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Larval performance and skeletal deformities in farmed gilthead sea bream (*Sparus aurata*) fed with graded levels of Vitamin A enriched rotifers (*Brachionus plicatilis*)

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Abstract:

Several nutritional studies have found a direct effect of several vitamins in chondrogenic and osteogenic development during early life stages of marine fish species. In the present study, the effect of vitamin A (VA) in gilthead sea bream skeletogenesis was evaluated by means of four different dietary regimes (enriched rotifers) containing increasing levels of total VA (75, 109, 188 and 723 ng total VA mg^{-1} DW). Dietary treatments were offered to larvae during the rotifer-feeding phase (4–20 days after hatching), while later all groups were fed with Artemia nauplii and weaned onto the same inert diet. Different dietary doses of VA affected gilthead sea bream larval growth, survival, performance (maturation of the digestive system) and quality (incidence of skeletal deformities). Higher levels of dietary VA than those included in the commercial emulsion for rotifer enrichment led to different levels and typologies of skeletal deformities, indicating that gilthead sea bream larvae were very sensitive to small increases in dietary VA. The degree of ossification was affected by the level of VA in enriched rotifers: the higher amount of VA in the diet, the higher number of skeletal pieces ossified ($R = 0.585$, $P = 0.04$). Dietary VA affected the normal process of bone formation and skeletogenesis, the skeletal structures mostly affected by high amounts of dietary VA were those from the cranial skeleton (splanchnocranium), vertebral centrums and caudal fin complex. The premaxilla, maxilla and dentary bones were the cranial structures affected by dietary VA levels, resulting in a large incidence of animals with compressed snout. Dietary VA also affected the normal development of the opercular complex, and a dose–response dependant effect was observed in relation to the incidence of specimens with incomplete operculum. Body shape was also affected by the level of dietary VA, increasing the incidence of specimens with lordosis, kyphosis and/or scoliosis with the dose of VA, being the prehaemal and caudal vertebrae the most affected regions of the vertebral column with this kind of abnormalities. The caudal fin complex was the most affected region of the skeleton affected by dietary treatments as seen by the high incidence of skeletal deformities in fish fed different doses of dietary VA. Deformities affected all skeletal elements composing the caudal fin, although the most affected ones were, in order of importance, the epurals, hypurals, parahypural, neural arch and uroneurals. Differences in sensitivity to dietary VA amongst caudal fin skeletal elements might be due to their differential ontogenetic development and differences in the exposure time to VA.

An excess of dietary VA also accelerated the intramembranous ossification process of vertebral centrums leading to one or two supranumerary vertebrae, and a high incidence of fused and compressed vertebral centrums. The sensibility of the developing skeletal structures to dietary VA levels should incline us to test lower doses of VA in live preys enrichments during early larval stages and higher doses afterwards.

Keywords: Gilthead sea bream; *Sparus aurata*; Larval quality; Vitamin A; Skeleton; Deformities

1. Introduction

Gilthead sea bream is one of the most important marine fish species farmed in the Mediterranean region with a total production of 71,355 t (FAO, 2005). In this area, a high competence between aquaculture companies and a reduction of the gilthead seabream market price due to overproduction during recent years, have forced aquaculture industry to reduce their production costs and improving their larval rearing efficiency. Skeletal deformities and their incidence are one of the most important factors affecting fish farmer's production costs, determining the external morphology, growth and fish survival rate (Matsusato, 1986; Divanach et al., 1997; Koumoundouros et al., 2002). In the aquaculture industry, losses due to deformities occur at two levels. At hatcheries, reducing larval survival rate and growth efficiency in malformed fish; and at on-growing farms, where malformed market size fish have to be discarded or sold at lower values than market prices. The levels of losses at either point are different, depending on the species and the husbandry practices followed. In whatever circumstance, these losses are substantial, in terms of productivity and profitability, since skeletal deformities might affect up to the 30 % of the production. This fact represents one of the bottlenecks in actual marine aquaculture. The yearly production of more than 500 million has a survival of less than 15-20%. High mortalities during the first stages of development, which are typical for marine aquaculture, are responsible for a loss of several millions of euros (Subasinghe, 1997). Thus, reducing the incidence of larval deformities would reduce the economic cost of production, both in the hatcheries and in the out-growing production sectors, and improve the quality of the products.

Most of skeletal deformities appear during the larval and juvenile stages, where many biological processes take place for organogenesis, morphogenesis and metamorphosis in a very short time. The development of skeletal disorders is linked to a poorly understood relationship between nutrition, environment and genetic factors. The larval stage is a very sensitive period where the harmonious larval development depends on the physiological, environmental, genetic, xenobiotic and nutritional factors (see review in Lall and Lewis, 2007).

Among them, larval nutrition at first feeding is one of the key parameters that affect skeletogenesis during early development. In this sense, several studies have demonstrated that nutrients are responsible for the appearance of skeletal deformities when their level and/or form of supply in the diet are inappropriate or unbalanced (Cahu et al. 2003, Lall and Lewis, 2007). The solution of the problem is strongly related to the understanding of the species- and stage-specific environmental preferences and nutritional requirements of the fish larvae, as well as to the ontogeny of the skeletogenesis and anatomy of each deformity type.

The effects of nutrition on bone development and remodelling have been deeply studied in terrestrial vertebrates, whereas this information in fish is fragmentary depending on the nutrient considered (Cahu et al., 2003; Lall and Lewis, 2007). In this sense, recent advances in the composition of starter diets for marine finfish larvae and enriching emulsions for live preys have identified several nutrients, particularly minerals, vitamins and lipids that can be critical for normal skeletogenesis. Vitamin A (VA), a morphogenetic nutrient, includes all compounds that posses the same biological activity of retinol, playing a key role in morphogenesis, cellular differentiation and proliferation processes. VA determines normal growth, body patterning, nervous system development, and differentiation of pigment cells, limbs and skeleton along vertebrate early development (Ross et al., 2000). The fish are not able to synthesize VA, thus they have to take it from diet and any excess or deficiency of this nutrient in the diet resulted in abnormal growth and development (Dedi et al*.,* 1995; Tarui et al., 2006; Takeuchi et al., 1998; Haga et al., 2002 a, b; Villeneuve et al., 2005). The impact of dietary VA on fish larvae development will depend on both the dietary dose and the developmental status of the larvae at first feeding. It is then necessary to fine-tune this particular relationship for each species.

The objective of the present study was to evaluate the effect on larval performance (growth, survival and maturation of the digestive function) and quality (incidence and typology of skeletal deformities) of graded levels of dietary VA on gilthead sea bream larval development.

2. Materials and methods

2.1 Larval rearing and diets

Gilthead sea bream larvae were obtained from a Spanish private hatchery and shipped to the IRTA facilities. After their acclimation, larvae were distributed (initial density: 100 larvae L^{-1}) in 24 cylindrical tanks (100 L) connected to a recirculation unit (Carbó et al., 2002). Water conditions were as follows: 18-19ºC, 35 ppt salinity, pH between 7,8-8,2, 20% daily water exchange and with gently aeration and oxygenation (> 4 mg l^{-1}) Photoperiod was 12L:12D, and light intensity of 500 lux at water surface.

Larvae were fed from day 4 post hatch (dph) to 20 dph enriched rotifers (*Brachionus plicatilis*, lorica length: 178±30 µm length), whose density was progressively increased from 5 to 10 rotifers ml^{-1}. *Artemia* nauplii (EG, INVE, Belgium) were offered to larvae from 16 to 22 dph, in increasing density from 0.5 to 2 nauplii ml^{-1}, and 2 days enriched-metanauplii from 20 to 40 dph (1 to 5 metanauplii ml^{-1}). From 36 dph to the end of the experiment (60 dph), larvae were progressively weaned onto dry feed, first with Proton 1/2 and 1/4 (INVE, Belgium) and then with Gemma Micro (size range: 75 to 500 µm; Skretting, Spain).

The effect of VA in gilthead sea bream skeletogenesis was evaluated by means of four different dietary regimes containing graded levels of VA using enriched rotifers. As rotifers and *Artemia* nauplii accumulate VA in different patterns (Giménez et al., 2007), it was not possible to maintain the same levels of VA during all the life prey-feeding period. Thus, we decided to focus our study during only the rotifer-feeding phase. The graded VA levels on live prey were obtained adding retinol palmitate (1,600,000 IU g^{-1}, Sigma-Aldrich, Spain) to a commercial enriching emulsion, Easy Selco™ (ES, INVE, Belgium). Theoretically, experimental emulsions contained 450, 900, 2,250 and 4,500 ng retinol equivalents mg^{-1} of emulsion in wet weight (Table 1). Dietary treatments were named as R450, R900, R2,250 and R4,500 according to the theoretical level of retinol contained in the enriching emulsion (wet weight). For comparative

purposes, the emulsion containing 450 µg retinol equivalents g^{-1} (R450) was considered as the control group (ES without retinol palmitate addition).

Live prey (rotifers and *Artemia* nauplii) were enriched according to Gimenez et al. (2007), rotifers were enriched during two hours with 0.15 g of each experimental emulsion per litre, and *Artemia* metanauplii for 18 h with 0.6 g ES l^{-1}. After enrichment, rotifers and *Artemia* were gently siphoned from enriching tanks, collected in a mesh, and washed in freshwater to reduce the bacterial load and rests of the enrichment emulsions. Live preys were introduced into the rearing tanks three times per day in order to assure an optimal live prey density in the water column, and their appropriate nutritional value.

The effects of graded levels of VA on gilthead sea bream larval performance and quality was evaluated by quintuplicate (three tanks were used for regular sampling and two for final survival). Larvae were sampled at 18 and 60 dph, coinciding with the end of the rotifer-feeding phase and the end of the weaning period, respectively. For sampling purposes, larvae were sacrificed with an overdose of anaesthetic (Tricaine methanesulfonate, MS-222, Sigma).

2.2 Biochemical analysis

Retinoids on enriching emulsions and live prey were analyzed by HPLC using a modification of the method proposed by Takeuchi et al. (1998). After sampling, live prey were washed with distilled water to remove marine salts and bacterial load, and samples were frozen at -80ºC until posterior analysis. Lipids were extracted with a chloroform:methanol mixture (C:M, 2:1) according to the Folch method (Folch et al., 1957), and stored in C:M:BHT (2:1:0.01) at 20 mg l^{-1} at −20 °C until their analysis. Then, samples were evaporated and redissolved on methanol:acetone (1:1 v/v) prior to their HPLC analysis. The HPLC system (Thermo Separation Products, San Jose, CA, USA) was equipped with a Lichrospher C-18 reverse phase column (Merck, Darmstadt, Germany) and a UV–visible detector set at a wavelength of 325 nm. The mobile phase was a mixture (85:15 v/v) of 98% methanol with 0.5% ammonium acetate, and chloroform. The flow rate was 1.5 ml min^{-1} and the elution time was 18 min. The concentration

of each retinoid was calculated from the calibration curves constructed with the peak area ratios of their external standards and an internal standard of retinol acetate added to the samples. All the reference retinoids were purchased to Sigma-Aldrich (Spain).

2.3 Larval growth and survival rate

Sampled larvae (*n* = 15) from each tank were washed with distilled water to avoid marine salts and used for body size and dry weight determination. Larval standard length (Ls) was measured with digital camera connected to a binocular microscope Nikon SMZ 800, AnalySIS (Soft Imaging Systems, GmbH). Once larvae were measured in length, they were dried at 60ºC until their weight was constant. Samples were weighted with an analytic microbalance Sartorius BP211D. At the end of the experiment, the total length of 150 fishes from each rearing tank was measured to evaluate the effects of VA on size dispersion. Survival rate was calculated as the percentage of final surviving fish in relation to the initial number at the beginning of the trial.

2.4 Maturation of the digestive system

The activity of two intestinal brush border enzymes (alkaline phosphatase and aminopeptidase N) and two pancreatic enzymes (trypsin and amylase) was used to assess the degree of development and maturation of the digestive system of larvae fed graded levels of VA. Enzyme activity was measured at 18 and 60 dph (*n* = 50 and 10 larvae per tank, respectively).

