Temperature influences growth, digestive system ontogeny and lipids deposition in the liver in gilthead seabream (*Sparus aurata*) larvae and juveniles

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Ethics approval statement

The experimental protocol with the larval rearing was performed at the AQUALABS facilities of the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Centre for Marine Research (HCMR), Heraklion, Crete, Greece, (Registration No EL91-BIObr-03 and EL91-BIOexp-04).

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Abstract

Sparus aurata is one of the most important species in the Mediterranean region. Temperature is considered the main factor affecting the ontogenetic rates of rearing fish. We examined effects of temperature (16°C, 19°C, and 22°C) on growth (total length, T.L.), ontogeny of the digestive system, and lipid deposition in the liver (area covered with lipid vacuoles, ACLV%) from hatching to metamorphosis. Three groups were analyzed, and larvae were collected daily until 15 days after hatching (dah) every two days between 15-35 dah and every three days between 35-60 dah. The development of the digestive system's was completed (stomach with gastric glands) at 33 (T.L. 9.55±0.1.47 mm, 726-degree days), 35 (T.L. 11.00±0.78 mm, 665-degree days), and 60 dah (T.L. 12.86±1.68 mm, 960-degree days) in the groups 22°C, 19°C, and 16°C, respectively. Total length was higher in higher temperature groups from hatching until 15 dah. The 19°C and 22°C exhibited 1.5-two-fold higher growth than the 16°C group. The ACVL percentages in the first phase were higher in the 16°C and 19°C groups on most days. During the second phase, the 16°C group showed higher lipid accumulations. Finally, in the third phase, there was variation among groups. In the fourth phase, the 22°C and 16°C groups showed higher liver lipid accumulations. The most appropriate rearing temperature for seabream is 19°C, constituting a cost-effective protocol for larval rearing. The effectiveness of the histological liver lipid analysis indicates an accurate method to evaluate feeding conditions during seabream larval rearing, indicating critical feeding periods with precision.

Keywords: Seabream; larvae; fish development; digestive tract; liver

1. Introduction

Gilthead Seabream (*Sparus aurata*) is a Sparidae species commonly found throughout the Mediterranean Sea and less frequently in the Black Sea and the Atlantic Ocean in the Great Britain Island to Cape Verde and around the Canary Islands (Basurco et al., 2011). This species is a protandrous hermaphrodite, euryhaline, sedentary, and mainly carnivorous fish that spawns in the winter and spring (December to April) (Cataudella et al., 1995; Zohar et al., 1995).

It is a high-value fishery product with a high commercial value (Yúfera et al., 1995). Combined with European seabass (*Dicentrarchus labrax*) production, it is now the most important aquaculture industry in the Mediterranean region and the second most important in the European Union (Llorente et al., 2020). Seabream has been produced at an industrial level since the 1080s, and its production has increased continuously, reaching almost 186.000 tons in 2016 (Llorente et al., 2020).

The production increases due to the control of some important steps in captivity, such as reproduction (Basurco et al., 2011), a feeding conversion rate between 1.5-2, some nutritional requirements (Lupatsch et al., 2003), and aspects of larval rearing and juvenile stages (Basurco et al., 2011). The larval rearing protocols must be improved to optimize seabream production in order to obtain better production performances (survival rates, homogenous growth, low cannibalism rate, and skeletal deformities). Thus, it is essential to study exogenous and endogenous factors that affect all these parameters, and temperature is the main exogenous factor that affects larval rearing success; the latter exerts a remarkable effect on fish larvae development (Jobling, 1997). Temperature affects almost all physiological parameters during development, such as size at hatching, yolk resorption metabolism, growth, feeding rate, metamorphosis, and digestion and metabolic rates (Blaxter, 1988; Kamler, 2002), and different process

levels, such as chemical process (oxygen consumption), and ecological aspects (swimming speed) (Hochaka and Somero, 2001), in addition to aspects at organ and organism levels (Blaxter, 1991; Prakoso et al., 2019; Wendelaar Bonga, 1997). Shortening growth periods and cost-effective protocols are always on demand in a hatchery; temperature modulates these processes. In some cases, the temperature during hatchery may also have a long-term effect on fish growth (Lee et al., 2017), affecting growth and muscle fibers (Johnson and Andersen, 2008), flesh quality (Lee et al., 2017), and digestive system ontogeny (Kamler, 2002).

Previous works have evaluated rearing temperatures for seabream, and the best rearing conditions (hatching rate, low abnormalities, success from fertilization to mouth opening, and higher survival rates) were obtained within the range of 16 to 22°C (Polo et al., 1991). Also, some hatcheries in the Mediterranean region use borehole water for biosecurity reasons, since borehole water is sterile but stable at a constant temperature of around 19°C, therefore being cost-effective provided there is no need of heating or cooling the water during larval rearing.

It is well known that temperature affects the ontogenetic rhythms and the developmental status of fish larvae. Temperature is the primary factor affecting digestive system development (Kamler, 2002) and is directly related to digestion and metabolism (Hochaka and Somero, 2001; von Herbing, 2002). Detailed information regarding the ontogenetic development of the digestive tract and the digestive capability of organisms during the early life stages continues to be a key area for aquaculture-related research on fish larvae (Yúfera and Darias 2007; Papadakis et al., 2009; Rønnestad et al., 2013; Zadmajid et al., 2019; Papadakis et al., 2009).

Larvae undergo profound anatomical and physiological changes. However, this process does not end with the transition from the endogenous to exogenous feeding; rather, it

106 continues until the juvenile phase (Gisbert et al., 2008). During this process, other morphological and physiological alterations occur, such as musculature reorganization,
108 gill and skeletal differentiation, stomach functionality with acid digestion, pyloric caeca development, and liver appropriate function and maturation (Gisbert et al., 2008;
110 Papadakis et al., 2013; Sarasquete et al., 1995).

The liver plays a significant role in metabolism, working on glycogen and lipid
112 metabolism and storage. The liver's morphology reveals the larvae's nutritional status, and it is an essential tool to evaluate the physiological condition, reflecting whether the
114 diet is appropriate and/or whether there is any feed unbalance or food deprivation (Gisbert et al., 2008). The liver's morphological aspect accurately reflects the nutritional
116 status, and these changes are observed in cell size, cell volume, hepatic glycogen, and lipid content in the hepatocyte (Gisbert et al., 2008; Papadakis et al., 2013).
118 The digestive system's ontogeny and hepatocyte morphology are similar among teleosts, but the timing of appearance is species-specific and considerable differences can be
120 observed. Also, such a plasticity is influenced by environmental factors such as temperature (Kamler, 2002), and it may affect the ontogeny and morphology of organs
122 such as the liver (Ibarz et al., 2007; 2010). Although effects of temperature on fish and seabream development have been evaluated before (Koumoundouros et al., 2001,
124 Sarasquete et al., 1995; Tandler et al., 1989), studies linking temperature to digestive system ontogeny and the liver as a nutritional quality indicator are still scarce.
126 The present study aims to describe the effects of different temperatures on a) growth performance, b) ontogeny of the digestive system, and c) lipid deposition in the liver
128 from hatching to metamorphosis. The different rearing temperatures were evaluated in terms of digestive system ontogeny linked to growth in order to provide insights about
130 the appropriate rearing temperature that may reflect the best temperature regime for

larvae. This study thus describes a specific protocol to improve commercial seabream larval rearing aiming to increase its production.

