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Research Article

THE INHIBITORY SITUATIONAL ANALYSIS OF SOME FEED INGREDIENTS FOR MEAGRE, *Argyrosomus regius* (Asso 1801) LARVAE AND EVALUATION FOR DIET FORMULATIONS

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ABSTRACT

Meagre, *Argyrosomus regius* (Asso 1801) is an important alternative species in aquaculture. The *in vitro* assay provides practical assessments for the evaluation of feed ingredients. In this study, the inhibition degrees of feed ingredients (fish meal-FM, fish hydrolysate-FH, krill meal-KM, soybean meal-SM, wheat gluten-WG, corn gluten-CG and sunflower meal-SF) on protease activities of meagre larvae were determined. Larvae were sampled from the first day of opening the mouth (3 days after hatching-DAH) until the end of the weaning (32 DAH) from the Egemar Hatchery (Aydın-Turkey). Larvae of the total length were measured as 3.19 ±0.02-21.61 ±0.22 mm and weights were calculated as 0.53 ±0.02-118.00 ±1.09 mg at 3 and 32 DAH, respectively. Protease activities of larvae were the lowest as 5.95 ±0.60 U/mg protein (15 DAH) and the highest as 211.21 ±12.56 U/mg protein (7 DAH), respectively (P<0.05). The lowest inhibitions degrees of feed ingredients were observed at 15 DAH except for SF. The use of FH in the diet formulations of meagre larvae should be paid attention. While CG and SF are advised, SM does not seem to be suitable.

Keywords: *Argyrosomus regius,* Meagre, Protease activities, Inhibitions, Feed ingredients

Introduction

Mediterranean marine aquaculture production in Turkey, Greece, France, Italy, Spain, Croatia and Cyprus based on total juvenile production reached to 1.3 billion number in gilthead bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) production (FEAP, 2016). Aquaculture requires low cost inputs and high productivity and needs to focus on the introduction of new candidate species. Meagre is an important species in diversification of Mediterranean aquaculture (El-Shebly et al., 2007; Monfort, 2010; Kružić et al., 2016). Because of its high pace of growth, it has significant advantages in aquaculture (Quemener, 2002; Duncan and Myrseth, 2011; Parisi et al., 2014). The sciaenid meagre is a Mediterranean species and distributed in the Atlantic coasts of Europe and the northwest coast of Africa (Whitehead et al., 1986; Haffray et al., 2012).

A better understanding of the nutrient requirement of larvae, the absolute requirement of nutrient concentrations and the determination of optimal intervals will provide significant contributions to larval feeding studies (Person-Le Ruyet and Bergot, 2001; Holt et al., 2011; Southgate, 2012). *In vivo* methods used in the aquaculture feeding experiment expressed that the evaluation of nutritional value of the feed is time-consuming and the results can be affected by environmental factors. *In vitro* methods were described as rapid, reproducible and allowed only small quantities of raw materials to be used (Ezquerra et al., 1997; Garciá-Ortega et al., 2000). *In vitro* methods commonly used in the evaluation of nutrition and nutritional qualities of humans and terrestrial animals had the potential to be used in determining the feed components and production methods of fish larvae (Holt et al., 2011; Moyano et al., 2015). It was found that *in vitro* methods of extracting larval digestive enzymes and mixing with food and hydrolysis measured were used to determine the digestibility of fish larvae (Holt et al., 2011). *In vitro* techniques were reported to be important for the development of larval artificial feeds and in recent years *in vitro* techniques were assessed for pre-protein digestibility of larval microcapsules (Cahu and Zambonino Infante, 1994). Inhibitors of proteases in fish diet revealed different sensitivity for the preliminary evaluation of the usability of the ingredients in feed (Moyano et al., 1998; Alarcón et al., 1999). The effects of feed ingredients on protease activities of *Sparus aurata* larvae and shrimps were determined (Alarcón et al., 1997, 1999). In addition, trials were carried out to evaluate cheap and sustainable alternative protein sources such as soybean meal in diets. However, the main obstacles to the use of high amounts of plant protein sources in fish diets were at low protein quality due to the amino acid imbalances and the availability of antinutritional component decreasing the activity of enzymes (Tacon, 1997; Krogdahl et al., 2003).