Sampled fish were washed with distilled water to avoid marine salts and stored at -80ºC prior to enzyme activity analysis. The whole 18 dph larvae were homogenized for enzymatic assays, since they were too small to dissect, while older fish were dissected to separate pancreatic and intestinal segments as described by Cahu and Zambonino-Infante (1994). Samples were homogenized (Ultra-Turrax T25 basic, IKA© - Werke) in five volumes (v/w) of ice-cold Milli-Q water, centrifuged at 3,300 g for 3 min at 4ºC and the supernatant removed for enzyme quantification. For determination of intestinal enzymes, samples were homogenized in

cold Mannitol 50 mM, Tris-HCl 2 mM buffer, pH 7.0. Intestinal brush border membranes were purified according to the method developed for intestinal scrapping (Crane et al., 1979).

Trypsin (E.C. 3.4.21.4) activity was assayed according to Holm et al. (1988), at 25ºC using BAPNA (N-α-benzoyl-DL-arginine *p*-nitroanilide) as substrate. Amylase (E.C. 3.2.1.1) activity was measured according to Métais and Bieth (1968), using soluble starch (0.3%) dissolved in Na_2HPO_4 buffer pH 7.4 as substrate.

Alkaline phosphatase (E.C. 3.1.3.1) was quantified at 37ºC using 4-nitrophenyl phosphate (PNPP) as substrate (Bessey et al., 1946). Aminopeptidase N (E.C.3.4.11.2) was determined at 25ºC according to Maroux et al. (1973), using sodium phosphate buffer 80 mM (pH = 7.0) and L-leucine *p*-nitroanilide as substrate (in 0.1 mM DMSO). Enzymatic activities were expressed as the specific activity, milli-units per milligram of protein (mU/mg protein), and soluble protein of crude enzyme extracts was quantified by means of the Bradford's method (Bradford, 1976) using bovine serum albumin as standard. All the assays were conducted in triplicate.

2.5 Skeletal deformities analysis, observations and measurements

To identify and quantify the skeletal deformities on larvae from different dietary treatments, 50-60 larvae per each tank were sampled at the end of the experiment, and fixed in formaldehyde solution (10%) until their double staining. Then, animals were stained for bone and cartilage on whole mounts using a modification of the method described by Klymkowsky and Hanken (1991). In brief, specimens were rehydrated two times in distilled water during 5 minutes and then placed in alcohol 95º. Specimens were stained with alcian blue solution with 80% alcohol 95º and 20% glacial acetic acid during 24 hours, rehydrated through a graded series of alcohol (95%–25%) and macerated using a 1% aqueous solution of KOH with 3% hydrogen peroxide (9:1 in volume) until skeletal elements were clearly visible. Then, specimens were placed between 6 and 20 hours in an aqueous solution saturated in sodium borate containing 0.3-0.5 g trypsin, and stained with alizarin red S (stock solution: 1% alizarin red in 1% KOH) during 24

hours. Staining time was variable and depended on the size of the specimen. Finally, fish were washed with distilled water, followed by a series of baths in 1% KOH to remove the excess of dye in soft tissues, and placed through graded series of glycerine-KOH solutions.

After staining, fish were placed on their right side, in order to observe meristic characters and skeletal abnormalities in the cranium, vertebral column and caudal fin complex. Skeletal structures were identified and named according to Faustino and Power (1998, 1999, 2001). The study was focused on the mean number of vertebra and frequency of individuals with abnormal number of vertebrae. Special emphasis was placed on the deformities occurring in the cranial region (upper and lower jaws), vertebral column and caudal fin complex (hypurals and parahypurals, epurals, uroneural, and specialized neural arch). In particular, we calculated the frequency of individuals with lordosis, scoliosis or kyphosis, the total sum of deformities in the vertebral column, and the incidence of vertebral compression and fusion.

In order to assess the degree of ossification of gilthead sea bream juveniles and establish a potential relationship with the dietary regimen during the larval stages, the percentage of juveniles in different stages of ossification was determined at the end of the experiment. Those stages were defined according to the ossification of selected bony structures that better describes the ossification process in this species (Faustino and Power, 1998). In brief, stage I corresponded to the early ossification of some vertebral centrums; at stage II, all vertebral centrums were completely ossified; and at the stage III, dorsal fin rays were completely ossified. At stage IV, caudal fin rays were ossified, and at stage V, the hypurals and parahypural started to ossify. The stage VI was characterized by the complete ossificatifon of most of the skeletal structures with the exception of the uroneural 2 and the haemal spines 2 and 3, which completed their ossification at older ages (larger sizes).

2.6 Statistical analysis

Results are given as means and standard deviations. Data expressed as percentage (survival, incidence of skeletal deformities) were previously arcsin($x^{1/2}$)-transformed. Results were

compared by means of One Way ANOVA (data normally distributed, Kolmogorov–Smirnov test) and when significant differences were detected the Tukey multiple-comparison test was used to detect differences among experimental groups (Zar, 1974). The test of Kolgomorov-Smirnov was used to evaluate the distribution of fish size at the end of the study. Correlation between different variables was evaluated with the Pearson Product Moment Correlation test. In all statistical analyses, the level of significant difference was set at $P < 0.05$. All the statistical analyses were conducted using SigmaStat 3.0 (SPSS, Richmond, USA).

3. Results

3.1 Retinoid content in experimental emulsions and live prey

Total lipid and retinoid content in emulsions and rotifers enriched with graded levels of retinol palmitate are presented in Tables 1 and 2, respectively. No statistically significant differences were detected in the total lipid content of experimental emulsions and enriched rotifers with different levels of VA (ANOVA, $P > 0.05$). Total vitamin A content in emulsions and enriched rotifers increased with increasing levels of retinyl palmitate incorporated into the emulsion (ANOVA, $P < 0.05$). Analysis of retinoid content of enriched rotifers showed that the real incorporated level of total VA was 75, 109, 188 and 723 ng mg^{-1} DW for live prey enriched with R450, R900, R2,250 and R4,500 experimental emulsions, respectively (Table 2). Retinyl palmitate (VA ester) was the dominant form of retinoid detected in emulsions and enriched rotifers, whereas retinol (VA alcohol) was also detected but at a minor concentration. Either retinal (aldehyde form of vitamin A) was not detected in emulsions or rotifers enriched with graded levels of vitamin A, whereas low levels of retinoic acid (0.8-1.1 ng mg^{-1} DW) were only detected in enriched rotifers, although they were not significantly different amongst rotifers enriched with graded levels of VA (ANOVA, $P > 0.05$).

3.2 Larval growth and survival

Table 3 contains the results of growth in length and DW of gilthead seabream larvae fed different levels of VA. At 18 dph, no statistically significant differences were observed in DW in larvae fed graded levels of VA during the rotifer-feeding phase, while fish fed rotifers enriched with the control emulsion showed the best growth in length (ANOVA, $P < 0.05$). At the end of the trial, larvae fed R450 and R900 were larger in length and DW (ANOVA, $P < 0.05$). Larvae fed rotifers enriched with higher doses of total VA (R2,250 and R4,500) weighted 12 and 21% less than the other groups. Similarly, the length of those fish was 3 and 7% smaller.

The frequency distribution of final Ls classes in experimental groups fed rotifers enriched with R450, R900 and R2,250 followed a normal distribution. The Kolmogorov-Smirnov test, however, revealed statistically significant differences between the final distribution of Ls of fish fed rotifers enriched with the highest dose of total VA (R4,500), skewing the distribution towards sizes classes comprised between 15 and 17 mm ($P < 0.05$; Fig. 1).

Dietary levels of VA significantly affected fish larval survival (Table 3; ANOVA, $P < 0.05$). Final survival ranged from 2.9 to 9.1% depending on the experimental group. The highest survival results were obtained in larvae fed R450 and R900, while higher dietary levels of VA (R2,250 and R4,500) significantly reduce their viability.

3.3 Maturation of the digestive system

At the end of the rotifer-feeding period, the different VA levels in enriched rotifers significantly affected the trypsin activity (ANOVA, $P < 0.05$, Fig. 2a), while no significant differences were detected in amylase secretion (ANOVA, $P > 0.05$, Fig. 2b). At 18 dph, the specific trypsin in larvae fed R450 was 2 times higher than in larvae from the other dietary treatments. At the end of weaning (60 dph), no statistical significant differences in specific trypsin and amylase

306 activities were detected among larvae from different experimental groups (ANOVA, $P > 0.05$,
307 Fig. 2c, d*).*

308 The specific activity of intestinal brush border enzymes was also affected by the dietary
309 content of VA in enriched rotifers, although the trend in the specific activity of both enzymes
310 was different depending on sampling date. At the rotifer-feeding phase, the highest alkaline
311 phosphatase specific activity was measured in larvae fed the highest dose of VA in enriched
312 rotifers (R4,500) (ANOVA, $P < 0.05$; Fig. 3a), while specific activity of aminopeptidase N in the
313 above-mentioned group was the lowest amongst all four tested experimental groups (ANOVA,
314 $P < 0.05$; Fig. 3b). At 60 dph, the alkaline phosphatase specific activity in fish fed R450 and
315 R900 was 80% higher than those fed higher levels of VA (ANOVA, $P < 0.05$; Fig. 3c).
316 Aminopeptidase N specific activities followed the same trend than alkaline phosphatase. The
317 highest activities were recorded in fish fed R450 and R900, which were 4 times higher than
318 those in larvae fed R2,250; while intermediate values were detected in fish fed rotifers
319 containing the highest dose of VA (Fig. 3d).
320
321
322 *3.4 Skeletal deformities: typology and frequencies*
323 The typology and incidence of skeletal deformities in gilthead sea bream juveniles fed different
324 levels of VA during the rotifer-feeding phase are shown in Figures 4-11.

325 The degree of ossification was affected by the level of dietary VA in enriched rotifers
326 (Figure 4): the higher amount of VA in the diet, the higher number of skeletal pieces ossified (R
327 $= 0.585$, $P= 0.04$). Fish fed the highest dose of VA showed the highest frequency of specimens
328 in most advanced stages of ossification (69.6% in stages V and VI) in contrast with those fed
329 the control diet, which only showed the 39.2% of specimens. However, the above-mentioned
330 data were not significantly different due to large variability in the ossification process between
331 replicates from the same dietary treatment.

The presence of cranial skeletal deformities in the jaw apparatus was strongly correlated to the level of retinoids in enriched rotifers (R = 0.789, P = 0.002) (Fig. 6a). The premaxilla, maxilla and dentary bones were the cranial structures more affected by different levels of dietary VA, resulting in large number of specimens with compressed snout (pugheadness, underdevelopment of the maxilla and premaxilla, Fig 5). The incidence of jaw deformities in larvae fed the highest doses of VA (R2,250 and R4,500) were significantly higher than those observed in juveniles fed lower levels of VA (ANOVA, $P < 0.05$; Fig. 6a). No statistical significant differences were detected regarding the incidence of jaw deformities between larvae fed R450 and R900 (ANOVA, $P > 0.05$). However, fish fed R900 showed a higher incidence of deformities in both jaws in the same specimen, while this type of deformation was not observed in the control group (Fig, 6b).

The incidence of opercular deformities was significantly correlated to the dose of VA in enriched rotifers (R = 0.843, P = 0,001; Fig. 6c). While the control group had 16.5 ± 1.64% of the individuals with an incomplete operculum, the incidence of this kind of deformity reached 39.2 ± 3.05%, in those fish fed R4,500 (ANOVA; $P < 0.05$). Significant statistical differences were also detected in the incidence of deformed operculum depending on the fish side considered, indicating that the expression of this type of deformity was side-dependent. In all experimental groups, the frequency of abnormal operculum was higher in the left than in the right side, independently of the level of dietary VA (t-test, $P < 0.05$; Fig. 6d). However, no statistically significant differences were detected in the frequency of individuals with bilateral or right-sided opercular complex deformities (ANOVA, $P > 0.05$).

