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

(Article begins on next page)

1   **The incidence of different pellet size on growth, gut evacuation, feed digestibility**

2   **and feed waste in gilthead sea bream (*Sparus aurata*)**

3   Serena Busti[a], Alessio Bonaldo[a], Alessia Diana[a], Simone Perfetti[a], Cinzia Viroli[b], Ramon

4   Fontanillas[c], Tommy Berger Eriksen[c], Pier Paolo Gatta[a], Luca Parma[a*]

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7   [a]Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra

8   50, 40064 Ozzano Emilia, Bologna, Italy

9   [b] Department of Statistical Sciences "Paolo Fortunati", University of Bologna, Via delle

10   Belle Arti 41, 40126 Bologna, Italy

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

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14   *Corresponding author:* Luca Parma, Department of Veterinary Medical Sciences,

15   University of Bologna, Viale Vespucci 2, 47042 Cesenatico, FC, Italy.

16   E-mail address: luca.parma@unibo.it

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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1. Introduction

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

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

73   2018). Few studies tried to calculate feed loss in sea bream caused by chewing. Among

74   these, Ballester-Moltó et al. (2016) quantified the loss rate by mean of using mesh screen

75   and taking into account full pellets disaggregation and leaching. The author found that

76   feed loss increases with the increase not only of feed size but also of fish size. They

77   hypothesized that although several studies stated that the appropriate pellet size should

78   represent from 25% to 50% of the fish mouth amplitude (Linnér and Brännäs, 1994;

79   Smith et al., 1995), alternative feeding regimes in which even large fish are fed with

80   small- size feeds (e.g. 2 mm) could help to reduce feed waste. Pellet size could also play

81   a significant role in gut evacuation potentially affecting feed efficiency and growth

82   performance (Aguado-Giménez, 2020; Andrew et al., 2004a; Ballester-Moltó et al., 2016;

83   Mazumder et al., 2020). Ballotini beads (inert metal powders incorporated in feed) have

84   been used as a feed marker to estimate feed intake (FI) and to study the trophic and

85   behavioural dynamics of fish (Andrew et al., 2004b; Talbot and Higgins, 1983).

86   Moreover, it could become a valid tool for obtaining a precise quantification of feed waste

87   relative to different pellet sizes. The aims of this work are i. to study the effects of 3

88   different pellet sizes 2mm, 4mm, and 6mm, on growth, feed efficiency, and gut

89   evacuation during the on-growing stage of gilthead sea bream; ii. to quantify the

90   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 85ºC 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 ± 1.0 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 ≤0.1 mg L^{-1}) and nitrite ($NO_2^- \leq 0.2$ 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 ADC = 100*(1− (dietary Y2O2 level/ faecalY2O2 level)) *((faecal nutrient or energy

171 level/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 ballotini 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   ballotini beads. The number of ballotini beads was used in the equations of calibration

207   straight lines to quantify the amount of feed lost by chewing via the formula:

208   *% loss by chewing on feed eaten* = feed chewed, g /feed eaten, g %

209   where feed chewed (g) is calculated as: (feed administered, g – feed left, g - feed ingested

210   estimated from ballotini 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     $$W_t = A\,e^{-rt}\,t^s \quad \text{or} \quad \ln W_t = \ln A - rt + s \ln t$$

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
$$[\,\ln 100 - \ln (100\text{-}p)\,] = rt - s - s \ln (s/rt).$$

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 segement. 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 ± 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

4. Discussion

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

Gut evacuation has already been studied in Mediterranean fish species, and it is known it could be affected by several factors, including plant-based dietary ingredients (Bonvini et al., 2018 ; Adamidou et al., 2009; Zhou et al., 2004); difference in ingredient processing (Venou et al., 2003); high lipid levels; high starch content (Fountoulaki et al., 2005; García-Meilán et al., 2014); food type (pellet or natural prey) (Pedro Andrade et al., 1996); physiological and species-specific factors (stomach physiological properties, 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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433

435

436 **References**

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

Ingredient, % of the diets with and without ballotini		
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

Proximate composition, % on a wet weight basis	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

[1]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.

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

		Experimental Diets		
		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 absorbtion 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.

Diet	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
% loss by chewing	-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 × (ln FBW- ln IBW) / days.

FI = Feed intake (% ABW^{-1} day^{-1}) = ((100*total ingestion)/(ABW))/days)).

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

PER = Protein efficiency ratio = ((FBW-IBW)/protein intake).

GPE (%) Gross protein efficiency = 100 × [(% final body protein × FBW) − (% initial body protein × IBW)]/total protein intake fish.

GLE (%) Gross lipid efficiency = 100 × [(% final body lipid × FBW) − (% initial body lipid × IBW)]/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).

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

Diet	S (2mm)	M (4mm)	L (6mm)	*P* 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 $(n = 3) \pm$ 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.