2. Materials and Methods

2.1. Experimental design, larval rearing management, and sampling procedure

The experiment was conducted in the AQUALABS facilities of the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Center for Marine Research (HCMR), Iraklion, Crete, Greece. Fifty thousand eggs were obtained from natural spawning at 18°C and stocked in 500-L tanks. The daily water renewal volume was initially 10% and increased to 100% at the end of the experiment.

Three experimental temperatures (16°C, 19°C, and 22°C) were analyzed in duplicate. A concentration of $6.5 \pm 3 \times 10^5$ cells ml^{-1} of phytoplankton of *Chlorella minutissima* was added daily. For larvae swim bladder inflation, a surface skimmer was installed in each tank to remove the lipids from the surface while the phytoplankton was provided.

Table 1 shows the feeding protocol performed during the larvae rearing procedure. The rotifers were enriched with DHA Protein Selco (INVE S.A., Belgium) and were added to the rearing tank four times a day. The rotifer density varied from 5 ind ml^{-1} (first feeding) to 7 ind ml^{-1}. The nauplii of *Artemia* was enriched with Easy DHA Selco (Easy SUPER Selco, INVE S.A., Belgium). The *Artemia nauplii* density was adjusted to 0.5 ind ml^{-1}. The transition to dry food (Proton, Alfa, INVE S.A., Belgium) started when the fish were approximately 15 mm T.L. The bottom of the tanks was daily cleaned by siphoning. A planktonic mesh was placed in the tank's outlet to collect and finally remove the excess rotifers from the rearing water (50 μm mesh size).

From day 0 (day 0 was the hatching day), the sampling of larvae (n=10) was performed daily until 15 dah, then every two days between 15 and 35 dah, and every three days

from 35 to 60 dah. The sampled larvae were anesthetized, measured in T.L., and preserved for histological analysis (Mcdowell and Trump, 1976).

2.2. Histological analysis

Larvae samples (n=4) were dehydrated in ethanol solutions at progressively concentrations from 70 to 96%. After dehydration, larvae were embedded in methacrylate resin (Technovit 7100®, Heraeus, Kulzer, Germany). Serial sections of 3 µm were obtained with a microtome (RM2245, Leica, Germany). Sections were stained with methylene blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic Fuchsin (Poliscience, USA) following the protocol of Bennet et al. (1976). For the description of the digestive system and accessory glands ontogeny, all sections were examined under a compound optical microscope (Nikon, Eclipse 50i, 256 Japan) with a digital camera (Jenoptik progress C12 plus, Germany) mounted on top, which was used to examine and photograph the sections.

2.3. Digestive system ontogeny and area covered with lipid vacuoles in the liver (ACLV%)

All larvae evaluated as for the digestive system ontogeny were also assessed for ACLV, according to the method of Papadakis et al. (2009, 2013). The ACLV was obtained after ten dah due to the significantly reduced size of the liver and for each sampling day. Briefly, four larvae per group in each day of sampling were used for histology and six microphotographs per larva were obtained at 100X magnification from sections obtained from different areas of the liver. Photographs were transformed to grayscale in order to convert lipid vacuoles' color to white, and the area covered with lipid vacuoles (%) was calculated using an image analysis software (Image J, NIH, USA).

182 **2.4. Statistical analysis**

All values were expressed as mean ± mean standard error ($\overline{X} \pm MSE$). The samples
184 were compared between the temperature regimes on the different days of development with one-way ANOVA. The differences were considered significant at $P<0.05$ (Holm-
186 Sidak test). These analyses were performed using the statistical program Sigma Stat for Windows (Systat Software, San Jose, CA).

188

3. Results

190 **3.1. Total length**

The growth rate of seabream larvae under the different temperature treatments are
192 described by the equations (16°C) $TL = 3.1660e^{0.0217x}$ ($R^2 = 0.9697$), (19°C) $TL = 2.9227e^{0.0351x}$ ($R^2 = 0.9875$), and (22°C) $TL = 2.9945e^{0.0359x}$ ($R^2 = 0.9867$).
194 Temperature presented a slightly positive effect on growth in higher temperature groups from hatching until 15 dah (Figure 1). In the 16°C, 19°C and 22°C groups, the total
196 length was 4.20±0.45 mm (240 degree days), 4.88±0.37 mm (285 degree days), and 5.18±0.42 mm (330 degree days), respectively. However, from 15 dah onwards,
198 temperature showed a more prominent role in growth in higher temperature groups since the 19°C and 22°C groups presented a total length longer than larvae in the 16°C
200 group. Higher temperature groups showed a similar growth trend between them and higher than the 16°C group. This pattern was observed until the end of the experiment,
202 where the 19°C and 22°C groups showed a remarkable growth of 1.5-two-fold total length higher than the 16°C group at 60 dah.

204

3.2. Ontogeny of the digestive tract

The organs and structures of the digestive system appeared earlier in the group reared at 22°C, followed by the group 19°C and lastly the group 16°C.

From hatching until 2 dah, the alimentary canal appeared in all groups as an undifferentiated straight and narrow tube dorsally positioned to the yolk sac (data not shown).

The mouth opening was observed at 2 dah (T.L. 3.78±0.05 mm, 44 degree days), 3 dah (T.L. 3.69±0.13 mm, 57 degree days), and 4 dah (T.L. 3.73±0.13 mm, 64 degree days) in response to temperature regimes at 22°C, 19°C, and 16°C (figure 3a). The yolk sac in all three groups occupied a large volume in the abdominal region with a single and circular lipid droplet positioned in the posterior region of the yolk sac (Figure 3a), where it is also possible to observe the syncytial layer (figure 3a). The taste buds (figure 3b) also were observed in the group 22°C, 19°C, and 16°C at 10 dah (T.L. 3.93±0.30 mm, 220 degree days), 13 dah (T.L. 4.70±0.39 mm, 247 degree days), and 16 dah (T.L. 4.85±0.27 mm, 256 degree days), respectively. After these days, they increased in number and size in all groups (data not shown). The pharyngeal teeth (figure 3b) were also observed from the highest temperature group to the lowest, similar as the taste buds, but at 9 dah (T.L. 4.00±0.29 mm, 198 degree days), 11 dah (T.L. 4.26±0.20 mm, 209 degree days), and 16 dah (T.L. 4.85±0.27 mm, 256 degree days), respectively.

