Reduction of skeletal anomalies in meagre (*Argyrosomus regius*, Asso, 1801) through early introduction of inert diet

Short title: Skeletal analysis of meagre with early weaning

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Abstract

The consolidation of meagre (*Argyrosomus regius*) in aquaculture requires an understanding and optimization of larval rearing and nutritional conditions. The aim of this study was to analyse the effects of an early introduction of inert diets during larval rearing, on growth performance, digestive enzymes activity and development of skeletal anomalies. This study evaluated the effects of three different timings for the introduction of inert diet during larval rearing: a control group (CTRL) where inert diet was initiated at 14 days after hatching (DAH) and two treatment groups that had an earlier introduction of inert diet at 8 DAH (T1) and 11 DAH (T2). Meagre larvae exhibited similar pancreatic and intestinal enzymatic activities among the different dietary treatments. No differences in the overall prevalence of anomalies were observed between treatments at 25 or 50 DAH, however a significant reduction was observed in all groups with the transition from larval to juvenile stage. The precocious introduction of inert diet shifted the distribution of vertebral anomalies to a more anterior vertebral column region. Altogether, this study shows that earlier introduction of inert diets in meagre hatcheries can be beneficial for meagre production in aquaculture.

Keywords: marine fish, digestive enzymes, skeletal development; skeletal anomalies, larval rearing, weaning.

1. INTRODUCTION

Meagre (*Argyrosomus regius*) is a highly valued species with increasing interest for aquaculture, particularly in the Mediterranean and Southern Europe (Cardeira et al., 2012; Chatzifotis et al., 2010; Poli et al., 2003; Pousão-Ferreira et al., 2013; Roo,

Hernández-Cruz, Borrero, Schuchardt, & Fernández-Palacios, 2010; Saavedra et al., 2016). Several studies have demonstrated that meagre is a strong candidate for the European aquaculture diversification due to its high commercial value and suitability for intensive production (Cardeira et al., 2012; Chatzifotis et al., 2010; Poli et al., 2003; Ribeiro et al., 2015; Roo et al., 2010; Saavedra et al., 2016; Schiavone, Zilli, Storelli, & Vilella, 2012). Moreover, meagre has high larval survival rates, excellent biological performances (~1 kg/year), and more importantly, high resistance to captivity and environmental stress such as temperature and salinity conditions (Fountoulaki et al., 2017).

The knowledge on meagre optimal larvae rearing in terms of environmental and nutritional conditions is still limited and essential for the optimization of this species production. The ontogeny of the external morphology (Cárdenas, 2011) and skeletal development events (Cardeira et al., 2012) in early life stages of meagre have been previously described, however there are scarce reports regarding the occurrence and the typologies of skeletal anomalies (Campoverde et al., 2017). Meagre production is affected by severe vertebral anomalies (Soares, Fernández, Costas, & Gavaia, 2014), thus affecting the external appearance and rendering the fish unsuitable for commercialization. It is widely recognized that skeletal anomalies constitute a major economic bottleneck in aquaculture (Boglione, Gavaia, et al., 2013a; Boglione, Gisbert, et al., 2013b; Sfakianakis, Georgakopoulou, Kentouri, & Koumoundouros, 2006) that can affect up to 20% of juvenile production and reaching occasionally values up to 45-100% in species such as *Sparus aurata* (Boglione, Gagliardi, Scardi, & Cataudella, 2001; Koumoundouros, 2010). Morphological anomalies are rare in the wild, however they are abundant in captivity (Gavaia et al., 2009; Georgakopoulou, Katharios, Divanach, & Koumoundouros, 2010) and considered an important stress

bio-indicator (Boglione et al., 2006). It is common to find anomalies in aquaculture produced species affecting preferably structures like opercula, mandible, fins and axial malformations such as vertebral curvatures (kyphosis, scoliosis and lordosis). It is also common to find caudal fin anomalies and vertebral compressions and fusions, as well as alterations in the number of arches and spines (Bardon et al., 2009; Divanach, Papandroulakis, Anastasiadis, Koumoundouros, & Kentouri, 1997; Gavaia, Dinis, & Cancela, 2002; Koumoundouros et al., 1997; Mazurais et al., 2009). Fish with a high accumulation of anomalies or showing severe alterations in fish morphology are discarded or downgraded in aquaculture, which represents a relevant loss of profit (Boglione et al., 2009; Boglione, Gavaia, et al., 2013a; Koumoundouros, 2010).

In aquaculture, fish diet constitutes the highest cost (50-70%) of the total economical investment of fish production (Naylor et al., 2000). Consequently, to achieve a sustainable fish production, the improvement of diets and feeding protocols is essential to ensure high quality larvae and improve growth rates (Saavedra et al., 2016). The use of life feeds during larval production is considered the major investment, since its production is much more expensive when compared to the use of inert diets (Campoverde et al., 2017). Therefore, the improvement of meagre larval feeding protocol aims the reduction of live feeds, by early introduction of inert diets, which constitutes a powerful tool to reduce feeding costs. Additionally, this strategy also aims at ensuring fish welfare and normal development. The objective of this work was to assess the effect of early introduction of inert diet during larval rearing on survival, growth performance, digestive capacity (enzymatic activity) and incidence of skeletal anomalies.

2. MATERIAL AND METHODS

2.1 Rearing conditions

Eggs were collected during the natural reproductive season for meagre (May), from a wild captured broodstock previously adapted to captivity (6 lots of 14 breeders at a ratio of 1:1 females and males), and larval rearing was conducted at the Aquaculture Research Station of the Portuguese Institute for the Sea and Atmosphere (IPMA, Olhão, Portugal). Eggs were incubated in 200 L fiberglass tanks and after hatching larvae were distributed in nine (n = 9) cylindroconical fiber glass tanks (200 L) at a density of 44 ± 0.3 larvae/L in a semi-open system, where they were maintained until 25 days after hatching (DAH). From 25 to 50 DAH the larvae were reared in 300 L fiberglass tanks in open system. The tanks were supplied with pre-filtered, UV sterilized sea water and water turnover was adjusted according to growth. During the larval rearing period the temperature, oxygen, salinity and mortality were monitored daily (Table 1).

