a slower gastric evacuation of L (6mm) diet (21.45 hours) compared to M (4mm) and S  
365 (2mm) diets (respectively 17.23 and 18.38 hours) was found, probably because greater  
366 pellets needed more time for gastric moisturization. Also, no significant differences were  
367 found in growth performance among diets, and this seems to confirm that in sea bream  
368 the gastric activity plays a lower role in digestibility than in the intestine (foregut and  
369 hindgut) (Gilannejad et al., 2020). Also, contrary to what is found in literature, according  
370 to which small pellets have faster gut evacuation times (Azaza et al., 2010; Hossain et al.,  
371 2000), in this study it was observed that diet L (6mm) and diet S (2mm) have a similar  
372 foregut evacuation rate, which was slower compared to that of diet M (4mm). The gastric  
373 evacuation rate recorded in the present study could also be related to sea bream chewing  
374 behaviour. Since we found chewing processing only on diets M (4mm) and L (6mm), it  
375 is possible that this feeding activity influenced on the actual size of the pellets arriving in  
376 the stomach and, consequently, gut evacuation.

377 Data on feed waste, highlighted how loss by chewing on feed eaten is practically  
378 absent for the S (2mm), while the M (4mm) and L (6mm) diets presented more chewed  
379 waste in a similar quantity of feed. In fact, for the diet S (2mm), negative values of %loss  
380 by chewing were found in the three tanks to which the diet was administered. Since the  
381 standard deviation presented an absolute value higher than the mean value, then the %

382 loss by chewing was within a range ( $-3.8 \pm 5.4$ ) which included zero. As zero is one of  
383 the possible loss by chewing values, the value calculated (-3.8) is not to be considered  
384 significantly different from zero. Consequently, it is possible to state that for the S (2mm)  
385 diet the loss by chewing was absent.

386 Previous studies postulated that in general the larger the pellet size, the greater the  
387 resulting waste (Ballester-Moltó et al., 2016), because sea bream has to subject the feed  
388 to a considerable oral manipulation to reduce its size before swallowing, losing feed  
389 fragments in the meantime (Aguado-Giménez, 2020). Our data are in agreement with  
390 Ballester-Moltó et al. (2016), who in supporting the aforementioned thesis identified in  
391 their results that fish with an average weight greater than 300 grams produce more chewed  
392 waste if fed with 4mm than 6 mm pellets, while this does not happen with smaller fish  
393 and/or smaller pellet size. One possibility is that the mouth apparatus of fish of this size  
394 is able to greatly reduce the size of the 6mm pellets and completely break up the 4 mm  
395 pellets, losing many fragments during the chewing process. As postulated by Andrew et  
396 al. (2003), the larger the fish, the longer the manipulation and the more effective the  
397 mastication, which results in greater feed wastes. Data of dentition analysis recently  
398 conducted on seabream, showed that large pellet size tended to produce fish with the  
399 lowest number of teeth on the dentary, while specimens fed with small pellet size  
400 presented the smallest teeth area. However, no significant differences were found in  
401 general dentition among fish fed with the three different pellet sizes (de Azevedo et al.,  
402 2021).

403 It could also be taken into account that differences in physical quality characteristics  
404 related to pellet size (i.e., density, durability) could have exerted an effect on chewing

405 activity. In particular, a lower density in larger pellets could be one of the factors that  
406 makes these diets more prone to breakage and therefore easier to chew.

407

## 408 **5. Conclusion**

409 In conclusion, no significant effects of different pellet size on growth (final body  
410 weight and SGR) were observed. Pellets diameters had no effects on feed digestibility  
411 and feed efficiency parameters (FCR, PER, GPE, GLE) even if differences in the gastric  
412 evacuation rate were detected at different pellet size. At this regard, the shape-rate model  
413 developed to estimate the gastrointestinal evacuation pattern evidenced a slower gastric  
414 evacuation rate in the 6 mm diet, while no differences in foregut evacuation rate were  
415 observed. Data on feed waste, highlighted how feed losses by chewing was absent in the  
416 S (2mm) diet while in the M (4mm) and L (6mm) diets 24.3 and 17.3 % of the entire meal  
417 was losses by chewing activity, respectively. The study reinforces previous observation  
418 that feeding pellets size of 4 and 6 mm in gilthead sea bream within 200-450g could  
419 induced an excess of feed waste by chewing activity with economic and environmental  
420 implication. Despite the reduced feed intake observed, pellets size of 2 mm did not lead  
421 to any feed losses by chewing and was able to guarantee similar growth compared to the  
422 other diets. Further studies considering intermediate pellets size (3 mm) maybe useful in  
423 order to further optimize the pellet size choice during the on-growing phase of this  
424 species.