The number of vertebrae most frequently observed in gilthead sea bream is twenty-four. At the end of the experimental period, statistically significant differences in the mean number of vertebrae were detected between larvae fed R900 and the rest of dietary treatments (ANOVA, $P < 0.001$; Fig. 7). Fish from this experimental group showed a higher frequency of specimens with twenty-five (69.1%) and twenty-six vertebrae (24.2%). Those supranumerary vertebrae

were observed in the caudal region, between the urostyle and vertebra number 23, and were significantly correlated to vertebral fusion and compression disorders (R = 0.959, P < 0.0001).

The incidence of specimens with lordotic, kyphotic or scoliotic bends in their vertebral column tended to increase with increasing levels of total VA in enriched rotifers, although this trend was not significant (R = 0.564, P = 0.056) due to the large variability of replicates. Similarly, no significant differences could be detected between experimental groups (ANOVA, P > 0.05; Fig. 8a, 9).

The frequencies of deformities in vertebral centrums were significantly affected by the level of VA in the diet (Fig. 8b). In all treatments, the compression of vertebral centrums was more frequent than their fusion with the adjacent ones (Fig, 8c, d). In particular, larvae fed R900 showed the highest incidence of compressed (80%) and fused (40%) vertebral bodies than the rest of the experimental groups (ANOVA, P < 0.05). The incidence of both types of deformities in vertebral centrums was 65 and 33% more frequent in the above-mentioned group than in larvae fed rotifers enriched with the control emulsion. Regarding the haemal and neural spines of vertebrae, in the haemal region of the vertebral column, haemal spines were significantly affected by the level of VA in the diet, increasing the incidence of twisted haemal spines with the dose of dietary VA (ANOVA, P < 0.05). In contrast, no differences were detected in the incidence of deformities in the neural spine (ANOVA, P > 0.05), although high variability between replicates and dietary groups was detected.

The incidence of skeletal deformities along the vertebral column in gilthead sea bream juveniles is shown in Figure 10. Independently of the level of VA in the diet, the caudal region was the area most affected by compression and fusion of the vertebral centrums; although the group fed R900 showed the highest percentage of compressed and fused vertebrae in this region; mostly affecting the urostyle (30%) and vertebra number 23 (90%). Vertebrae from the cephalic and prehaemal regions were also affected by the level of VA in the diet, thus juveniles fed rotifers containing high levels of VA during the rotifer-feeding phase showed a higher incidence of skeletal deformities in these regions (>10%; ANOVA, P < 0.05).

385 The effect of different levels of dietary VA on the skeletogenesis of the caudal fin
386 complex is presented in Figure 11. Only the 29% of gilthead sea bream juveniles from the
387 control group had at least one skeletal anomaly per fish in the caudal fin complex, while more
388 than 90% of specimens fed higher levels of dietary VA had one or more types of deformities
389 per specimen (Fig. 11a). The skeletal elements in the caudal fin complex most affected were
390 the specialized neural arch, epurals, hypurals and parahypurals, and the uroneural. In most
391 cases, deformities consisted in twisted or undeveloped skeletal elements and their fusion with
392 adjacent ones. In particular, dietary levels of VA significantly affected the frequency of
393 specimens with deformed specialized neural arch (ANOVA, $P < 0.05$; Fig. 11b). Juveniles fed
394 R900 showed the highest frequency (50%) of specimens with this bonny element deformed in
395 comparison to the other dietary treatments containing higher levels of VA, while none of the
396 fish from the control group was detected with this type of skeletal deformity. The frequency of
397 individuals with deformed epurals, hypurals and parahypurals was significantly higher in those
398 treatments fed higher doses VA than in the control group (ANOVA, $P < 0.05$; Fig. 11c, d). The
399 increase in VA in the diet increased up to four and eight times the incidence of juveniles with
400 deformed epurals, hypurals, and parahypurals, respectively. The incidence of deformities in the
401 uroneural was lower than in the other caudal fin complex bonny pieces, affecting less than 5%
402 of fish (Fig. 11e), although no statistically significant differences were detected in the frequency
403 of specimens with deformed uroneural between different treatments due to the large variability
404 observed amongst replicates and experimental groups (ANOVA, $P > 0.05$).
405 Other minor skeletal deformities were also detected in gilthead sea bream juveniles,
406 such as supranumerary predorsal fin rays (<30%), and dorsal and ventral fin ray fusion (<13%),
407 although no statistical significant differences between experimental groups were detected in
408 the frequency of specimens with such skeletal anomalies due to the large variability observed
409 between dietary treatments and replicates. Due to their minor effect on the external
410 appearance and fish quality, data on these deformities was deliberately not included.
411

Discussion

Generally, marine fish larvae hatch much earlier in their development than other vertebrates, suggesting that the spatiotemporal sequences of the skeletal development in teleosts are quite different from those of higher vertebrates (Haga et al., 2002a). In gilthead sea bream larvae, these developmental processes still continue after hatching, and this particularity facilitates studies of the effects of nutrition on morphogenesis. In particular, several authors have described the morphogenesis and osteogenesis processes in gilthead sea bream (Faustino and Power, 1998, 1999, 2001; Koumoundouros et al., 1997a, b; 2002), while others have focused their objectives in describing and quantifying the typology and incidence of skeletal deformities in this species under different rearing conditions (Koumoundouros et al., 1997b, 2002; Chatain, 1994a; Andrades et al., 1996; Boglione et al., 2001). However, none of these studies have evaluated the effect of the diet on skeletogenesis and appearance of skeletal deformities during early ontogeny. In this sense, it has been recently demonstrated that the morphogenesis of marine fish larvae could be perturbed by inappropriate dietary levels of different nutrients (Takeuchi et al., 1998; Haga et al., 2002a, b; Villeneuve et al., 2005a, b; Hernandez et al., 2006; Tarui et al., 2006). Thus, in the present study, we aimed to evaluate the effects of different dietary levels of VA on the incidence of skeletal deformities and larval performance in gilthead sea bream fed rotifers enriched with graded levels of this morphogenetic nutrient.

The feeding protocol used in the present study makes difficult to perform accurate nutritional studies, because of the variability of the nutrient content in live prey (Giménez et al., 2007). However, the use of a balanced compound diet for this kind of study, as it has been previously used in European sea bass (Villeneuve et al., 2005a, 2006), was discarded, since a compound microdiet is not completely developed for first feeding gilthead sea bream. Lipid content in rotifers after enrichment with graded levels of total VA was similar in all treatments. This result indicates that the differences observed in total VA content in the live prey were only

due to the levels of retinyl palmitate and retinol incorporated into the experimental emulsions and cannot be related to the emulsion preparation and/or the enrichment conditions. Under the present experimental conditions, total VA levels in rotifers increased proportionally to the content of retinyl palmitate in the enriching emulsion. In agreement with Takeuchi et al. (1998) and Giménez et al. (2007), the increase in retinol and retinoic acid, this last form of retinoid not present in the enriching emulsion, indicated that rotifers were able to absorb, digest and metabolize the retinyl palmitate contained in the enriching emulsion. Although retinoic acid is the most active form of VA (Ross et al., 2000), the similar concentration of this retinoid in all batches of enriched rotifers with experimental emulsions suggested that the observed effects of VA on gilthead sea bream larval development were not due to its content in rotifers, rather than the accumulation and transformation of different forms of VA in larvae.

At the end of the present experiment, , the different levels of dietary VA used during the rotifer-feeding phase (4-18 dph) significantly affected gilthead sea bream larval growth and survival, indicating that early larval nutrition exerted a strong effect on the further larval performance. Larvae that showed the highest growth in length and dry weight, and survival were those fed rotifers enriched with R450 and R900 emulsions, while higher dietary doses of VA dramatically reduced larval performance. Similar results have been reported in other fish species, such as Japanese flounder (Dedi et al*.* 1995; Takeuchi et al., 1995, 1998; Haga et al. 2003), European sea bass (Villeneuve et al., 2005a), red sea bream (Hernández et al., 2006) and Atlantic salmon (Ørnsrud et al., 2002) where high dietary doses of VA led to a lower growth and survival. However, the results from the above-mentioned studies are not directly comparable due to different experimental dietary levels of VA, feeding protocols and diets (live prey and inert diets). Nevertheless, survival and growth results observed in gilthead sea bream larvae fed the control diet were similar to those obtained in commercial hatcheries (Tandler et al*.,* 1995; Başaran et al., 2004), ensuring that valid physiological and nutritional observations could be drawn from this study.

Correct maturation of the larval digestive system allows larvae digesting and assimilating the ingested diet, incorporating the needed amount of nutrient required for normal growth and harmonious development. Pancreatic and brush border intestinal enzyme activities have been widely used in nutritional studies as markers of larval fish development (Zambonino-Infante and Cahu, 2001). In this study, low trypsin specific activity at 18 dph in gilthead sea bream larvae fed high levels of VA, might be indicative of a delay in the maturational process of the exocrine pancreas, as Villeneuve et al. (2005a) already reported in European sea bass fed inert diets containing different levels of dietary VA. However, dietary effects of VA administrated during the rotifer feeding phase (4-18 dph) were not evident after larval weaning at 60 dph, which might indicate that larvae fed high levels of VA were able to recover the normal digestive status regarding the pancreatic enzymes, once the excess of VA was eliminated from their diet (*Artemia* feeding and inert diet phases). In contrast to the results reported by Villeneuve et al. (2006) with European sea bass fed high levels of VA, the specific activity of amylase in gilthead sea bream was not affected by the dietary levels of VA, although numeric values tended to decrease with dietary doses of VA, but they were not significant due to large variability between replicates and experimental treatments.

The specific activity of brush border intestinal enzymes was also affected by the dietary dose of VA. Alkaline phosphatase is considered to serve as a marker for the maturation of the brush border of enterocytes: the greater its activity, the better the level of intestinal maturation (Zambonino-Infante and Cahu, 2001). However, high recorded activities of this brush border enzyme at 18 dph in fish fed the highest dose of VA (R4500) in comparison to the rest of dietary treatments might be attributed to an increase in cell proliferation (hyperplasia) induced by dietary VA (Reifen et al., 1998; Uni et al., 2000), rather than a more advanced stage of maturation of the intestinal mucosa, as aminopeptidase N specific activities indicated (three times lower than in the other groups). At the end of the study, the low brush border enzyme activity (alkaline phosphatase and aminopeptidase N) found in larvae from R2250 and R4500 groups, indicated dietary VA interfered with the normal development of the intestinal mucosa

and consequently, this might have impaired normal larval growth and further development. It has been reported in different vertebrate species that VA influences enterocyte proliferation and maturation, and decreases brush border enzyme-specific activity (Reifen et al., 1998; Uni et al., 2000; Villeneuve et al., 2005a).

Dietary VA also affected the normal process of bone formation and skeletogenesis in gilthead sea bream. The skeletal structures most affected by high levels of dietary VA were those from the cranial skeleton (splanchnocranium), vertebral centrums and caudal fin complex. Many authors have reported that the operculum complex, premaxilla, maxilla and dentary bones were the cranial structures mostly affected by skeletal deformities (Barahona-Fernandes, 1982; Chatain, 1994b; Andrades et al., 1996; Francescon et al., 1988; Boglione et al., 2001; Faustino and Power, 2001; Villeneuve et al., 2005). In this study, the premaxilla, maxilla and dentary bones were the cranial structures affected by dietary VA levels, resulting in a large incidence of animals with compressed snout. This kind of deformity is quite common in gilhead sea bream intensive larval rearing conditions (Andrades et al., 1996; Loy et al., 1999; Boglione et al., 2001) and it has been also reported in other finfish species (Barahona-Fernandes, 1982; Haga et al., 2003; Villeneuve et al., 2005a, 2006). The high incidence of this kind of deformity under current experimental conditions might be linked to the ontogeny of the splanchnocranium formation in gilthead sea bream, since the maxillar, premaxillar and dentary are some of the first skeletal structures to appear and ossify due to their important functional roles (Faustino and Power, 2001), although the frequency of the detection might be influenced by the fatal nature of these kinds of deformities (Barahona-Fernandes, 1982).