Researchers focused on growth, survival and larval rearing of meagre, the histology and ontogeny of digestive system of larval meagre and the effects of different levels of plant proteins on juvenile meagre (Fernández-Palacios et al., 2007; Roo et al., Arda, 2011; 2010; Estévez et al., 2011; Schiavone et al., 2012; Papadakis et al., 2013; Vallés and Estévez, 2013, 2015). Also, digestive enzymes of marine fish larvae such as European seabass, gilthead seabream, Senegalese sole, white seabream, redbanded seabream, meagre were studied (Zambonino Infante and Cahu, 1994; Moyano et al., 1996; Ribeiro et al., 1999; Cara et al., 2003; Moyano et al., 2005; Süzer et al., 2013; Solovyev et al., 2016).

We identified studies on inhibition effects of feed ingredients and microdietes related to marine fish (Kuzu and Naz, 2012; Naz and Yúfera, 2012; Yıldız et al., 2012; Yılmaz et al., 2012; Haközü, 2014). We could only find few studies on the inhibitory effects of microdiets and feed ingredients on protease activities of meagre larvae (Diken et al., 2016a, b, c; 2017; Diken et al., 2018). Therefore, the aim of this research was to determine the potential inhibitory effects of commonly used feed ingredients on protease activities of meagre larvae using *in vitro* techniques and suggested for microdiet formulations of larvae of meagre.

Material and Methods

Larvae Culture and Sampling

Larval rearing was conducted in EGEMAR Aquaculture Food Industry and Commercial Incorporated Company (Aydın/TURKEY). Eggs were obtained by hormone injection which fertilized ones were incubated at conical fiberglass tank and 23.6±0.5ºC (GnRH; 20 µg/kg ♀ and 10 µg/kg ♂). Larvae were fed between 0-15 DAH, in 7 m^3 ellipsoidal fiberglass tank and the rate of 75-80 larvae/L in the larva unit. Larvae weaning were taken at 16-32 DAH, in 27 m^3 raceway made of concrete and the rate of 10-12 larvae/L in the weaning unit. The water used in the aquaculture was filtered by sand, bag and UV filters. Environmental conditions of larval cultures were determined at 20.8-24.1ºC temperature, 27.0-40.0 g/L salinity, 8.4-14.4 mg/L O_2, and 7.5-7.9 pH. Air and water was entered from the surface until 15 DAH and it was applied to 18light:6dark photoperiod (18L:6D h). The feeding protocol is at Table 1. Prior to feeding, samples were taken from 3, 5, 7, 10, 12, 15, 17, 20, 22, 25, 27, 30, 32 DAH larvae triplicates and taken to protection in liquid nitrogen tank (-196 ºC).

In Vitro Assay

Extracts of larvae

The larvae were thawed by maintaining the cold chain and rinsed in distilled water, and the whole body was homogenized (400 mg/mL in distilled water) and centrifuged (16,000 g, 30 minutes at 4 °C) to extract the larvae.

Extracts of feed ingredients

Extracts of feed ingredients (fish meal, fish hydrolysate, krill meal, soybean meal, wheat gluten, corn gluten, and sunflower meal) were prepared by homogenization (100 mg/mL in distilled water) followed by centrifugation (15,000 g, 10 minutes).

Determination of protease activities of larvae

Total protease activities of larvae were measured as described by Walter (1984), using casein (10 mg/mL) in 50 mM Tris-HCl buffer at pH 8.5 as the substrate. The mixtures including extracts of larvae and substrate were incubated and then the reaction was stopped by addition of 500 µL trichloroaceticacid (TCA) (concentration of TCA, 120 g/L). Total protease activities were determined as spectrophotometrically (Shimadzu UV mini 1204). One unit of enzyme activity was defined as 1 µg of tyrosine release per minute. The soluble protein concentrations of larvae were determined according to Bradford (1976).

Effects of feed ingredients on protease activities of larvae

The inhibitory effects of feed ingredients on protease activities of meagre larvae were determined by measuring the reduction in protease activity of extracts using a modification of the method described by García-Carreno (1996). The method was based on the measurement of residual protease activity remaining after preincubation with feed ingredients. Values were calculated as inhibition degrees %.