425

## 426 **Declaration of Competing Interest**

427 The authors declare that they have no known competing financial interests or personal  
428 relationships that could have appeared to influence the work reported in this paper entitled

429 “The incidence of different pellet size on growth, gut evacuation, feed digestibility and  
430 feed waste in gilthead sea bream (*Sparus aurata*)”.

431

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593

**Table 1.** Ingredients of the diets with and without ballotini beads, and proximate composition of all the experimental diets

<i>Ingredient, % of the diets with and without ballotini</i>						
Wheat	12.85	12.85				
Corn gluten	4.93	4.93				
Soy bean meal	18.21	18.21				
Wheat gluten	5.50	5.50				
Soya protein concentrate	18.77	18.77				
Fish meal	20.00	20.00				
Rapeseed oil	9.72	9.72				
Fishoil	9.72	9.72				
Min Premix <sup>1</sup>	0.10	0.10				
Vit premix <sup>1</sup>	0.11	0.11				
Ytrium oxide	0.10	0.10				
Ballottini beads	0.00	0.20				
<i>Proximate composition, % on a wet weight basis</i>	S (2mm)	Sb	M (4mm)	Mb	L (6mm)	Lb
Moisture	6.04	7.16	6.33	7.34	6.09	7.04
Protein	43.6	43.7	44.4	44.6	44.0	44.7
Lipid	23.3	22.7	23.3	20.4	23.2	20.9
Ash	6.17	6.87	6.13	6.79	6.62	7.16

<sup>1</sup>Vitamin and mineral premix; Skretting, Stavanger, Norway (fulfilling recommendations for marine fish species given by NRC, 2011)  
Sb= Diet S (2mm) with 0.2 % of ballotini glass beads inclusion; Mb= Diet M (4mm) with 0.2 % of ballotini glass beads inclusion; Lb= Diet L (6mm) with 0.2 % of ballotini glass beads inclusion.

**Table 2.** Physical pellet quality characteristics of the three experimental diets.

		<i>Experimental Diets</i>		
		S (2mm)	M (4mm)	L (6mm)
Bulk density (g/L)		660	630	610
Durability (%)	Broken	2	1.3	2.1
	Dust	1.1	0.9	3.3
	Total breakage	3.1	2.2	5.4
Oil leaking (%)		2.28	0.95	0.56
Water stability (%)		9	9	12
Floating rate (%)		13	18	73.5
Water absorption index (%)	2h	95.8	110.2	129.4
	4h	101.5	103.2	112.4
	6h	121	117.3	122.3
	8h	131.9	131.3	135.8
	16h	129.3	142.9	162.4
	18h	136.5	122.2	133.2
	20h	145	133.2	131
	22h	126.5	150.8	153.6
	24h	143.1	166.2	145.3
	24+h	154.6	157.4	125.1

Bulk density: the mass of particles of a granular material divided by the total volume they occupy (g/L).

Durability: the mechanical stress resistance of a feed sample.

Oil leaking: the extent of oil leakage from each of the feeds.

Water stability: (weight of retained whole pellets/initial total weight of pellets) \*100.

Floating rate: the percentage of buoyancy.

Water absorption index: the volume occupied by a granular material after swelling in excess of water.

**Table 3.** Growth performance, nutritional indices of sea bream fed experimental diet over 122 days, and feed chewed estimation.

<i>Diet</i>	S (2mm)	M (4mm)	L (6mm)	P value
IBW (g)	216.4±3.3	216.1 ± 0.9	215.1 ± 0.8	0.744
InBW(g)	326.78±18.95	325.61±21.08	315.91±6.49	0.28
FBW (g)	438.9±14.6	465.9 ± 19.7	443.0 ± 19.1	0.217
SGR	0.57±0.02	0.63 ± 0.03	0.59 ± 0.03	0.213
FI	0.90±0.03a	0.98±0.01b	1.01±0.02b	0.002
<i>% loss by chewing</i>	-3.8±5.4a	24.3±9.5b	17.3±5.5b	0.006
FCR	1.63±0.06	1.65±0.09	1.81±0.16	0.159
PER	1.42±0.06	1.40±0.07	1.28±0.09	0.172
GPE	26.32±1.67	25.27±1.18	23.93±1.83	0.255
GLE	51.84±2.13	51.55±4.11	46.30±4.29	0.190

Data are given as the mean (n=3) ± SD. Different superscript letters indicate significant differences among treatments (One-way Anova  $p \leq 0.05$ ).

IBW = Initial body weight.

FBW = Final body weight.

InBW= Intermediate body weight for ballotini calculation

SGR = Specific growth rate (% day<sup>-1</sup>) =  $100 \times (\ln \text{FBW} - \ln \text{IBW}) / \text{days}$ .