At the beginning of the esophagus, the characteristic was a lamellar epithelium with a small lumen inside it, with irregular cells in the border part covered by a thin muscular layer (data not shown). After that, the main structures started to differentiate on the second day after hatching, but the esophagus folds (figure 3c) and goblet cells (figure 3c) were observed in the 22°C, 19°C, and 16°C groups at 10 dah (T.L. 3.93±0.30 mm, 220 degree days), 16 dah (T.L. 5.38±0.12 mm, 304 degree days), and 23 dah (T.L. 4.87±0.34 mm, 368 degree days), 10 dah (T.L. 3.93±0.30 mm, 220 degree days), 13 dah

(T.L. 4.70±0.39 mm, 247 degree days), and 23 dah (T.L. 4.87±0.34 mm, 368 degree days), respectively. They increased progressively in size and number after that (data not shown).

Stomach compartmentalization was observed first in the group 19°C and after in the 22°C and 16°C groups, when the cardiac sphincter (figure 3d) appeared at 4 dah (T.L. 3.70±0.22 mm, 76 degree days), 5 dah (T.L. 3.71±0.08 mm, 110 degree days), and 8 dah (T.L. 3.70±0.28 mm, 128 degree days), respectively. Thereafter, the pyloric sphincter (figure 3d) appeared at 4 dah (T.L. 3.70±0.22 mm, 76 degree days), 5 dah (T.L. 3.71±0.08 mm, 110 degree days), and 9 dah (T.L. 3.54±0.21 mm, 144 degree days), respectively. At the same period, there was a differentiation between the midgut and the foregut (figure 3e). The gastric glands (figure 3F) were observed at 33 dah (T.L. 9.55±0.1.47 mm, 726 degree days), 35 dah (T.L. 11.00±0.78 mm, 665 degree days), and 60 dah (T.L. 12.86±1.68 mm, 960 degree days) in the groups 22°C, 19°C, and 16°C, respectively.

At 1 dah, the intestine was a straight tube dorsally positioned to the yolk sac and undifferentiated between the anterior and posterior part. However, following development and yolk sac resorption, it changed in shape and structure (data not shown). The differentiation of the intestine started at 2 dah (T.L. 3.78±0.05 mm, 44 degree days) when the ileorectal valve appeared (figure 3g) in the group 22°C. Thereafter, at 3 dah (T.L. 3.69±0.13 mm, 57 degree days) and 4 dah (T.L. 3.73±0.13 mm, 64 degree days), it appeared in the 19°C and 16°C groups, respectively. The ileorectal valve appeared on the same day as the anus opening for all groups (data not shown). The goblet cells in the hindgut were observed at 17 dah (T.L. 5.63±0.39 mm, 374 degree days), 22 dah (T.L. 6.55±0.53 mm, 418 degree days), and 32 dah (T.L. 5.85±0.47 mm, 512 degree days) in the 22°C, 19°C, and 16°C groups, respectively.

Thereafter, they increased in number and size (data not shown). The goblet cells in the midgut (figure 3h) were observed at 10 dah (T.L. 3.93±0.30 mm, 220 degree days), 19 dah (T.L. 5.83±0.43 mm, 361 degree days), and 39 dah (T.L. 7.92±0.54 mm, 624 degree days) in the groups 22°C, 19°C, and 16°C. Thereafter, it increased in size and number in all groups (data not shown). The supranuclear bodies in the midgut were first observed at 5 dah (T.L. 3.71±0.08 mm, 110 degree days) in the 22°C group, at 5 dah (T.L. 3.63±0.21 mm, 95 degree days) in the 19°C group, and at 8 dah (T.L. 3.70±0.28 mm, 128 degree days) in the 16°C group.

The liver and pancreas are present since birth, but they were not detectable on the first day of development, and both were observed histologically at 2 dah (T.L. 3.78±0.05 mm, 44 degree days) in the 22°C group, at 3 dah in the 19°C group (T.L. 3.69±0.13 mm, 57 degree days), and 3 dah in the 16°C group (T.L. 3.96±0.09 mm, 48 degree days) (figure 3d, e, g).

3.3. Ontogeny of the digestive system linked to fish size

The ontogeny of the digestive system associated with total fish length revealed that most of the digestive system structures appeared in all groups when the fish presented a similar total length. However, the goblet cells in midgut and hindgut in the 22°C (T.L. 3.93±0.30 mm) and 19°C (T.L. 5.83±0.43 mm) groups were different from those of the 16°C (T.L. 7.92±0.54 mm) group ($P<0.05$) (figure 4).

3.4. Area covered with lipids vacuoles in the liver (ACLV%)

The liver's ACLV evolution was characterized in four different periods identified by the lipids pattern during the development days (figure 5). In the first period (from 8 to 16 dah), the ACLV in groups 16°C and 19°C at 10 dah was significantly higher than the

values for the 22°C group (P=0.001). At 11 dah, the 22°C group showed an increase in ACLV, the 19°C group showed a decrease, and the 16°C group was almost at the same level, but they were all different among themselves (P=0.001). At 13 dah, the 22°C group was different from the 16°C group (P=0.001), but both were similar to the 19°C group; at 16 dah, the 16°C and 22°C groups showed an increase in ACLV percentages, reaching the same percentage as the 19°C group did. On this day, all groups presented similar ACLV percentages (P=0.900). On the second period (from 19 to 35 dah), at 19 dah, the 19°C group demonstrated an increase in ACLV percentage significantly different from the other groups (P=0.001). From 23 dah until 35 dah, the 16°C group presented significantly higher levels than the 22°C and 19°C groups did, but the ACLV levels of all groups showed fluctuating percentages (at 23, 25, 32 and 35, P=0.001, but at 35 dah, P=0.0007). On the third period (from 39 to 45 dah), at 39 dah, the 16°C group's ACLV levels decreased dramatically, but were still significantly higher than those of the 19°C and 22°C groups (P=0.001). At 41 dah, the 22°C group showed a significant increase, reaching the maximum ACLV levels of this group (P=0.001). At 45 dah, the 22°C group showed a significant decrease, and all groups were different (P=0.001), with the group 16°C presenting higher percentages and the group 22°C presenting lower ACLV percentages. On the fourth period (47 to 60 dah), at 47 dah, the groups 16°C and 19°C showed similar ACLV levels but higher than those of the 22°C group (P=0.001). At 54 dah, the 22°C group showed another significant increase in ACLV percentages with higher percentages than those of the 16°C and 19°C groups (P=0.036). Finally, at 60 dah, the 22°C and 19°C groups showed significantly higher ACLV percentages than those of the 16°C group (P=0.008).

4. Discussion

306 The present study evaluated three different temperatures during seabream larval rearing and its effects on growth, digestive system's ontogeny, and lipid deposition in the liver
308 during the first 60 dah.