Statistical Methods

Larval measurements were made on 30 samples. *In vitro* assays were performed in triplicates. The experimental data, the larval total length and weight and larvae's protease and the inhibition degrees of feed ingredients were subjected to one-way ANOVA and mean ±standard error (SE) differences were calculated by using SPSS software (v21, IBM, USA) statistical package. Statistical significance of larvae's protease was tested by Duncan test at P=0.05 content level.

Results and Discussion

Meagre is an important species because of market preferences and product diversity in various sizes with rapid development production in Mediterranean marine aquaculture (Monfort, 2010). Studies on this important and potential cultivation have been continuing at a considerable level in recent years (Fernández-Palacios et al., 2007; Gil Oviedo and Gracia and Jofre, 2013; Bodur et al., 2014; Vargas-Chacoff et al., 2014; Velazco-Vargas et al., 2014; Candeias-Mendes et al., 2015; Saavedra et al., 2016; Campoverde et al., 2017). Among these studies, diet and nutrition relationship are the main research topics. In addition, meagre is a species that has the potential to evaluate vegetable feed ingredients (Bestin et al., 2014; Dias et al., 2014; Ribeiro et al., 2015). Our study, is a research that supports investigations in which we conducted *in vitro* studies to determine the feed ingredients of the meagre larvae. *In vitro* techniques have been used and recommended by many investigators (Eid and Matty 1989; Ezquerra et al., 1998; Alarcón et al., 1999; Ali et al., 2009; Kuzu and Naz, 2012; Yıldız et al., 2012; Yılmaz et al., 2012). Significant results have been achieved with this method for determining potential inhibitory effects of feed ingredients (fish meal, fish hydrolysate, krill meal, soybean meal, wheat gluten, corn gluten, and sunflower meal) on protease activities of larvae of meagre.

Total length and wet weight gains were obtained from 3 DAH to 32 DAH of the meagre larvae (Figure 1, 2). It determined that these values have increased from 0.53 ±0.02 mg to 118.00± 1.09 mg and 3.19 ±0.02 mm to 21.61 ±0.22 mm, respectively. These results indicated that the larval stage of meagre is a species with a high rate of development. The results of the study revealed that larvae had high growth rates. These results were similar to the larval stage results of Gamsız and Neke (2008), Arda (2011) and Papadakis et al., (2013) and support the expression that Quemener (2002), and Gamsız and Neke (2008)'s meagre larvae had a high rate of development.

Changes in protease activity of larvae of meagre were calculated at the highest value at 7 DAH (211.21 ±12.56 U/mg protein) and the lowest at 15 DAH (5.95 ±0.6 U/mg protein) (Figure 3). Sharper decreases and increases were observed in protease activity changes on days 3, 5, 7, and 10, and these values were determined at statistical differences at significant levels on the 4 measurement days (P<0.05). From 10 DAH to 32 DAH, there were no significant differences in protease activity changes and calculated lower before 10 DAH. The protease activity changes of meagre larvae supported that the fluctuations in the protease activities of the larvae of gilthead seabream was not related to the decrease in enzyme synthesis but reflected an increase in tissue proteins (Zambonino Infante and Cahu, 2001).

