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).

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94   Among them, larval nutrition at first feeding is one of the key parameters that affect

95   skeletogenesis during early development. In this sense, several studies have demonstrated

96   that nutrients are responsible for the appearance of skeletal deformities when their level and/or

97   form of supply in the diet are inappropriate or unbalanced (Cahu et al. 2003, Lall and Lewis,

98   2007). The solution of the problem is strongly related to the understanding of the species- and

99   stage-specific environmental preferences and nutritional requirements of the fish larvae, as well

100   as to the ontogeny of the skeletogenesis and anatomy of each deformity type.

101   The effects of nutrition on bone development and remodelling have been deeply studied

102   in terrestrial vertebrates, whereas this information in fish is fragmentary depending on the

103   nutrient considered (Cahu et al., 2003; Lall and Lewis, 2007). In this sense, recent advances in

104   the composition of starter diets for marine finfish larvae and enriching emulsions for live preys

105   have identified several nutrients, particularly minerals, vitamins and lipids that can be critical for

106   normal skeletogenesis. Vitamin A (VA), a morphogenetic nutrient, includes all compounds that

107   posses the same biological activity of retinol, playing a key role in morphogenesis, cellular

108   differentiation and proliferation processes. VA determines normal growth, body patterning,

109   nervous system development, and differentiation of pigment cells, limbs and skeleton along

110   vertebrate early development (Ross et al., 2000). The fish are not able to synthesize VA, thus

111   they have to take it from diet and any excess or deficiency of this nutrient in the diet resulted in

112   abnormal growth and development (Dedi et al., 1995; Tarui et al., 2006; Takeuchi et al., 1998;

113   Haga et al., 2002 a, b; Villeneuve et al., 2005). The impact of dietary VA on fish larvae

114   development will depend on both the dietary dose and the developmental status of the larvae

115   at first feeding. It is then necessary to fine-tune this particular relationship for each species.

116   The objective of the present study was to evaluate the effect on larval performance

117   (growth, survival and maturation of the digestive function) and quality (incidence and typology

118   of skeletal deformities) of graded levels of dietary VA on gilthead sea bream larval

119   development.

120

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

146   purposes, the emulsion containing 450 µg retinol equivalents g^{-1} (R450) was considered as the

147   control group (ES without retinol palmitate addition).

148   Live prey (rotifers and *Artemia* nauplii) were enriched according to Gimenez et al. (2007),

149   rotifers were enriched during two hours with 0.15 g of each experimental emulsion per litre, and

150   *Artemia* metanauplii for 18 h with 0.6 g ES l^{-1}. After enrichment, rotifers and *Artemia* were

151   gently siphoned from enriching tanks, collected in a mesh, and washed in freshwater to reduce

152   the bacterial load and rests of the enrichment emulsions. Live preys were introduced into the

153   rearing tanks three times per day in order to assure an optimal live prey density in the water

154   column, and their appropriate nutritional value.

155   The effects of graded levels of VA on gilthead sea bream larval performance and quality

156   was evaluated by quintuplicate (three tanks were used for regular sampling and two for final

157   survival). Larvae were sampled at 18 and 60 dph, coinciding with the end of the rotifer-feeding

158   phase and the end of the weaning period, respectively. For sampling purposes, larvae were

159   sacrificed with an overdose of anaesthetic (Tricaine methanesulfonate, MS-222, Sigma).

160

161   *2.2 Biochemical analysis*

162   Retinoids on enriching emulsions and live prey were analyzed by HPLC using a modification of

163   the method proposed by Takeuchi et al. (1998). After sampling, live prey were washed with

164   distilled water to remove marine salts and bacterial load, and samples were frozen at -80ºC

165   until posterior analysis. Lipids were extracted with a chloroform:methanol mixture (C:M, 2:1)

166   according to the Folch method (Folch et al., 1957), and stored in C:M:BHT (2:1:0.01) at 20 mg

167   l^{-1} at −20 °C until their analysis. Then, samples were evaporated and redissolved on