2.2 Feeding protocols

In this study, two feeding protocols were used where inert diet was introduced at early stages compared to the standard protocol: 8 DAH (T1 - treatment group 1) and 11 DAH (T2 - treatment group 2), when compared to standard feeding protocol (CTRL) where diet is introduced at 14 DAH (Pousão-Ferreira et al., 2013). Feeding protocols were tested in triplicate (n = 9), and for all protocols inert diet was used in co-feeding with live preys until 25 DAH (Figure 1). Briefly, larvae were fed 4 times a day between 3 and 9 DAH and afterwards fed 6 times a day, between 10 DAH to the end of the experimental trial. Larvae were first fed with enriched rotifers (*Brachionus sp.*) four times a day, from 3 to 15 DAH, with an initial concentration of 5 rotifers/ml, which was increased by 2.5 rotifers/ml per day until 10 DAH. At 10 DAH, the concentration

of rotifers were decreased in order to replace rotifers by enriched *Artemia* (*Instar II*) at a concentration of 0.5 metanauplii/ml, which was increased by 2.5 metanauplii/ml per day between 10 and 20 DAH. After 20 DAH this concentration was gradually reduced and completely eliminated at 25 DAH, being replaced by inert diet. In T1 and T2 the concentrations of live feeds were adjusted according to the time of introduction of inert diets to ensure a uniform amount of feed in all treatment groups. The initial daily amount of dry feed was approximately 1 g per tank, and increased by approximately 0.5 g per day during all the experiment. Fish were fed *ad libitum* and the granulometry of the pellets (CAVIAR, Bernaqua™, Belgium) were adjusted according to development of fish mouth apparatus.

Both rotifers and *Artemia* (*Instar II*) (SepArtTechnology™, INVE, Dendermonde, Belgium) were previously enriched with Red pepper™ (Bernaqua, Belgium), according to the manufacturer´s specifications. Green water technique (Papandroulakis, Divanach, & Anastasiadis, 2001) was performed between 3 DAH until 23 DAH, by adding phytoplankton (*Nannochlosopsis sp.* and *Isochrysis sp.*) to the rearing tanks. This procedure was accomplished by adding a concentration of 120 – 160 x 10^3 cell/ml that were reduced from day 20 DAH onwards and completely removed at 23 DAH.

Survival was calculated at 50 DAH by determining the number of surviving fish relatively to the number introduced in each tank at the beginning of the trial (Table 1). Larval total length and weight were analysed at regular intervals until the end of the experimental period at 50 DAH (Table 2).

2.3 Sample Collection

Samplings were performed by random collection of 15 specimens from each tank at 25, 30, 40 and 50 DAH to determine total length (TL), weight and growth indexes (Table 2). At 25 DAH, before feeding, a group of 45 larvae were collected, from each tank, to perform digestive enzymes analysis. Larvae were rinsed with distilled water and immediately frozen in liquid nitrogen, being stored at -80ºC until further analysis. A supplementary sampling of 30 specimens per replicate was performed at 25 and 50 DAH for analysis of skeletal anomalies. The number of collected specimens for each of the analytical procedures were taken in account on the calculation of mortality rates, except for the specimens collected at the end of the experiment at 50 DAH.

The specimens were sacrificed with an overdose of 2-phenoxyethanol (400 ppm) (Sigma-Aldrich, Spain), photographed for analysis of TL using a stereomicroscope (Leica MZ6, Wetzlar, Germany) equipped with a digital camera Sony cybershot PSC-P71 (Sony, Japan). The fish were weighed and fixed with 4% paraformaldehyde at pH 7.4 (Sigma-Aldrich, Spain) for whole-mount double staining at 25 DAH, while at 50 DAH the specimens were immediately stored at -30ºC for radiographic analysis.

2.4 Calculation of growth performances

The growth performances were calculated with TL and weight analysed at specific periods of growth (30 to 50 DAH). Growth was characterized in terms of weight gain (WG, %), daily growth (DGI, %) according to (Silva et al., 2010), and specific growth rate (SGR, %) according to (Rane & Markad, 2013):

Weight gain (WG)

=100*(final body weight – initial body weight) / (initial body weight)

Daily growth index (DGI)

$$=100*((\text{final body weight}^{1/3}) - (\text{initial body weight}^{1/3})) / \text{days}$$

Specific growth rate (SGR)

$$=100*(\ln(\text{final body weight}) - \ln(\text{initial body weight})) / \text{days}$$

2.5 Digestive enzymes determinations

Fish larvae were dissected, on a glass maintained at 0ºC, to obtain the abdominal cavity used for digestive enzymes determinations, following the methodology described by (Guerreiro et al., 2010). The collected tissue was homogenized in 30 volumes (w/v) of ice cold Manitol (50 mM), Tris-HCl buffer (2 mM) at pH 7. A known volume of the homogenate was used for the purification of the brush border membranes according to the methodology described by (Cahu & Zambonino-Infante, 1994) for fish larvae. The remaining volume was centrifuged at 3300 g at 4ºC for 3 min.

Digestive enzymes were determined following the methodology described in (Guerreiro et al., 2010). Briefly, trypsin (E.C.3.4.21.4) activity was measured at 25ºC using BAPNA (Nα-Benzoyl-DL-arginine-p-nitroanilide) as substrate in trizma-CaCl2 buffer (20 mM) at pH 8.2. Amylase (E.C.3.2.1.1) activity was assayed using starch as the substrate dissolved in NaH2PO4 buffer (0.07 M) at pH 7.4. Lipase (E.C.3.1.1) activity was measured using p-nitrophenyl myristate (0.53 mM) as a substrate dissolved in Tris-HCl buffer (0.25 M) at pH 9.0, 2-methoxyethanol (0.25 mM), and sodium cholate (5 mM). Acid protease activity was determined using bovine haemoglobin dissolved in HCl (1M) as substrate at pH 2. Alkaline phosphatase (E.C.3.1.3.1) activity was assayed using pNPP 5 mM (p-nitrophenylphosphate) as substrate in a solution of carbonate buffer (30 mM) at pH 9.8. Leucine-alanine peptidase (E.C.3.4.11) activity was measured using leucine-alanine peptidase (0.01

M) as substrate in LAOR (L-amino oxidase, horseradish peroxidase, o-dianisine) and was dissolved in a Tris-HCl 50 mM buffer at pH 8.0.

Enzyme activities were calculated as micromoles of substrate hydrolysed per min (i.e., U) at 37ºC for alkaline phosphatase and leucine-alanine peptidase, and at 25ºC for trypsin. Amylase activity was expressed as the equivalent enzyme activity which was required to hydrolyse 1 mg of starch in 30 min at 37ºC. Acid protease activity was expressed as specific activity with 1 U representing 1 mM equivalent of tyrosine liberated per minute per mg of protein at 37ºC. One unit of lipase activity was defined as 1 μmoL of p-nitrophenol released per min at 30ºC. Protein was determined by the Bradford method (Bradford, 1976). Enzyme activities were expressed as specific activities, i.e., U/mg protein or mU/mg protein.

2.6 Whole-mount double staining and radiographic analysis

The identification of skeletal anomalies at 25 DAH was performed using a modified acid free double staining procedure with alcian blue 8 GX (Sigma-Aldrich, Spain) and alizarin red S (Sigma-Aldrich, Spain) according to (Walker & Kimmel, 2007) and (Gavaia, Sarasquete, & Cancela, 2000). Stained samples were photographed in a stereomicroscope (Leica MZ6, Wetzlar, Germany) equipped with a digital camera Sony cybershot PSC-P71 (Sony, Japan).