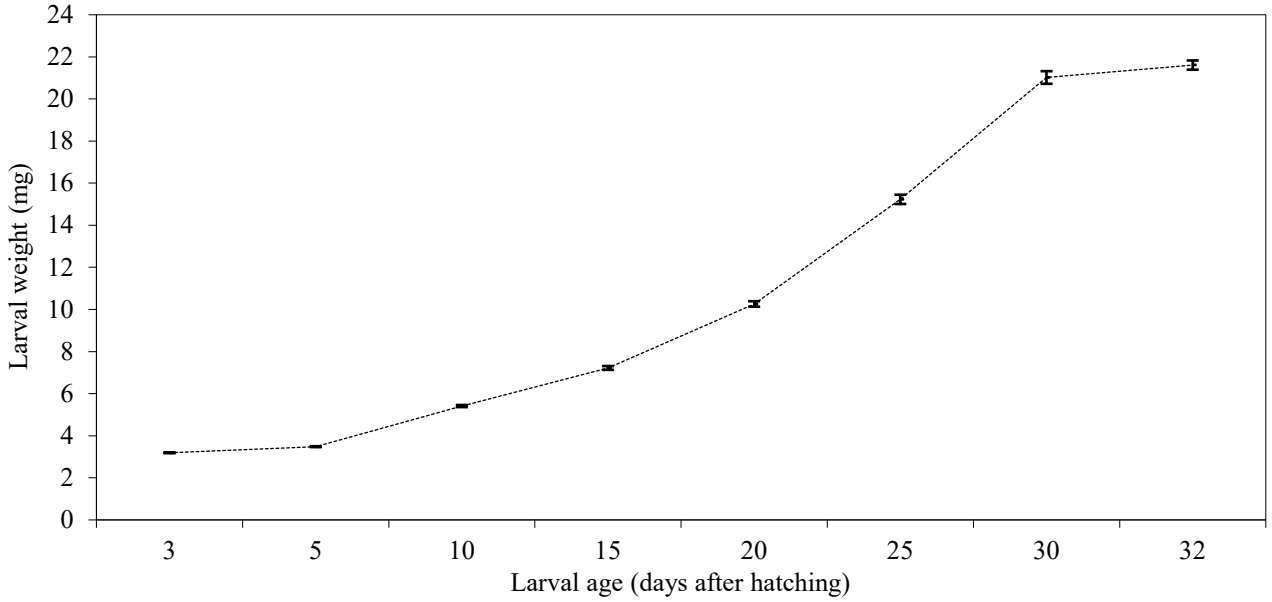

Figure 1. Weight changes of larvae of meagre (*A. regius*) (wet weight mean ±SE mg n=30)

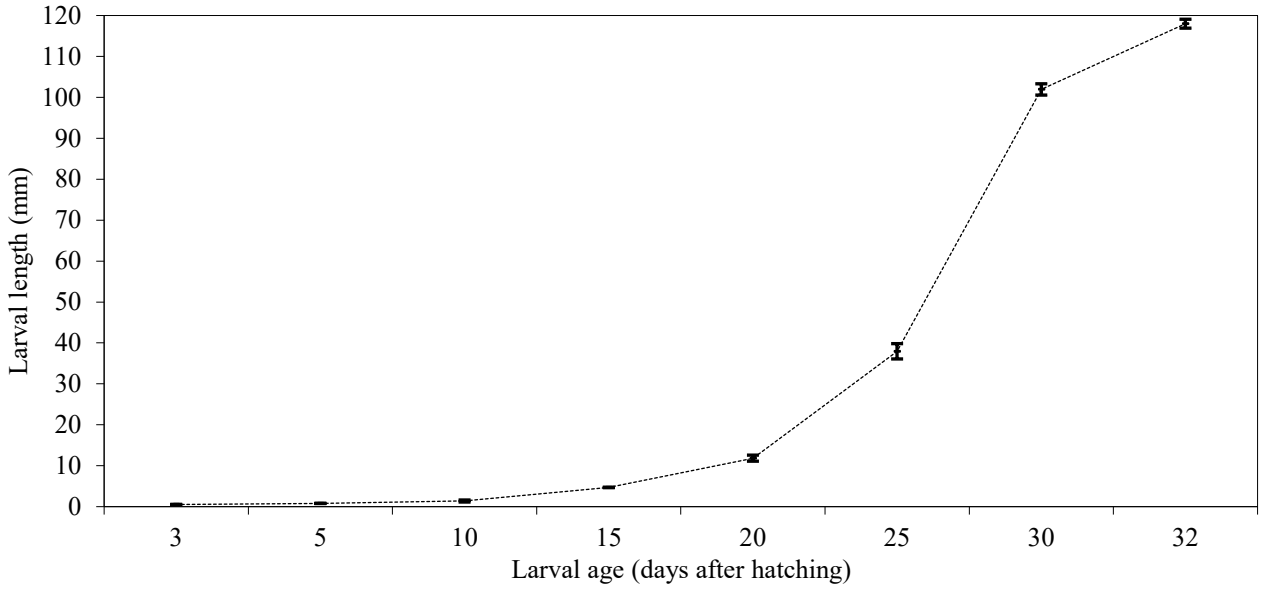

Figure 2. Lenght changes of larvae of meagre (*A. regius*) (total lenght mean ±SE mm n=30)