Temperature played an essential role in seabream growth, digestive tract ontogeny, and
310 lipid deposition in the liver, which directly affected all evaluated parameters. The growth performance was higher in the 19°C and 22°C groups and almost all digestive
312 tract organs appeared first in the 22°C group and later on the 19°C and 16°C groups, respectively. However, the two higher temperature groups (22°C and 19°C) showed a
314 similar development. Seabream growth was slightly higher in higher temperatures groups than in the lower temperature group from hatching until 15 dah, a fact that
316 occurred for different Sparidae species when exposed to higher temperatures during the larval phase, such as the Australian snapper *Pagrus auratus* (Fielder et al., 2005) and
318 the blackspot seabream *Pagellus bogaraveo* (Silva et al., 2011).

From 15 to 60 dah, the 19°C and 22°C groups showed similar total lengths, with 1.5-
320 two-fold longer sizes than the 16°C group. Previous studies showed that when reared at 19°C seabream showed a better development from hatching onwards and a better
322 growth compared within a range of temperatures (12-30°C) (Polo et al., 1991). Another work observed a similar growth rate and almost the same fish size at 16 dah, with an
324 average of about 5.7 mm at a 18°C rearing temperature (Russo et al., 2007). After 15 dah, the temperature was determinant in increasing the fish growth in the groups 19°C
326 and 22°C.

The ontogenetic development of the digestive system of seabream was similar as those
328 of other marine fish species, such as dusky grouper (*Epinephelus marginatus*) (Mello et al., 2018), sharpsnout seabream (*Diplodus puntazzo*) (Micale and Muglia, 2011), white
330 seabream (*Diplodus sargus*) (Ortiz-Delgado et al., 2003), and seabream (*Sparus aurata*)

(Sarasquete et al., 1995). Compared to other commercial marine fish, seabream can be considered a species with a slow digestive system development. From the ontogenetical point of view, the gastric glands, which mark the functional stomach (Stroband and Kroon, 1981), can be considered a criterion to differentiate larvae from juveniles (Sarasquete et al., 1995; Tanaka, 1971). In species that are considered to have slow growth, the gastric glands had a delayed development. For example, they were observed around 36 dah in yellowtail flounder (*Pleuronectes ferrugínea*) (8-10°C rearing temperature) (Baglole et al., 1997), 30 dah in sharpsnout seabream (*Diplodus puntazzo*) 28 dah in common pandora (*Pagellus erythrinus*) (19-21.5°C rearing temperature) (Micale and Muglia, 2011), on 60 dah in seabream (18-19°C rearing temperature) (Elbal et al., 2004). Another study with seabream also which did not observe gastric glands until 30 dah (19.5°C rearing temperature) (Sarasquete et al., 1995). In the present study, the gastric glands were observed at 33 dah in the 22°C group, which was the group with a faster digestive system development. On the other hand, in fast-growing species, the gastric glands can be observed much earlier, for example, at 9-10 dah in cobia (*Rachycentron canadum*) (25.9°C rearing temperature) (Faulk et al., 2007), 15 dah in meagre (*Argyrosomus regius*) (19-23°C rearing temperature) (Papadakis et al., 2013), 11 dah in Pacific Bluefin tuna (*Thunnus thynnus*) (25°C rearing temperature) (Kaji et al., 1996), and 15 dah in yellowtail kingfish (*Seriola lalandi*) (24°C rearing temperature) (Chen et al., 2006).

Besides all differences among treatments during the ontogeny of the digestive system, when correlated to fish size only the goblet cells in the midgut showed significant differences in which the highest temperature group (22°C) was significantly different from the lower temperature groups (19 and 16°C). These cells secrete a very diverse group of mucosubstances (MS) from neutral to acid characteristics in the digestive tract,

and it can act as a morphological adaptation to replace functional stomach, such as in a gastric fish (Jaroszewska et al., 2008). Also, this MS plays an important role in the absorption process and transport of macromolecules (Zambonino et al., 2008). Neutral MS are related to enzymatic digestion and absorption of small substances, and acid MS can act as a lubricant and improve/facilitate ingestion (Gisbert et al., 2004; Micale et al., 2008). Besides that, the goblet cells in the midgut help the displacement of food particles and protect intestinal mucosa from injuries, such as mechanical injuries (Zambonino et al., 2008). Therefore, the presence of these cells can show an improvement in effectiveness of the digestive capacity of seabream larvae in fish with smaller sizes, showing an adjustment of the fish intestinal morphology for improving digestion process and consequently improving feed assimilation.

Temperature showed an effect during the endogenous feeding phase. The 22°C group was ready to capture preys and start its exogenous feeding life at 2 dah, followed by the 19°C group at 3 dah, a result that was also observed in previous work with seabream at the same temperature (Sarasquete et al., 1995). For sharpsnout seabream (*Diplodus puntazzo*) (Micale et al., 2008), the value was the same as for the 16°C group, when larvae were ready to feed exogenously at 4 dah. The lower temperature regimes delayed the appearance of pharyngeal teeth, esophagus folds, and goblet cells, which are the structures that affect the ability of causing the first mechanical damage to the food and of swallowing. Pharyngeal teeth appeared later compared to meagre (*Argyrosomus regius*), for example, when it was observed at 6 dah (Papadakis et al., 2013), in common dentex (*Dentex dentex*) at 7 dah (Santamaria et al., 2004) and yellowtail kingfish (*Seriola lalandi*) at 8 dah (Chen et al., 2006). In dusky grouper (*Epinephelus marginatus*), which is considered a very slow-growing species, this structure appeared only at 17 dah (Mello et al., 2018), such as happened in the 16°C group.

The esophagus structures, such as goblet cells that act in the mucus secretion, which protects against food abrasion (Gisbert et al., 1999) and facilitates food ingestion (Abol-Munafi et al., 2006), and longitudinal folds, which distend and expand for food ingestion, appeared very late in the development of sea bream. In fast growth developing species such as meagre (*Argyrosomus regius*), goblet cells and longitudinal folds were observed at 5 and 3 dah (Papadakis et al., 2013); for sharpsnout seabream (*Diplodus puntazzo*), both structures appeared at 5 dah (Micale et al., 2008; Micale and Muglia, 2011); in common pandora (*Pagellus erythrinus*), at 20 dah (Micale and Muglia, 2011). In dusky grouper (*Epinephelus marginatus*), a slow-developing species, both structures appeared at 11 and 13 dah (Mello et al., 2018). The appearance of longitudinal folds in similar periods can be related to the ability for enlargement that may aid in the transition of food particles to the subsequent sections of the digestive system (Diaz et al., 2008). The appearance of longitudinal folds in haddock (*Melanogrammus aeglefinus*) has been associated with the initiation of the exotrophic feeding phase (Hamlin et al., 2000).