Skeletal analysis in the specimens collected at 50 DAH was performed through radiographic analysis, with a low energy digital X-Ray apparatus (Kodak DXS4000 System, USA) with 2 min of exposure, FOV 15 mm, KVP 35, POV 120 mm. The images were further analysed using Carestream Molecular Imaging software (Caresteam, USA). The prevalence of skeletal anomalies (% of abnormal specimens), load of anomalies (number of anomalies / number abnormal specimens) and load of

fusions and compressions (number of fusions or compressions/number of abnormal specimens) were calculated in the different experimental groups. An analysis of the distribution of the anomalies was made according to the different affected structures. Nomenclature for skeletal elements was performed using the description of meagre skeletogenesis by (Cardeira et al., 2012).

2.7 Statistical analysis

Data were expressed as mean ± standard deviation (SD). All data was tested for normality, homogeneity and independence prior analysis. Differences in TL, weight of meagre, prevalence of anomalies and digestive enzymes activity were analysed through one-way ANOVA and significant differences among treatment groups were detected using a Tukey´s HSD test ($p < 0.05$). The remaining results were analysed by Person´s Chi-squared test ($p < 0.05$). Statistical analysis was performed in IBM SPSS® 25.0 software.

3. RESULTS

3.1 Growth performance and survival

The experimental groups with precocious diet introduction (T1 at 8 DAH and T2 at 11 DAH) showed higher growth in length and weight when compared to the control group (CTRL at 14 DAH) at 25 DAH, while at 50 DAH only T1 showed a significantly higher weight (Table 1 and 2). Moreover, data showed that earlier introduction of inert diet (T1) led to higher growth performance when compared to the groups that started feeding inert diet later on (T2 and CTRL) (Table 2).

3.2 Digestive enzymes activities

The precocious introduction of inert diets did not affect the digestive capacity of meagre larvae (Figure 2). Similar values for digestive enzymatic activities were observed among treatments, namely for pepsin, pancreatic (trypsin, amylase, lipase) and intestinal (alkaline phosphatase and leucine-alanine peptidase) enzymes ($P>0.05$).

3.3 Skeletal analysis

The most common types of skeletal anomalies observed in meagre rearing were those affecting the vertebral column. These include fusions and compressions of two or more vertebrae causing the deformation of skeletal axis. The cephalic vertebrae were mostly affected by fusions, while the remaining vertebral regions - pre-haemal and haemal vertebrae - were affected by both fusions and compressions (Figure 3). No differences in prevalence of anomalies were found between groups at each sampling point analysed. However, a higher prevalence of anomalies was observed in all groups at 25 DAH compared to 50 DAH (Figure 4).

The treatment groups under different feeding protocols presented variation in the structures affected by bone anomalies. In T2 specimens, the cephalic vertebrae were less affected by anomalies than in CTRL. T1 showed a lower prevalence of anomalies in haemal vertebrae, in comparison with the other treatment groups at 25 DAH (Figure 5A). However, those differences in distribution of anomalies were not observable at 50 DAH by radiographic analysis, since no statistical differences were found among the different areas (Figure 5B). At 50 DAH, there were no anomalies affecting the caudal fin vertebrae. The control group showed no anomalies in cephalic vertebrae (Figure 5B). At 25 DAH, there were more than 4 vertebral anomalies per specimen both in control and T2 groups. However, the load of anomalies between treatment groups was not significant. Considering only the deformed individuals sampled at 25

DAH (Figure 6A) the fish showed one or two affected areas while at 50 DAH only one area was mostly affected (Figure 6B).

A detailed analysis throughout the vertebral column showed that, at 25 DAH, deformed vertebral structures in the CTRL and T2 groups had higher prevalence in the haemal vertebrae (Figure 7A and 7C) while in the T1 group were mostly located in the pre-haemal region (Figure 7B). At 50 DAH, most of the anomalies in the CTRL were located in the pre-haemal and first haemal vertebrae (v6 – v15) (Figure 7A), while in T1 and T2 groups the anomalies were distributed more anteriorly, centred on the cephalic and pre-haemal vertebrae (v3-v12) (Figure 7B and 7C). Considering the load of fusions, no differences were observed between treatment groups at 25 DAH. However, there were differences in the load of compressions, with T2 presenting a higher prevalence in comparison with CTRL and T1 treatments (Figure 8A). At 50 DAH, no statistical differences were observed in the load of fusions and compressions among treatments (Figure 8B).

4. DISCUSSION

The successful implementation of meagre in aquaculture with standardized procedures depends on the development and optimization of production methodologies that result in higher survival and growth rates to obtain high quality progeny (Saavedra et al., 2016). This study showed that an earlier introduction of inert diets at 8 DAH in the meagre hatchery production cycle is viable and promotes improved biological performances when compared to later introductions (12 and 14 DAH). This method decreased skeletal anomalies without compromising growth performance and digestive activity. The growth rate observed in our study is in agreement with the

results by Papadakis, et al., (2013) that presented results of production in a mesocosm system with temperature conditions similar to those used in the present study.

Growth rate is highly dependent on a multiplicity of factors and fish larval weaning can only be performed with success when the anatomical and physiological structures enable the larvae to metabolize correctly the diet. In our study fish length at 8 DAH (SL, 4.1 ± 0.2) were similar to previously described by Campoverde et al. (2017) at 12 DAH. In both cases, an adequate larval development was observed. Consequently, we propose that an SL of 4 mm is an adequate indicator to initiate inert diet introduction in meagre. The transition from live feeds to inert diets must be performed according to development of the mouth apparatus which is generally length dependent (Kestemont et al., 2003). Still, the introduction of inert diet occurs during larval development and might interfere with the digestive mechanisms, decreasing the activity of digestive enzymes or delaying its appearance (Zambonino-Infante et al., 2008). In this study, similar digestive enzymes activities were observed at 25 DAH despite the differences in the timing of inert diet introduction. Pancreatic enzymes trypsin, amylase and lipase exhibited similar activity levels, indicating that the precocious diet introduction did not affect the establishment of digestive processes. The magnitude of activities for these digestive enzymes was higher than the values reported by Campoverde et al. (2017) where a co-feeding regime was performed with inert diet and *Artemia* at 12, 15 and 20 DAH. Our data for larval length is in agreement with the results presented in that study. However, in our work we observed higher enzymatic activities and no differences were observed between treatments, validating the improvement obtained with the methodology here described. The differences between enzymatic activities might be justified by differences on inert diet composition and/or the amount of live feed used. Being a voracious and fast-growing