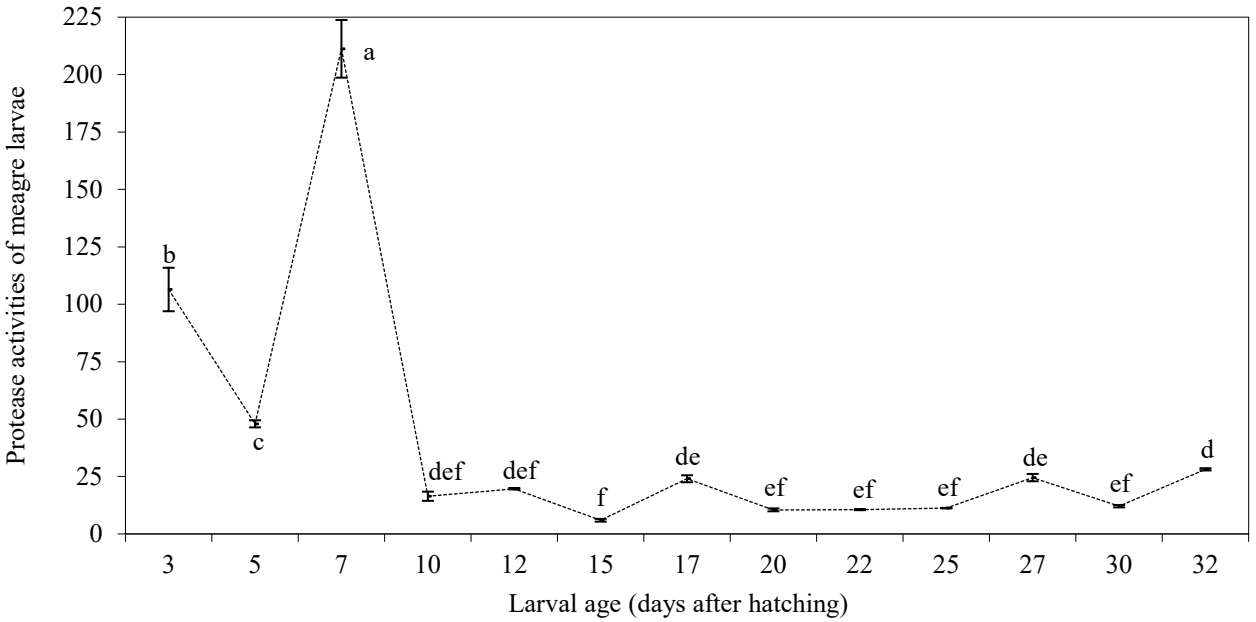

Figure 3. The changes of protease activities of larvae of meagre (*A. regius*) (U/mg protein mean ±SE mg). Different superscripts show significant differences between means of protease activities

The mean values of inhibition levels of feed ingredients at 3-32 DAH were calculated high in fish hydrolysate from animal sources and in soybean meal and wheat gluten from vegetable sources (Figure 4, 5). However, in these assessments, 3, 5, 7, 10, 12, 15, 17, 20, 22, 25, 27, 30, and 32 DAH of the analysis should be evaluated separately.

Results of inhibition analysis revealed that fish meal had low inhibitions until 10 DAH while it was expected that larvae offered fish hydrolysate would exhibit worse performance than fish meal (Figure 4). Fish meal, fish hydrolysate and krill meal showed the lowest inhibitions at 15 DAH and then, followed by a sharp increase from 15 to 17 DAH. The inhibitions of fish meal and krill meal tended to increase until 20 DAH but not fish hydrolysate. After 20 DAH, fish hydrolysate had the highest inhibitions. Fish meal exhibited lower inhibitions at 17, 22, 27, and 32 DAH than those of both fish hydrolysate and krill meal. However, fish hydrolysate at 12, 20, 25, and 30 DAH exhibited lower inhibitions than those of fish meal. Kolkovski and Tandler (2000) reported even 50% replacement of the dietary protein with hydrolysed squid meal was associated with a decline in seabream larval growth. This study clearly reveals the suitability of fish meal for critical larval stages but not fish hydrolysate except for the mentioned days above.