Regarding the taste buds, which provide the fish with the sense of taste, temperature affect prey selection. Compared to other produced marine fish, such as meagre (*Argyrosomus regius*), this structure appeared at 3 dah (Papadakis et al., 2013), at 8 dah in shi drum (*Umbrina cirrosa*) (Zaiss et al., 2006), at 11 dah in sharpsnout seabream (*Diplodus puntazzo*) (Micale et al., 2008, Micale and Muglia, 2011), at 11 dah in common pandora (*Pagellus erythrinus*), at 14 dah in common dentex (*Dentex dentex*), and at 14 dah dusky grouper (*Epinephelus marginatus*) (Mello et al., 2018).

The digestive tract's compartmentalization segments the digestive tract into foregut, midgut, and hindgut, which functionally is divided into bucco-pharynx, esophagus, stomach, intestine, and anus (Ronnestad et al., 2013). Cardiac and pyloric sphincter,

406 which delimits the stomach from the esophagus and gut, provides the stomach with epithelium conditions to differentiate them morphologically. Gastric glands start to
408 develop and acid digestion begins, being this the last step of gut differentiation, which allows digestion of inert diets, for example (Mitra et al., 2015). The ileorectal valve also
410 separates the terminal part of the gut. Therefore, all these structures improve the digestive tract's enzymatic activity by providing an adequate environment for digestion.
412 Previous studies have shown that gastric glands appeared in seabream larvae reared at 19°C with total length of larvae between 15-20 mm at 60 dah (Elbal et al., 2004). In the
414 present study, larvae showed gastric glands when reared at 22°C with 9.5±1.4 mm at 33 dah; when reared at 19°C, they appeared with 11.0±0.7 mm at 35 dah; finally, when fish
416 were reared at 16°C, they appeared with 12.8±1.6 mm at 60 dah.

Accessory organs as the liver, which are present since birth, play an essential role in
418 larvae development, not only in the bile production but also in the metabolic function since this organ starts to store glycogen (Hoehne-Reitan and Kjorsvik, 2004) and lipids
420 (Gisbert et al., 2008; Papadakis et al., 2013) in a species-specific time sequence (Ronnestad et al., 2013). Also, the morphological pattern of the liver, such as lipid
422 vacuolization, is directly affected by feeding management and in seabream larvae. The feeding protocol has a direct effect on liver lipid accumulation. Moreover, the liver
424 morphological analysis can accurately reveal any dietary imbalances since this can be easily observed in the lipid deposition of hepatocytes (Gisbert et al., 2008).
426 The liver area covered with lipid vacuoles was affected by the feeding protocol and temperature since temperature affected fish size. Fish size is related to the type and the
428 amount of food that a bigger or a smaller fish can consume. In response to low temperatures, seabream juveniles can mobilize lipid from different tissues, such as
430 mesenteric and white muscle, to the liver (Melis et al., 2016); apparently, from 9 dah,

there is a lipid deposition in lower temperature groups (16°C and 19°C) (3.5±0.2 mm and 3.9±0.6 mm, respectively) than in the highest temperature group (22°C) (4.0±0.2 mm). This deposition lasts until 16 dah, when groups presented a similar ACLV. All groups ingested only rotifers until 11 dah, but on the 12 dah, the group 22°C (4.4±0.3mm) started to ingest *Artemia nauplii* and at 13 dah, the group 19°C (4.7±0.3mm) started the transition to *Artemia nauplii*. Therefore, this increase in the ACLV in both groups until 16 dah occurs due to the higher lipid content of the enriched *Artemia nauplii*, as observed for meagre (*Argyrosomus regius*) (Papadakis et al., 2013) and grey mullet (*Mugil cephalus*) (Loi et al., 2020). This was also observed for sea horse (*Hipocampus gutttatus*) fed on enriched artemia accumulated lipids in the liver at higher rates than those fed on copepods (Randazzo et al., 2018). The 16°C group showed high ACLV lipids due to temperature; however, from 10 dah (3.8±0.2 mm) onwards, this group showed a constant decrease of ACLV until 19 dah (4.6±0.3 mm), possibly due to the low lipid content of rotifers in comparison to the enriched *Artemia nauplii*. Besides a slight effect of temperature on growth between groups during the first 15 dah, temperature affected the 16°C group that started the transition to *Artemia nauplii* at 16 dah (4.2±0.8 mm). The effect of enriched *Artemia nauplii* is observed from 19 dah onwards, as observed for the sea horse *H. guttatus* (Randazzo et al., 2018) due to the higher energetic supply of enriched *Artemia*, which in turn increased the ACLV levels up to 50% at 35 dah (the highest percentage in this group), values more than two-fold higher than those of the 19°C and 22°C groups. Around 13 dah, the group 19°C (4.8±0.3 mm) started the transition to ingest *Artemia* nauplii and an ACLV increase until 19 dah (5.8±0.4 mm) was observed probably due to the high energy demand from *Artemia* nauplii as observed for meagre (*Argyrosomus regius*) (Papadakis et al., 2013), grey mullet (*Mugil cephalus*) (Loi et al., 2020), and the sea horse *H. guttatus* (Randazzo

et al., 2018). This result is significant for enrichment with rotifers and *Artemia* nauplii and adequacy with the rearing temperature for seabream larvae to provide balanced amounts of lipids that will directly affect fish development (Lubzens et al., 2001) and liver lipid deposition.

Even though larvae consumed enriched *Artemia* nauplii from 19 dah to 39 dah, the ACLV decreased probably due to the higher metabolic rates shown by higher temperatures groups, which decreased the lipid content in the liver.

The group reared at 16°C reached the higher values of ACLV at 35 dah (5.9±0.5 mm). However, after this day and until 39 dah (7.9±0.5 mm), there was a dramatic decrease, followed by a slight reduction of ACLV percentages maintenance around 20% until 54 dah (11.0±1.1 mm). This prolonged effect of low temperature can be related to a lower feed intake or a fasting period, which could accelerate lipid liver metabolism (Ibarz et al., 2005; 2007). This was also observed in seabream juveniles exposed to prolonged low temperatures and fasting, which showed low somatic liver indexes and total liver weight and metabolized the liver lipids polar fraction under prolonged low temperatures (Ibarz et al., 2007). Around 45 dah (8.6±0.7 mm), this group started the weaning phase, considered critical (Hamlin et al., 2000). Even with this important transitional period, this group showed a slight increase in ACLV from 54 (11.0±1.1 mm) to 60 dah (12.8±1.6 mm), responding positively, ingesting a higher amount of inert food, and consequently, accumulating energy in the liver.