	Experimental diets		
	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.
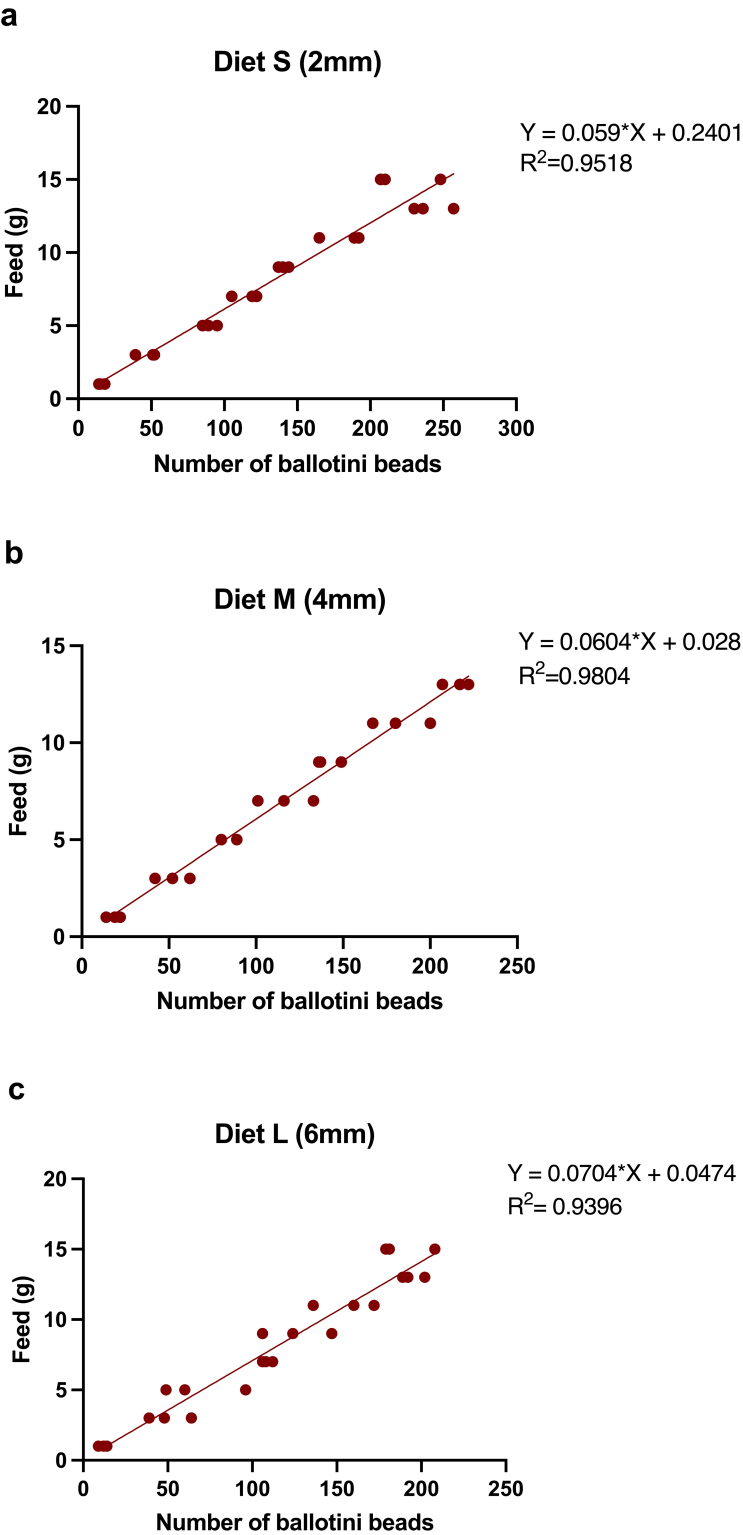GET= gastric evacuation time; FET= foregut evacuation time; HET= hindgut evacuation time.
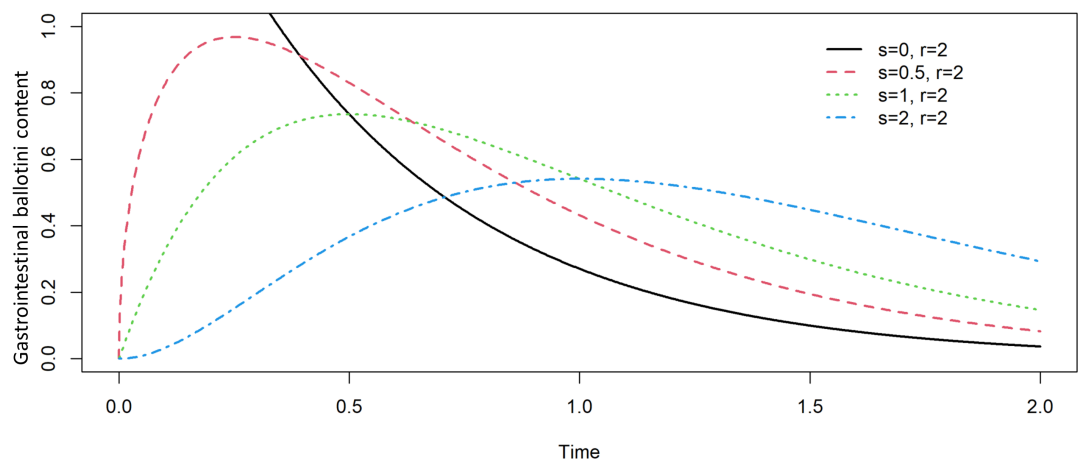
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614 **Figure captions**

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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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638   **Figure 1**

a

Diet S (2mm)

$Y = 0.059*X + 0.2401$
$R^2=0.9518$

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b

Diet M (4mm)

$Y = 0.0604*X + 0.028$
$R^2=0.9804$

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c

Diet L (6mm)

$Y = 0.0704*X + 0.0474$
$R^2= 0.9396$

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644 **Figure 2**

645