Under intensive rearing conditions, opercular abnormalities in gilthead sea bream can affect up to 80% of the population, seriously compromising both fish morphology and biological performance (Koumoundouros et al., 1997b). Considering that defects in the opercular complex are frequent and have been reported in many different fish species (Barahona-Fernandes, 1982; Beraldo et al., 2003; Fraser and Nys, 2005), this structure seems to be fundamentally fragile and easily alterable during early development stages (Beraldo et al.,

519 2003). In the present study, opercular abnormalities in gilthead sea bream fed the control diet
520 were 15.0% and close to values reported by Galeotti et al. (2000). However, the level of dietary
521 VA affected the incidence of abnormal opercula, since the incidence of reduced opercula
522 increased with the levels of VA in enriched rotifers. Earlier studies on opercular deformities in
523 gilthead sea bream concluded that unilateral deformation was side-independent and hence it
524 was the result of a fluctuating asymmetry model (Koumoundouros et al., 1997b; Galeotti et al.,
525 2000; Beraldo et al., 2003). Surprisingly, under the present experimental conditions a high
526 frequency of reduced opercula was detected in the left side of the head. These results are in
527 agreement with those reported by Verhaegen et al. (2007) and suggested a directional
528 asymmetry model. Fluctuating asymmetry is believed to be a consequence of environmental
529 factors that have an effect on developmental instability during the early life stages (Barahona-
530 Fernandes, 1982; Koumoundouros et al., 1997b), while directional asymmetry is believed to be
531 an inherited factor. As Verhaegen et al. (2007) reported, the existing literature on the genetic
532 influence on the asymmetric development of opercular deformities is contradictory depending
533 on the species and study. Although a basic assumption of asymmetry research is that left and
534 right side experience identical environmental factors, this may not be the case under the
535 present rearing conditions where the hydrodynamics of the experimental tanks with a central
536 outlet might have caused a different opercular development on both sides of the body.
537 However, the aetiology of this type of skeletal deformity remains unclear and further studies
538 have to be conducted to elucidate if the cause is due to environment, to genetics or both.
539 Under the present experimental conditions, the levels of dietary VA affected the normal
540 skeletogenesis process of the vertebral column and the number of vertebrae. Other authors
541 have previously reported that the number of vertebrae in fish can be influenced by factors other
542 than nutrition, such as triploidy in trout (Kacem et al., 2004), or temperature in halibut (Lewis et
543 al., 2004). In gilthead sea bream, the mean number of vertebrae is twenty-four, although there
544 is some discrepancy in the literature about the frequency of individuals with one vertebra more
545 or less. In this sense, Boglione et al. (2001) found 75% of fish with twenty-three and twenty-five

546 vertebrae, while the prevalence of fish with twenty-five vertebrae was only 5% according to
547 Faustino and Power (2001). Under the present experimental conditions, larvae fed the control
548 diet showed most part of fish with twenty-four vertebrae (75%) and a low incidence of vertebral
549 deformities. Differences between these data might be due to other factors than nutritional
550 conditions, and could be related to different rearing conditions (*e.g.* extensive and intensive
551 rearing systems) and origin of larvae (*e.g.* egg quality and/or broodstock diet). However, the
552 level of dietary VA had a marked effect on the normal process of morphogenesis of the
553 vertebral column, since higher levels of VA than those from the control diet resulted in a higher
554 incidence of individuals with supranumerary vertebrae and a higher incidence of vertebral
555 deformities (compression and/or fusion of vertebral centrums). The morphogenetic effects of
556 VA on the normal development of the vertebral column have also been reported in Japanese
557 flounder (Haga et al*.,* 2002a), European sea bass (Villeneuve et al., 2006) and red sea bream
558 Hernández et al*.* (2006). High levels of dietary VA in gilthead sea bream were responsible for a
559 higher incidence of specimens with a supranumerary vertebra in the caudal region of the
560 vertebral column, while in European sea bass larvae resulted in a loss of one vertebra. As
561 vertebrae from the caudal region are the last to ossify in gilthead sea bream (Faustino and
562 Power, 1998), an excess of dietary VA might have accelerated the normal differentiation
563 pattern of vertebral centrums and their osteogenesis, resulting in one or two supranumerary
564 vertebrae. Our hypothesis is supported by recent results (Mazurais et al., 2008) showing that
565 the dietary level of vitamins positively influence osteogenesis differentiation. These differences
566 in the effect of high levels of dietary VA in the number of vertebral centrums between both
567 species might be due to differences in the timing of notochord segmentation and vertebral
568 centrums formation, although further studies considering the expression pattern of genes
569 involved in larval morphogenesis and skeletogenesis are needed for comparing results from
570 both studies.

571 Body shape was also affected by the level of dietary VA, increasing the incidence of
572 specimens with lordosis, kyphosis and/or scoliosis with the dose of VA in the diet, being the

573 prehaemal and caudal vertebrae the most affected regions of the vertebral column with this
574 kind of abnormalities. These results were similar to those reported in European sea bass
575 larvae, where the authors found a statistically significant linear correlation between the dietary
576 level of VA and the frequency of animals with deformities in their vertebral column (Villenueve
577 et al., 2005a). These kinds of deformities affecting the normal development of the vertebral
578 column have been described to affect larval performance and survival (Faustino and Power,
579 2001; Koumoundouros et al., 2002); since they have been reported in specimens presenting a
580 smaller body size in comparison to non-affected fish (Koumoundouros et al., 2002). This kind
581 of skeletal deformities can also be induced by unfavourable rearing conditions, such as tank
582 hydrodynamics (Divanach et al., 1997), water temperature (Sfakianakis et al., 2006) or non-
583 inflation of the swimbladder (Chatain, 1994a). However, the absence of lordotic or kyphotic fish
584 from the control group indicated that the experimental rearing conditions were optimal for the
585 proper development of the skeleton, and consequently, skeletal abnormalities might be
586 attributed to the effect of dietary VA. Our data indicate that these anomalies in body shape
587 resulted from deformities (compression and fusion) of one or two vertebral centrums, as it was
588 previously reported in Japanese flounder (Takashima, 1978; Dedi et al., 1995) and red sea
589 bream (Hernandez et al., 2006). Deformities in vertebral centrums in the haemal and caudal
590 region of the vertebral column have been found to be significantly correlated with the number of
591 vertebrae. The appearance of supernumerary vertebrae reduced the space for the normal
592 development of the other centrums, which ended compressing the vertebrae (also referred to
593 as platyspondyly). In this sense, Faustino and Power (2001) showed a clear relationship
594 between the incidence of lordotic gilthead sea bream and the frequency of specimens with
595 twenty-three vertebrae. The appearance of vertebral fusions might be attributed to a defect of
596 notochord segmentation and disruption of vertebral centrum differentiation, which might be a
597 result of a VA-induced accelerated skeletogenesis. In addition, the higher incidence of vertebral
598 deformities in the cephalic and prehaemal vertebrae (15-18%) in fish exposed to the highest
599 VA level (R4,500) in contrast to the other dietary regimes (<5%), showed that high doses of VA

accelerated the normal differentiation pattern of vertebral centrums of these regions that appear chronologically earlier (Faustino and Power, 1998).

The caudal fin complex was the most affected region of the gilthead sea bream skeleton affected by dietary treatments as seen by the high incidence of skeletal deformities in fish fed different doses of dietary VA. Deformities affected all skeletal elements composing the caudal fin, although the most affected were, in order of importance, the epurals, hypurals, parahypural, neural arch and uroneurals. The skeletal elements that compose the caudal fin (urostyle and fin elements) are formed either by endochondral or by intramembranous ossification (Gavaia et al., 2002). The first group included the parahypural, hypurals, epurals, uroneurals and the specialized neural arch; and the second group included the vertebra 23, the urostyle and the caudal fin rays. The present results indicate that independently of the ossification process type that takes place in the different skeletal elements that compose the caudal fin, it seems that the effect of dietary VA is the same, and might be more related to the intense ossification process induced by the VA that disrupted the normal and harmonious development of the above-mentioned skeletal elements. Differences in sensitivity to dietary VA amongst caudal fin skeletal elements might be due to their differential ontogenetic development, and to differences in the exposure time to VA. Thus, the skeletal structures more sensitive to dietary VA were those that differentiated earlier, such as epurals, hypurals and parahypurals, whose cartilages appeared between 4.4 - 5.1 mm Ls and were not completely ossified until 14.7 – 16.0 mm Ls (Faustino and Power 1998). In contrast, the uroneurals, which appeared at larger sizes (10.4 – 16.0 mm Ls, Faustino and Power 1998) when all the elements of the caudal complex were already ossified, were less affected by dietary treatments (<5%) due to its late ossification process (Koumoundouros et al. 1997a; Faustino and Power 1998).

Although dietary VA affected the incidence of skeletal deformities in the caudal fin complex, these abnormalities were not lethal but seriously affected the external appearance and quality of larvae and juveniles, confirming the data reported by Koumoundouros et al. (1997a). These results are in agreement with those already published from wild-caught and

627 hatchery reared gilthead sea bream (Koumoundouros et al., 1997a; Boglione et al., 2001),
628 although differences in the typology and frequencies of different skeletal deformities exist
629 amongst studies. The incidence of skeletal deformities in the caudal complex was affected by
630 the levels of dietary VA, the higher the VA dose, the higher the incidence of deformed skeletal
631 elements. Similar results have been obtained in summer flounder (Martínez et al., 2007), and
632 Japanese flounder exposed to different levels and forms of this nutrient by means of balneation
633 (Haga et al., 2002a) and dose-response dietary trials (Dedi et al., 1998; Haga et al., 2002b). In
634 contrast to Haga et al. (2002a, b), we did not find any case of partial or complete absence of
635 the caudal fin when larvae were exposed to high levels of dietary VA. Such differences
636 between different studies might be due to different levels of retinoic acid to which larvae were
637 exposed, independently to the experimental approach used (balneation or dose-response trial).
638 According to the former authors, retinoic acid would inhibit cartilage differentiation before the
639 commencement of chondrocytes formation, causing the loss of the hypural, which would result
640 in the loss of the caudal fin.

641 In conclusion, different dietary doses of VA affected gilthead sea bream larval growth,
642 survival, performance (maturation of the digestive system) and quality (incidence of skeletal
643 deformities). Higher levels of dietary VA than those included in the commercial emulsion for
644 rotifer enrichment led to different levels and typologies of skeletal deformities, indicating that
645 gilthead sea bream larvae were very sensitive to dietary levels of VA (an increase of only 1.5
646 times of total VA in enriched rotifers, significantly increased the incidence of skeletal
647 deformities). Dietary VA affected the normal process of bone formation and skeletogenesis, the
648 skeletal structures mostly affected by high amounts of dietary VA were those from the cranial
649 skeleton (splanchnocranium), vertebral centrums and caudal fin complex. An excess of dietary
650 VA also accelerated the intramembranous ossification process of vertebral centrums leading to
651 a supranumerary vertebra, and a high incidence of fused and compressed vertebrae. Further
652 studies are needed to evaluate the effect of VA on larval quality and the molecular mechanisms
653 involved in skeletogenesis. Moreover, the sensibility of the developing skeletal structures to

dietary VA levels should incline us to test lower doses of VA in live preys enrichments during early larval stages and higher doses afterwards.

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841 **Figure captions**

842 Figure 1. Final size distribution of gilthead sea bream larvae fed rotifers enriched with graded
843 levels of vitamin A.

844

845 Figure 2. Specific enzyme activity in larvae 18 dph fed with the different diets for trypsin (a) and
846 amylase (b) expressed in U mg^{-1} prot DW, and in larvae at 60 dph for trypsin in mU mg^{-1} prot
847 DW (c) and for amylase in U mg^{-1} prot DW (d). Different indexed letters show significant
848 differences between treatments (ANOVA, $P < 0.05$).