species, the acquisition of acidic digestion is important for meagre to cope with high developmental rates. In the present study, similar values for pepsin-like activities were obtained independently of the inert diet introduction period, suggesting that meagre were able to rely on acidic digestion at a total length equal or higher than 13 mm (13 mm – CTRL; 16 mm - T1 and T2). This observation is in agreement with the results of Papadakis et al., (2013) that described the presence of gastric glands and y-shaped stomach respectively at 14 DAH and 18 DAH. At this point meagre larvae reached 7 (14 DAH) and 10 mm (18 DAH) of total length, respectively (Papadakis et al., 2013). A similar trend was observed for intestinal enzymes where both cytosolic (reflected by leucine-alanine peptidase) and brush border levels (reflected by alkaline phosphatase) were similar between experimental groups. Consequently, the earlier introduction of inert diet did not affect the maturation of the intestinal epithelium. The variability observed within replicates contributed to eliminate any potential differences on the activity of digestive enzymes for the different experimental groups. This variability is often related with the different length of meagre larvae on the pool, as also observed by Campoverde et al. (2017). Overall the similarity in the activities of different digestive enzymes obtained with meagre reared under different feeding protocols, reflect that despite differences on the age of inert diet introduction, meagre larvae were able to handle the introduction of microdiets as early as 8 DAH.

Early weaning in fish production is performed commonly with a co-feeding period to avoid larval stress (Engrola et al., 2009). A co-feeding period of 11 days was previously described in meagre (Campoverde et al., 2017), however we observed that a co-feeding period of 17 days results in higher larval length and weight. In this species, it was previously reported that short co-feeding regimes can induce stress, leading to cannibalism and high mortality Campoverde et al. (2017). In our study all

the treatments showed lower mortality rates (CTRL, 20.3±11.4%; T1, 27.4±4.9%; T2, 20.4±7.7%) when compared to the previous study (3.9±0.5%). These differences can be partly explained by the lower density of meagre larvae (44±0.3 larvae/L) used in our study when compared to previously reported by Campoverde et al. (2017) (100 larvae/L). Cannibalism is one of the major challenges in meagre larval rearing, since it is a voracious predator that relies naturally on this feeding strategy when food resources are limited (Kestemont et al., 2003). Our results suggest that low rearing densities can be used as a successful strategy to improve survival rates, as observed in other cannibalistic and voracious species (Fox, 1975; Baras & Jobling, 2002; Kestemont et al 2003).

The adaptation to inert diets during larval development might increment stress levels and influence nutrient digestibility. This can directly impact survival rates and affect bone formation and homeostasis, and consequently increase the prevalence of bone anomalies. Inert diets are more energetic than live feeds and may influence fish performance promoting an unbalance between muscular and skeletal development, thus leading to higher prevalence of skeletal anomalies (Boglione, Gavaia, et al., 2013a). However, in our study there was no observed increase in the prevalence of skeletal anomalies with the precocious introduction of inert diets, when compared to the remaining feeding protocols. This result suggest that meagre larvae development was appropriate for weaning and the diets could supply the necessary nutrients for development. The most common anomalies detected were fusions and compressions (of two or more vertebrae). These anomalies are predominantly located in the haemal region of vertebral column. This pattern of location of skeletal anomalies in the haemal region was previously observed in this species (Campoverde et al. 2017). The precocious introduction of inert diet changed the distribution of skeletal anomalies to

the anterior vertebra column region. Meagre mineralization in the axial skeletal region occurs in an anterior to posterior pattern (Cardeira et al. 2012). Our data showed a predominant occurrence of skeletal anomalies in a more anterior axial region in early weaning experimental groups (T1 and T2) when compared to CTRL group. This fact suggests that a higher growth and mineralization pattern occurs on the moment of the diet introduction, promoting an increase in the occurrence of skeletal anomalies. Therefore, future studies should focus on the optimization of meagre dietary requirements, decreasing the incidence of skeletal anomalies and promoting fish welfare.

The development and maturation of gastric glands and the enzymatic activity on teleost larvae is necessary for appropriate digestion of inert diets (Rønnestad et al., 2013). Our data revealed that an introduction of inert diets at 11 DAH (TL, 4.93 ± 0.43 mm) promoted an increase in the load of compressions throughout the vertebral column. This might be associated to increment of stress during the appearance of the first gastric glands. The changes in the digestive tract that occur between the 4.6 to 6.8 mm SL (Solovyev et al., 2016) influence the enzymatic activity and consequently decrease the assimilation of nutrients, thus promoting an imbalance in skeletal development. We observed a decrease in the prevalence of anomalies at 50 DAH, a period when all the skeletal structures were completely developed (Cardeira et al., 2012). The skeletal evaluation at 50 DAH reflect the prevalence of anomalies observed in later life stages. Therefore, we suggest that 50 DAH is an adequate timing to evaluate skeletal anomalies as quality biomarker. Our results show that whole-mount double staining (25 DAH) is more accurate for the detection of skeletal anomalies when compared to radiographic analysis (50 DAH). Radiographic analysis presents a loss of sensitivity in the detection of small skeletal anomalies, inherent to the lower

resolution. Nevertheless, radiography is a much faster, non-lethal technique and allows the processing of multiple specimens simultaneously, which might be easier to implement in aquaculture production conditions.

This study showed that precocious introduction of inert diets at 8 DAH (SL, 4.1 ± 0.2 mm) in a co-feeding regime (17 days) is advantageous for meagre production in aquaculture. Additionally, this feeding methodology improved meagre survival rates. We were able to reduce the skeletal anomalies in meagre larvae with this methodology, without compromising growth performance or pancreatic and intestinal digestive activities. The assessment of skeletal anomalies is a valuable larval quality biomarker and we propose that 50 DAH is the most adequate developmental stage for the evaluation of bone anomalies in meagre. Overall this contribution provides valuable insights and management tools to increase the profitability and sustainability of meagre aquaculture.

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Figure legends

Figure 1 – Feeding protocol used in *A. regius* larvae during hatchery rearing under different feeding protocols with dry feed introduction at 8 DAH (T1), 11 DAH (T2) and 14 DAH (CTRL).

Figure 2 – Digestive enzymes activities in *A. regius* larvae at 20 DAH, from experimental rearing under different feeding protocols (8 DAH (T1), 11 DAH (T2) and 14 DAH (CTRL)). A – trypsin, B - Leucine-alanine peptidase, C – Pepsin, D - Alkaline phosphatase, E – Amylase, F - Alkaline phosphatase BB, G – Lipase and H - Acid phosphatase. Values are presented as mean ± SD.

