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

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 ± 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^{-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.,

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

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

(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 ± 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 m^3, RAS utilized and water flow rate according to Busti et al. (2020). The oxygen level was maintained at 8.0 ± 1.0 mg L^{-1} through a liquid oxygen system connected to a software controller (B&G Sinergia snc, Chioggia, Italy); temperature was kept at 24 ± 1.0 °C during the entire trial, salinity (25 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 ≤0.1 mg L^{-1}) and nitrite (NO_2^- ≤ 0.2 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.

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

per tank) and immediately kept at −20 °C until analysis (Busti et al., 2020). ADC was calculated as follows:

ADC = 100*(1− (dietary Y2O2 level/ faecalY2O2 level)) *((faecal nutrient or energy level/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 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°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 ballotini 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^{-1}. 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 ballotini beads. The number of ballotini beads was used in the equations of calibration straight lines to quantify the amount of feed lost by chewing via the formula:

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

where feed chewed (g) is calculated as: (feed administered, g – feed left, g - feed ingested estimated from ballotini 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 lnWt= lnA - rtwhere $A=W_0$ 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:

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

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

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

represents the ballotini content. In other term, the time of maximum ballottini content is $t=s/r$. Since $\ln W_0 - \ln W_t = rt - s - s \ln (s/rt)$, the GET of this model can be estimated by solving the nonlinear equation as function of t:

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

In order to check the effect of the diet on evacuation time, the model has been applied to each gut segment separately on the weights of the sea breams distinguished by diets. Let's W_{ti} the weight means of all fishes at time t and for diet i, with i=1,2,3, corresponding to the three diets S (2mm), M (4mm) and L (6mm) and for a specific gut segement. The model with rate-varying parameter is $\ln W_{ti} = \ln A - r_i\, t + s \ln t$.

2.8.2 *Performance parameters calculation*

The formulae employed were as follows:

Specific growth rate (SGR) (% day^{-1}) = 100 * (ln FBW- ln IBW) / days (where FBW and IBW represent the final and the initial body weights). Feed intake (FI) (% ABW^{-1} day^{-1}) = ((100 ∗ total ingestion)/(ABW))/days)) (where average body weight, ABW = (IBW + FBW)/2; Feed conversion ratio (FCR) = feed intake / weight gain. Protein efficiency rate (PER) = (FBW – IBW) / protein intake. Gross protein efficiency (GPE) (%) = 100 * [(% final body protein * FBW) - (% initial body protein * IBW)] / total protein intake fish. Gross lipid efficiency (GLE) (%) = 100 * [(% final body lipid * FBW) - (% initial body lipid * IBW)] / total lipid intake fish.

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 ± 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

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

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);

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 %

loss by chewing was within a range (-3.8 ± 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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References

Aas, T.S., Oehme, M., Sørensen, M., He, G., Lygren, I., Åsgård, T., 2011. Analysis of pellet degradation of extruded high energy fish feeds with different physical qualities in a pneumatic feeding system. Aquacultural Engineering, 44 (1), 25-34. https://doi.org/10.1016/j.aquaeng.2010.11.002.

Adamidou, S., Nengas, I., Alexis, M., Foundoulaki, E., Nikolopoulou, D., Campbell, P., Karacostas, I., Rigos, G., Bell, G.J., Jauncey, K., 2009. Apparent nutrient digestibility and gastrointestinal evacuation time in European seabass (Dicentrarchus labrax) fed diets containing different levels of legumes. Aquaculture 289, 106–112. https://doi.org/10.1016/j.aquaculture.2009.01.015

Aguado-Giménez, F., 2020. Effect of Feed Delivery Rate and Pellet Size on Rearing Performance, Feed Wastage and Economic Profitability in Gilthead Seabream (Sparus Aurata) Ongrowing. Water 12, 954. https://doi.org/10.3390/w12040954

Alcaraz, R., Hernandéz-Contreras, A., Iglesias, P., Hernandéz, M.D., 2021. Effect of the inclusion of microalgae on the physical properties of extruded feed for gilthead seabream (Sparus aurata L.) Algal Research, 53, 102167. https://doi.org/10.1016/j.algal.2020.102167.