849

850 Figure 3. Specific enzyme activity in 18 dph larvae fed with the different diets measured in U
851 mg^{-1} prot DW for alkaline phosphatase (a) and aminopeptidase N (b), and in larvae at 60 dph
852 (c) and (d), respectively. Different indexed letters show significant differences between
853 treatments (ANOVA, $P < 0.05$).

854

855 Figure 4. Degree of ossification (Stages I-VI) of larvae fed different levels of VA-enriched
856 rotifers. The description of each stage of ossification is presented in the text.

857

858 Figure 5. Different types of mandibular deformities. Fish with normal developed upper and
859 lower jaws (a), fish with lower jaw (dentary) deformed (b), fish with the upper jaw (premaxillar)
860 deformed (c). Abbreviations: An, angular; De, dentary; Fr, frontal; Mx, maxillar; Nas, nasal; Pm,
861 premaxillar; Ps, parasphenoid; Sc, sclerotic.

862

863 Figure 6. Cranial deformities in gilthead sea bream fed different levels of VA. Frequencies of
864 fish with mandibular (a, b) and opercular complex (c, d) skeletal deformities. Different letters
865 show significant differences between treatments (ANOVA, $P < 0.05$).

866

867 Figure 7. Frequency of gilthead sea bream larvae fed different doses of dietary VA with
868 different number of vertebrae.
869
870 Figure 8. Frequency of fishes fed different levels of VA with lordotic, kyphotic or scoliotic
871 vertebral column (a), with at least one vertebral deformity (b), fused vertebral centrums (c) and
872 compressed vertebral centrums (d). Different letters show significant differences between
873 treatments (ANOVA, $P < 0.05$).
874
875 Figure 9. Fish with severe lordosis and kyphosis on their vertebral column.
876
877 Figure 10. Incidence of vertebral deformities (vertebral compression and fusion) along the
878 vertebral axis in larvae fed rotifers enriched with R450 (a), R900 (b), R2,250 (c) and R4,500 (d)
879 emulsions.
880
881 Figure 11. Incidence of deformities in the caudal fin complex in gilthead sea bream fed graded
882 levels of VA. Percentage of specimens with at least one deformity in the caudal fin (a),
883 specialized neural arch (b), epurals (c), hypurals and parahypural (d) and uroneural (e).
884 Different indexed letters show significant differences between treatments (ANOVA, $P < 0.05$).
885
886 Figure 12. Different typologies of skeletal deformities in the caudal region of gilthead sea
887 bream fed different levels of VA. Normal caudal fin complex developed (a), caudal fin complex
888 with vertebral centra compressed (b), caudal fin complex with fussed preural centra 2 and 3 (c),
889 caudal fin complex with fussed hypurals and straight urostyle (d) and caudal fin complex
890 severe deformed with several vertebral centrum deformities (e). Abbreviations: Ep, epural;
891 Haem, haemal spine; Hyp, hypural; Na, specialized neural arch; Neur, neural spine; Un,
892 uroneural; Phyp, parhypurapophyses; PU_2, PU_3, preural centra 2 and 3; Vert, vertebral
893 centrum.

894

895

Table 1

Table 1. Total lipid and retinoid content (retinyl palmitate, retinol and total VA) in experimental live prey enriching emulsions. Total lipid content is expressed as % DW and retinoid content is expressed as ng mg^{-1} DW. Different letters within the same column show statistical significant differences between emulsions (ANOVA, $P < 0.05$).

Emulsion	Total lipids	Retinyl palmitate	Retinol	Total VA
R450	86.8±11.9	1690.9±295.91^{d}	6.6±1.05^{b}	1698.3±297.13^{d}
R900	94.8±5.73	3219.6±386.14^{c}	5.6±1.48^{b}	3226.3±383.95^{c}
R2,250	95.5±1.92	7973.2±768.84^{b}	6.8±0.25^{b}	7980.8±768.05^{b}
R4,500	96.2±2.90	16931.6±44.09^{a}	11.4±1.23^{a}	16946.7±443.18^{a}

Table 2

Table 2. Total lipid and retinoid content (retinyl palmitate, retinol, retinoic acid and total VA) in rotifers enriched graded levels of retinol palmitate. Total lipid content is expressed as % DW and retinoid content is expressed as ng mg^{-1} DW. Different letters within the same column show statistical significant differences between dietary groups (ANOVA, $P < 0.05$).

Emulsion	Total lipids	Retinyl palmitate	Retinol	Retinoic acid	Total VA
R450	8.3±0.78	66.7±12.20 ^{c}	7.6±0.84 ^{c}	1.0±0.17	75.4±38.72 ^{c}
R900	7.8±0.66	100.3±17.21 ^{b,c}	8.2±0.60 ^{c}	0.8±0.29	109.2±18.10 ^{c}
R2,250	7.1±0.59	139.1±0.90 ^{b}	49.8±7.72 ^{b}	1.1±0.28	187.6±9.95 ^{b}
R4,500	8.7±1.43	681.9±65.54 ^{a}	68.1±7.72 ^{a}	0.9±0.28	723.3±26.21 ^{a}

Table 3

Table 3. Larval size in standard length (Ls) and dry weight (DW), and survival rate of gilthead seabream larvae fed different levels of vitamin A. Values are mean ± standard deviation. Different letters within the same column show statistical significant differences.

	Ls (mm)		DW (mg)		
	18dph	**60dph**	**18dph**	**60dph**	**Survival (%)**
R450	5.33±0.48 [a]	16.90±2.82[a]	0.087±0.028 [a]	74.61±8.84[a]	9.1±1.9 [a]
R900	4.94±0.06[bc]	17.23±2.36[a]	0.094±0.017 [a]	69.31±10.24[ab]	8.0±0.2 [a]
R2,250	4.66±0.75 [c]	16.40±2.48[ab]	0.097±0.043 [a]	65.61±8.18[b]	2.9±0.3 [b]
R4,500	5.11±0.84 [ab]	15.66±2.03[b]	0.115±0.027 [a]	56.86±11.27[c]	3.3±0.5 [b]

Figure 1

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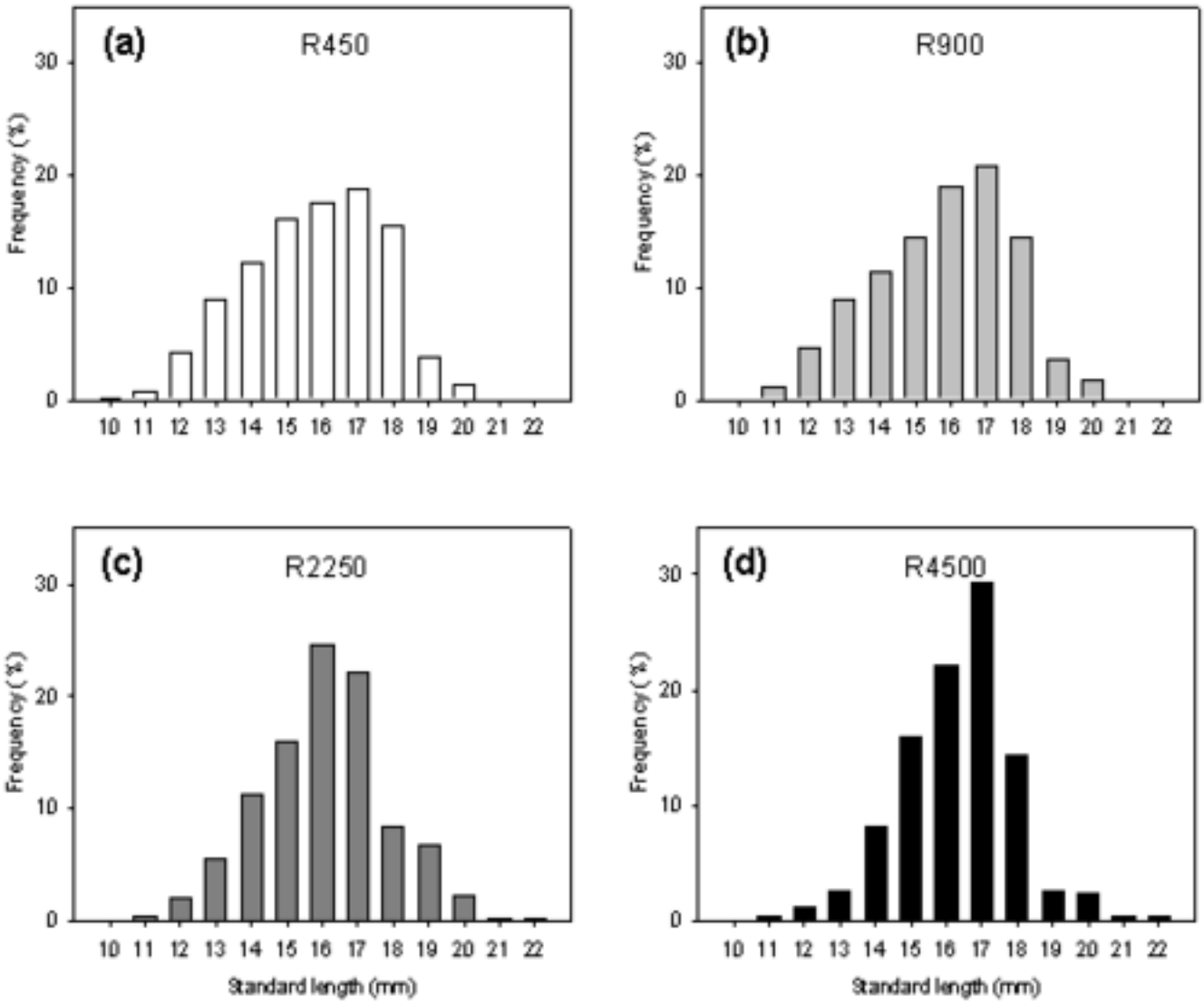

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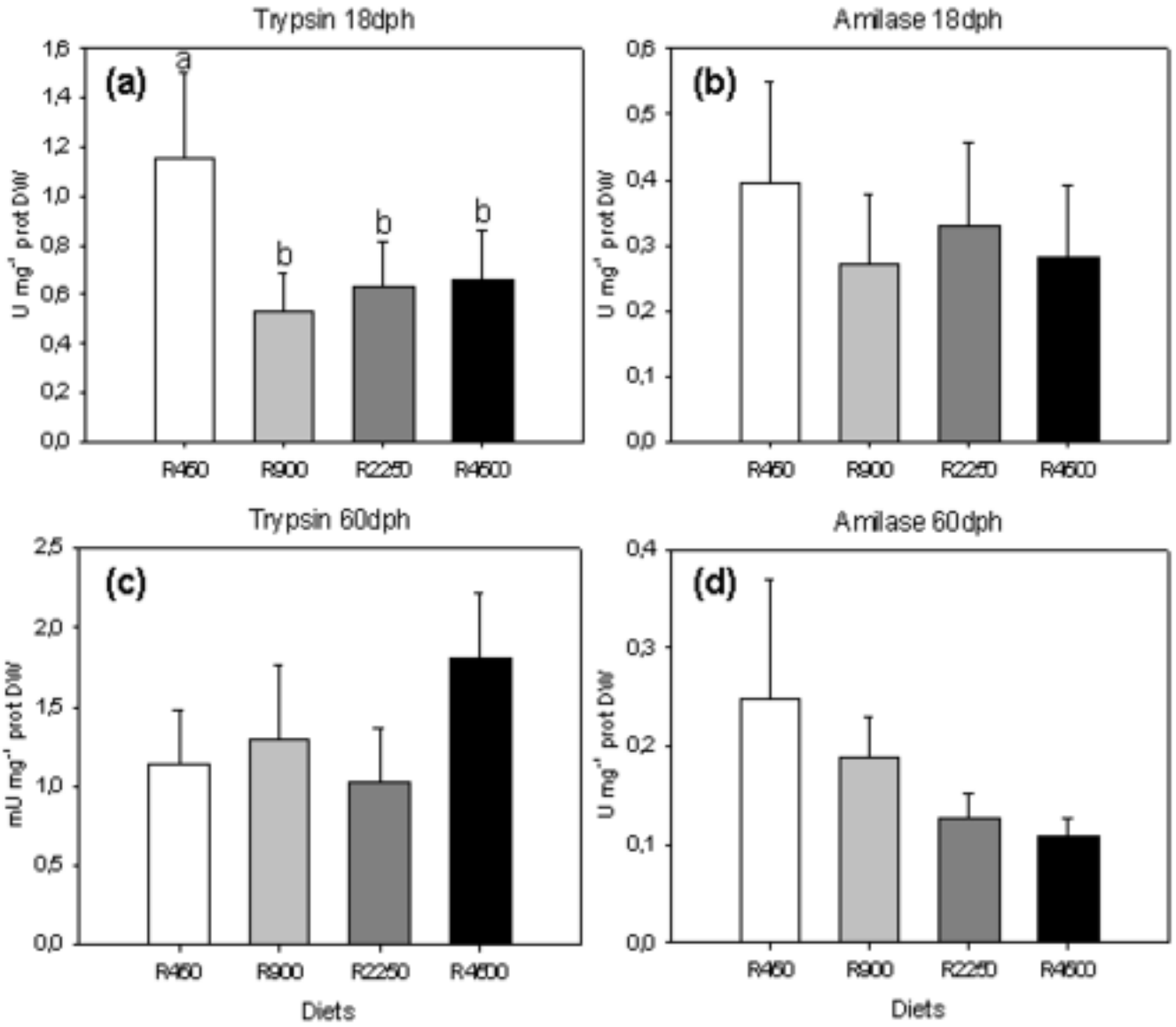