Figure 3 – Anomalies observed in *A. regius* larvae during hatchery rearing under different feeding protocols at 25 DAH (A-C) and 50 DAH (D-F). A - Detail of fusions in the cephalic vertebrae 3-4 (F1) and in pre-haemal vertebrae 7-8 (F2), and malformed centra 6 (C) in T1; B - Vertebral fusions and compressions affecting pre-haemal vertebrae 7-8 (F) and haemal vertebrae 11-13 (C) with malformed arches in T2; C - Detail of multiple fusions affecting pre-haemal vertebrae 8-11 (F1) and haemal vertebrae 12-15 (F2) also affecting haemal and neural arches in T2; D - Fusion in the pre-haemal vertebrae 10-11 (F) and compressions of pre-haemal vertebrae and haemal vertebrae 9-12 (C) in T2; E - Fusion and compression of pre-haemal vertebrae 8-10 leading to a mild lordosis-kyphosis (KL) and fusion of haemal vertebrae 13-14 with compression of haemal vertebrae 12-14 (F/C) in CTRL; F - Fusion and compression of pre-haemal of vertebrae 7-10 (F/C) in T1. Scale bars: A - C = 0.05 cm; D - F = 0.5 cm.

Figure 4 – Prevalence of skeletal anomalies in *A. regius* during hatchery rearing (25 DAH and 50 DAH), treated with different feeding protocols (8 DAH (T1), 11 DAH (T2) and 14 DAH (CTRL)). Values are presented as mean ± SD of the 3 replicates for each treatment. Statistical differences ($p < 0.05$) are represented with different superscripts.

Figure 5 – Distribution of skeletal anomalies by affected structures in *A. regius* at different life stages. A – 25 DAH and B – 50 DAH. Dietary treatments during hatchery rearing with dry feed introduction at 8 DAH (T1), 11 DAH (T2) and 14 DAH (CTRL). Structures are defined as cephalic (VC), pre-haemal (VP-H), haemal (VH), caudal vertebrae (VCD). Values are presented as mean ± SD of the 3 replicates for each treatment. Statistical differences ($p < 0.05$) are represented with different superscripts.

Figure 6 – Load of skeletal anomalies in *A. regius* at different life stages. A – 25 DAH and B – 50 DAH. Dietary treatments during hatchery rearing with dry feed introduction at 8 DAH (T1), 11 DAH (T2) and 14 DAH (CTRL). Load of skeletal anomalies represented by specimens presenting 1 DF – 1 deformity, 2 DF - 2 deformities, 3 DF - 3 deformities and ≥ 4 DF (above 4 deformities). Values are presented as mean ± SD of the 3 replicates for each treatment. Statistical differences ($p < 0.05$) are represented with different superscripts.

Figure 7 – Distribution of skeletal anomalies in the vertebral column of *A. regius* for each of the experimental treatments at 25 and 50 DAH. Dietary treatments with dry feed introduction at 8 DAH (T1), 11 DAH (T2) and 14 DAH (CTRL).

Figure 8 – Load of vertebral fusions and compressions in the vertebral column of *A. regius* from the different dietary treatments with dry feed introduction at 8 DAH (T1), 11 DAH (T2) and 14 DAH (CTRL). A - 25 DAH and B - 50 DAH. Values are presented as mean ± SD of the 3 replicates for each treatment. Statistical differences ($p < 0.05$) are represented with different superscripts.

Table 1- Environmental and zootechnical conditions used for *A. regius* during hatchery rearing under different feeding protocols with dry feed introduction at 8 DAH (T1), 11 DAH (T2) and 14 DAH (CTRL).

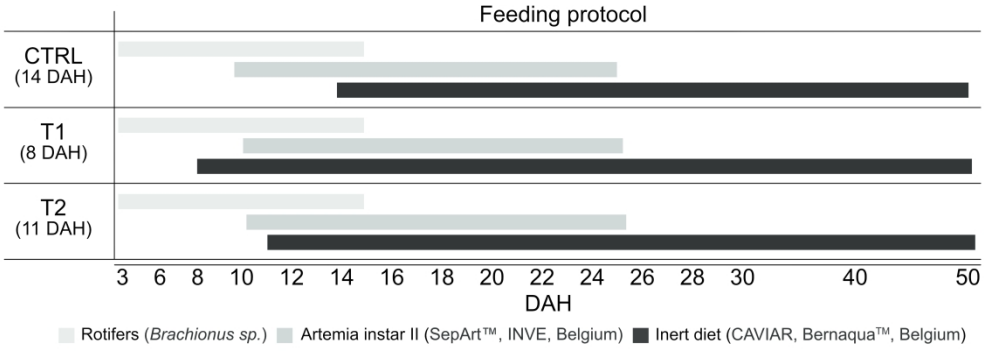
Condition	CTRL	T1	T2
Temperature (°C)	22.74 ± 2.04	22.75 ± 2.04	22.69 ± 2.00
Dissolved Oxygen (ppm)	6.20 ± 1.0	6.08 ± 1.20	6.15 ± 1.03
Salinity (ppt)	37.1 ± 1.0	37.1 ± 1.0	37.1 ± 1.0
Water renewal (L/hr)	36	36	36
Light intensity (lux)	1500	1500	1500
Photoperiod (hr/day)	14	14	14
Fish density (larvae/L)	44 ± 0.3	44 ± 0.3	44 ± 0.3
Survival rate (%)	20.3 ± 11.4	27.4 ± 4.9	20.4 ± 7.7

Values are presented as means ± SD.

Table 2– Growth parameters for *A. regius* during hatchery rearing under different feeding protocols with dry feed introduction at 8 DAH (T1), 11 DAH (T2) and 14 DAH (CTRL).

Condition	CTRL	T1	T2
TL (25 DAH, cm)	1.28 ± 0.10^{a}	1.57 ± 0.41^{b}	1.62 ± 0.58^{b}
TL (50 DAH, cm)	6.44 ± 0.59^{ab}	6.75 ± 0.65^{a}	6.25 ± 0.81^{b}
Weight (25 DAH, g)	0.15 ± 0.02^{a}	0.20 ± 0.04^{b}	0.11 ± 0.01^{c}
Weight (50 DAH, g)	3.40 ± 0.26^{a}	4.09 ± 0.35^{b}	3.15 ± 0.11^{a}
WG (25-50 DAH, %)	2176.63 ± 463.17	2043.75 ± 488.61	2672.44 ± 276.87
SGR (25-50 DAH, %)	12.41 ± 0.85	12.16 ± 0.88	13.27 ± 0.41
DGI (25-50 DAH, %)	3.87 ± 0.25	4.07 ± 0.25	3.92 ± 0.11

Values are presented as means ± SD. Total length (TL); Weight gain (WG); Specific growth rate (SGR); Daily growth index (DGI) Values are presented as means ± SD. Different superscripts represent statistical differences (One-way ANOVA followed by Tukey´s HSD test $p < 0.05$).