FI = Feed intake (% ABW<sup>-1</sup> day<sup>-1</sup>) =  $((100 \times \text{total ingestion}) / (\text{ABW})) / \text{days}$ .

FCR = Feed conversion rate = feed intake / weight gain.

PER = Protein efficiency ratio =  $((\text{FBW} - \text{IBW}) / \text{protein intake})$ .

GPE (%) Gross protein efficiency =  $100 \times [(\% \text{ final body protein} \times \text{FBW}) - (\% \text{ initial body protein} \times \text{IBW})] / \text{total protein intake fish}$ .

GLE (%) Gross lipid efficiency =  $100 \times [(\% \text{ final body lipid} \times \text{FBW}) - (\% \text{ initial body lipid} \times \text{IBW})] / \text{total lipid intake fish}$ .

% loss by chewing on feed eaten: feed chewed, g / feed eaten estimated, g %.

Feed chewed (g): (feed administered, g – feed left, g - feed ingested from ballotini calculation, g).

Feed eaten estimated (g): (feed administered, g – feed left, g).

**Table 4.** Feed digestibility of gilthead sea bream fed diets with three different pellet sizes.

Diet	S (2mm)	M (4mm)	L (6mm)	<i>P</i> value
Dry matter	95.0 ± 0.6	95.8 ± 0.5	95.6 ± 0.7	0.253
Protein	83.3 ± 3.7	80.9 ± 3.7	79.7 ± 4.4	0.543

Data are given as the mean (n = 3) ± SD. No significant differences among treatments (One-way Anova  $p > 0.05$ ).

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**Table 5.** Estimated parameters in the different gastric traits for each experimental diet. GER, FER and HER denote the rates and GES, FES and HES the estimated shapes. The evacuation times (in hours) are estimated for  $p=50\%$ ,  $75\%$  and  $90\%$ . In brackets standard errors are reported.

	<i>Experimental diets</i>		
	S (2mm)	M (4mm)	L (6mm)
<b>Stomach</b>			
GER		0.148 (0.019)a	
GER additive effect	-0.009 (0.013)		-0.029 (0.012)b
GES	0.051 (0.108)	0.051 (0.108)	0.051 (0.108)
GET 50% (h)	6.41	6.01	7.48
GET 75% (h)	11.62	10.89	13.56
GET 90% (h)	18.38	17.23	21.45
R2	0.93	0.93	0.93
<b>Foregut</b>			
FER		0.312 (0.033)a	
FER additive effect	-0.053 (0.022)b		-0.091 (0.022)a
FES	0.503 (0.188)b	0.503 (0.188)b	0.503 (0.188)b
FET 50% (h)	7.12	5.91	8.33
FET 75% (h)	10.54	8.76	12.34
FET 90% (h)	14.71	12.22	17.22
R2	0.92	0.92	0.92
<b>Hindgut</b>			
HER		0.171 (0.025)a	
HER additive effect	-0.014 (0.016)		-0.014 (0.016)
HES	0.296 (0.139)c	0.296 (0.139)c	0.296 (0.139)c
HET 50% (h)	7.92	8.57	9.34
HET 75% (h)	12.4	13.41	14.61
HET 90% (h)	17.96	19.42	21.16
R2	0.89	0.89	0.89

Superscript letters indicate significant difference.

Significance levels: 0.01 'a' 0.05 'b' 0.1 'c'

GER= gastric evacuation rate; FER= foregut evacuation rate; HER= hindgut evacuation rate.

GES= gastric evacuation shape; FES= foregut evacuation shape; HES= hindgut evacuation shape.

GET= gastric evacuation time; FET= foregut evacuation time; HET= hindgut evacuation time.



614 **Figure captions**

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616 **Figure 1 a-c.** Calibration straight lines for each pellet size at 0.2% ballotini beads  
617 concentration. The straight lines indicate the quantity of ballottini contained in a very  
618 specific quantity of feed, from 1 to 15 grams.

619 **Figure 2.** Possible patterns as the shape parameter (s) varies and rate parameter is  $r=2$ .