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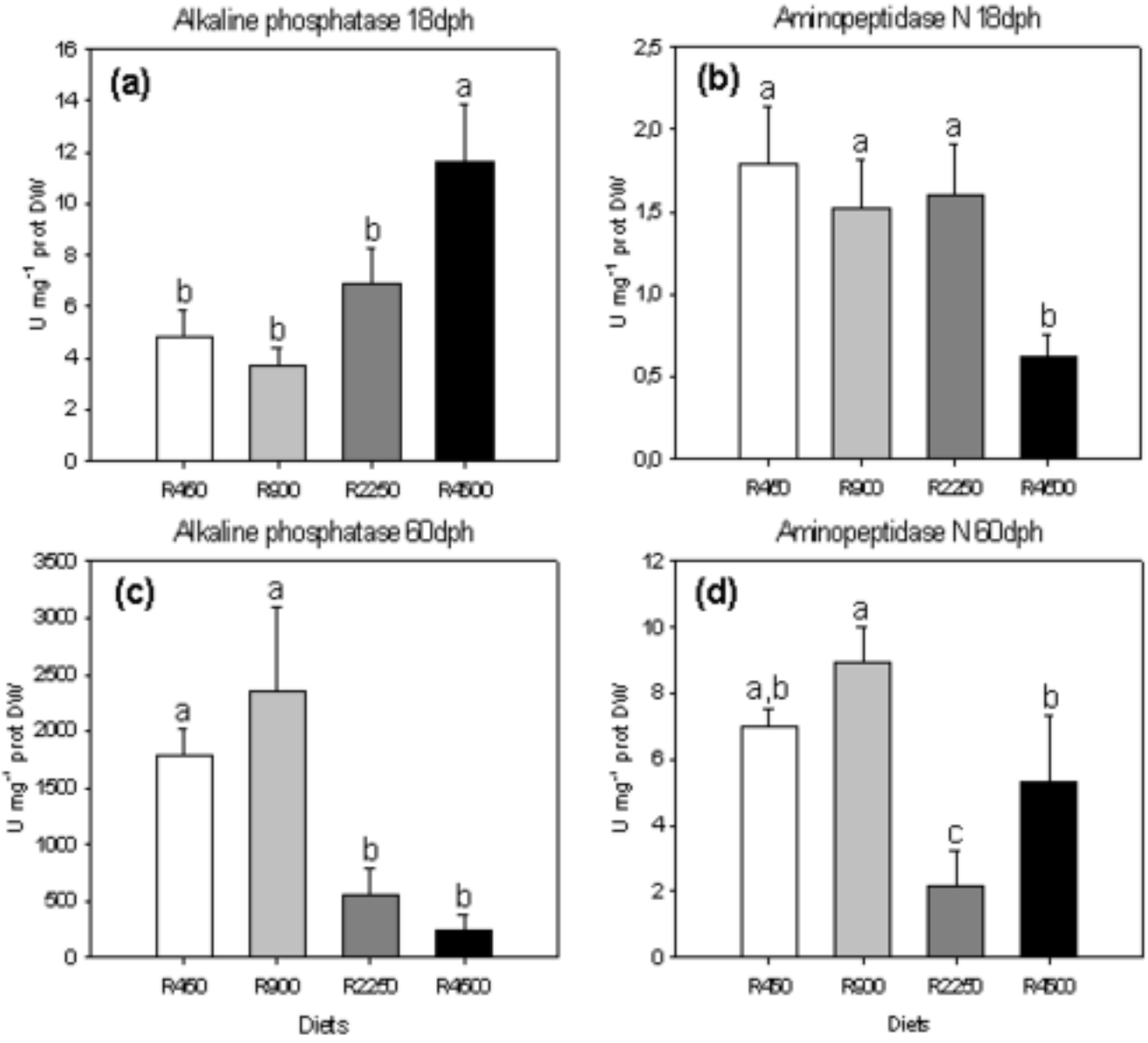

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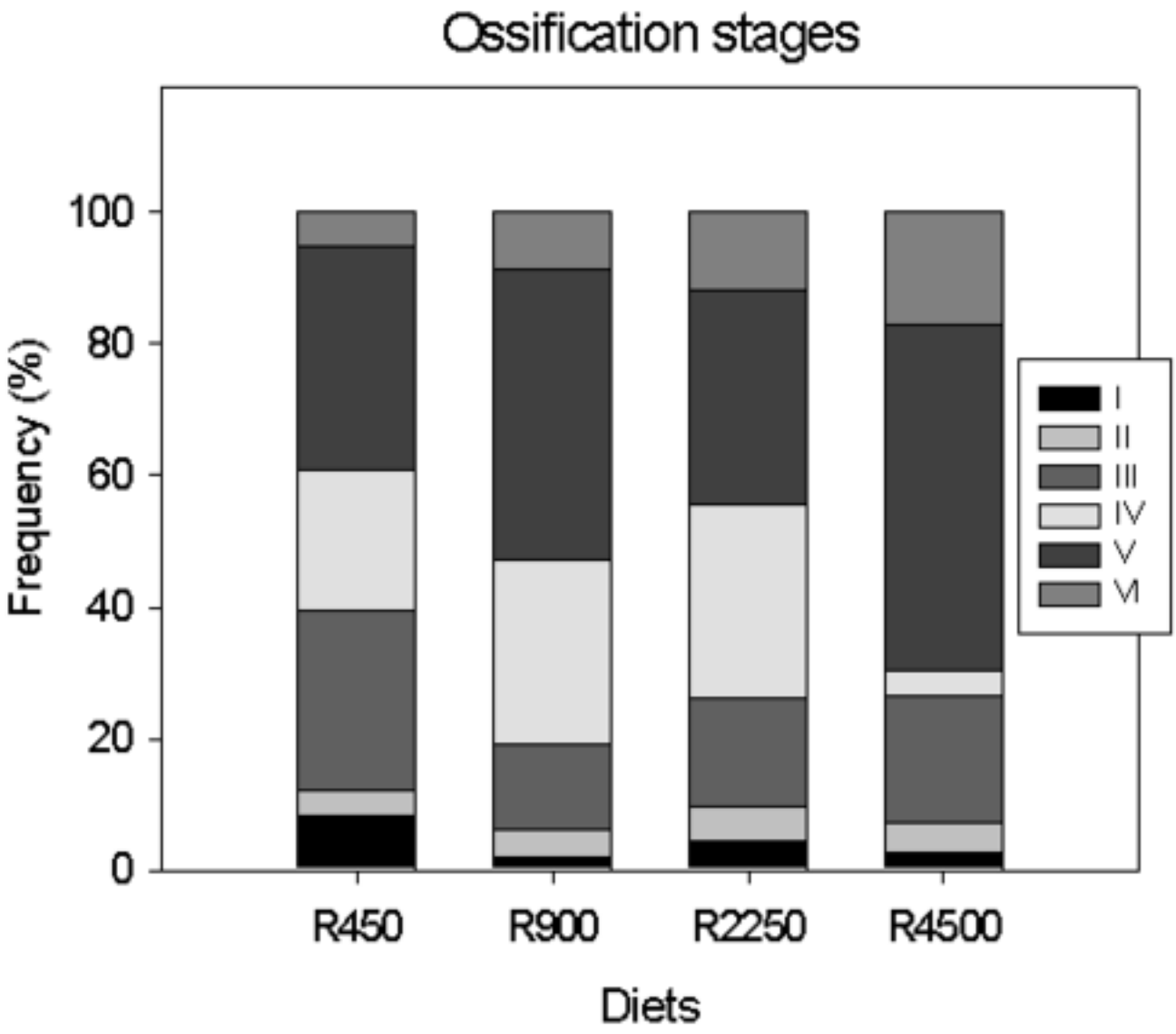

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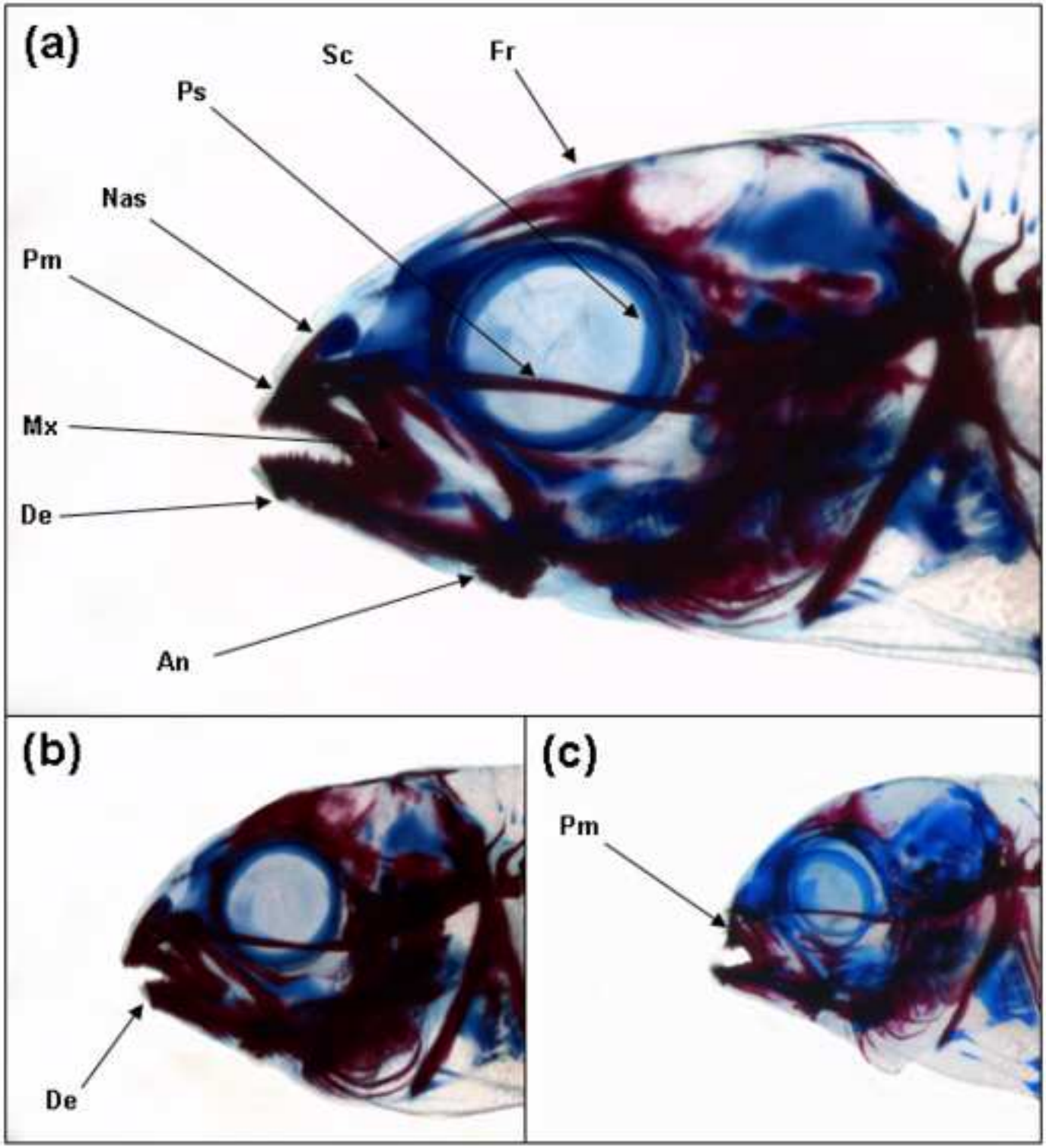

