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The effect of different co-feeding protocols on greater amberjack (*Seriola dumerili*, Risso 1810) larvae

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Abstract

Optimizing the growth and survival of marine fish larvae, while reducing the cost of production, is important for the development of viable hatcheries on an industrial scale. This study was aimed to determine whether *Artemia* sp. use could be minimized while maintaining high growth and survival, and low skeleton anomalies occurrence in greater amberjack (*Seriola dumerili*) during the weaning period. Five co-feeding protocols, named as 1% A, 25% A, 50% A, 75% A and 100% A, according to different initial *Artemia* sp. densities provided (0.02, 0.5, 1, 1.5 and 2 *Artemia* sp. ml^{-1} day^{-1}, respectively) were tested from 32 to 48 days post-hatching (dph). Growth of larvae fed with protocols 1% A, 25% A and 50% A were significantly higher than larvae fed with protocols 75% A and 100% A. Survival increased as the effect of *Artemia* sp. increased. The incidence of total skeletal severe anomalies was high in larvae fed protocols 1% A and 25% A. Besides, the expression of growth and stress-related genes were higher at 40 dph and then decreased significantly at 48 dph in all the co-feeding protocols. The results obtained from this study suggest that the amount of *Artemia* sp. utilized can be significantly reduced during the weaning phase for this species.

KEYWORDS
Artemia sp., co-feeding, Greater amberjack, larvae, skeletal anomalies, stress, weaning

1 | INTRODUCTION

Greater amberjack (*Seriola dumerili*) is a high-value candidate for marine fish aquaculture production. This cosmopolitan fish species is mainly produced in Japan, Spain, Italy and recently in Vietnam (Matsunari et al., 2013; Nijssen et al., 2019; Sicuro & Luzzana, 2016). It is of great interest to the European aquaculture sector due to its excellent flesh quality, high economic value and high consumer acceptance (Mazzola et al., 2000; Sicuro & Luzzana, 2016). Its rapid growth and large size make this species very suitable for diversified marine products and the development of value-added products (Nijssen et al., 2019). As in many marine fish species, the mortality, cannibalism and variable size distribution in greater amberjack larvae constitute the main bottleneck for efficient commercial production of fingerlings (Hashimoto et al., 2015; Miki et al., 2011). Previous studies have investigated the optimization of abiotic factors such as photoperiod or temperature to increase larval survival (Hirata et al., 2009). Likewise, it is well understood that nutrition is a critical factor for proper larval development, and some studies have been carried out on determining the nutritional requirements of greater amberjack during the live food stages to boost survival rates (Matsunari et al., 2013; Yamamoto et al., 2008). A more recent study has shown

that levels of n-3 highly unsaturated fatty acids (n-3 HUFAs) in live food can also affect growth, survival and skeletal anomalies occurrence in greater amberjack (Roo et al., 2019). The feeding protocol adapted to greater amberjack is based on the use of rotifers (*Brachionus* sp.) from the first feeding, followed by the use of brine shrimp (*Artemia* sp. nauplii and metanauplii) appropriately enriched when larvae increase in size and are weaned onto artificial diets at a later stage of development (Hamasaki et al., 2009; Matsunari et al., 2013; Roo et al., 2019; Yamamoto et al., 2013). Significant progress on weaning has also been achieved for greater amberjack (Navarro-Guillén et al., 2019; Papandroulakis et al., 2005). However, even though greater amberjack larval development and larval rearing techniques have been studied, weaning to dry diets remains to be an important bottleneck for this species.

Weaning is a process to replace live feeds with artificial diets at a very critical moment during larval development (Rosenlund et al., 1997). It is not simply a change from one food to another but is a phase of adaptation where both types of food are provided for some time with a slow decrease in the administration of live prey and a progressive increase in microdiet (MD) (Chèvre et al., 2011; Williot et al., 2011). Moreover, weaning is considered a critical period and it is very stressful to fish. In this context, the best weaning protocol is based on a good balance of the combination of several parameters, such as high survival and larval growth, and low size dispersion and incidence of skeletal anomalies, having a direct impact on fingerlings quality during the on-growing phase (Gisbert et al., 2018). In addition to the above-mentioned biological variables, other parameters need also to be considered such as labour, production costs and the use of facilities. A prolonged period of using live foods is costly and may cause nutritional deficiency since it does not contain an adequate nutrition content for growth and development of fish larvae (Callan et al., 2003; Ma et al., 2015). Moreover, live food is a potential vector for protozoan parasites as well as infectious viruses and bacteria (Bonaldo et al., 2011; Makridis et al., 2000). Thus, reducing the reliance on live feeds will reduce material and labour costs, further increasing profitability margins.

In this sense, to minimize the use of live foods by using MD and maximize survival in the greater amberjack larval weaning, this study had the following objective: to find out whether reducing the inclusion level of *Artemia* sp. in daily feeding protocol from 32 up to 48 dph affects growth, survival, fatty acids composition, skeleton anomalies occurrence, hepatocyte vacuolization in the liver and the expression of growth and stress-related genes. If the use of *Artemia* sp. in the larval diet can be minimized, then substantial savings in food cost, floor space and labour can be achieved. The results obtained from this investigation will provide basic information that would be valuable in the development of hatchery techniques for greater amberjack production.

2 | MATERIAL AND METHODS

All the experiment mentioned below was conducted at the Marine Scientific and Technological Park of ECOAQUA University Institute of the University of Las Palmas de Gran Canaria (Las Palmas, Canary Islands, Spain), with an official aquaculture facility register number (REGA): ES350260026567.

2.1 | Ethics statement

All animal experiments described in this manuscript fully comply with the recommendations in the Guide for Care and Use of Laboratory Animals of the European Union Council (2010/63/EU), with the general guidelines approved by the project 'DIVERSIFICACIÓN DE LA ACUICULTURA ESPAÑOLA MEDIANTE LA OPTIMIZACIÓN DEL CULTIVO DE SERIOLA (*Seriola dumerili*) – SERIOLA', within the National Agricultural Aquaculture Plans 2016–2018 (Ref. 23.17.415A.741).

2.2 | Larval rearing

Greater amberjack larvae of 32 dph (N = 400; total length 11.54 ± 1.15 mm); fresh mass 23.57 ± 2.83 mg (mean ± standard deviation) were randomly distributed into 15 light grey colour cylindrical fibreglass tanks (five triplicate treatments) of 200 L. All tanks were equipped with continuous aeration and supplied with filtered UV-sterilized seawater (salinity 37 psu). Continuous water flow was maintained at a 25% per hour exchange rate from 32 to 35 dph and then increased to achieve a 50% per hour exchange rate at the end of the experiment, to guarantee a good water quality during the trial. Water entered the tanks at the bottom and exited at the surface. During the experimental period, tanks were siphoned daily to remove dead larvae, uneaten food and faeces. Dissolved oxygen (6.1 ± 0.4 g L^{-1}) and temperature (22.3 ± 0.6°C) were monitored daily. An artificial fluorescent light above each tank provided a surface light intensity ranging between 1000 and 1500 lux (digital Lux Tester YF-1065; Powertech Rentals, Osborne, Australia) at the centre of each rearing tank for a photoperiod of 12 h light/12 h darkness cycle with lights on from 07:30 a.m. to 07:30 p.m., local time.

2.3 | Feeding trial

Five different co-feeding protocols named 1% A, 25% A, 50% A, 75% A and 100% A according to different initial *Artemia* sp. densities provided 0.02, 0.5, 1, 1.5 and 2 *Artemia* sp. ml^{-1} day^{-1}, respectively, and the same amount of MD for all treatments, were tested from 32 to 48 dph (Tables 1 and 2). *Artemia* sp. (EG type; INVE Aquaculture, Dendermonde, Belgium) were enriched for 18 h in 100 L tanks (250,000 individuals L^{-1}) maintaining 28 °C seawater, with an experimental emulsion (GIA, own formula), under vigorous aeration and oxygen supply. During the experimental period, *Artemia* sp. feeding frequency was gradually reduced from four to one fed per day (Table 1). Additionally, MD (Gemma Micro; Skretting, France) was used by mixing (1:1) two different particle sizes, 150 μm and 300 μm. The MD was manually fed to the larvae 7–10 times

TABLE 1 Co-feeding protocols using *Artemia* sp. and MD for larval greater amberjack

	Treatment				
	1% A	25% A	50% A	75% A	100% A
From 32 to 35 dph					
Artemia sp. day^{-1}[a]	4000	100,000	200,000	300,000	400,000
MD g day^{-1}	0.75	0.75	0.75	0.75	0.75
Energy content (kJ) per day	16.92	18.03	19.18	20.33	21.49
From 36 to 39 dph					
Artemia sp. day^{-1}[b]	2000	50,000	100,000	150,000	200,000
MD g day^{-1}	1.25	1.25	1.25	1.25	1.25
Energy content (kJ) per day	28.15	28.70	29.28	29.85	30.43
From 40 to 48 dph					
Artemia sp. day^{-1}[c]	1000	25,000	50,000	75,000	100,000
MD g day^{-1}	1.50	1.50	1.50	1.50	1.50
Energy content (kJ) per day	33.76	34.04	34.33	34.61	34.90

[a]*Artemia* sp. was offered to the larvae four times per day per tank.
[b]Twice per day.
[c]Once per day.

a day (approximately every hour). The amount of MD was always offered to assure enough feed, based on estimated consumption and growth, and periodically adjusted based on visual inspection to avoid a large excess of uneaten food. The feeding quantity of the MD for each tank was 0.75 g day^{-1} at the first co-feeding days (32 to 35 dph), increased to 1.25 g day^{-1} at 36 to 39 dph, and reached to 1.50 g day^{-1} at 40 dph until the end of the experiment (Table 1). Proximate analysis and fatty acids composition of *Artemia* sp. and MD are shown in Table 2.