168   methanol:acetone (1:1 v/v) prior to their HPLC analysis. The HPLC system (Thermo Separation

169   Products, San Jose, CA, USA) was equipped with a Lichrospher C-18 reverse phase column

170   (Merck, Darmstadt, Germany) and a UV–visible detector set at a wavelength of 325 nm. The

171   mobile phase was a mixture (85:15 v/v) of 98% methanol with 0.5% ammonium acetate, and

172   chloroform. The flow rate was 1.5 ml min^{-1} and the elution time was 18 min. The concentration

173   of each retinoid was calculated from the calibration curves constructed with the peak area

174   ratios of their external standards and an internal standard of retinol acetate added to the

175   samples. All the reference retinoids were purchased to Sigma-Aldrich (Spain).

176

177   *2.3 Larval growth and survival rate*

178   Sampled larvae ($n = 15$) from each tank were washed with distilled water to avoid marine salts

179   and used for body size and dry weight determination. Larval standard length (Ls) was

180   measured with digital camera connected to a binocular microscope Nikon SMZ 800, AnalySIS

181   (Soft Imaging Systems, GmbH). Once larvae were measured in length, they were dried at 60ºC

182   until their weight was constant. Samples were weighted with an analytic microbalance Sartorius

183   BP211D. At the end of the experiment, the total length of 150 fishes from each rearing tank

184   was measured to evaluate the effects of VA on size dispersion. Survival rate was calculated as

185   the percentage of final surviving fish in relation to the initial number at the beginning of the trial.

186

187   *2.4 Maturation of the digestive system*

188   The activity of two intestinal brush border enzymes (alkaline phosphatase and aminopeptidase

189   N) and two pancreatic enzymes (trypsin and amylase) was used to assess the degree of

190   development and maturation of the digestive system of larvae fed graded levels of VA. Enzyme

191   activity was measured at 18 and 60 dph ($n = 50$ and 10 larvae per tank, respectively).

192   Sampled fish were washed with distilled water to avoid marine salts and stored at -80ºC

193   prior to enzyme activity analysis. The whole 18 dph larvae were homogenized for enzymatic

194   assays, since they were too small to dissect, while older fish were dissected to separate

195   pancreatic and intestinal segments as described by Cahu and Zambonino-Infante (1994).

196   Samples were homogenized (Ultra-Turrax T25 basic, IKA© - Werke) in five volumes (v/w) of

197   ice-cold Milli-Q water, centrifuged at 3,300 g for 3 min at 4ºC and the supernatant removed for

198   enzyme quantification. For determination of intestinal enzymes, samples were homogenized in

199 cold Mannitol 50 mM, Tris-HCl 2 mM buffer, pH 7.0. Intestinal brush border membranes were

200 purified according to the method developed for intestinal scrapping (Crane et al., 1979).

201   Trypsin (E.C. 3.4.21.4) activity was assayed according to Holm et al. (1988), at 25ºC

202 using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide) as substrate. Amylase (E.C. 3.2.1.1)

203 activity was measured according to Métais and Bieth (1968), using soluble starch (0.3%)

204 dissolved in Na_2HPO_4 buffer pH 7.4 as substrate.

205   Alkaline phosphatase (E.C. 3.1.3.1) was quantified at 37ºC using 4-nitrophenyl

206 phosphate (PNPP) as substrate (Bessey et al., 1946). Aminopeptidase N (E.C.3.4.11.2) was

207 determined at 25ºC according to Maroux et al. (1973), using sodium phosphate buffer 80 mM

208 (pH = 7.0) and L-leucine p-nitroanilide as substrate (in 0.1 mM DMSO). Enzymatic activities

209 were expressed as the specific activity, milli-units per milligram of protein (mU/mg protein), and

210 soluble protein of crude enzyme extracts was quantified by means of the Bradford's method

211 (Bradford, 1976) using bovine serum albumin as standard. All the assays were conducted in

212 triplicate.

