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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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**Abstract**

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

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

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

## 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<sup>-1</sup> 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.,

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.

## **2. Materials and methods**

### *2.1 Experimental Diet*

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

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

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

## *2.2 Feed Calibration straight lines*

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

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

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

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

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

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

### *2.3 Fish and feeding trial*

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



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

#### *2.4 Sampling for growth parameters*

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

#### *2.5 Digestibility experiment*

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

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

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

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

## *2.6 Gastrointestinal evacuation experiment*

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

## *2.7 Estimation of feed loss by chewing*

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

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

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

## 2.8 Calculations

### 2.8.1 Gastrointestinal evacuation pattern calculation

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

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

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

When  $s=0$  the model coincides with the Elliott exponential curve; for  $s>0$  the curve can take different shapes, as shown in Figure 2. This shape-rate curve is essentially equivalent to fitting a Gamma probabilistic model on the normalized ballotini content as a function 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

### 2.9.1 X-ray analyses

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

### 2.9.2 Proximate composition

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

### 2.10 Statistical analysis

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

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

### 3. Results

#### 3.1 Growth and physical pellet quality

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

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

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

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

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

### 3.2 Digestibility

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

### 3.3 Gastrointestinal evacuation rate and time

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



#### 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);

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

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

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

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

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

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

## **5. Conclusion**

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

## **Declaration of Competing Interest**

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

“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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**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				
Yttrium 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
% 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<sup>-1</sup>) =  $100 \times (\ln \text{FBW} - \ln \text{IBW}) / \text{days}$ .

FI = Feed intake (% ABW-1 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.

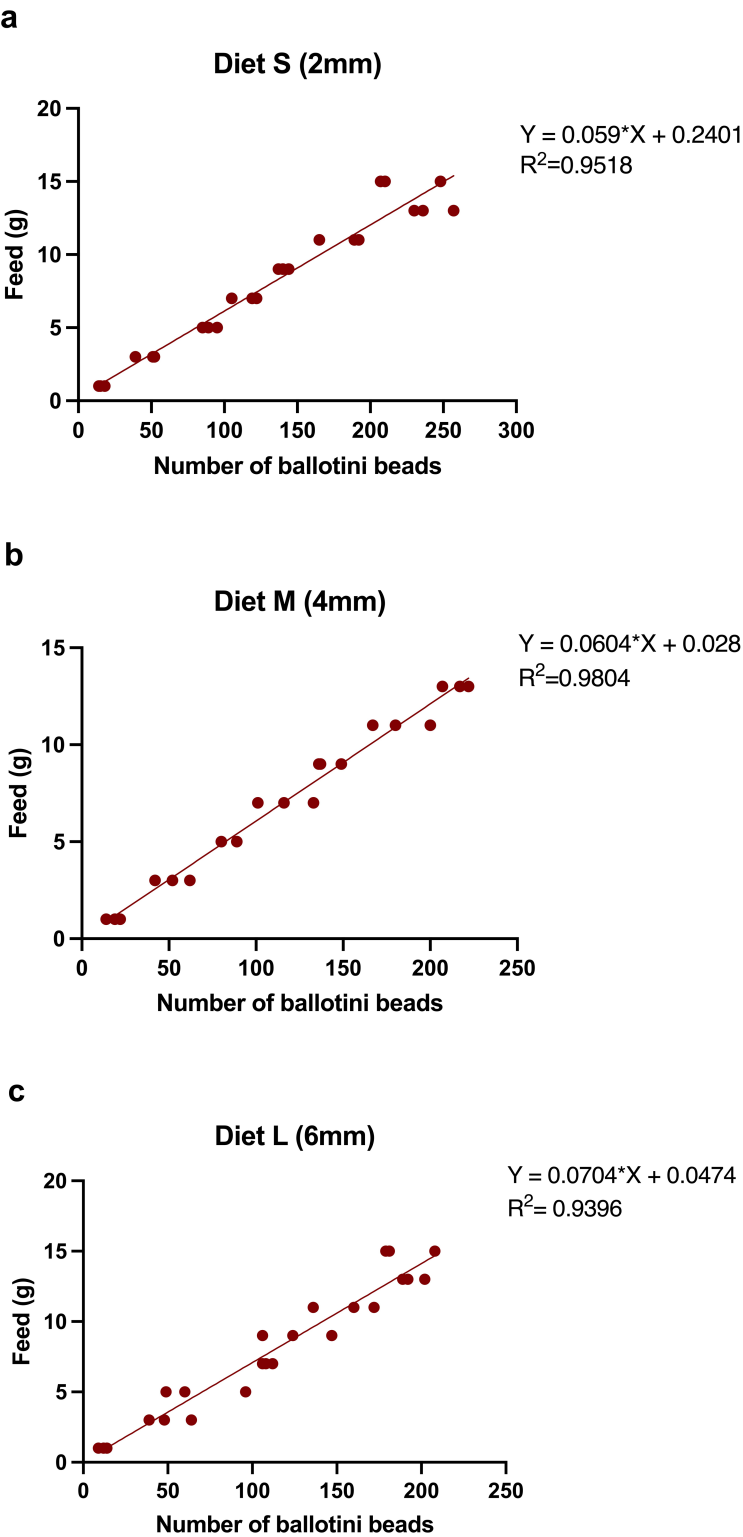


**Figure captions**

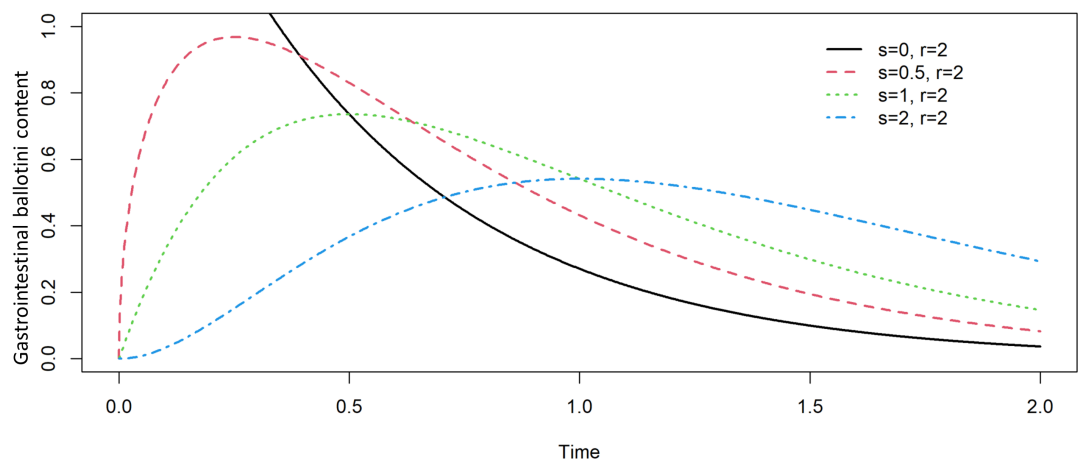
**Figure 1 a-c.** Calibration straight lines for each pellet size at 0.2% ballotini beads concentration. The straight lines indicate the quantity of ballottini contained in a very specific quantity of feed, from 1 to 15 grams.

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

638     **Figure 1**



644 **Figure 2**



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