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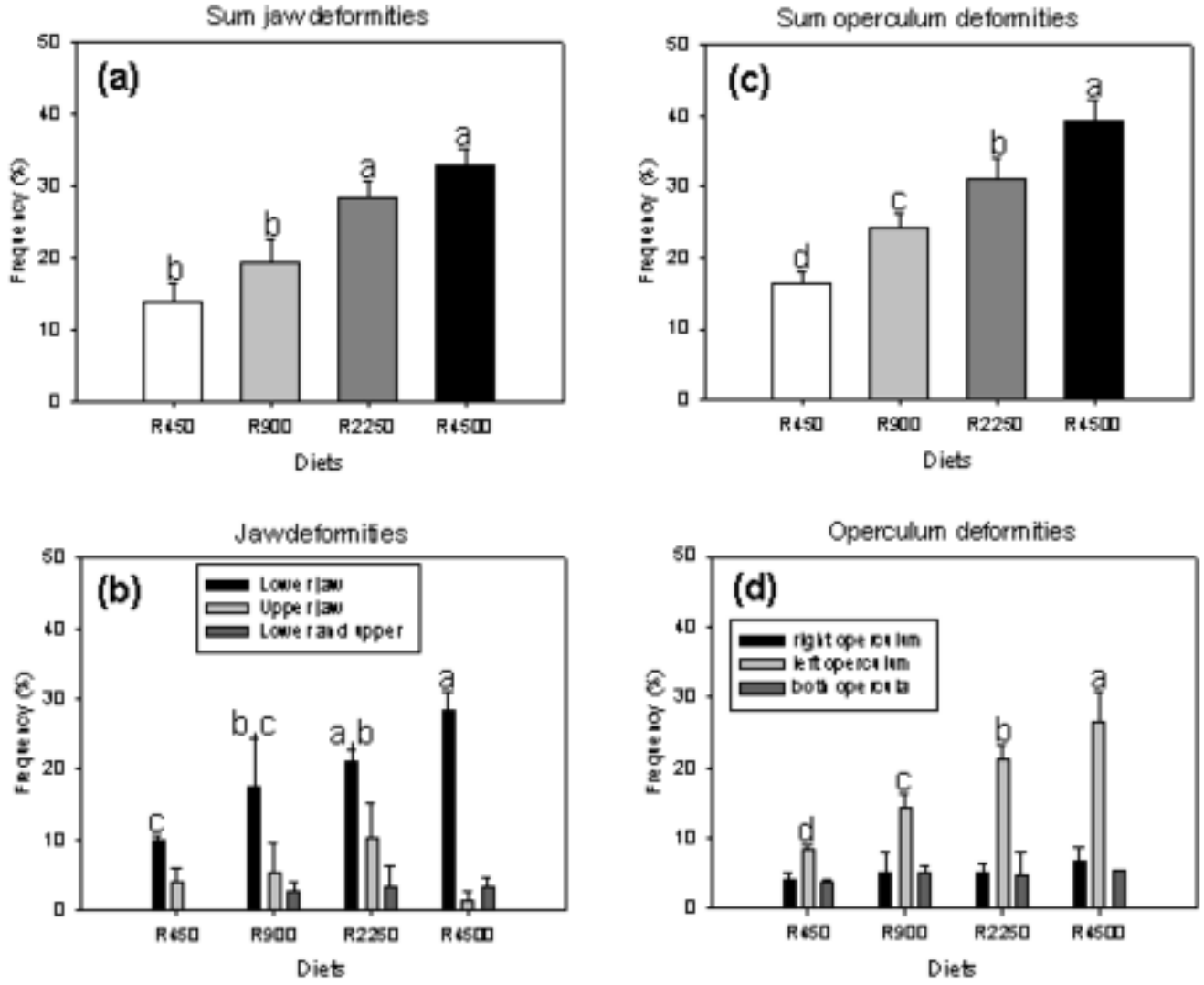

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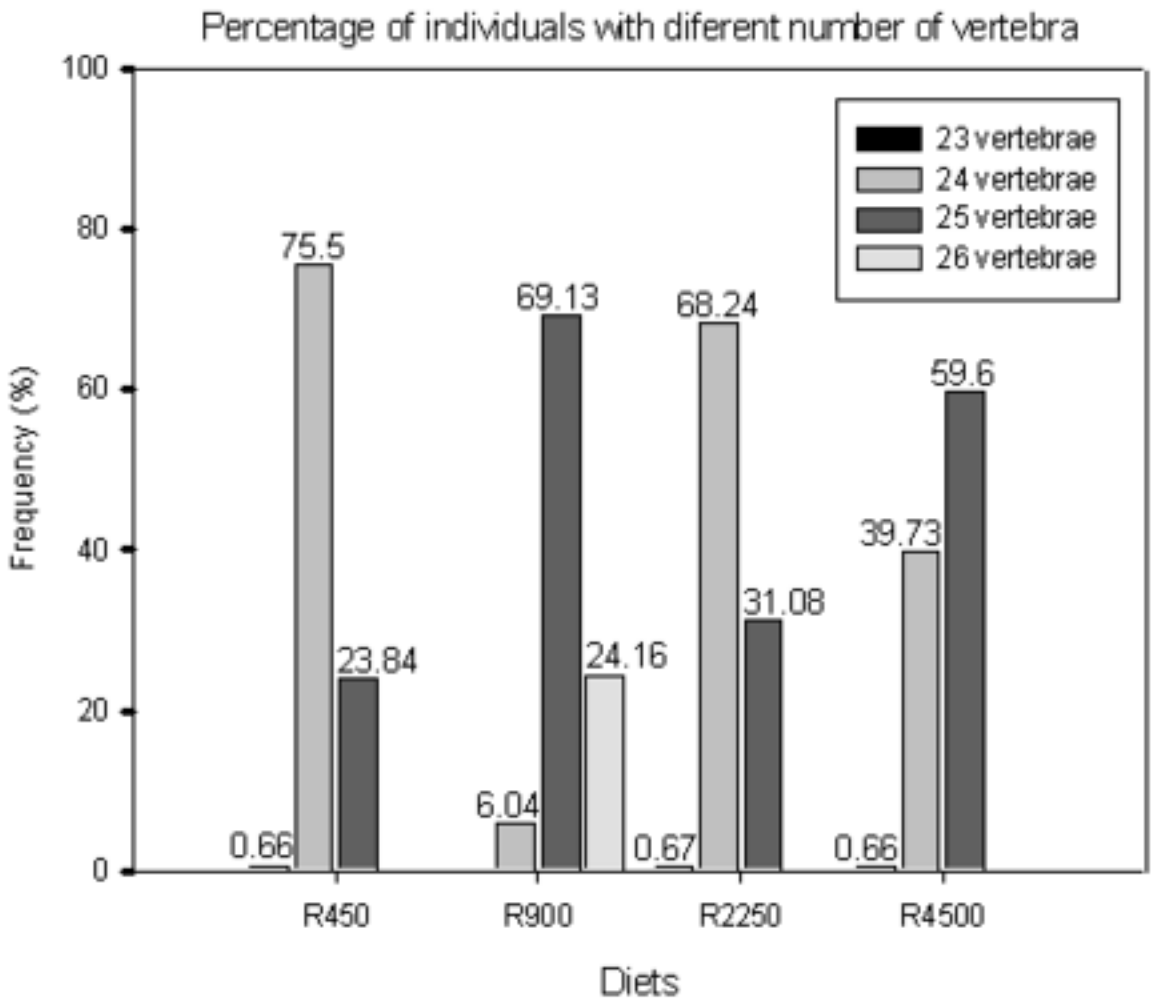

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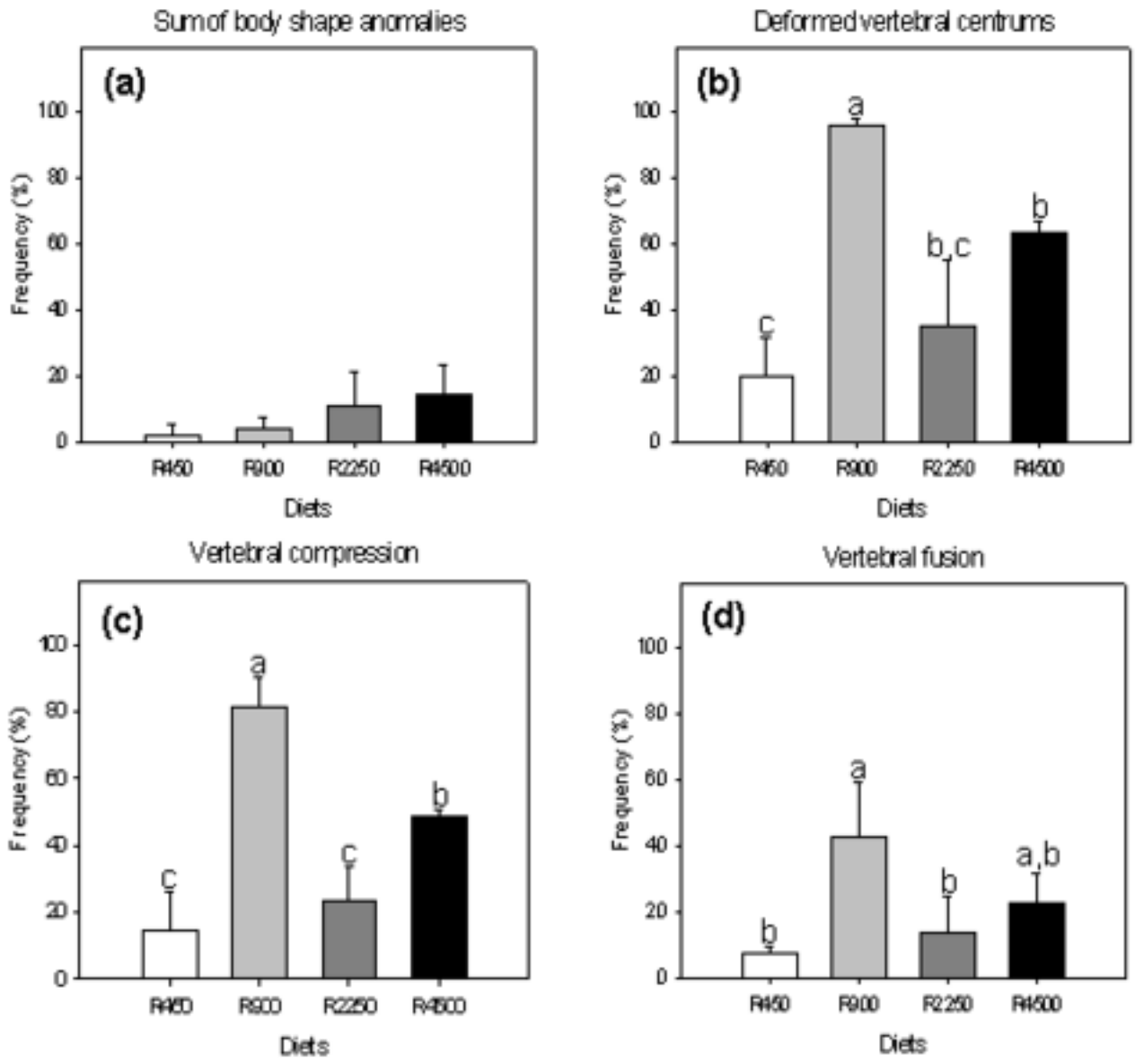