213

214 *2.5 Skeletal deformities analysis, observations and measurements*

215 To identify and quantify the skeletal deformities on larvae from different dietary treatments, 50-

216 60 larvae per each tank were sampled at the end of the experiment, and fixed in formaldehyde

217 solution (10%) until their double staining. Then, animals were stained for bone and cartilage on

218 whole mounts using a modification of the method described by Klymkowsky and Hanken

219 (1991). In brief, specimens were rehydrated two times in distilled water during 5 minutes and

220 then placed in alcohol 95º. Specimens were stained with alcian blue solution with 80% alcohol

221 95º and 20% glacial acetic acid during 24 hours, rehydrated through a graded series of alcohol

222 (95%–25%) and macerated using a 1% aqueous solution of KOH with 3% hydrogen peroxide

223 (9:1 in volume) until skeletal elements were clearly visible. Then, specimens were placed

224 between 6 and 20 hours in an aqueous solution saturated in sodium borate containing 0.3-0.5

225 g trypsin, and stained with alizarin red S (stock solution: 1% alizarin red in 1% KOH) during 24

226   hours. Staining time was variable and depended on the size of the specimen. Finally, fish were

227   washed with distilled water, followed by a series of baths in 1% KOH to remove the excess of

228   dye in soft tissues, and placed through graded series of glycerine-KOH solutions.

229   After staining, fish were placed on their right side, in order to observe meristic

230   characters and skeletal abnormalities in the cranium, vertebral column and caudal fin complex.

231   Skeletal structures were identified and named according to Faustino and Power (1998, 1999,

232   2001). The study was focused on the mean number of vertebra and frequency of individuals

233   with abnormal number of vertebrae. Special emphasis was placed on the deformities occurring

234   in the cranial region (upper and lower jaws), vertebral column and caudal fin complex (hypurals

235   and parahypurals, epurals, uroneural, and specialized neural arch). In particular, we calculated

236   the frequency of individuals with lordosis, scoliosis or kyphosis, the total sum of deformities in

237   the vertebral column, and the incidence of vertebral compression and fusion.

238   In order to assess the degree of ossification of gilthead sea bream juveniles and

239   establish a potential relationship with the dietary regimen during the larval stages, the

240   percentage of juveniles in different stages of ossification was determined at the end of the

241   experiment. Those stages were defined according to the ossification of selected bony

242   structures that better describes the ossification process in this species (Faustino and Power,

243   1998). In brief, stage I corresponded to the early ossification of some vertebral centrums; at

244   stage II, all vertebral centrums were completely ossified; and at the stage III, dorsal fin rays

245   were completely ossified. At stage IV, caudal fin rays were ossified, and at stage V, the

246   hypurals and parahypural started to ossify. The stage VI was characterized by the complete

247   ossificatifon of most of the skeletal structures with the exception of the uroneural 2 and the

248   haemal spines 2 and 3, which completed their ossification at older ages (larger sizes).

249

250   *2.6 Statistical analysis*

251   Results are given as means and standard deviations. Data expressed as percentage (survival,

252   incidence of skeletal deformities) were previously $arcsin(x^{1/2})$-transformed. Results were

253   compared by means of One Way ANOVA (data normally distributed, Kolmogorov–Smirnov

254   test) and when significant differences were detected the Tukey multiple-comparison test was

255   used to detect differences among experimental groups (Zar, 1974). The test of Kolgomorov-

256   Smirnov was used to evaluate the distribution of fish size at the end of the study. Correlation

257   between different variables was evaluated with the Pearson Product Moment Correlation test.

258   In all statistical analyses, the level of significant difference was set at $P < 0.05$. All the statistical

259   analyses were conducted using SigmaStat 3.0 (SPSS, Richmond, USA).