The results demonstrate that krill meal had low inhibitions in critical larval stage except for 5 DAH. After 15 DAH, krill meal showed better performance than those of fish hydrolysate except for 20 DAH and also, fish meal at 25 and 30 DAH. Kolkovski et al. (2000) reported that feed attractants such as krill can play an important role in acceptance of dry diets in fish larvae during the weaning period as well as enhancing growth due to higher consumption. They showed that, coating dry diets with liquid krill hydrolysate can improve dry diet attractiveness, increase in larval growth and can potentially decrease the duration of weaning period. Our results revealed that krill meal is a good candidate to be used in microdiets of meagre larvae except for the mentioned days above.

The inhibitions of soybean meal on protease activities of larvae were high except for 15 and 25 DAH (Figure 5). In addition, wheat gluten on protease activities of larvae had the high inhibitions except for 15, 20, 22, 25, and 30 DAH. Corn gluten had lower inhibitions than those of soybean meal and also wheat gluten except for 30 DAH. Sunflower meal had low inhibitions except for 5, 15, and 25 DAH. The lowest inhibition of sunflower meal was measured at 3 DAH. The lowest inhibitions of soybean meal, wheat gluten, and corn gluten were determined at 15 DAH except for sunflower meal. The highest inhibitions of soybean meal, wheat gluten were observed at 3 DAH and corn gluten at 17 DAH. Soybean meal and wheat gluten exhibited high inhibitions until 12 DAH. However, corn gluten and sunflower meal in these days had better performance except for 5 DAH of sunflower. Inhibition results indicated that soybean meal is not suitable

for microdiets of meagre larvae. However, corn gluten could be used as feed ingredient in microdiets of meagre larvae. Soy protein concentrate and corn gluten have been researched as possible fish meal replacement in aquaculture feeds. Complete substitution of fish meal with soy protein has been achieved only in rainbow trout (Kaushik et al., 1995; Rodehutscord et al., 1995). In addition, a similar study has been reported that soy protein concentrate and vegetable protein concentrate do not appear to be suitable for meagre larvae (Diken et al., 2016c). The use of soy protein or corn gluten as the sole protein source in diets for gilthead seabream is not recommended (Kissil and Lupatsch, 2004). Hence the replacement of fish meal with a mixture of several vegetable protein sources is a common approach in order to minimize the amino acid deficiencies in fish diet and meet the nutritional requirements of fish species (De Francesco et al., 2007). Kissil and Lupatsch (2004) reported that processing of soybean meal (heating, defattening or germination) does not guarantee the elimination of antinutritional factors. Moyano et al. (1999) showed that protease inhibitors in soybeans and corn gluten reduce the activity of proteolytic enzymes in seabream and also soybean inhibitors have a stronger effect than those in corn gluten. Results obtained positive effects of corn gluten and negative effects of soybean meal on protease activities of meagre larvae and was supported by Moyano et al. (1999). On the other hand, it was reported that meagre larvae of soybean meal (defatted soybean meal) can be used at the level that can substitute fish meal in their growing feed (Velazco-Vargas et al., 2013).

Wheat gluten was researched as possible fish meal replacement in aquaculture feeds. Complete substitution of fish meal with wheat gluten was achieved only in rainbow trout (Kaushik et al., 1995; Rodehutscord et al., 1995). Kaushik et al. (2004) recently suggested that the use of wheat gluten in combination with other plant proteins may be economically feasible as a fish meal substitute for European seabass. Results of the present study indicated that wheat gluten is not suggested until 12 DAH. After 12 DAH, wheat gluten can be used up to 32 DAH except for 17 and comparatively 27 DAH. Sunflower meal and corn gluten had lower inhibitions when compared with other plant protein sources. According to the results of this study, sunflower meal is moderately advisable as feed ingredient in microdiets of meagre larvae except for 5 and 25 DAH.