453 Andrew, J.E., Anras, M.L.B., Kadri, S., Holm, J., Huntingford, F.A., 2003. Feeding
454 Responses of Hatchery-Reared Gilthead Sea Bream (Sparus aurata L.) to a
455 Commercial Diet and Natural Prey Items. null 36, 77–86.
456 https://doi.org/10.1080/1023624031000109864
457 Andrew, J.E., Holm, J., Huntingford, F.A., 2004a. The effect of pellet texture on the
458 feeding behaviour of gilthead sea bream (Sparus aurata L.). Aquaculture 232,
459 471–479. https://doi.org/10.1016/S0044-8486(03)00490-3
460 Andrew, J.E., Holm, J., Kadri, S., Huntingford, F.A., 2004b. The effect of competition
461 on the feeding efficiency and feed handling behaviour in gilthead sea bream
462 (Sparus aurata L.) held in tanks. Aquaculture 232, 317–331.
463 https://doi.org/10.1016/S0044-8486(03)00528-3
464 Azaza, M.S., Dhraief, M.N., Kraiem, M.M., Baras, E., 2010. Influences of food particle
465 size on growth, size heterogeneity, food intake and gastric evacuation in juvenile
466 Nile tilapia, Oreochromis niloticus, L., 1758. Aquaculture 309, 193–202.
467 https://doi.org/10.1016/j.aquaculture.2010.09.026
468 Ballester-Moltó, M., Sanchez-Jerez, P., Cerezo-Valverde, J., Aguado-Giménez, F., 2017.
469 Particulate waste outflow from fish-farming cages. How much is uneaten feed?
470 Marine Pollution Bulletin 119, 23–30.
471 https://doi.org/10.1016/j.marpolbul.2017.03.004
472 Ballester-Moltó, M., Sanchez-Jerez, P., García-García, B., García-García, J., Cerezo-
473 Valverde, J., Aguado-Giménez, F., 2016. Controlling feed losses by chewing in
474 gilthead sea bream (Sparus aurata) ongrowing may improve the fish farming
475 environmental sustainability. Aquaculture 464, 111–116.
476 https://doi.org/10.1016/j.aquaculture.2016.06.018

Bligh, E.G., Dyer, W.J., 1959. A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. Can. J. Biochem. Physiol. 37, 911–917. https://doi.org/10.1139/o59-099

Bonvini, E., Bonaldo, A., Parma, L., Mandrioli, L., Sirri, R., Grandi, M., Fontanillas, R., Viroli, C., Gatta, P.P., 2018. Feeding European sea bass with increasing dietary fibre levels: Impact on growth, blood biochemistry, gut histology, gut evacuation. Aquaculture 494, 1–9. https://doi.org/10.1016/j.aquaculture.2018.05.017

Busti, S., Bonaldo, A., Dondi, F., Cavallini, D., Yúfera, M., Gilannejad, N., Moyano, F.J., Gatta, P.P., Parma, L., 2020. Effects of different feeding frequencies on growth, feed utilisation, digestive enzyme activities and plasma biochemistry of gilthead sea bream (Sparus aurata) fed with different fishmeal and fish oil dietary levels. Aquaculture 529, 735616. https://doi.org/10.1016/j.aquaculture.2020.735616

Cho, C.Y., Bureau, D.P., 2001. A review of diet formulation strategies and feeding systems to reduce excretory and feed wastes in aquaculture. Aquaculture Research 32, 349–360. https://doi.org/10.1046/j.1355-557x.2001.00027.x

Davis, D. A., editor. Feed and feeding practices in aquaculture. Woodhead Publishing; 2015 May 12.

de Azevedo, A.M., Fontanillas, R., Owen, M.A.G., Busti, S., Parma, L., Bonaldo, A., Witten, P.E., Huysseune, A., 2021. A quantitative analysis of gilthead seabream (Sparus aurata) juvenile dentition as a tool to assess the effect of diet. Can. J. Zool. 99, 548–556. https://doi.org/10.1139/cjz-2020-0284

Elliot, J.M., 1972. Rates of gastric evacuation in brown trout, Salmo trutta L. Freshwater Biology 2, 1–18. https://doi.org/10.1111/j.1365-2427.1972.tb01575.x