2.4 | Growth and survival

Larval growth was assessed by estimating the total length (TL) and wet body mass (WW) at 32, 40 and 48 dph, in 30 randomly selected larvae per tank, 1 h before the first feeding at 32 and 40 dph and after the night starvation at 48 dph, to avoid any influence of gut contents on the nutrient content of the larvae as much as possible. Larvae were previously anaesthetized with clove oil at 1% and TL was measured under a profile projector (Mitutoyo, PJ-A3000, Japan). The same larvae were used to estimate the wet body mass, previously washed with distilled water and weighed in an analytical balance (Gibertini Elettronica, E50 S/2, Milano, Italy). Specific growth rate (SGR) was calculated in relation to total length at the end of the co-feeding period using the following formula: SGR = 100*(ln (TLf) - ln (TLi))/Δt, where TLf was the final larval total length (mm), TLi was the initial larval total length (mm) and Δt was the time between sampling days (Hopkins, 1992; Lugert et al., 2014). The coefficient of variation (CV) of total length was calculated according to the formula: CV = 100*(standard deviation/mean).

At the end of the experiment, the dead and live larvae count allowed us to calculate the survival and cannibalism rates per tank. Survival rate = 100* Nf/(Ni-Ns) and estimated cannibalism rate = 100* (Ni-Nc-Nf)/Ni, where Nf was the number of living larvae at the end of the experiment, Ns was the number of larvae sampled during the trial, Ni was the initial larvae numbers stocked in each tank, and Nc was the accumulated number of dead larvae during the trial (Cortay et al., 2019). The total yield from each treatment was determined by average larval weight multiplied by average live larvae.

2.5 | Biochemical analysis

To analyse the proximate and fatty acid composition, a sample of 48 dph larvae from each tank was washed with distilled water and kept at −80°C. Proximate composition was conducted following standard procedures (Association of Official Analytical Chemists [AOAC], 2016). Moisture was determined by thermal dehydration until constant mass at 105°C. Ash content was determined by combustion at 600°C for 12 h. Crude protein content (*N* x 6.25) was determined by the Kjeldahl method, and crude lipid was extracted following the Folch method (Folch et al., 1957). Fatty acid methyl esters profiles were obtained by transmethylation of total lipids (Christie & Han, 2010) and separated by gas-liquid chromatography (GC-14A; Shimadzu, Tokyo, Japan) following the conditions described by Izquierdo et al., (1990) and identified by comparison to previously characterized standards and GLC-MS (Polaris QTRACETM Ultra; Thermo Fisher Scientific). All analyses were conducted in triplicate.

TABLE 2 Proximate (% dry matter) and fatty acids composition (% total fatty acids, TFA) of *Artemia* sp. and MD

	Artemia sp.	MD
Proximate analysis (% dry matter)		
Lipids	20.77 ± 1.00	18.78
Ash	10.00 ± 1.55	13.73
Proteins	63.29 ± 0.78	63.91
Moisture	91.62 ± 0.16	14.20
Energy content (kJ g^{-1})[a]	23.06	22.50
Fatty acid content (% TFA)		
Total SFA	19.85 ± 2.02	19.46
Total MUFA	28.38 ± 1.52	38.21
Total n-3	38.63 ± 3.27	33.41
Total n-6	11.23 ± 0.84	7.80
Total n-9	18.74 ± 0.87	19.57
Total n-3 PUFA	12.90 ± 2.00	29.62
14:0	0.33 ± 0.11	1.10
16:0	11.29 ± 1.43	14.17
16:1 n-7	1.57 ± 0.15	4.47
18:0	7.64 ± 0.64	3.73
Oleic (18:1 n-9)	17.65 ± 0.86	15.14
18:1 n-7	6.34 ± 0.39	5.57
18:2 n-6	4.85 ± 0.13	6.17
18:3 n-3	22.62 ± 1.33	1.25
20:1 n-9	0.10 ± 0.02	3.16
ARA (20:4n-6)	2.52 ± 0.35	0.81
EPA (20:5n-3)	3.04 ± 0.38	14.36
DHA (22:6n-3)	6.77 ± 1.53	13.27
DPA (22:5n-6)	2.04 ± 0.32	0.18
DHA/DPA	3.31 ± 0.33	72.07
ARA/EPA	0.83 ± 0.05	0.06
DHA/EPA	2.23 ± 0.42	0.92
DHA/ARA	2.72 ± 0.71	16.29
Oleic/DHA	2.72 ± 0.74	1.14
Oleic/n-3 PUFA	1.40 ± 0.28	0.51
n-3/n-6	3.45 ± 0.35	4.28

Note: Proximate analysis and fatty acid content data of *Artemia sp.* represent means ± SD, $n = 3$.

Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, long-chain polyunsaturated fatty acids; SFA, saturated fatty acids.

[a]Energy content was estimated for: Lipid × 39.5 kJ g^{-1}; Protein × 23.6 kJ g^{-1} (New, 1987).

2.6 | Analysis of skeletal anomalies

To identify and quantify larval skeletal anomalies, 100 larvae per each tank (300 larvae per treatment) were randomly sampled at 48 dph, preserved in 10% neutral-buffered formalin, stained with alizarin red and examined under a Stereoscope (Leica, M125, Wetzlar, Germany). Photographs were taken using a Leica DFC295 digital camera (Leica, Wetzlar, Germany) and processed using the Leica application suite (LAS 32167, Leica, Wetzlar, Germany) to characterize skeletal anomalies occurrence (Vandewalle et al., 1998) by two independent observers. Several skeletal anomalies were determined in the different regions of the axial column according to the method described by Boglione et al., (2014). The percentage of high-quality larvae produced per tank was estimated as follows: Survival rate at the end of the experiment multiplied by the percentage of non-malformed larvae.

2.7 | Histological analysis

At the end of the experiment, ten greater amberjack larvae per tank were sampled and fixed in 10% neutral-buffered formalin. After 48 h, tissues were dehydrated with an increased graded series of ethanol, submerged in xylene, and embedded in paraffin blocks. Paraffin-embedded larvae were cut at 4 μm on a microtome (Mod. Jung Autocut 2055; Leica, Nussloch, Germany) and stained with haematoxylin and eosin (H&E) (Martoja & Martoja-Pierson, 1970). The mounted sections were examined under light microscopy using an Olympus CX41 binocular microscope (Olympus, Hamburg, Germany) connected to an Olympus XC30 camera (Olympus), which was linked to a computer using Image Capturing Software (CellB®; Olympus). Tissue morphology of hepatic was examined by two independent observers. Hepatocyte vacuolization was assessed by using a three-point scoring system where 1, 2 and 3 represented no, mild and severe hepatic vacuolization, respectively (Betancor et al., 2012).

2.8 | RNA isolation and gene expression analysis

Total RNA was extracted from whole greater amberjack larvae after RNAlater® (Ambion, Applied Biosystems) was eliminated using lint-free laboratory wipes, employing a Polytron PT 1200 E with a dispersing tool PT-DA 03/2EC-E050 (Kinematica AG), or an Ultra Turrax® T25 (IKA®-Werke) with a dispersing tool S25N-8G, and the NucleoSpin® kit (Macherey-Nagel). In all cases, a digestion step with RNase-free DNase was performed to eliminate or reduce the genomic DNA contamination and, finally, samples were stored at −80°C. RNA concentration was measured with a Qubit® 2.0 fluorimeter and a Qubit™ RNA BR kit, while its quality was assessed with a Bioanalyzer 2100 and an RNA 6000 Nano kit (Agilent Technologies, LifeSciences). Reverse transcription was performed with the qScript™ cDNA synthesis kit (Quanta BioSciences) using only samples which had an RNA integrity number (RIN) greater than 7.0 and 500 ng of total RNA. Each reaction was carried out in a volume of 20 μL, according to manufacturer's instructions, and it was diluted 1/10th with 10 mM Tris-HCl, 0.1 mM EDTA (pH = 8) to obtain a final concentration of 2.5 ng μL^{-1}.

