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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 ± 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⁻¹ 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 ± 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 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 ± 1.0 °C during the entire trial, salinity (25 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⁻¹ 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°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 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°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⁻¹. 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 $\text{GET } p\% = [\ln 100 - \ln (100-p)] / r$.

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⁻¹) = 100 * (ln FBW - ln IBW) / days (where FBW and

253 IBW represent the final and the initial body weights). Feed intake (FI) (% ABW⁻¹ day⁻¹)

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 ± 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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435

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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 ¹	0.10	0.10				
Vit premix ¹	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

¹Vitamin 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⁻¹) = $100 \times (\ln \text{FBW} - \ln \text{IBW}) / \text{days}$.

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

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)
Stomach			
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
Foregut			
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
Hindgut			
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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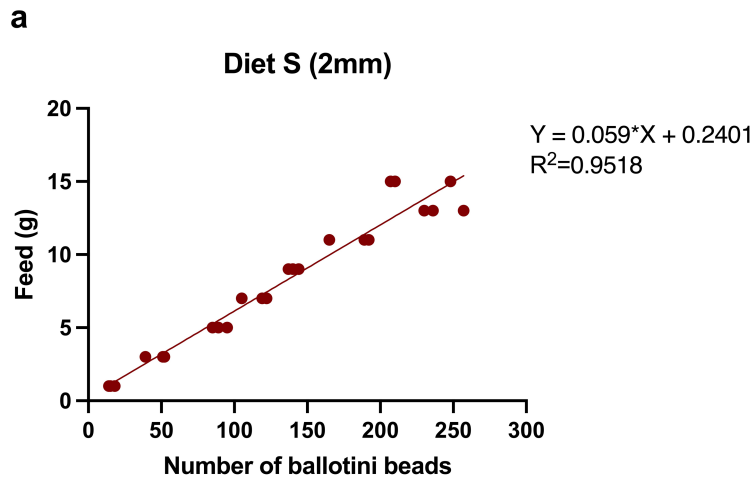
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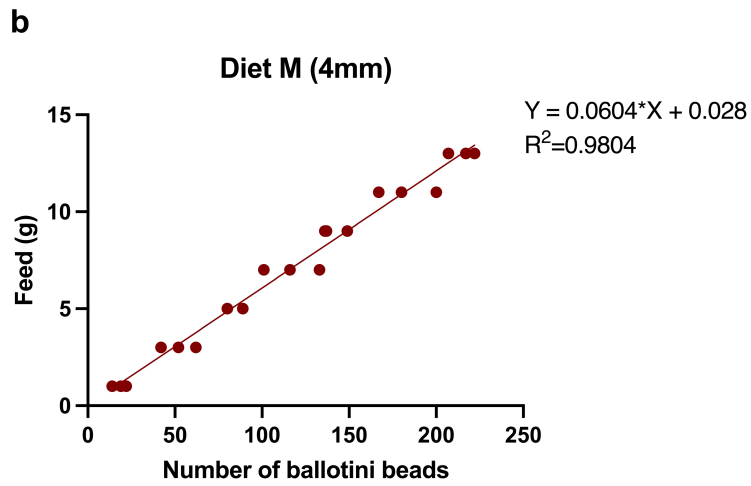
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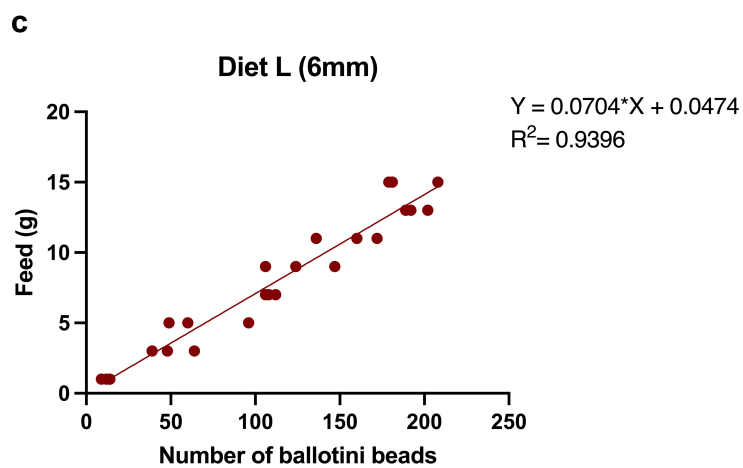
638 **Figure 1**



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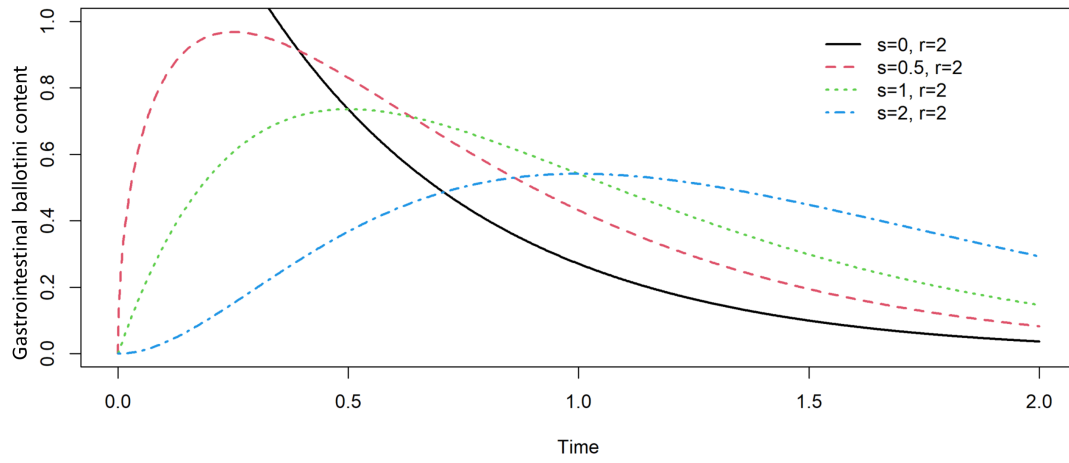


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644 **Figure 2**



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