500 Fountoulaki, E., Alexis, M.N., Nengas, I., Venou, B., 2005. Effect of diet composition on
501 nutrient digestibility and digestive enzyme levels of gilthead sea bream (Sparus
502 aurata L.). Aquac Research 36, 1243–1251. https://doi.org/10.1111/j.1365-
503 2109.2005.01232.x
504 García-Meilán, I., Ordóñez-Grande, B., Gallardo, M.A., 2014. Meal timing affects
505 protein-sparing effect by carbohydrates in sea bream: Effects on digestive and
506 absorptive processes. Aquaculture 434, 121–128.
507 https://doi.org/10.1016/j.aquaculture.2014.08.005
508 Gilannejad, N., Moyano, F., Martínez-Rodríguez, G., Yufera, M., 2020. The digestive
509 function of gilthead seabream juveniles in relation to feeding frequency.
510 Aquaculture 531. https://doi.org/10.1016/j.aquaculture.2020.735867
511 Gilannejad, N., Silva, T., Martínez-Rodríguez, G., Yúfera, M., 2019. Effect of feeding
512 time and frequency on gut transit and feed digestibility in two fish species with
513 different feeding behaviours, gilthead seabream and Senegalese sole. Aquaculture
514 513, 734438. https://doi.org/10.1016/j.aquaculture.2019.734438
515 Hossain, Haylor, Beveridge, 2000. The influence of food particle size on gastric emptying
516 and growth rates of fingerling African catfish, Clarias gariepinus Burchell, 1822.
517 Aquaculture Nutrition 6, 73–76. https://doi.org/10.1046/j.1365-
518 2095.2000.00131.x
519 Hughes, S.G., Barrows, R., 1990. Measurements of the abilities of cultured fishes to
520 moisturize their digesta. Comparative Biochemistry and Physiology Part A:
521 Physiology 96, 109–111. https://doi.org/10.1016/0300-9629(90)90049-X
522 Irungu, F.G., Mutungi, C.M., Faraj, A.K., Affognon, H., Kibet, N., Tanga, C., Ekesi, S.,
523 Nakimbugwe, D., Fiaboe K.K.M., 2018. Physico-chemical properties of extruded

524 aquafeed pellets containing black soldier fly (Hermetia illucens) larvae and adult
525 cricket (Acheta domesticus) meals. Journal of Insects as Food and Feed 4(1), 19-
526 30. https://doi.org/10.3920/JIFF2017.0008.
527 Khater, E.G., Bahnasawy, A.H., Ali, S.A., 2014. Physical and Mechanical Properties of
528 Fish Feed Pellets. Food Processing & Technology 5, 378. https://doi.org/
529 10.4172/2157-7110.1000378
530 Linnér, J., Brännäs, E., 1994. Behavioral Response to Commercial Food of Different
531 Sizes and Self-Initiated Food Size Selection by Arctic Char. null 123, 416–422.
532 https://doi.org/10.1577/1548-8659(1994)123<0416:BRTCFO>2.3.CO;2
533 Mazumder, S.K., Ghaffar, M.A., Das, S.K., 2020. Effect of temperature and diet on
534 gastrointestinal evacuation of juvenile malabar blood snapper (Lutjanus
535 malabaricus Bloch & Schneider, 1801). Aquaculture 522, 735114.
536 https://doi.org/10.1016/j.aquaculture.2020.735114
537 Nikolopoulou, D., Moutou, K.A., Fountoulaki, E., Venou, B., Adamidou, S., Alexis,
538 M.N., 2011. Patterns of gastric evacuation, digesta characteristics and pH changes
539 along the gastrointestinal tract of gilthead sea bream (Sparus aurata L.) and
540 European sea bass (Dicentrarchus labrax L.). Comparative Biochemistry and
541 Physiology Part A: Molecular & Integrative Physiology 158, 406–414.
542 https://doi.org/10.1016/j.cbpa.2010.11.021
543 Pedro Andrade, J., Erzini, K., Palma, J., 1996. Gastric evacuation and feeding in the
544 gilthead sea bream reared under semi-intensive conditions. Aquacult Int 4, 129–
545 141. https://doi.org/10.1007/BF00140594
546 Piedecausa, M.A., Aguado-Giménez, F., García-García, B., Ballester, G., Telfer, T.,
547 2009. Settling velocity and total ammonia nitrogen leaching from commercial