The cDNA sequences used in this work were obtained from greater amberjack brain, hypophysis, liver, and kidney samples sent to Bioarray (Spain), where an RNA-seq was performed by NGS mass sequencing, using the Ion Total RNA-Seq Kit v2 in the Ion Proton Sequencer from Life Technologies. The sequences were aligned with the Trinity software, annotating the transcripts with the Blast2GO software. Primers for real-time PCR were designed using Primer3 software v.0.4.0 (available at http://bioinfo.ut.ee/primer3/ (Table 1)). Two internal reference genes, *actin beta (actb)* and *eukaryotic elongation factor 1 alpha (eef1a)*, were used as internal reference genes, owing to their lower than 0.5 target stability M value and lower than 0.25 CVs. All reactions were performed in a CFX Connect™ and a CFX 96 Real-Time Detection System with BioRad CFX Maestro Software v1.1 (BIORAD Laboratories). A pool of cDNAs (CAL), derived from mixing the 5 RNA samples from the 32 dph group, was used to correct for inter-assay errors.

Before samples analyses, every primer was tested at final concentrations of 400 and 200 nM, and a temperature range of 55 to 60°C. Furthermore, 1:10 serial dilutions (from 10 ng to 100 fg) of cDNA were carried out to verify amplification efficiency and to produce a calibration curve. Those pairs of primers that showed an efficiency (E) between 90% and 110%, a determination coefficient (R^2) higher than .980, and a calibration curve interpolating at least two points over six, were chosen for real-time PCR reactions (Table 3). Negative control with RNA was used to check for the presence of genomic DNA contamination, and negative control with water was used to determine the existence of artefacts such as primer-dimers. Each reaction mixture contained 0.5 µl of each specific forward and reverse primers at their best-tested concentration, 5 µl of iTaq™ Universal SYBR Green Supermix (BioRad), and 4 µl of cDNA (10 ng). Reactions were accomplished in a volume of 10 µl using Hard-Shell® Low-Profile Thin-Wall 96 White-Well Skirted PCR plates (BioRad) covered with Microseal® B Adhesive Seals (BioRad). PCRs were performed with an initial denaturation and polymerase activation at 95°C for 10 min, followed by 40 cycles of denaturation in 15 s at 95°C, annealing and extension at 60°C for 30 s and finishing with a melting curve from 60 to 95°C increasing 0.5°C every 5 s. Relative gene quantification was performed according to the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001), corrected for efficiencies (Pfaffl, 2001) and normalized by geometric average of the two internal control genes (Vandesompele et al., 2002).

2.9 | Statistical analysis

Analyses were conducted by SPSS statistics (version 22.0 for Windows; Inc, IBM, Chicago, IL, USA) and visualized using SigmaPlot 12.0 (Systat Software, San José, USA). The significant level for all analyses was set at 5%. All data were tested for normal distribution with the one-sample Kolmogorov–Smirnoff test, as well as for homogeneity of the variances with the Levene test (Sokal & Rohlf, 2012). When the assumptions were passed, the one-way analysis of variance (ANOVA) test was performed, followed by post hoc Tukey's test and Student's t test for paired samples. When heterogeneity of

TABLE 3 Specifications of the real-time PCR assays including forward (F) and reverse (R) primers, efficiencies (Eff) of PCR reactions, length of amplicon and coefficient of determination (R^2)

Gene	Acronym	Eff (%)	Amplicon length (bp)	R^2	Primer sequence (5′–3′)	GenBank Accession No.
Actin beta	*actb*	94.2	145	.999	F: CAGTGGTTGGCGCATACTTAT R: GAAGAGGTCACGATTGGGTTT	MW311085.1 XM_022757055.1
Eukaryotic elongation factor 1 alpha	*ef1a α*	99.9	142	.998	F: CCCTGGATCACCTTCTCTGA R: TAAGAGGCACCGTCATGTGA	MW311086.1 XM_022744048.1
Corticotropin-releasing hormone	*crh*	92.6	110	.996	F: TCGGGAGATGATGGAGATGT R: TTGGCGGACTGGAAAGAG	MW311087.1 XM_022744291.1
Corticotropin-releasing hormone-binding protein	*crhbp*	104.2	103	.996	F: ATGGTGGTGAGGATGGTGTC R: CGTTGTTGAGTTTGATGGTCTG	MW311088.1 XM_022769490.1
Growth hormone	*gh*	100.3	105	.998	F: AGGCGAAGAGTTGCTGAGAC R: GGAGAGAGCCGACATTTAGC	MW311089.1 XM_022769709.1
Insulin-like Growth factor I	*igf1*	92.7	169	.998	F: GTCCCTCGGTGTATCTGGAA R: GCGGAGTCGTGAATGTCT	XM_022749759.1 MW311090.1
Insulin-like growth factor II	*igf2*	100.1	187	.997	F: TTTCCTCCTCCTCCATTGTG R: CACTGCCTGCTTTGTTGC	XM_022754221.1 MW311091.1
Proopiomelanocortin A	*pomca*	92.5	129	.996	F: CAAAGATGGACAGCAGCAGA R: GCTTCCCTGGCTAATGAGAA	MW311092.1 XM_022770028.1
Proopiomelanocortin B	*pomcb*	98.4	147	.996	F: CACTGCTCACGCTCTTCAAA R: AAGGACTTGTAGGCCGATCA	MW311093.1 XM_022757428.1
Steroid acute regulatory protein	*star*	109.5	103	.995	F: TCAGGCAAAGGATGGCTAAT R: TTTCTGCCACTGTTGAGTGC	MW311096.1 XM_022754985.1
Thyrotropin-releasing hormone	*trh*	106.3	82	.999	F: AGCACCCAGGTAAGCGGTAT R: GGCAGGTCTTCGTCTCCATC	XM_022739244.1 MW311097.1

variances and/or normality of distribution were not complied, the Kruskal–Wallis test was applied and differences among treatments were graphed with a box and whisker plot.

3 | RESULTS

3.1 | Growth, survival and cannibalism

Co-feeding protocols did not have a significant ($p < .05$) impact on greater amberjack wet body mass and total length during the first period, from 32 dph until 40 dph (Figure 1). Nevertheless, at the end of the experiment (48 dph), larvae in 1% A, 25% A and 50% A co-feeding protocols were significantly ($p < .05$) heavier and longer than larvae in 75% A and 100% A co-feeding protocol, while there were no significant differences ($p < .05$) among 1% A, 25% A and 50% A co-feeding protocols (Figure 1).

The highest SGR was found in 1% A co-feeding protocol ($3.57 \pm 0.48\%$ day^{-1}), whereas the lowest in the 100% A group ($2.56 \pm 0.17\%$ day^{-1}) at the end of the experimental period (Figure 2). Furthermore, no significant differences in CV of total length were observed among co-feeding protocols ($p < .05$) (Figure 3).

Larval survival varied from $21.53 \pm 4.04\%$ in 1% A co-feeding protocol, as the lowest, to $50.72 \pm 4.87\%$ in 100% A co-feeding protocol, as the highest (Figure 4a). Survival increased as an effect of *Artemia* sp. amount provided and was significantly higher ($p < .05$) in the 75% A and 100% A co-feeding protocols (Figure 4a). Besides, cannibalistic behaviour was most frequently observed in tanks that received reduced amounts of *Artemia* sp. (1% A and 25% A), compared with the treatments receiving higher amounts (50% A, 75% A and 100% A) (Figure 4b). In addition, the highest total yield was observed in 75% A co-feeding protocol while the lowest in 1% A (Figure 5).

3.2 | Proximate and fatty acids composition

At the end of the co-feeding trial, larval total lipids, proteins and moisture were significantly different ($p < .05$) among co-feeding protocols (Table 4). Regardless of the treatments, palmitic acid (16:0) accounted for the bulk of the saturated fatty acids (SFA), oleic acid (18:1n-9) for most of the monounsaturated fatty acids (MUFA), and docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) for most of the n-3 long-chain polyunsaturated fatty acids (PUFA) present in the larvae (Table 4). The fatty acid composition of total lipids from whole larval body lipids reflected the *Artemia* sp. and MD fatty acid profiles (Table 2). The increased amount of *Artemia* sp. was followed by increased arachidonic acid (ARA, 20:4n-6), α-linolenic acid (18:3n-3), and docosapentaenoic acid (DPA, 22:5n-6), together with reduced MUFA and n-9 fatty acids, particularly oleic acid (18:1n-9), in the larvae. The proportions of DHA and EPA in the total fatty acids of the greater amberjack larvae were similar in all the experimental groups (Table 4). However, an increase in *Artemia* sp. density from 0.02 (1% A) to 2 ml^{-1} day^{-1} (100% A) significantly ($p < .05$) increased the ARA/EPA ratio and decreased the DHA/ARA and DHA/DPA ratios (Table 4).