The weaning phase started at 35 dah in the groups 22°C (10.5±1.5 mm) and 19°C (11.8±0.7 mm). It is possible to observe an effect of the inert diet's ingestion in both groups. From 39 to 41 dah, the group 22°C (12.2±1.6 mm and 15.6±1.5 mm) showed a surprising increase in ACLV. Therefore, the inert diet ingestion provided energy supply and accumulation of lipids in the liver. At 45 (17.2±2.2 mm) and 47 dah (17.0±2.7 mm),

this group presented a drastic decrease in ACLV. Possibly, this is due to the transition between inert food pellets' size because fish can take a few days to accept feed and succeed in the transition. After the transition and fish adequately fed again, it is possible to observe an increase at 54 dah (24.2±3.6 mm) while keeping ACLV percentage levels at 60 dah, showing the importance of a correct feed management during the weaning phase. The 19°C group also showed an increase in the ACLV from 39 (13.0±1.2 mm) to 45 dah (16.0±1.4 mm), which can be explained by the high energy demand of inert food, which also provided high levels of energy and finally increased the lipid accumulation in the liver. These high levels were followed by a decrease in ACLV from 45 (16.0±1.4 mm) to 54 dah (18.0±1.5 mm), possibly the same as happened with the 22°C group, in which fish showed a low ingestion of food due to pellet size transition. This period was followed by an increase at 60 dah (19.8±1.3 mm), when this group showed almost the same ACLV as the 22°C group. This adequate feeding management must be perfectly adjusted since it is a key factor for aquaculture practices, first because it is a period with the highest cost and labor demand and secondly because it is imperative to avoid starvation periods, which consequently lead to malnourishment and affect larvae metabolism, negatively affecting the success of the weaning phase.

5. Conclusions

The present study indicates effects of high temperature on the seabream ontogenetic rates of the digestive system and the lipid deposition on the liver. The best rearing temperature for seabream larval rearing during the first 60 dah is 19°C since growth performance is very similar that of the 22°C group. The higher ontogenetical rates of the digestive system at 19°C in comparison to 16°C offers the larvae's digestive system the ability to quickly prepare, capture, digest, and assimilate feeds that are included in the

feeding rearing protocol. Additionally, as the rearing temperature of 19°C is in most cases used in hatcheries when sea bream broodstock lays eggs, it is not necessary to heat or cool the water during the larval rearing period. This would be a costly and non-energy-effective protocol for seabream larval rearing. This study also shows that liver lipid analysis is an accurate method for evaluating feeding conditions during fish development, indicating fast and detailed critical periods during the larval rearing procedure.

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726 **Tables and Figure captions**

728 **Table 1**. Feeding management for Gilthead seabream (*Sparus aurata*) larvae during larval rearing in relation to its size.

730

Figure 1. Total length (mm) (mean ± SD) of Gilthead seabream (*Sparus aurata*) larvae
732 during larval rearing in different temperature regimes in relation to time (days after hatching). 16°C is represented by the white round dots, 19°C is represented by the grey
734 round dots and 22°C is represented by the black round dots.

736 **Figure 2.** Schematic representation of the appearance (circles) of the main developmental structures examined in Gilthead seabream (*Sparus aurata*) larvae in the three different
738 temperature treatments, 16°C is the white round dot and dotted black line, 19°C is represented by the grey round dot and grey line and 22°C is the black round dot with the
740 black line. They are represented per days after hatching (dah, horizontal axis) and the digestive tract structures appearance (vertical axis).

742

Figure 3. Microphotographs of histological sections of Gilthead seabream (*Sparus*
744 *aurata*) larvae from group 22°C at different developmental stages. A. Larvae at 2dah showing mouth opening and different structures during mixotrophic phase evidencing
746 yolk sac resorption (bar represent 200µm) with the syncytial layer (insert - bar represent 50µm). B. Larvae at 3dah showning strucutures for capturing preys and gustative organs
748 (bar represent 100µm). C. Larvae at 45dah showing advanced development of esophagus and bucopharynx (bar represent 200µm). D. Larvae at 3dah showing beginning of
750 compartimentalization of digestive system (bar represent 100µm). E. Larvae 3dah showning the beginning of absortive capacity (bar represent 200µm) evidenced by the
752 presence of supranuclear vacuoles (Insert - bar represent 50µm). F. Larvae at 45dah showinig advanced development of the stomach with gastric glands and ingested food
754 (bar represent 500µm). G. Larvae at 2dah showing ileo-rectal valve and different digestive structures (bar represent 200µm). H. Larvae at 10dah showning goblet cel in the
756 midgut (bar represent 50µm) (Insert – bar represent 20 µm). BC = buccopharynx, CS = cardiac sphincter, EF = Esophagus, EY = Eye, FG = fore gut, Fd=Food, GC= goblet cells,
758 GG = gastric glands, L = liver, MG = median intestine, PS = pyloric sphincter, PA = pancreas, SV = supranuclear vacuoles, TB = taste buds, PT = pharyngeal teeth; Br=brain,
760 CS=cardiac sphincter, Es=esophagus, Es F=longitudinal fold at esophagus, FG= fore gut, GA=gill arch, H=heart, IV=ileo-rectal valve, GG=gastric glands, GC=goblet cells,
762 MG=anterior–median intestine, MO=Mouth open, LD=Lipid droplet, PT=pharyngeal teeth, PS=pyloric sphincter, PA=pancreas, P end=endocrine pancreas, St=stomach,

764 SynL=syncytial layer, SB=swim bladder, YS=yolk sac. The black bars represent xxxx
mm

766

Figure 4. Gilthead seabream (*Sparus aurata*) larvae size between different temperature
768 regimes and main organs and structures appearance during ontogeny of digestive tract.
Lower case letters above the bars means significant differences between temperatures
770 regimes.

772 **Figure 5.** Mean (±SD) Area Covered by Lipid Vacuoles (ACLV, %) in the liver of
Gilthead seabream (*Sparus aurata*) larvae at different temperature regimes during larval
774 rearing. Braces below the graph indicate the four periods with changes in ACLV. Lower
case letters aside the lines mean significant differences between temperatures regimes at
776 the same day after hatching.