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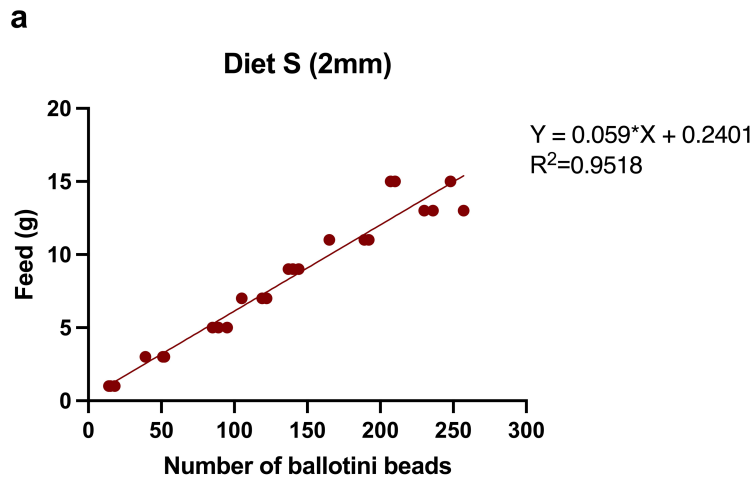
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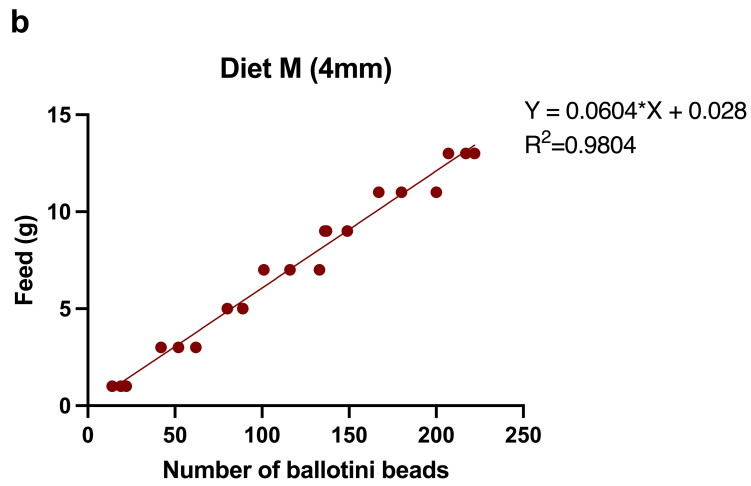
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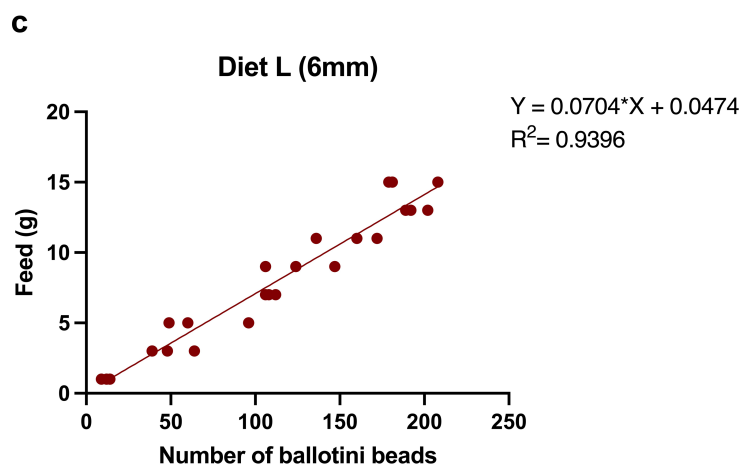
638 **Figure 1**



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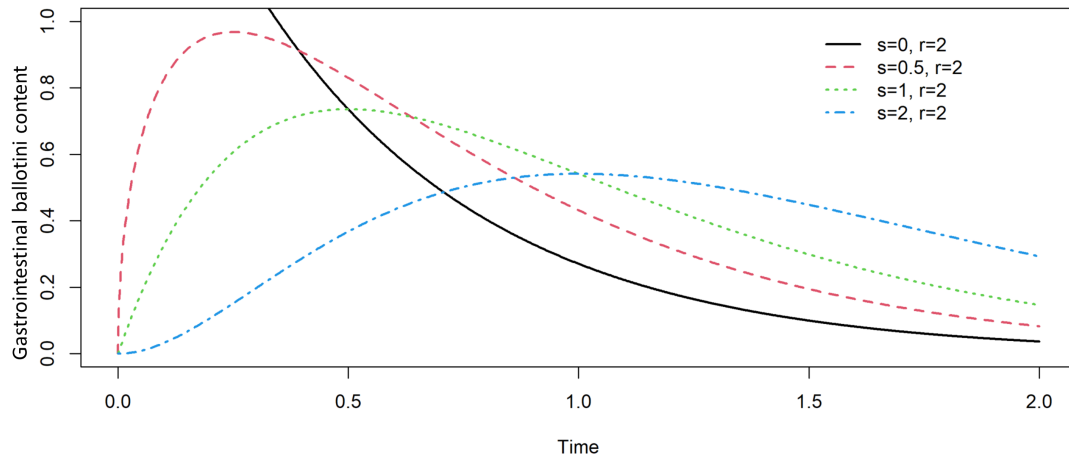


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644 **Figure 2**



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