3.3 | Skeletal anomalies

The highest incidence ($p < .05$) of total skeletal severe anomalies (lordosis, kyphosis, scoliosis, deformed vertebrae) was observed in 1% A and 25% A co-feeding protocols (Figure 6a). Most of the acute skeletal anomalies were alterations in the pre-haemal region, related mainly to the lordosis appearance, while in the haemal region, the most important affection was recorded as kyphosis and vertebral anomalies, particularly identified as shape anomaly and ossification ridges (Figure 6b,c). Furthermore, the co-feeding protocol had a significant ($p < .05$) effect on the percentage of high-quality larvae. The 1% A, 25% A and 50% A co-feeding protocols exhibited a lower rate of high-quality larvae (13.25%, 20.36% and 29.16%, respectively) while 75% A and 100% A co-feeding protocols showed higher rates of high-quality larvae with 43.44% and 47.34%, respectively.

3.4 | Hepatocyte vacuolization

Significant differences were determined in the hepatocyte vacuolization in the liver of larvae receiving different co-feeding protocols (Figure 7). The hepatocyte vacuolization decreased as an effect of *Artemia* sp. amount elevation and was significantly lower ($p < .05$) in the 75% A and 100% A co-feeding protocols (Figure 7). Furthermore, in the hepatocytes of greater amberjack larvae in 1% A, 25% A and 50% A co-feeding protocols, the infiltration of lipid vacuolization displaced the nuclei of cells from a central position to the periphery (Figure 8a). Contrarily, liver of larvae in 75% A and 100% A co-feeding protocols showed smaller hepatocytes, with spherical nuclei and mostly located at a central position in the cell (Figure 8b).

3.5 | Gene expression

The expression of growth (*gh*, *igf1*, *igf2*) and stress-related (*crh*, *crhbp*, *trh*, *pomca*, *pomcb*, *star*) genes in whole greater amberjack larvae from different co-feeding protocols at 32, 40 and 48 dph are shown in Figure 8. High *gh* and *igf2* expressions at 40 dph were followed by a significant decline at 48 dph in all co-feeding protocols (Figure 9a,c). Significant differences ($p < .01$) were also found in the expression of *igf1*, but only in the 25% A and 75% A co-feeding protocols, where higher values were obtained at 48 dph (Figure 9b). Within 40 and 48 dph, *gh* and *igf2* expression were significantly higher ($p < .05$) in 75% A and 100% A co-feeding protocols, while the lowest expression was observed in the 1% A co-feeding protocol (Figure 9a,c). However, no significant differences were found in the expression of *igf1* among co-feeding protocols, within 40 or 48 dph (Figure 9b).

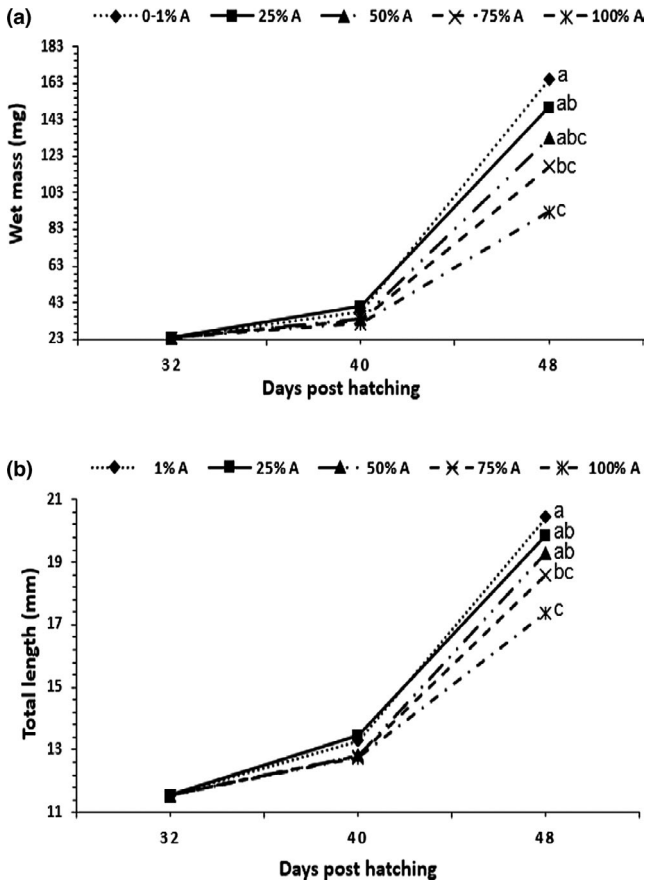


FIGURE 1 Growth parameters of greater amberjack larvae (a) wet mass (mg), and (b) total length (mm) from different co-feeding protocols at 32, 40 and 48 dph. Different letters denote significant differences among protocols ($p < .05$; one- way ANOVA; Post-hoc Tukey test)

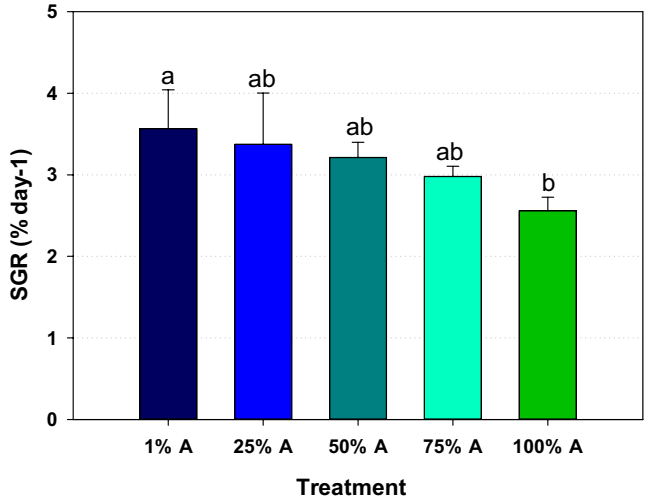


FIGURE 2 Specific growth rate (SGR, % day-1) in 48 dph greater amberjack larvae fed different co-feeding protocols (means ± SD, $n = 3$). Different letters above the bars indicate significant differences among protocols ($p < .05$; one- way ANOVA; Post-hoc Tukey test)

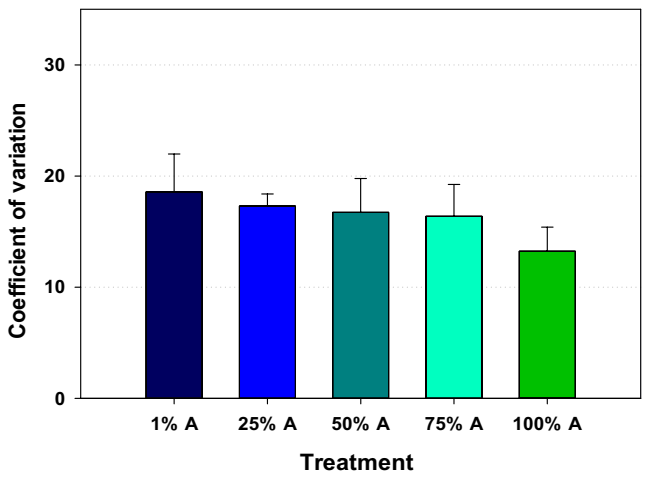
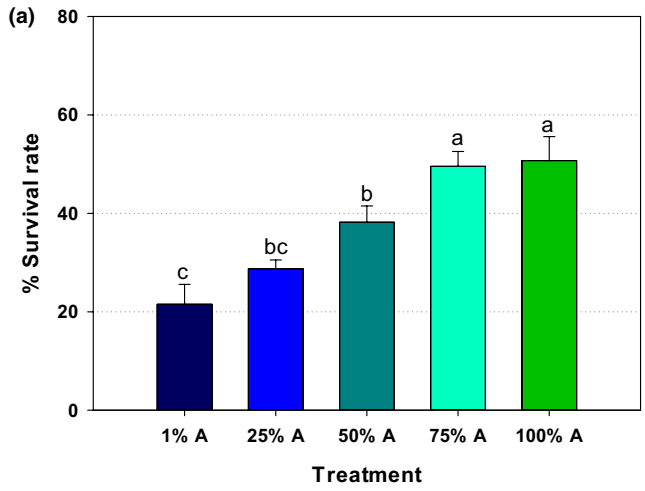


FIGURE 3 Coefficient of variation of total length in 48 dph greater amberjack larvae fed different co-feeding protocols (means ± SD, $n = 3$)

Expression of *crh* and *crhbp* at 40 dph was higher in all co-feeding protocols than those at 48 dph ($p < .01$); however, no significant differences were found among co-feeding protocols, within both 40 and 48 dph (Figure 9d,e). Similarly, the expression of *trh* was significantly higher in larvae at 40 dph compared with 48 dph in all co-feeding protocols, but significant differences were found among co-feeding protocols at 40 dph, with the lowest expression in 1% A (Figure 9e,f). Concerning *pomca* and *pomcb*, they showed significant differences among co-feeding protocols at different times. At 40 dph, *pomca* expression was significantly higher ($p < .05$) in 100% A, while 75% A co-feeding protocol showed the lowest levels (Figure 8g). At 48 dph, lower mRNA expression of *pomcb* was observed in the 1% A co-feeding protocol compared with the other protocols (Figure 9h). Similar to *pomca*, *star* was also affected by the co-feeding protocols at 40 dph, and the larvae of 100% A co-feeding protocol showed a marked increase in gene expression (Figure 9i).