On the protease activities of gilthead seabream larvae corn gluten, and on the protease activities of European seabass larvae wheat gluen and protease activities of both marine fish larvae soybean meal have not been recommended due to inhibition effects. However, wheat gluten for gilthead seabream larvae and corn gluten for European seabass larvae have been recommended (Kuzu and Naz, 2012; Yıldız et al., 2012). Our study results also suggest that soybean meal has not been recommended for meagre larvae, marine fish larvae are important for feed formulations. On the other hand, the preference for meagre larvae supports the availability of vegetable feed ingredients in microdiet formulations of the meagre larvae of corn and wheat gluten.

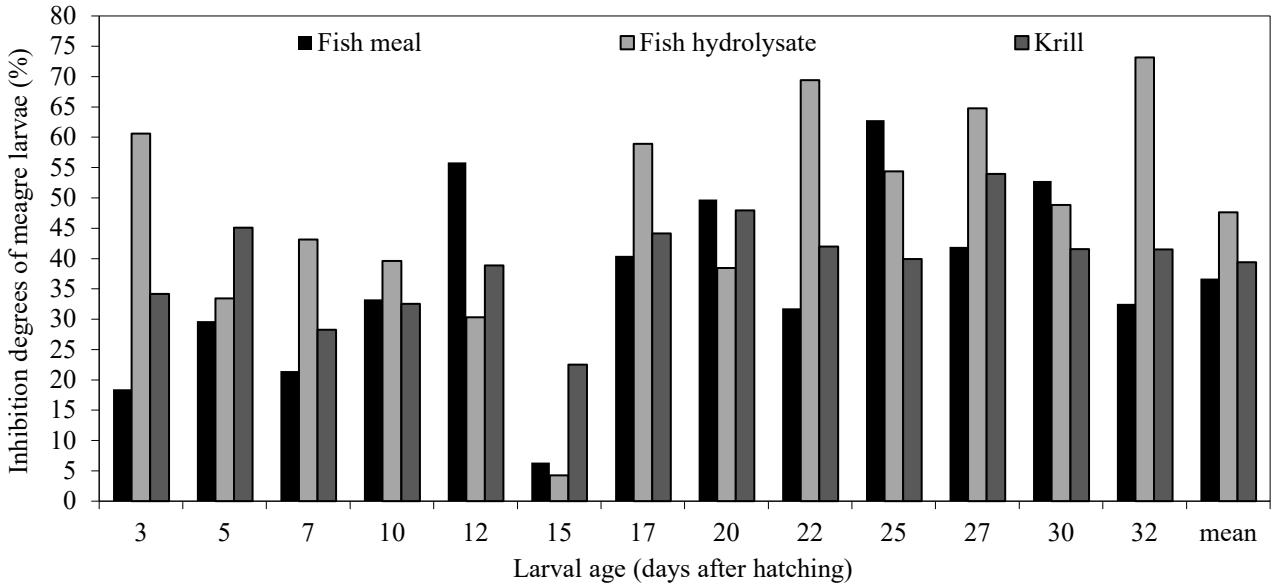

Figure 4. The inhibitory effects of feed ingredients such as fish meal, fish hydrolysate, and krill meal on protease activities of larvae of meagre (*A. regius*) (%)