548 feed and faecal pellets of gilthead seabream (Sparus aurata L. 1758) and seabass
549 (Dicentrarchus labrax L. 1758). Aquaculture Research 40, 1703–1714.
550 https://doi.org/10.1111/j.1365-2109.2009.02272.x
551 Robb, D.H.F., Crampton, V.O., 2013. On-farm feeding and feed management:
552 perspectives from the fish feed industry. Presented at the FAO Fisheries and
553 Aquaculture Technical Paper, Food and Agriculture Organization of the United
554 Nations (FAO), Rome, pp. 489–518.
555 Rosentrater, K.A., Muthukumarappan, K. and Kannadhason, S., 2009. Effects of
556 ingredients and extrusion parameters on aqua feeds containing DDGS and potato
557 starch. Journal of Aquaculture Feed Science and Nutrition 1, 22-38.
558 https://medwelljournals.com/abstract/?doi=joafsnu.2009.22.38
559 Smith, I.P., Metcalfe, N.B., Huntingford, F.A., 1995. The effects of food pellet
560 dimensions on feeding responses by Atlantic salmon (Salmo salar L.) in a marine
561 net pen. Aquaculture 130, 167–175. https://doi.org/10.1016/0044-
562 8486(94)00207-5
563 Sørensen, M., Morken, T., Kosanovic, M., Øverland, M., 2011. Pea and wheat starch
564 possess different processing characteristics and affect physical quality and
565 viscosity of extruded feed for Atlantic salmon. Aquaculture Nutrition, 17, 326-
566 336. https://doi.org/10.1111/j.1365-2095.2010.00767.x
567 Tabachek, J.-A.L., 1988. The effect of feed particle size on the growth and feed efficiency
568 of Arctic charr [Salvelinus alpinus (L.)]. Aquaculture 71, 319–330.
569 https://doi.org/10.1016/0044-8486(88)90201-3

570 Talbot, C., Higgins, P.J., 1983. A radiographic method for feeding studies on fish using
571 metallic iron powder as a marker. Journal of Fish Biology 23, 211–220.
572 https://doi.org/10.1111/j.1095-8649.1983.tb02896.x
573 Vandewalle, P., Saintin, P., Chardon, M., 1995. Structures and movements of the buccal
574 and pharyngeal jaws in relation to feeding in Diplodus sargus. Journal of Fish
575 Biology 46, 623–656. https://doi.org/10.1111/j.1095-8649.1995.tb01101.x
576 Venou, B., Alexis, M.N., Fountoulaki, E., Nengas, I., Apostolopoulou, M., Castritsi-
577 Cathariou, I., 2003. Effect of extrusion of wheat and corn on gilthead sea bream
578 (Sparus aurata) growth, nutrient utilization efficiency, rates of gastric evacuation
579 and digestive enzyme activities. Aquaculture 225, 207–223.
580 https://doi.org/10.1016/S0044-8486(03)00290-4
581 Wainwright, P., Friel, J., 2000. Effects of prey type on motor pattern variance in
582 tetraodontiform fishes. The Journal of experimental zoology 286, 563–71.
583 https://doi.org/10.1002/(SICI)1097-010X(20000501)286:63.0.CO;2-C
584 Wańkowski, J.W.J., Thorpe, J.E., 1979. The role of food particle size in the growth of
585 juvenile Atlantic salmon (Salmo salar L.). Journal of Fish Biology 14, 351–370.
586 https://doi.org/10.1111/j.1095-8649.1979.tb03530.x
587 Zhou, C., Xu, D., Lin, K., Sun, C., Yang, X., 2018. Intelligent feeding control methods
588 in aquaculture with an emphasis on fish: a review. Reviews in Aquaculture 10,
589 975–993. https://doi.org/10.1111/raq.12218
590 Zhou, Q.-C., Tan, B.-P., Mai, K.-S., Liu, Y.-J., 2004. Apparent digestibility of selected
591 feed ingredients for juvenile cobia Rachycentron canadum. Aquaculture 241,
592 441–451. https://doi.org/10.1016/j.aquaculture.2004.08.044
593