4 | DISCUSSION

Weaning the marine fish larvae at first feeding directly onto MD is quite challenging, being a species-specific process. However, good results could be obtained when used in combination with live food (Curnow et al., 2006; Curnow et al., 2006). The evaluation of five co-feeding protocols for greater amberjack made in this study revealed that the amount of *Artemia* sp. is an important factor affecting the growth performance, survival and nutritional condition of the larvae. In the current study, weaning was carried out by a gradual replacement of *Artemia* sp. with a commercial diet over a minimum of 16 days, and its conditions influenced the growth of the greater amberjack larvae. The reduction in *Artemia* sp., in 1%–50% A protocols, was reflected in higher larval growth, in terms of total length, wet body mass, and SGR, especially during the second feeding period, from 40 to 48 dph. Normally, under low live food provision, fish larvae spend more time to capture and ingest the prey, leading to slow



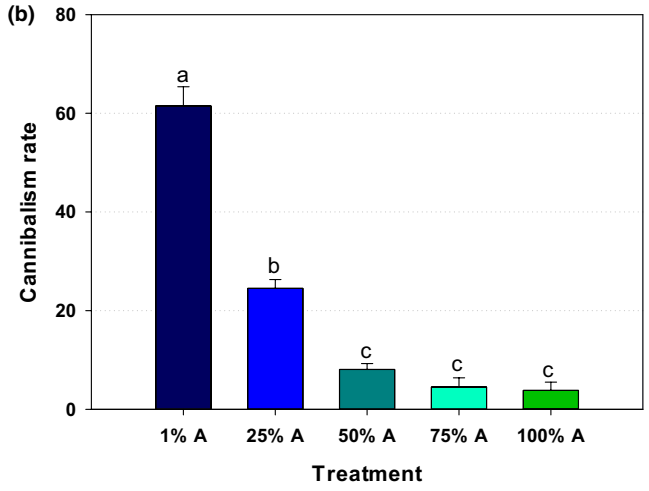


FIGURE 4 (a) Final survival rate and (b) Cannibalism rate in 48 dph greater amberjack larvae fed different co-feeding protocols (means ± SD, $n = 3$). Different letters above the bars indicate significant differences among protocols ($p < .05$; one- way ANOVA; Post-hoc Tukey test)

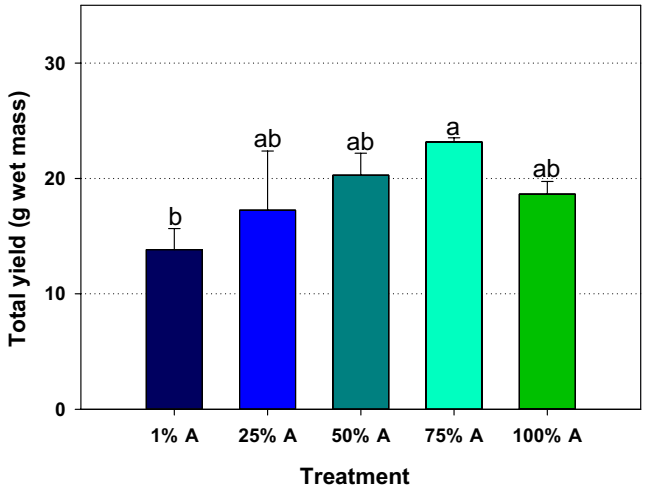


FIGURE 5 Total yield (g wet mass) in 48 dph greater amberjack larvae fed different co-feeding protocols (means ± SD, $n = 3$). Different letters above the bars indicate significant differences among protocols ($p < .05$; one- way ANOVA; Post-hoc Tukey test)

growth (Ma et al., 2013; Shaw et al., 2006). In yellowtail amberjack, the growth of fish larvae was improved by increasing the *Artemia* co-feeding (Ma et al., 2013). Similar results have been observed in Mulloway (*Argyrosomus japonicus*), in which the larvae grew faster at high *Artemia* concentrations (Ballagh et al., 2010). Therefore, the increased growth observed in 1% A and 25% A at the end of the present experiment is more likely linked to the significantly lower survival and higher cannibalism rates observed in low *Artemia* sp. co-feeding treatments. The higher growth observed in 1% A and 25% A larvae could also be attributed to the enhanced nutrition of the large larvae that were consuming the smaller ones, which are presumably very good food for the larger ones. Successful weaning of fish larvae is largely dependent on the larvae's ability to catch, select and digest the MD, which is also related to larval capacity to adapt to the new feed characteristics (no mobility, size, form or hardness, palatability among others). Delays in adaptation to the MD can lead to starvation and nutrient deficiency, resulting in growth retardation and even death (Gisbert et al., 2004). In this sense, in the present experiment, the larvae of the treatments that received lower amounts of *Artemia* suffered higher daily mortality during the first days of early weaning, mainly due to the fact that most of the larvae that did not accept well the MD, grew slowly and became vulnerable to predation by bigger larvae.

In fact, in low *Artemia* co-feeding treatments, the high cannibalism and low survival rates led to a lower larval density, especially from 40 to 48 dph. Generally, low larval density is associated with high growth in some marine species, such as meagre and red porgy (*Pagrus pagrus*) (Hernández-Cruz et al., 1999; Roo et al., 2010). Another aspect to consider is that the increase in larval density leads to high competition for food and space, a higher expenditure of energy deriving high metabolic rates and, therefore, a decrease in growth (Ellis et al., 2002; Thorarensen & Farrell, 2010). In the present study, lowering the amount of *Artemia* sp. from 2 Artemia ml^{-1} day^{-1} (100% A) to 1.5 Artemia ml^{-1} day^{-1} (75% A) did not affect the growth, survival or cannibalism incidence. Similarly, Ballagh et al., (2010) showed that it was possible to co-feed the mulloway (*Argyrosomus japonicus*) larvae a MD along with a reduced amount of *Artemia* (up to 50%) without compromising the growth. However, if *Artemia* was excluded from the diet, growth was reduced. Callan et al., (2003) indicated that similar results to live food controls could be achieved by weaning Atlantic cod (*Gadus morhua*) larvae early, while supplementing MD feed with reduced rations (25%–50% of the live food) of *Artemia*.

Optimization of prey density and its early replacement with MD are of great importance to improve the survival of marine fish larvae in general. Such optimization is of particular importance at the weaning period, as inadequate regimes during this critical transition could lead to high rates of mortality (Chen et al., 2007; Hamre et al.,

TABLE 4 Proximate (% dry matter) and fatty acids composition (% total fatty acids, TFA) of 48 dph greater amberjack larvae fed different co-feeding protocols (means ± SD, n = 3)