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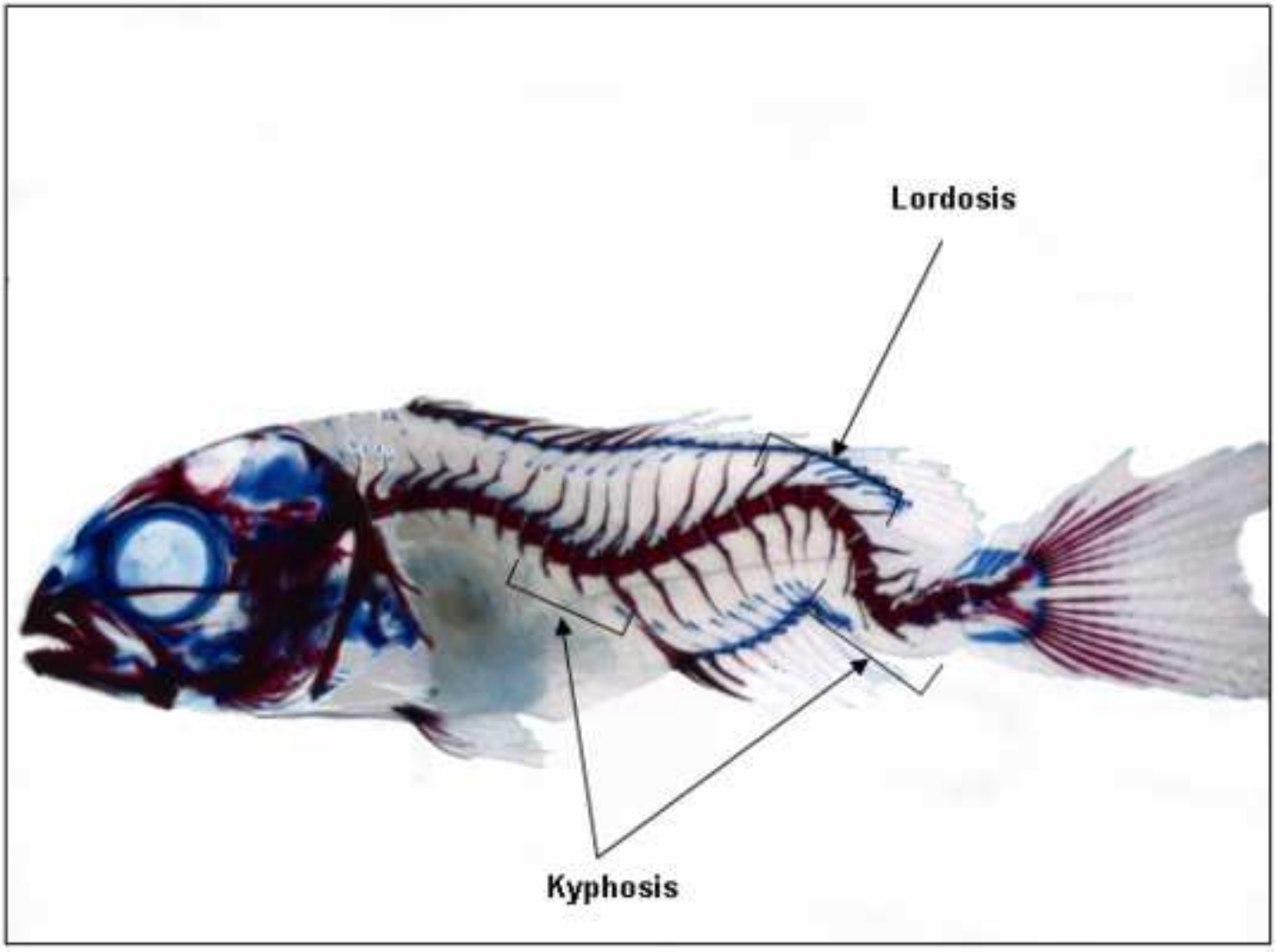

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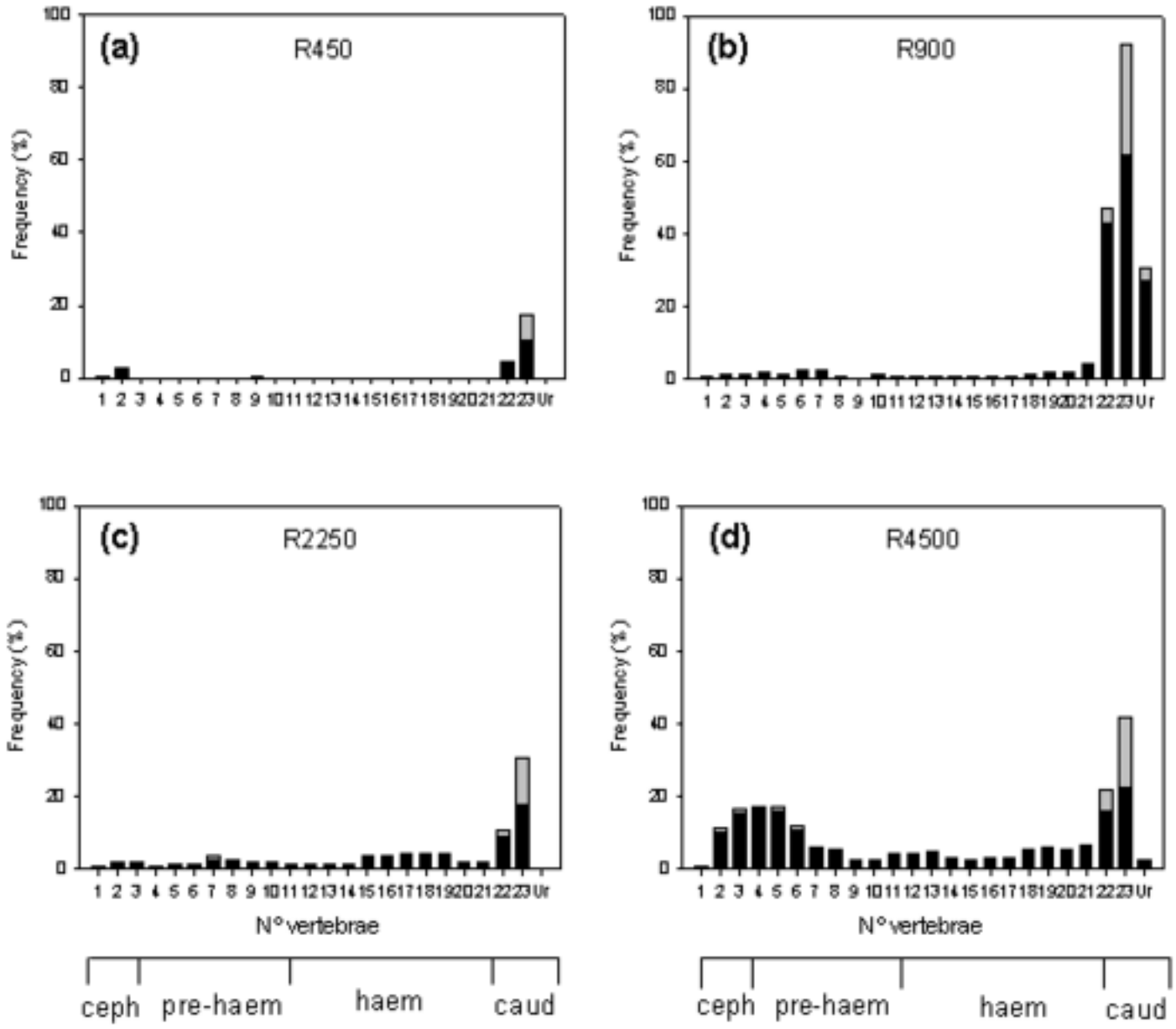

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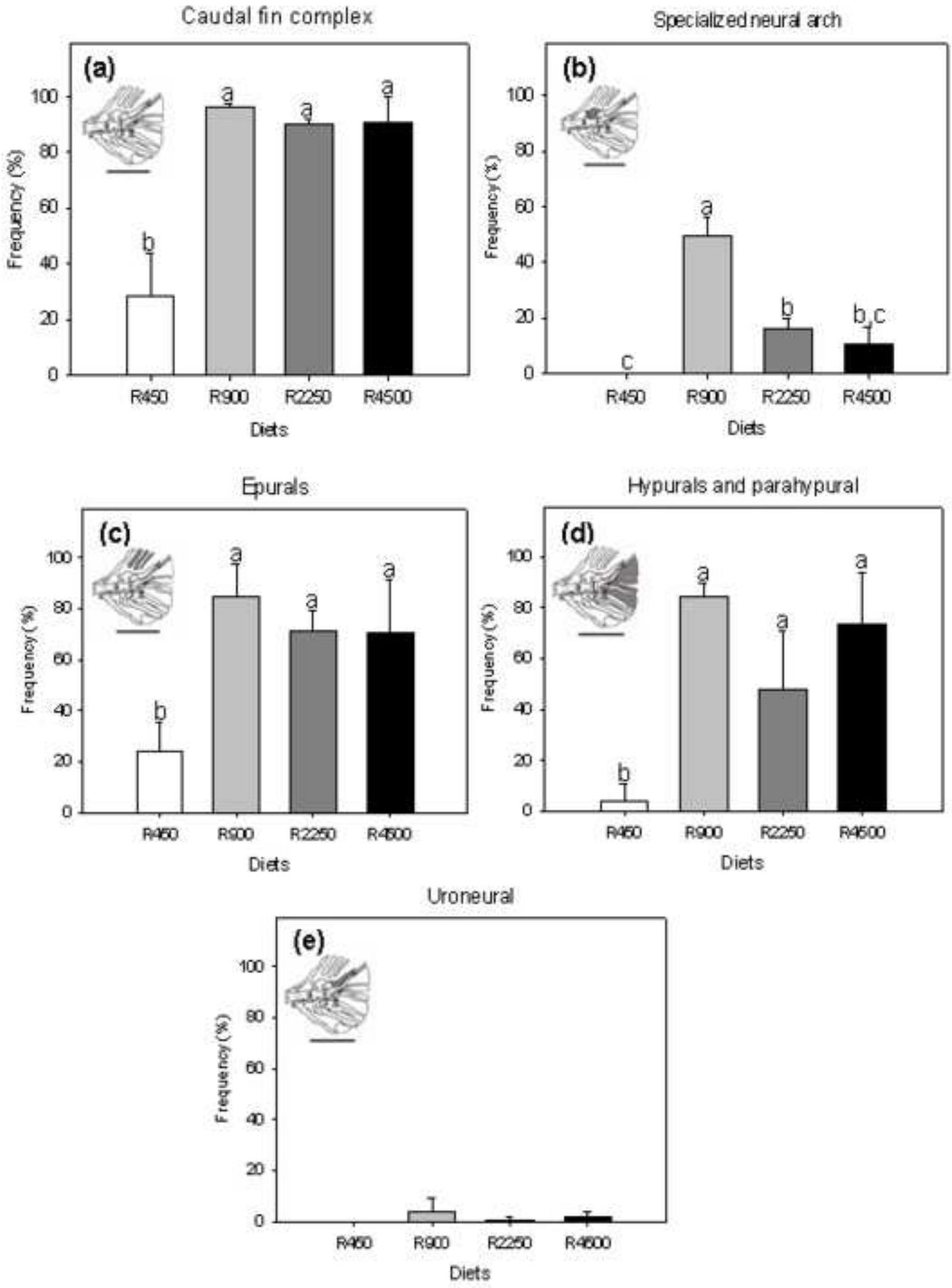

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