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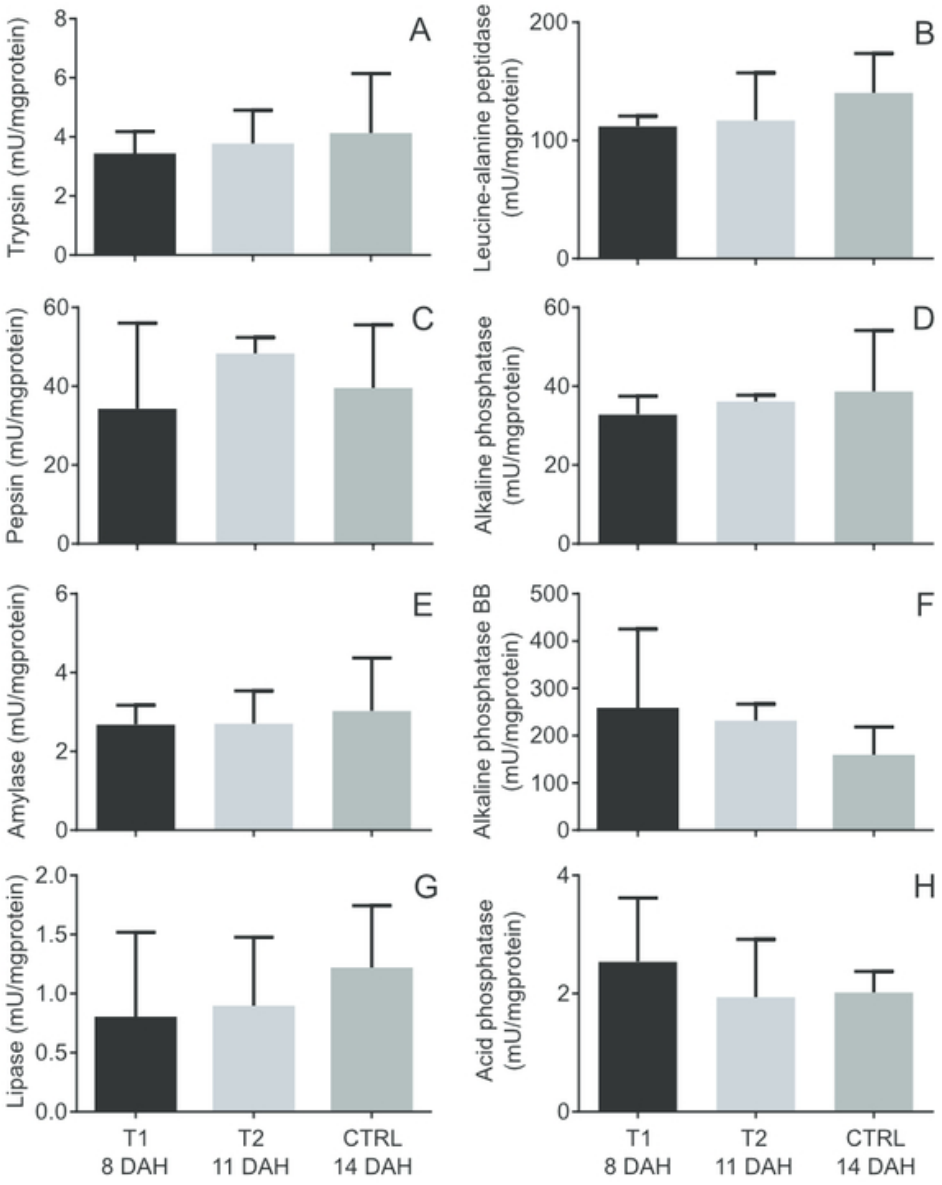

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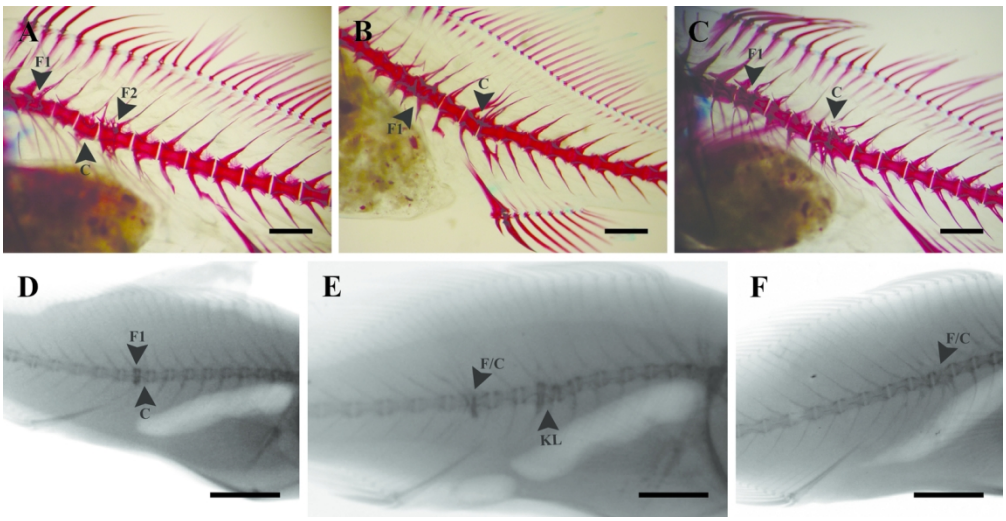

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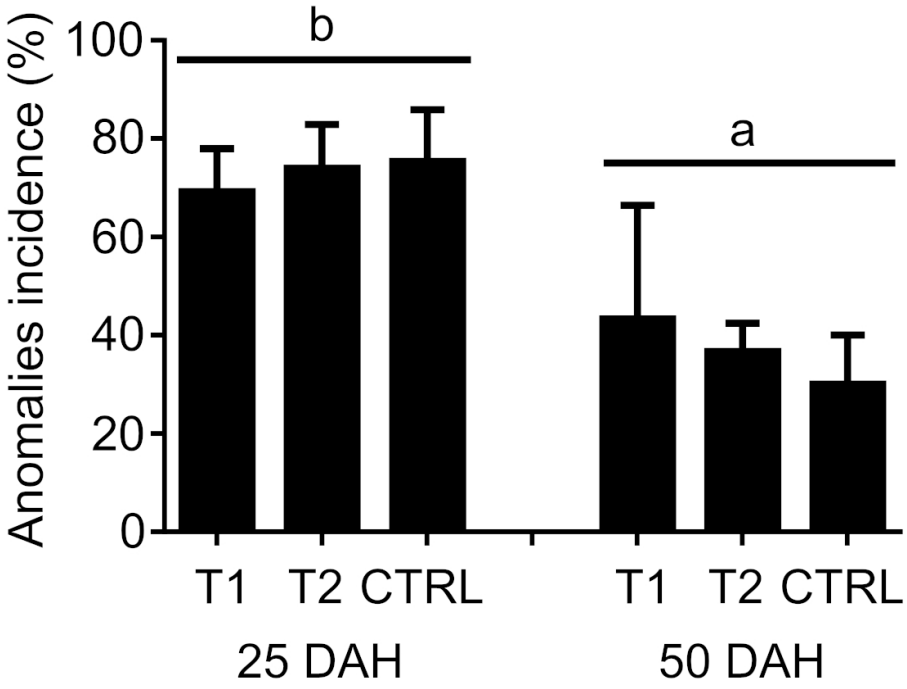