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Figures

Figure 1

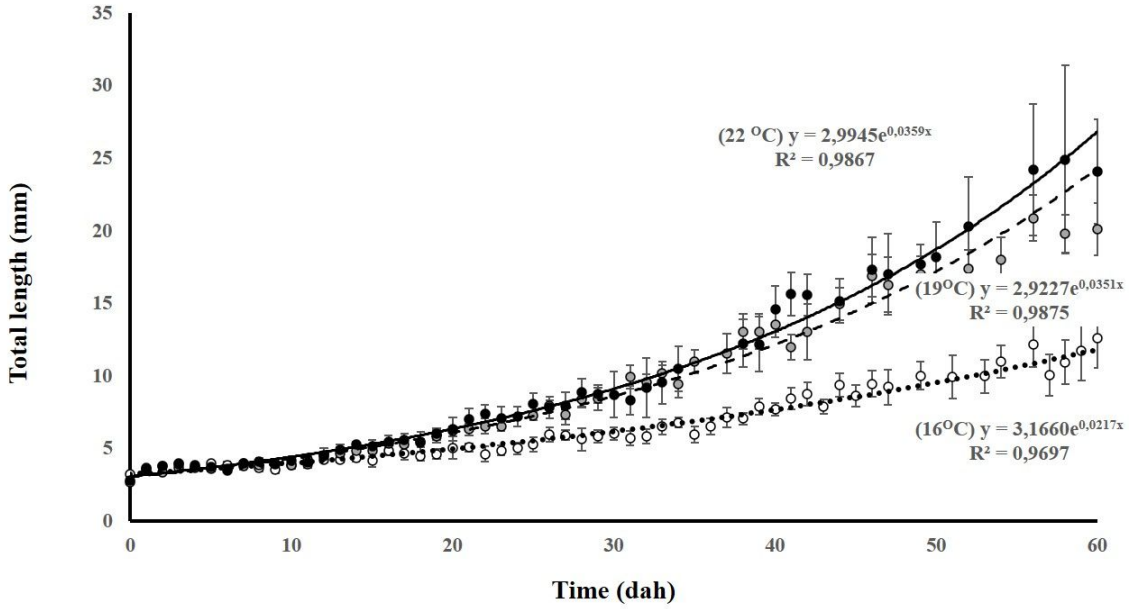

Figure 2

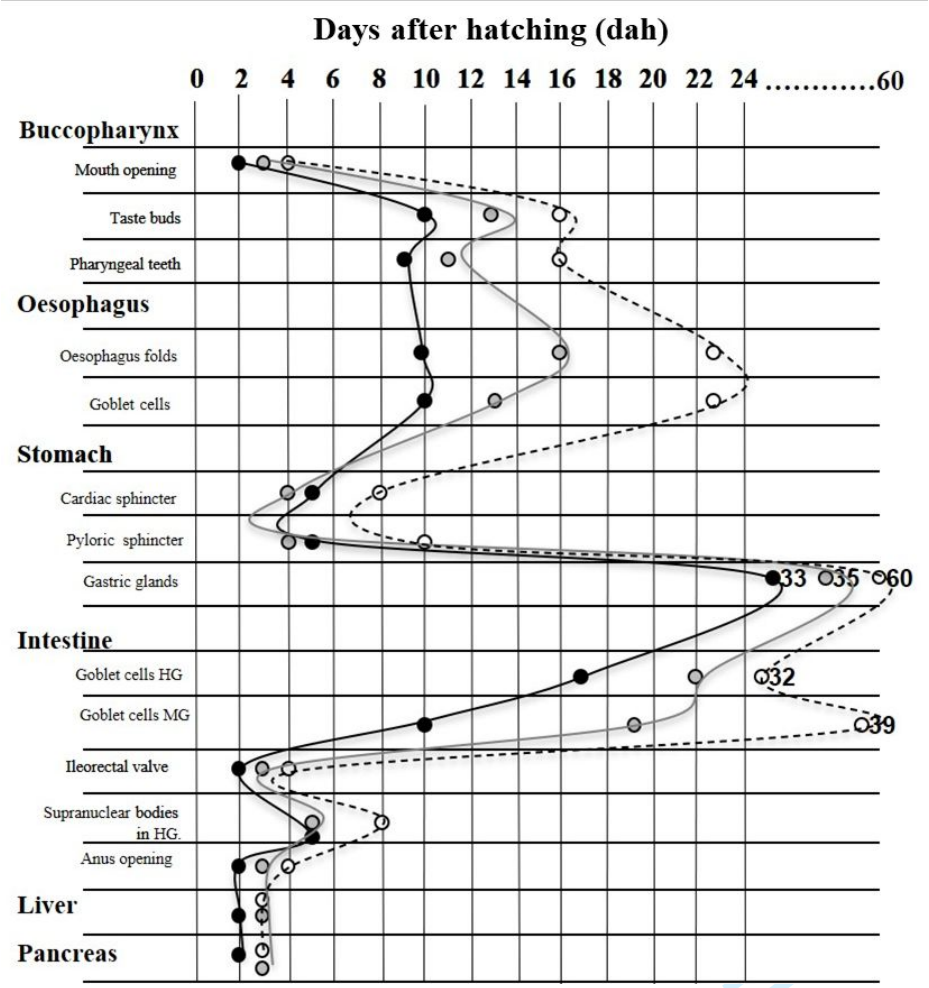
Days after hatching (dah)
0 2 4 6 8 10 12 14 16 18 20 22 24 60
Buccopharynx
Mouth opening
Taste buds
Pharyngeal teeth
Oesophagus
Oesophagus folds
Goblet cells
Stomach
Cardiac sphincter
Pyloric sphincter
Gastric glands
33 35 60
Intestine
Goblet cells HG
32
Goblet cells MG
39
Ileorectal valve
Supranuclear bodies in HG.
Anus opening
Liver
Pancreas

Figure 3

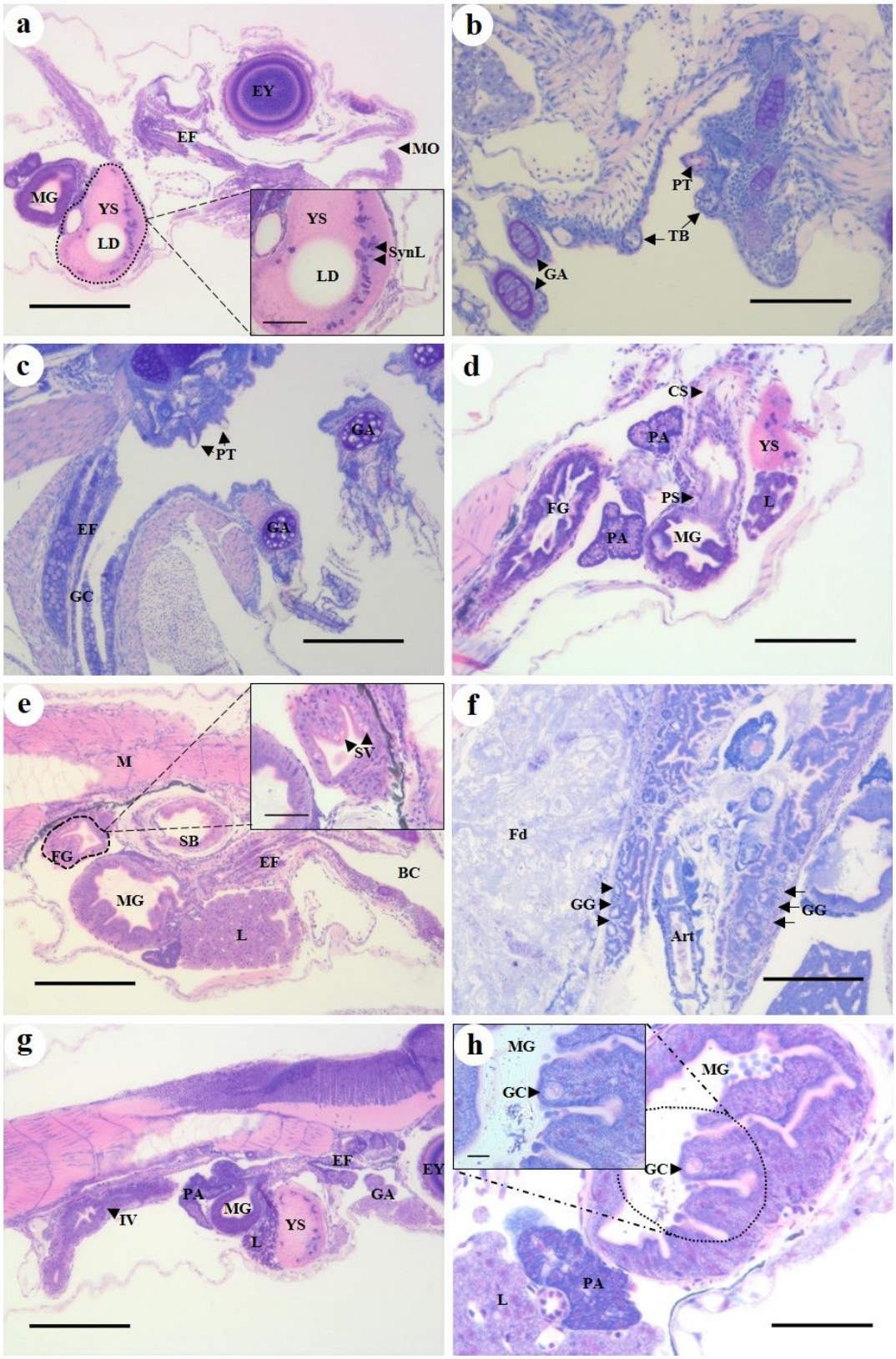
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Figure 4