	Treatment				
	1% A	25% A	50% A	75% A	100% A
Proximate analysis (% dry matter)					
Lipids	10.47 ± 1.04^{b}	11.89 ± 0.82^{b}	12.99 ± 1.29^{b}	17.34 ± 0.70^{a}	17.91 ± 0.84^{a}
Ash	13.04 ± 2.68	14.77 ± 1.92	15.66 ± 1.54	14.58 ± 1.72	13.81 ± 1.48
Proteins	71.71 ± 0.62^{ab}	77.90 ± 2.63^{a}	77.02 ± 2.72^{a}	77.89 ± 2.75^{a}	69.60 ± 2.42^{b}
Moisture	79.46 ± 1.29^{b}	81.46 ± 2.21^{ab}	81.30 ± 0.15^{ab}	82.87 ± 0.68^{ab}	83.74 ± 1.62^{a}
Fatty acid content (% TFA)					
Total SFA	23.27 ± 0.32	24.81 ± 1.90	26.59 ± 2.20	25.05 ± 1.83	23.15 ± 7.29
Total MUFA	26.72 ± 0.86^{a}	27.19 ± 0.50^{a}	23.97 ± 0.65^{b}	22.60 ± 1.66^{b}	22.86 ± 2.01^{b}
Total n-3	38.72 ± 0.56	36.09 ± 2.29	36.80 ± 2.91	40.16 ± 2.89	41.38 ± 8.10
Total n-6	9.56 ± 0.45	9.91 ± 0.20	10.44 ± 0.59	9.89 ± 0.56	10.34 ± 0.82
Total n-9	15.14 ± 0.41^{a}	15.86 ± 0.18^{a}	14.47 ± 0.30^{ab}	13.52 ± 0.96^{b}	13.81 ± 1.09^{b}
Total n-3 PUFA	36.31 ± 0.57	32.29 ± 2.34	33.08 ± 2.94	36.54 ± 3.28	37.21 ± 8.27
14:0	1.10 ± 0.28	1.11 ± 0.64	0.90 ± 0.20	0.81 ± 0.22	0.74 ± 0.49
16:0	14.71 ± 0.30	15.42 ± 1.68	16.36 ± 1.89	14.81 ± 1.66	13.06 ± 6.74
16:1n-7	2.90 ± 0.29	2.98 ± 0.59	2.36 ± 0.21	1.99 ± 0.28	1.78 ± 1.03
18:0	6.98 ± 0.31^{c}	7.75 ± 0.73^{bc}	8.79 ± 0.17^{ab}	8.90 ± 0.04^{a}	8.82 ± 0.29^{a}
Oleic (18:1n-9)	13.23 ± 0.26^{a}	13.11 ± 0.29^{a}	12.37 ± 0.64^{ab}	11.37 ± 0.87^{b}	11.56 ± 0.84^{b}
18:1n-7	4.55 ± 0.39	4.70 ± 0.15	4.46 ± 0.22	4.32 ± 0.29	4.46 ± 0.20
18:2n-6	6.57 ± 0.38	6.65 ± 0.17	6.69 ± 0.79	5.42 ± 0.61	5.41 ± 0.32
18:3n-3	1.09 ± 0.03^{c}	2.36 ± 0.18^{b}	2.45 ± 0.09^{ab}	2.43 ± 0.34^{ab}	2.93 ± 0.15^{a}
20:1n-9	2.05 ± 0.30^{a}	1.77 ± 0.16^{ab}	1.24 ± 0.20^{c}	1.29 ± 0.13^{bc}	$1.26 \pm 0.14b^{c}$
ARA (20:4n-6)	1.60 ± 0.12^{b}	1.73 ± 0.18^{b}	2.03 ± 0.12^{ab}	2.39 ± 0.07^{a}	2.56 ± 0.51^{a}
EPA (20:5n-3)	9.27 ± 0.31	8.34 ± 0.43	8.15 ± 0.63	8.20 ± 0.43	8.34 ± 0.87
DHA (22:6n-3)	21.91 ± 0.64	19.37 ± 1.82	20.60 ± 2.08	23.67 ± 2.55	24.18 ± 6.43
DPA (22:5n-6)	0.56 ± 0.07^{b}	0.66 ± 0.09^{b}	0.80 ± 0.13^{ab}	1.13 ± 0.08^{ab}	1.33 ± 0.43^{a}
DHA/DPA	39.40 ± 3.97^{a}	29.75 ± 3.03^{b}	26.03 ± 1.62^{bc}	21.01 ± 0.80^{cd}	18.46 ± 1.66^{d}
ARA/EPA	0.17 ± 0.01^{c}	0.21 ± 0.02^{bc}	0.25 ± 0.01^{b}	0.29 ± 0.01^{a}	0.30 ± 0.03^{a}
DHA/EPA	2.37 ± 0.12	2.32 ± 0.15	2.52 ± 0.13	2.89 ± 0.29	2.87 ± 0.45
DHA/ARA	13.71 ± 0.86^{a}	11.23 ± 0.70^{b}	10.13 ± 0.52^{b}	9.88 ± 0.83^{b}	9.39 ± 0.86^{b}
Oleic/DHA	0.56 ± 0.03	0.68 ± 0.08	0.61 ± 0.09	0.49 ± 0.08	0.50 ± 0.15
Oleic/n-3 PUFA	0.34 ± 0.02	0.41 ± 0.04	0.38 ± 0.05	0.31 ± 0.05	0.32 ± 0.08
n-3/n-6	4.06 ± 0.19	3.64 ± 0.22	3.54 ± 0.49	4.07 ± 0.41	3.98 ± 0.45

Note: Different superscripts within each row indicate significant differences among protocols ($p < .05$; one-way ANOVA; post hoc Tukey's test).
Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, long-chain polyunsaturated fatty acids; SFA, saturated fatty acids.

2013). Low amounts of *Artemia* negatively affected the survival rate in our study, 1% A and 100% A co-feeding protocol representing the minimum (21.53 ± 4.04%) and maximum rates (50.72 ± 4.87%), respectively. The present results showed an improvement in the survival rate compared with the previous reports by Roo et al., (2019), with 24%, and Papandroulakis et al., (2005), with 3.5%, for the same species. Moreover, these results were better than what has been obtained for other fast-growing species like yellowtail amberjack (*Seriola lalandi*; 10.08 ± 1.17%; Hu et al., 2017) or meagre (*Argyrosomus regius*; 2.8 ± 0.6%; Campoverde et al., 2017). Additionally, during the experiment, we observed that the activity of the larvae became more vigorous, increasing their predation and cannibalism activity, especially under low *Artemia* sp. co-feeding protocols. When resources are limited in natural environments, cannibalism is considered as an alternative feeding strategy that is adopted mainly by piscivorous larvae (Hecht & Pienaar, 1993). Cannibalism affects directly the survival or indirectly the larval growth, as small individuals are typically exposed to more potential predators and the ability

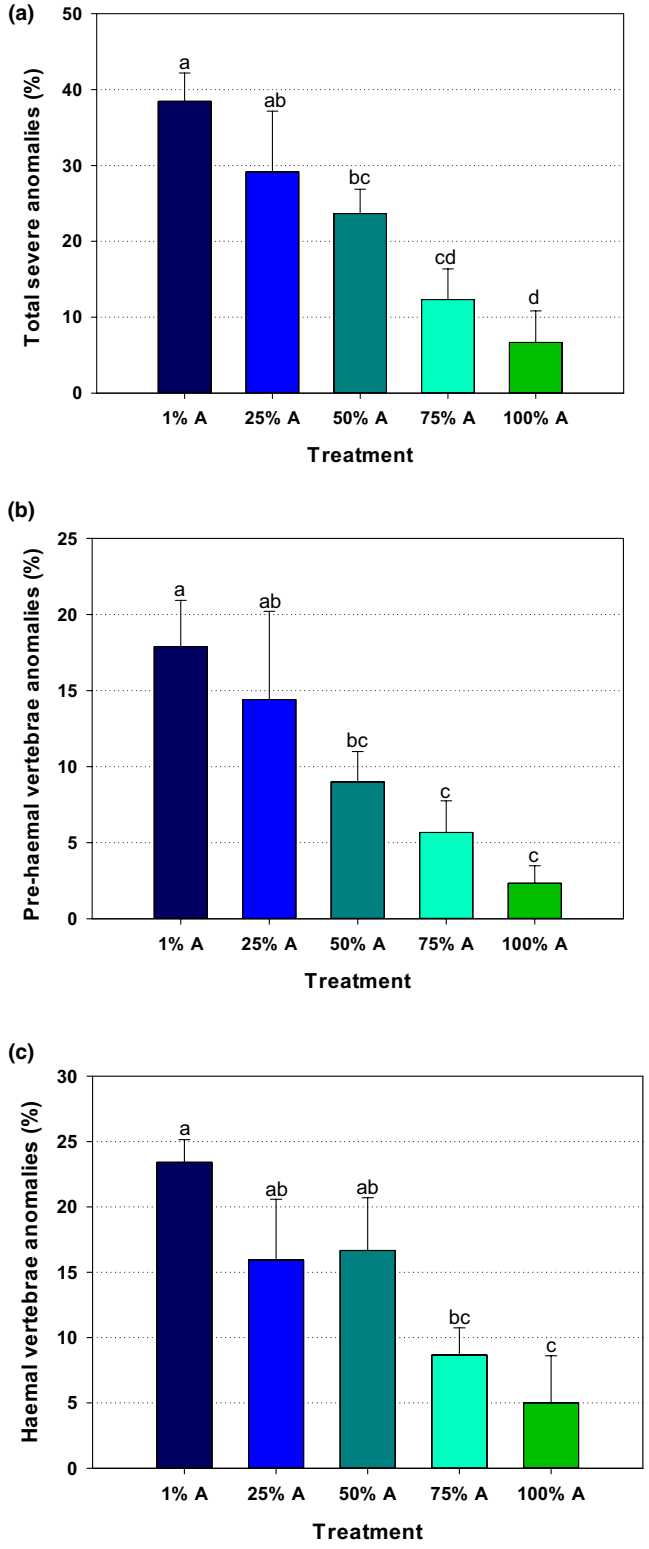


FIGURE 6 (a) Total skeleton severe anomalies (%), (b) pre-haemal vertebrae anomalies (%), (c) haemal vertebral anomalies (%) in larval greater amberjack at 48 dph fed different cofeeding protocols (means ± SD, $n = 3$). Different letters above the bars indicate significant differences among protocols ($p < .05$; one- way ANOVA; Post-hoc Tukey test)