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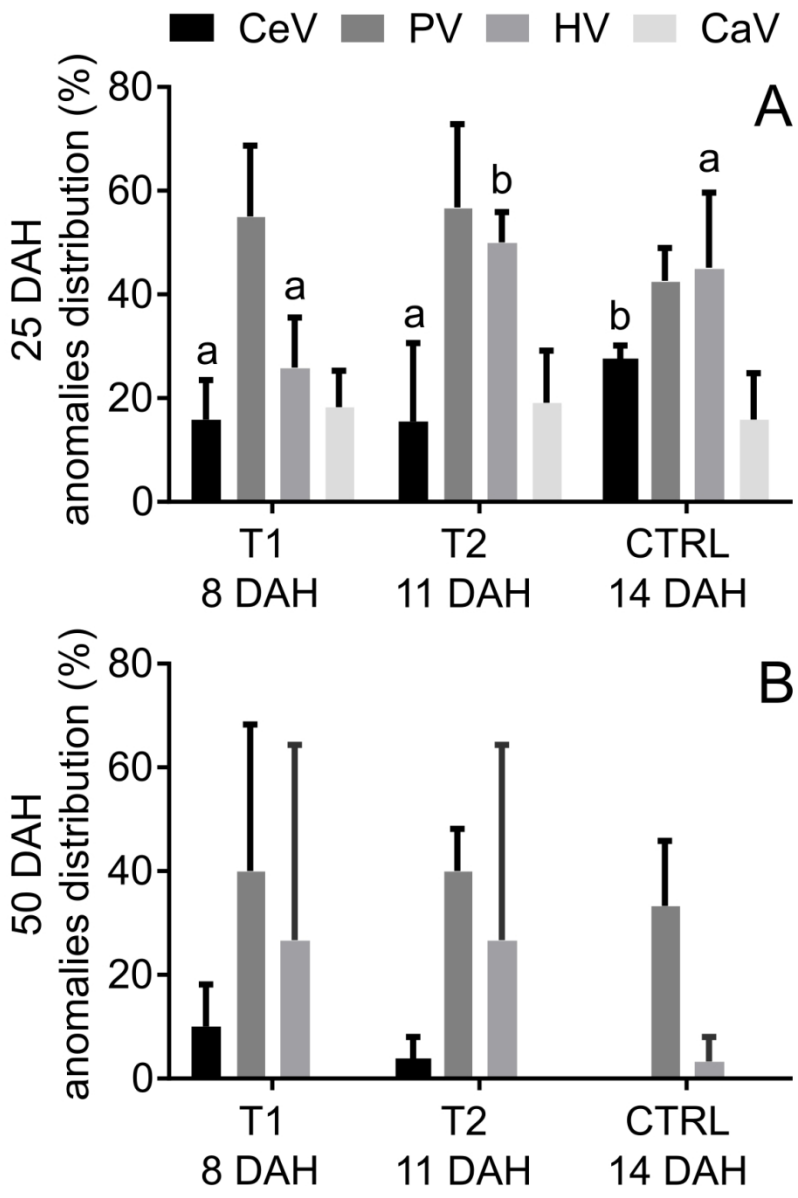

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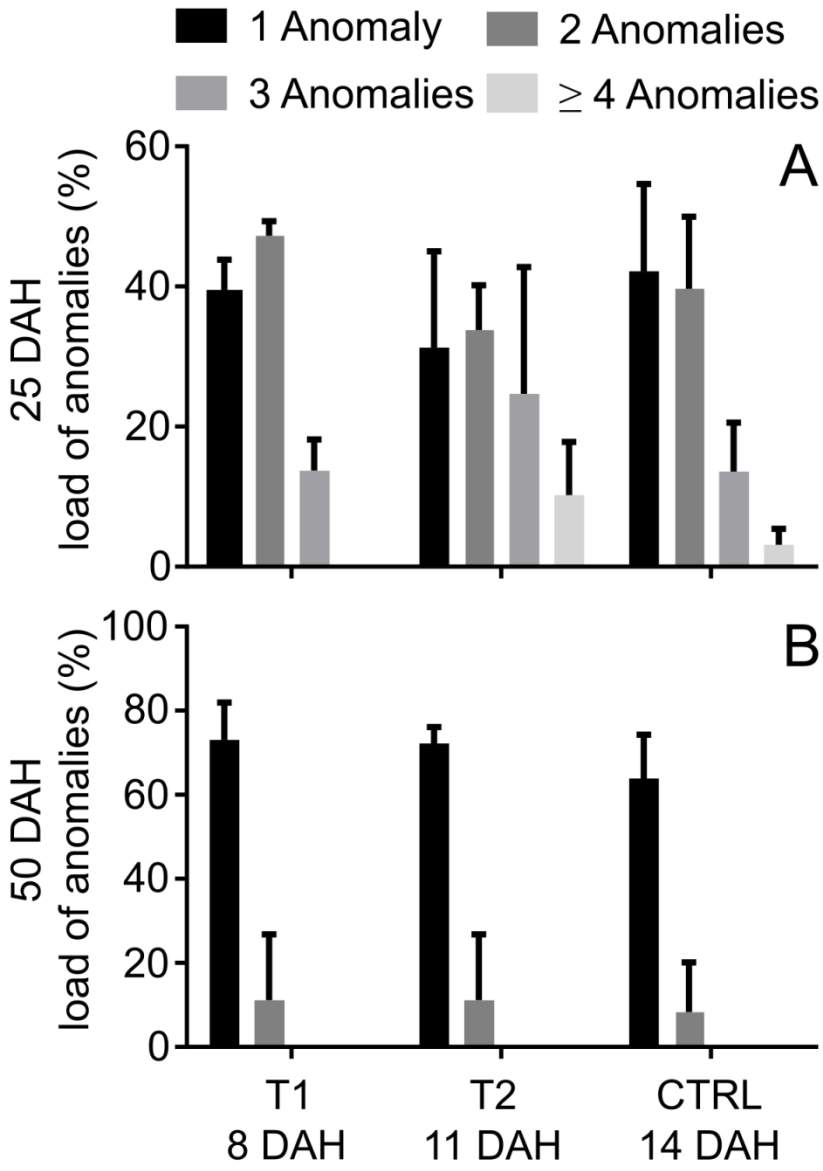