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) ± 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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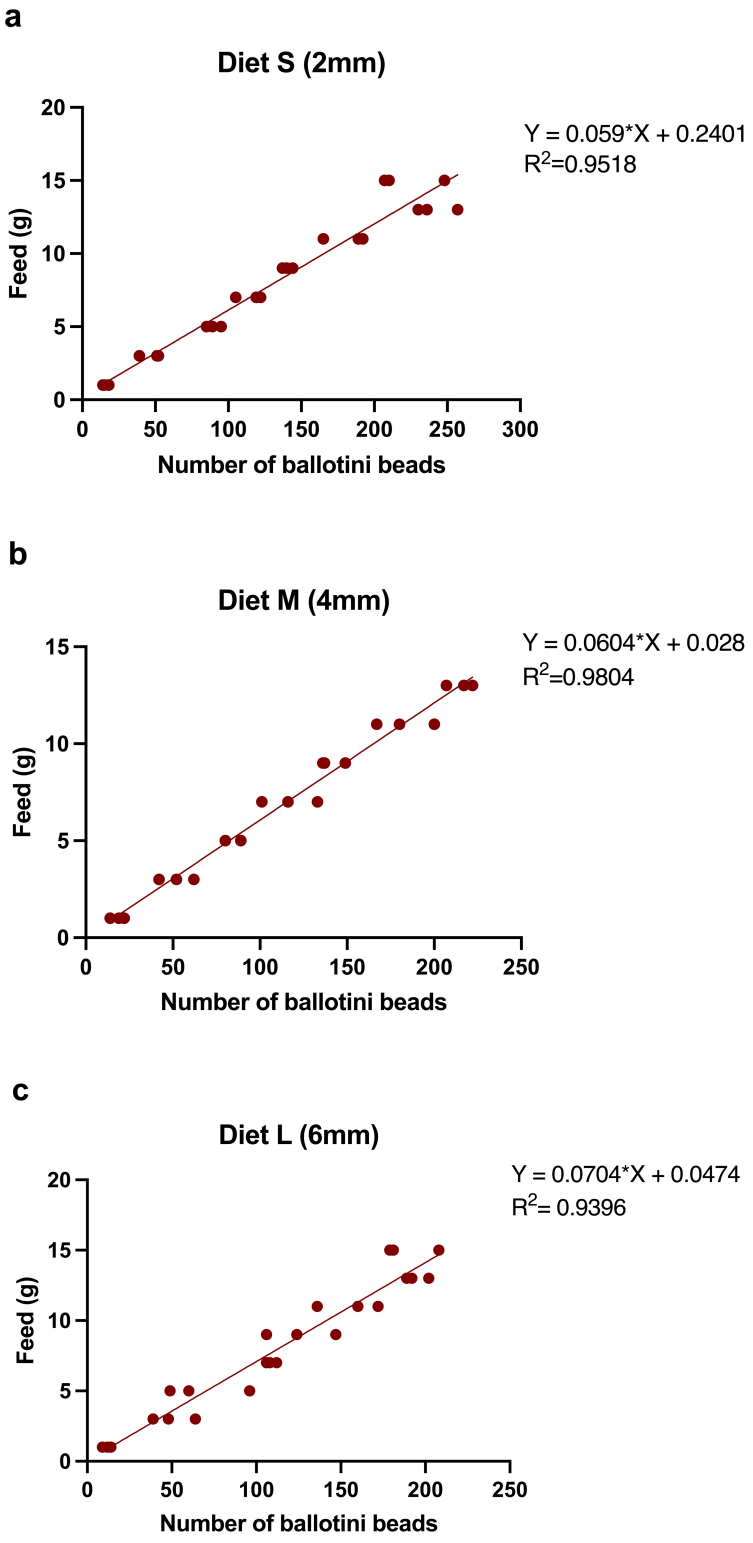
Figure captions

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**



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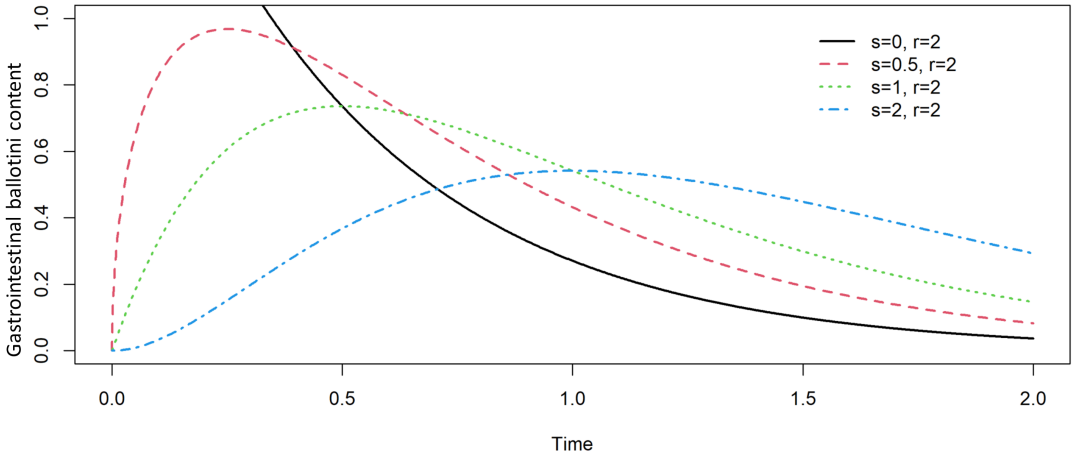
644 **Figure 2**



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