to evade predators generally increases with the body size (Bailey & Houde, 1989). This could explain why lowering the *Artemia* provision in 1% A and 25% A treatments affected survival. In fact, cannibalism was also higher in treatments that received smaller amounts of *Artemia* sp. Generally, there are two peaks of mortality in greater amberjack larval rearing (Miki et al., 2011). The first one is linked to the transition from endogenous to exogenous feeding and the swim bladder inflation. The second mortality peak, on the other hand, is caused by cannibalism during the weaning. In the present study, significantly increased mortality as an effect of *Artemia* sp. reduction implies the enhancement of cannibalistic behaviour. Similar findings have been reported for other fast-growing species such as meagre, where high cannibalism resulted in a reduction in survival rates and dispersion in larvae size during the period of reducing the *Artemia* density (Campoverde et al., 2017; Roo et al., 2010). Cannibalism occurs in other *Seriola* larvae such as yellowtail (*Seriola quinqueradiata*) (Sakakura & Tsukamoto, 1999) or yellowtail amberjack (Moran, 2007; Stuart & Drawbridge, 2013). The authors attributed the cannibalistic aggressions to high size differences within the cohorts and suggested that the frequency of such behaviours increases when the size differences between prey and predator are larger. Although not statistically significant, low amounts of *Artemia* tended to increase the size deviation in the present experiment. Besides, other factors, such as live prey regimes, could also affect the onset of cannibalism. Miki et al., (2011) indicated that restricted live prey feeds entailed aggressive behaviour in greater amberjack larvae. Similarly, in yellowtail amberjack, the introduction of *Artemia* sp. as a food source was correlated with the increase in size heterogeneity and cannibalism behaviour (Moran, 2007).

Increasing *Artemia* sp. replacement levels by MD (1% A, 25% A and 50% A) led to a higher incidence of skeleton anomalies appearance. In many fish species, skeletal anomalies have been frequently reported in larval culture, being often associated with a low survival of the fish larvae (Boglione, Gavaia, et al., 2013; Boglione, Gisbert, et al., 2013; Izquierdo et al., 2010; Mesa-Rodriguez et al., 2019; Roo et al., 2009, 2019). Greater amberjack larvae are particularly susceptible to the incidence of skeletal anomalies (Roo et al., 2019; Sawada et al., 2020). Despite the predominant number of anomalies in the pre-haemal and haemal vertebrae in all larvae, greater amberjack fed low *Artemia* sp. amount showed a significantly higher prevalence of skeletal anomalies compared with larvae fed high *Artemia* sp. amount. Various physiological and behavioural performances of fish larvae such as swimming, feed intake and feeding efficiency may be affected by anomalies in the pre-haemal and haemal vertebrae (Powell et al., 2009). The cause of skeleton anomalies during the weaning phase depends on several factors including genetic, nutrition and culture conditions. Nutrition has been considered an important factor affecting skeleton anomalies (Cahu et al., 2003; Hu et al., 2017; Roo et al., 2019). Feed supply with a reduction in *Artemia* sp. amount had an important nutritional implication related to lipids and fatty profile. In this regard, high substitution levels of this MD

may be insufficient to support normal bone development in a fast-growing species like greater amberjack. Roo et al., (2019) found that the occurrence of skeletal anomalies was associated with n-3 highly unsaturated fatty acids (HUFA) content in *Artemia*, and the occurrence of cranial anomalies was correlated with increased dietary n-3 HUFA levels. Likewise, previous studies with similar species such as longfin yellowtail (*Seriola rivoliana*) suggest that the use of MD with high levels of n-3 HUFA content could induce the appearance of skeleton anomalies (Mesa-Rodriguez et al., 2019).

Most of the research on larval lipid nutrition has been focused on essential fatty acid requirements, particularly HUFA, due to its importance for larval growth and quality (Hamre et al., 2013). Inadequate content of those essential fatty acids in live prey or MD brings about several biological symptoms in larvae such as reduced appetite, growth, swimming activity, survival and particularly skeleton anomalies (Cahu et al., 2003; Izquierdo, 1996, 2005; Roo et al., 2019). In greater amberjack, a recent study has shown that n-3 HUFA content in enriched *Artemia* should also be considered to satisfy the larval nutritional requirements (Roo et al., 2019). In that study, when larvae were fed *Artemia*, the best growth, survival and resistance to stress were obtained with an n-3 HUFA range of 12%–17% TFA, DHA content of 5%–8.5%, and EPA content of 4.3%–5.5% TFA. In the present study, the n-3 HUFA level in *Artemia* sp. (12.9 ± 2% TFA) was in the range reported by these authors. However, the n-3 HUFA level in MD (29.62%) was markedly higher, probably being related to the high incidence of severe anomalies. Roo et al., (2019) reported a higher incidence of total deformities when n-3 HUFA were higher than 17% in the diet. Regardless of the co-feeding treatment, n-3 HUFA, DHA and EPA contents in greater amberjack larvae were similar. Considering that n-3 HUFA content of greater amberjack larvae was more similar to the n-3 HUFA profile in the MD compared with the *Artemia* sp., these results suggest that either the larvae assimilated the MD efficiently or such similar profile was due to the high cannibalistic behaviour of the larvae at the end of the experiment.

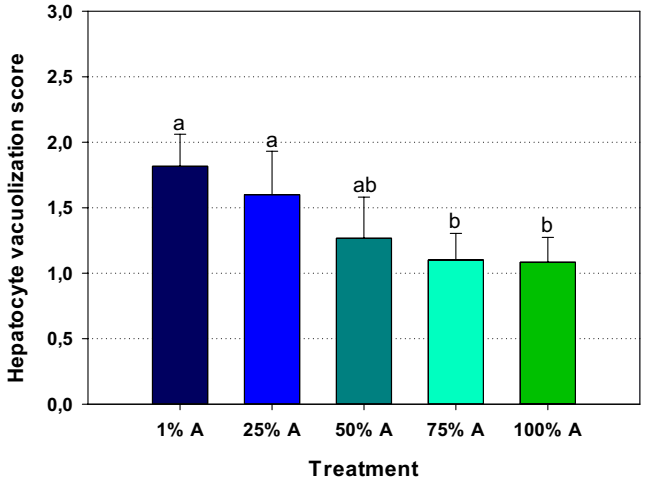


FIGURE 7 Hepatocyte vacuolization in 48 dph greater amberjack larvae fed different cofeeding treatments recorded on a 0–3 score (means ± SD, $n = 3$). Different letters above the bars indicate significant differences among protocols ($p < .05$; one-way ANOVA; Post-hoc Tukey test)

The liver is a good biomarker for nutritional effects of different diet composition and feeding regimes, as the hepatic energy stores respond sensitively and rapidly to the nutritional changes in fish larvae (Lazo et al., 2011). Fat deposition in hepatocytes reflects a physiological disorder originated from unsuitable feeding conditions or a nutritionally unbalanced diet (Gisbert et al., 2008; Lazo et al., 2011). In the present study, the co-feeding protocols with lower *Artemia* levels tended to increase the lipid content in the liver. The greater lipid accumulation might be due to higher digestive efficiency for this nutrient that results in greater lipid absorption from the intestine and storage in the liver (Boglino et al., 2012). Alternatively, such high hepatic lipid accumulation might be due to a poor nutritional condition of the larvae fed smaller amounts of *Artemia*. Similarly, in Senegal sole (*Solea senegalensis*) larvae reared with *Artemia* showed a normal histological pattern, while histological alterations were detected in those fed MD (Fernández-Diaz et al., 2006).

As in most vertebrates, growth endocrine regulation in fish is regulated by the growth hormone (Gh)/insulin-like growth-factor (Igf) axis (Bertucci et al., 2019; Reinecke, 2010). In this study, the expressions of some of the key genes from this axis, that is *gh*, *igf1* and *igf2*, were analysed. Both Gh and Igfs promote growth, although Igf1 seems to be particularly responsible for body growth (Triantaphyllopoulos et al., 2019). In the present study, high *gh and igf2* expressions at 40 dph were followed by a significant decline at 48 dph in all the co-feeding protocols. Evidence at the nutrition level has shown that prolonged starvation or fasting generally results in suppressed body growth, with a concomitant reduction in the levels

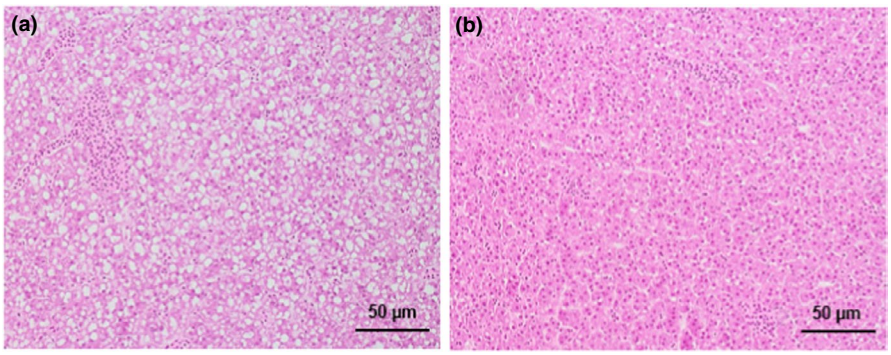


FIGURE 8 Different hepatocyte vacuolization found in 48 dph greater amberjack larvae stained with haematoxylin and eosin, (a) 1% A treatment (b) 100% A treatment