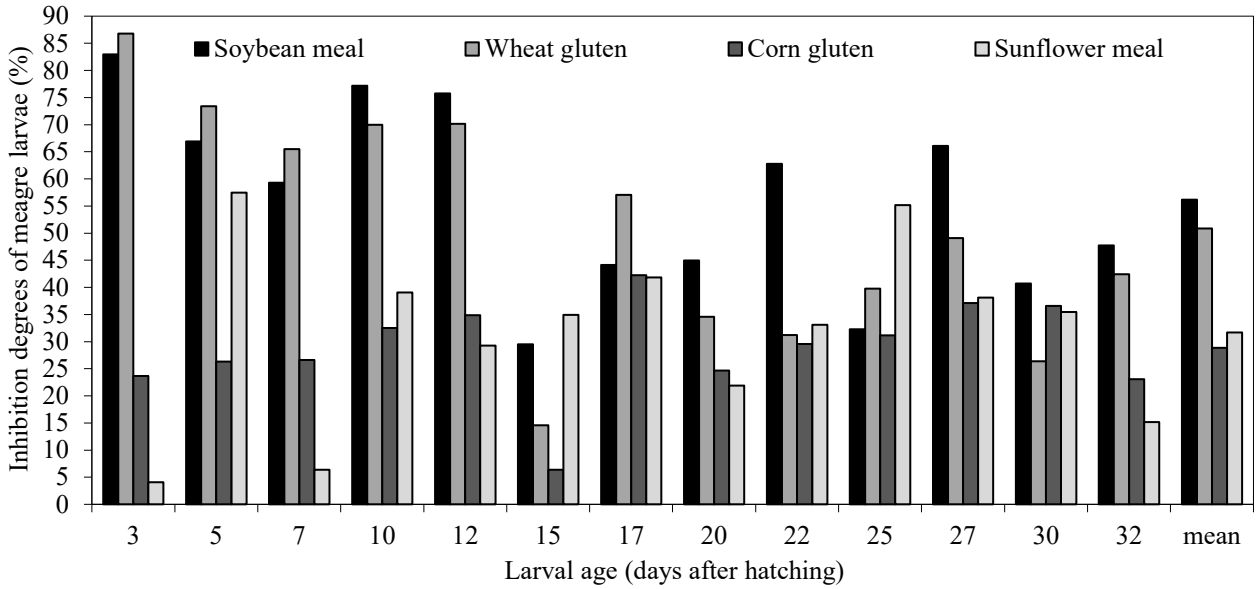

Figure 5. The inhibitory effects of feed ingredients such as soybean meal, wheat gluten, corn gluten, and sunflower meal on protease activities of larvae of meagre (*A. regius*) (%)

Table 1. Meagre (*A.regius*) larvae's feeding protocol

DAH	Practice
3-15	**Green water** (Commercial powder microalgae-Sanolife GWS; Inve Aquaculture, NV Hoogveld, 91 9200, Dendermonde, Belgium or ω3 Algae®; Bernaqua, NV Hagelberg, 3 B-2250, Olen, Belgium)
16-26	Sanolife GWS
	Live food
3-9	Rotifer, *Brachionus plicatilis* Culture (Commercial culture diets-Algamac Protein Plus; Aquafaune Bio-Marine Inc. Hawthorne USA and Sparkle, INVE Aquaculture) Enrich (Commercial enriched diet-Spresso; INVE Aquaculture) 10-15 prey/mL
6	*Artemia* nauplii (AF480; Inve Aquaculture) 2-4 prey/mL
10	*Artemia* metanauplii 1.5-6 prey/mL Enrich (Artemia EG; Great Salt Lake Brine Shrimp Cooperative Inc., Utah, USA), (Commercial enriched diets-Algamac 3050-Aquafaune, Red Papper-Bernaqua, and Spresso-INVE Aquaculture, 26 ºC and 28 g/L)
16-32	**Microdiets** (Orange Start-S,100-200 µ, Orange Start-L, 200-300 µ, Orange Nurse-XS, 300-500 µ, Orange Grow-S, 300-500 µ, Orange Grow-L, 500-800 µ; INVE Aquaculture)

Conclusion

In conclusion, fish meal and krill meal is advised to be used as feed ingredient in microdiets of meagre larvae but not for fish hydrolysate except for the mentioned days (12, 15, and 20 DAH) and the use of fish hydrolysate should be paid attention. Second, soybean meal seems not to be a good candidate as feed ingredient due to having higher inhibitions on protease activities. Third, wheat gluten is not recommended until 12 DAH. Fourth, corn gluten and sunflower meal could be used as feed ingredient in formulations of meagre larvae. Fifth, the highest resistance to protease inhibitors found in feed ingredients was observed at 15 DAH. When such data become available, they will serve the replacement of fish meal with alternative and sustainable feed ingredients. These results are also recommended in future studies, as it will be an important factor in determining inhibitory effects on the protease activities of marine fish larvae.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: In this study, it was approved by the Local Ethical Committee of Animal Experiments of the Süleyman Demirel University

Ref. Number: B.08.6.YÖK.2.SD.0.05.0.07.00-22

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