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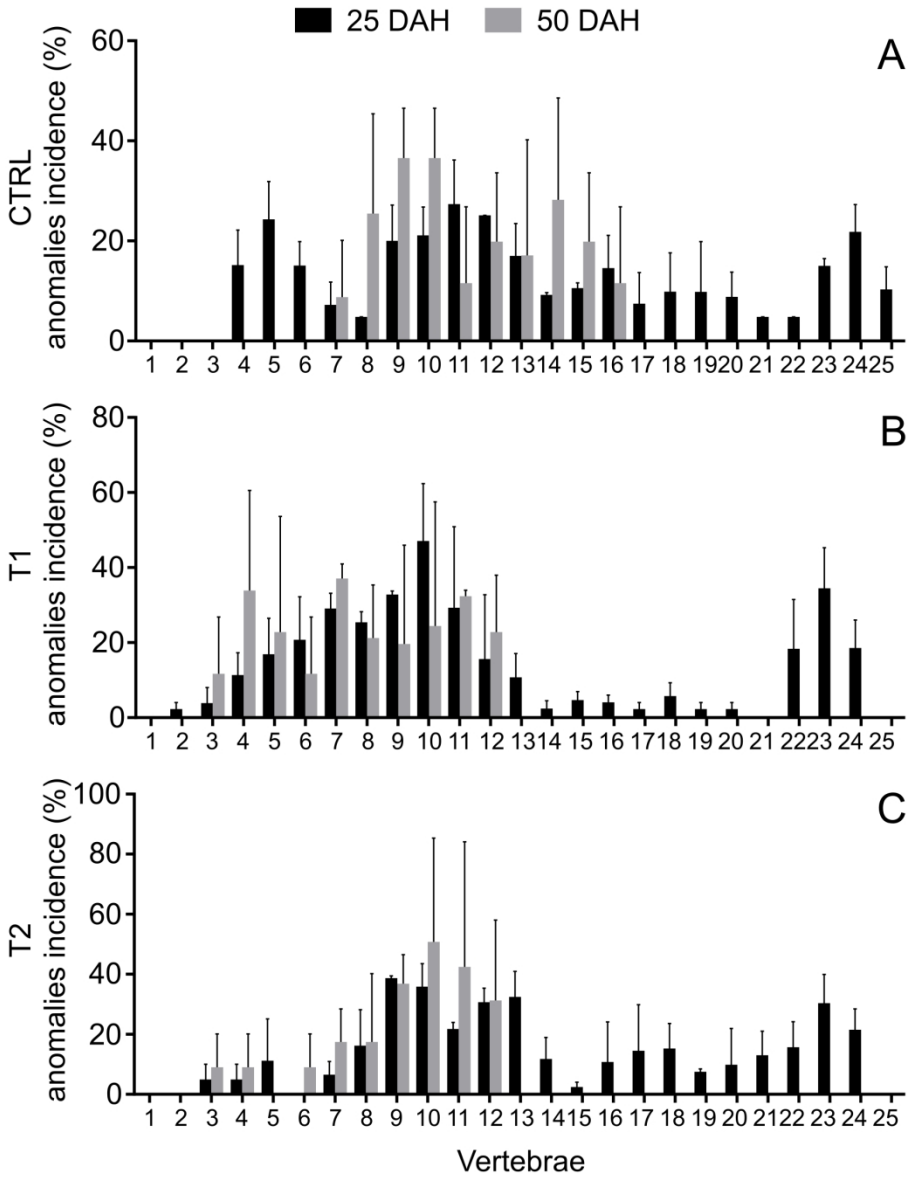

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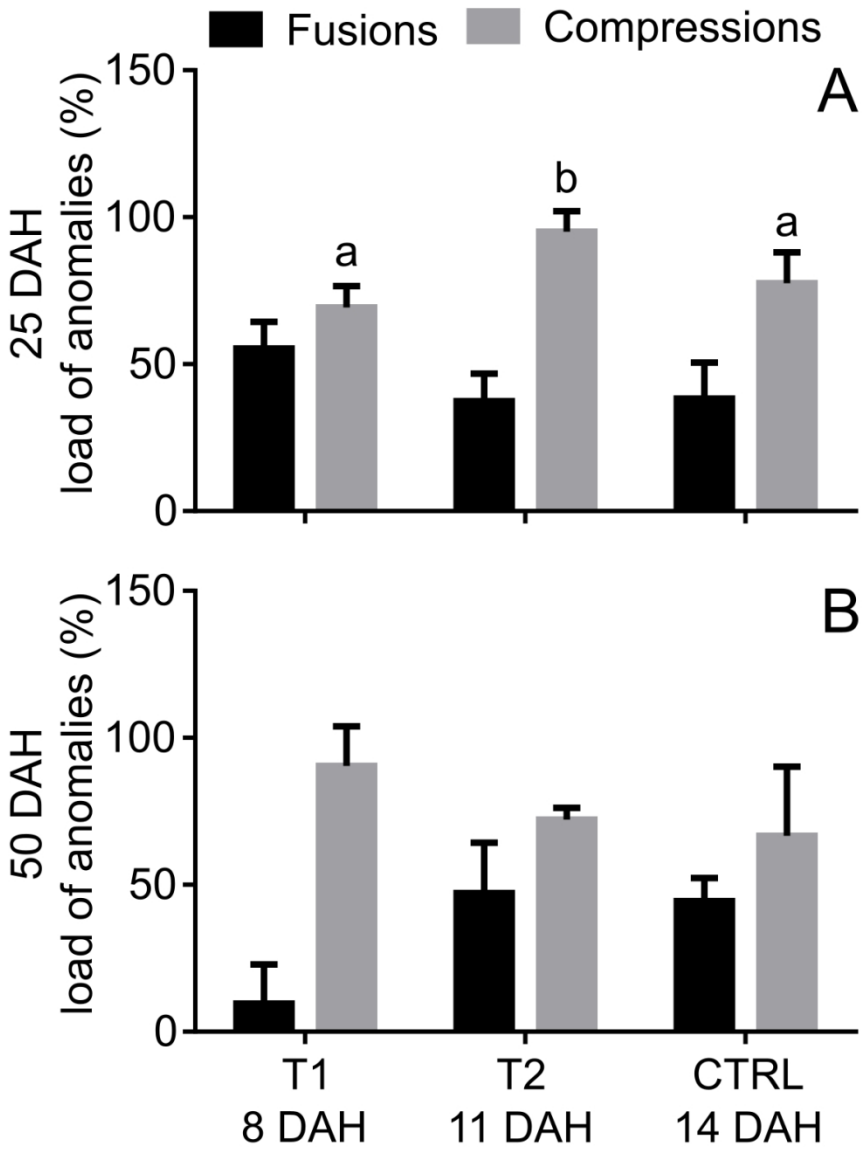

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