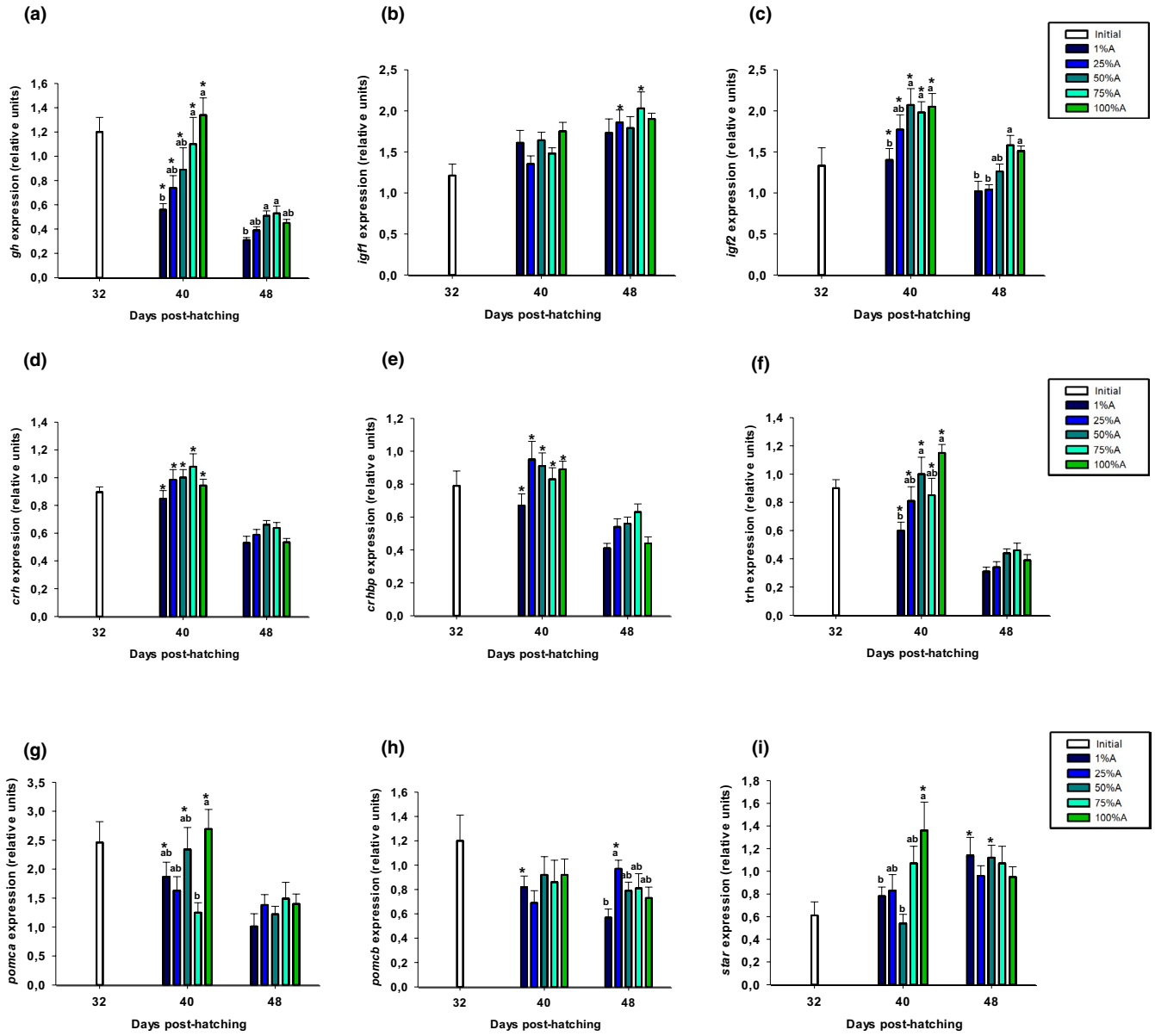


FIGURE 9 Relative gene expression levels determined in whole greater amberjack larvae from different co-feeding protocols, at 32, 40 and 48 dph (mean ± SEM, $n = 15$); (a) growth hormone (gh), (b) insulin-like growth factor I (igf1), (c) insulin-like growth factor II (igf2), (d) corticotropin releasing hormone (crh), (e) corticotropin releasing hormone binding protein (crhbp), (f) thyrotropin releasing hormone (trh), (g) proopiomelanocortin a (pomca), (h) proopiomelanocortin b (pomcb), and (i) steroidogenic acute regulatory protein (star). Different letters indicate significant differences among co-feeding protocols at the same sampling time ($p < .05$; one- way ANOVA; Post-hoc Tukey test), whereas asterisks indicate significant differences between 40 and 48 dph sampling times in the same co-feeding protocol ($p < .01$; Student's *t*-test)

of circulating *igfs* (Triantaphyllopoulos et al., 2019). Many studies have shown that fasting or poor nutritional status in larvae fish alters the mRNA expression of components of the Gh/Igf axis (Opazo et al., 2017; Piccinetti et al., 2017). But the most suitable explanation for this variation along ages could be the normal ontogenic variation of these genes in this species, as it has been already published in others, like grey mullet (*Chelon labrosus*) (Gilannejad et al., 2020).

The *gh* expressions at 40 and 48 dph were higher in smaller larvae than in the larger ones. Similarly, the mean of *gh* mRNA levels in larvae body of zebrafish (*Danio rerio*) was higher in smaller larvae and suggests that the lower growth rate observed in these larvae was associated with the poor nutritional status (Opazo et al., 2017). Comparing the different co-feeding protocols at 48 dph, it was observed that *gh* expression was higher in 50% A and 75% A protocols. Nevertheless, *igf2* expression was higher in the 75% A and 100% A protocols, which also showed the lowest specific growth rate and highest survival rate, leading to higher larval density. A recent study has found that the higher expression of *igfs* was associated with high densities in Siberian sturgeon (*Acipenser baerii*) larvae. Highest stocking densities could provoke crowding stress with a subsequent

increase in energy demand and utilization of energy reserves (Aidos et al., 2020). Moreover, the Gh/Igf axis is influenced by stress conditions, and any conditions that induce stress require the larvae to spend energy to maintain the homeostasis (Reindl & Sheridan, 2012; Sadoul & Vijayan, 2016). Stress responses associated with co-feeding protocols have been described in some aquaculture species (Liu et al., 2012; Piccinetti et al., 2012). In fish, in response to stressor exposure, the hypothalamic–pituitary–interrenal (HPI) axis is activated leading to the production of corticotropin-releasing hormone (crh) in the hypothalamus, whose levels are regulated by the Crh-binding protein (crhbp) and thyrotropin-releasing hormone (trh) (Gorissen & Flik, 2016; Ruiz-Jarabo et al., 2018). Crh stimulates the proopiomelanocortin (pomc) production in the pituitary, which in turn stimulates the cortisol synthesis in the steroidogenic cells located predominantly in the head kidney region in fish (Faught et al., 2016). Consequently, activation of this pathway enhances the activity of enzymes such as the steroidogenic acute regulatory protein (star), producing cortisol as the end-product (Montero et al., 2015). In this study, the changes in expression levels of hypothalamic factors (*crh*, *crhbp* and *trh*), pituitary hormonal precursors (*pomca* and *pomcb*) and the first enzyme involved in cortisol synthesis (*star*) showed that some components that play a role in the HPI axis are affected by the weaning period. High expression of genes related to the stress response in the mid-weaning period might indicate that forcing the larvae to consume artificial compound feed may have a stressful effect. Differences in the expression of stress-related genes are probably explained because greater amberjack has poor adaptability to stress and can be easily affected by the weaning conditions. Fish larvae are especially sensitive to non-optimal feeding conditions or nutritional stress factors because most tissues and organs are under progressive differentiation and development (Gisbert et al., 2008; Lazo et al., 2011). It has been shown that fish can become habituated to mild repetitive and predictable stressors (Madaro et al., 2016; Sánchez-Nuño et al., 2019). Therefore, a process of adaptation could explain the highest levels of stress genes in the mid-weaning period, being attenuated at the end of the experiment.

5 | CONCLUSIONS

In summary, considering our results as a whole, we can suggest that the density of *Artemia* sp. in greater amberjack larval co-feeding could be reduced at least to 25% of the standard protocol, 1.5 *Artemia* sp. ml^{-1} day^{-1} being the optimum concentration under such co-feeding protocols. These co-feeding protocols are expected to ensure high growth and survival rates while reducing the costs, stress response and occurrence of skeletal anomalies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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