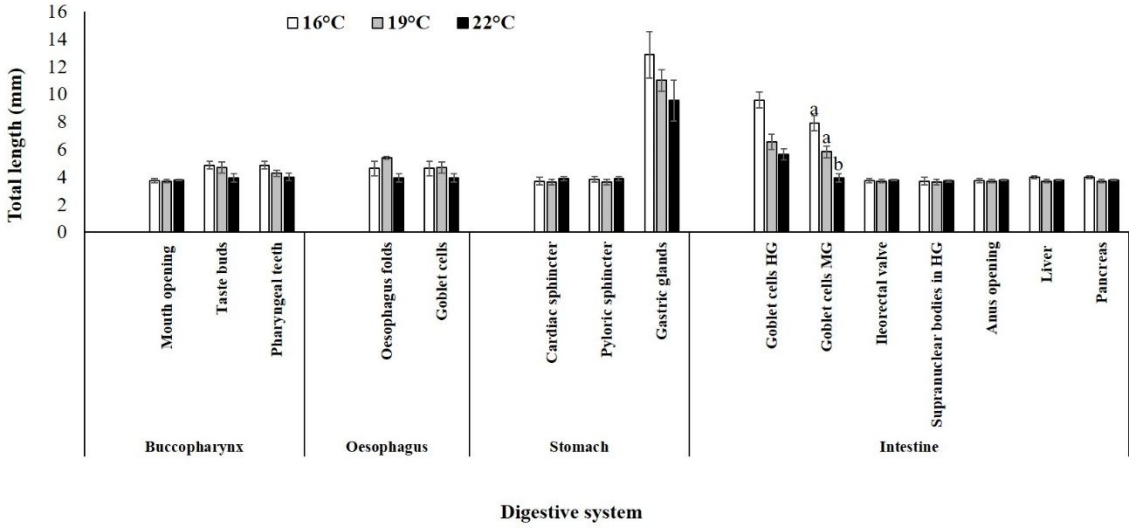
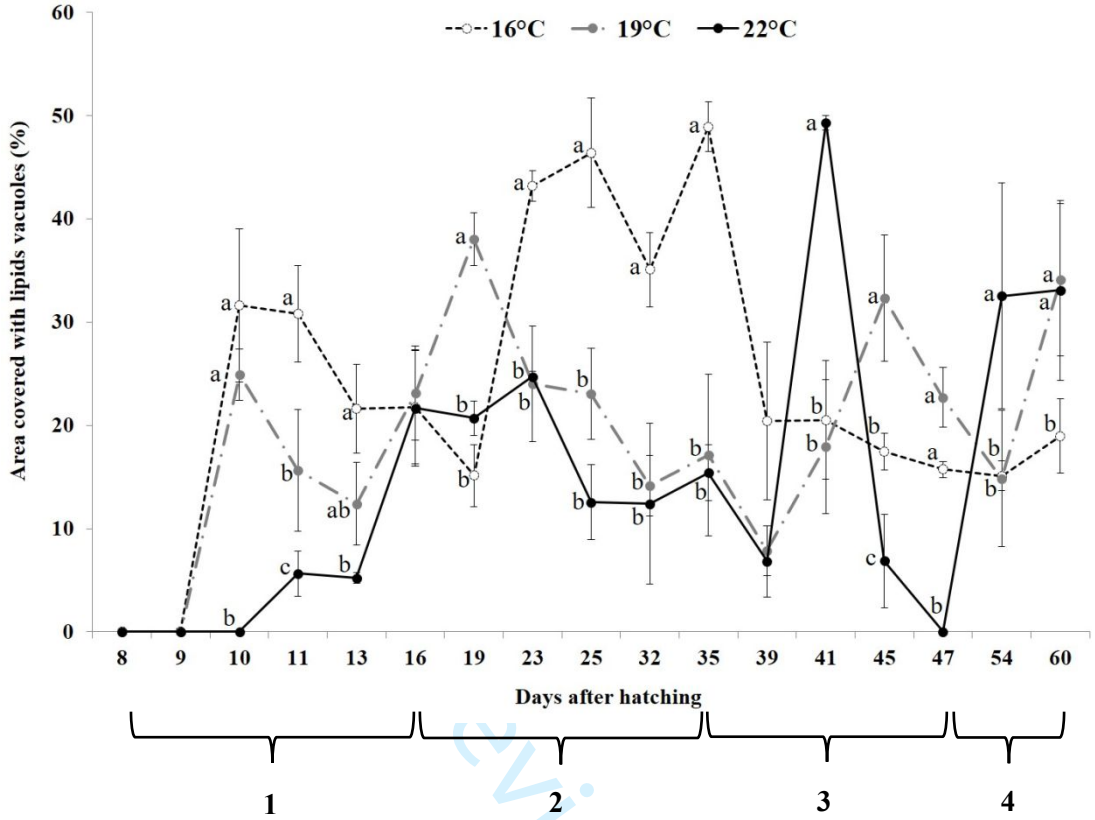


Figure 5



Tables

Table 1