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262

263   **3. Results**

264   *3.1 Retinoid content in experimental emulsions and live prey*

265   Total lipid and retinoid content in emulsions and rotifers enriched with graded levels of retinol

266   palmitate are presented in Tables 1 and 2, respectively. No statistically significant differences

267   were detected in the total lipid content of experimental emulsions and enriched rotifers with

268   different levels of VA (ANOVA, $P > 0.05$). Total vitamin A content in emulsions and enriched

269   rotifers increased with increasing levels of retinyl palmitate incorporated into the emulsion

270   (ANOVA, $P < 0.05$). Analysis of retinoid content of enriched rotifers showed that the real

271   incorporated level of total VA was 75, 109, 188 and 723 ng mg^{-1} DW for live prey enriched with

272   R450, R900, R2,250 and R4,500 experimental emulsions, respectively (Table 2). Retinyl

273   palmitate (VA ester) was the dominant form of retinoid detected in emulsions and enriched

274   rotifers, whereas retinol (VA alcohol) was also detected but at a minor concentration. Either

275   retinal (aldehyde form of vitamin A) was not detected in emulsions or rotifers enriched with

276   graded levels of vitamin A, whereas low levels of retinoic acid (0.8-1.1 ng mg^{-1} DW) were only

277   detected in enriched rotifers, although they were not significantly different amongst rotifers

278   enriched with graded levels of VA (ANOVA, $P > 0.05$).

279

280   *3.2 Larval growth and survival*

281   Table 3 contains the results of growth in length and DW of gilthead seabream larvae fed

282   different levels of VA. At 18 dph, no statistically significant differences were observed in DW in

283   larvae fed graded levels of VA during the rotifer-feeding phase, while fish fed rotifers enriched

284   with the control emulsion showed the best growth in length (ANOVA, $P < 0.05$). At the end of

285   the trial, larvae fed R450 and R900 were larger in length and DW (ANOVA, $P < 0.05$). Larvae

286   fed rotifers enriched with higher doses of total VA (R2,250 and R4,500) weighted 12 and 21%

287   less than the other groups. Similarly, the length of those fish was 3 and 7% smaller.

288   The frequency distribution of final Ls classes in experimental groups fed rotifers

289   enriched with R450, R900 and R2,250 followed a normal distribution. The Kolmogorov-

290   Smirnov test, however, revealed statistically significant differences between the final

291   distribution of Ls of fish fed rotifers enriched with the highest dose of total VA (R4,500),

292   skewing the distribution towards sizes classes comprised between 15 and 17 mm ($P < 0.05$;

293   Fig. 1).

294   Dietary levels of VA significantly affected fish larval survival (Table 3; ANOVA, $P <$

295   0.05). Final survival ranged from 2.9 to 9.1% depending on the experimental group. The

296   highest survival results were obtained in larvae fed R450 and R900, while higher dietary levels

297   of VA (R2,250 and R4,500) significantly reduce their viability.

298

299

300   *3.3 Maturation of the digestive system*

301   At the end of the rotifer-feeding period, the different VA levels in enriched rotifers significantly

302   affected the trypsin activity (ANOVA, $P < 0.05$, Fig. 2a), while no significant differences were

303   detected in amylase secretion (ANOVA, $P > 0.05$, Fig. 2b). At 18 dph, the specific trypsin in

304   larvae fed R450 was 2 times higher than in larvae from the other dietary treatments. At the end

305   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.

13

332   The presence of cranial skeletal deformities in the jaw apparatus was strongly

333   correlated to the level of retinoids in enriched rotifers ($R = 0.789$, $P = 0.002$) (Fig. 6a). The

334   premaxilla, maxilla and dentary bones were the cranial structures more affected by different

335   levels of dietary VA, resulting in large number of specimens with compressed snout

336   (pugheadness, underdevelopment of the maxilla and premaxilla, Fig 5). The incidence of jaw

337   deformities in larvae fed the highest doses of VA (R2,250 and R4,500) were significantly higher

338   than those observed in juveniles fed lower levels of VA (ANOVA, $P < 0.05$; Fig. 6a). No

339   statistical significant differences were detected regarding the incidence of jaw deformities

340   between larvae fed R450 and R900 (ANOVA, $P > 0.05$). However, fish fed R900 showed a

341   higher incidence of deformities in both jaws in the same specimen, while this type of

342   deformation was not observed in the control group (Fig, 6b).

343   The incidence of opercular deformities was significantly correlated to the dose of VA in

344   enriched rotifers ($R = 0.843$, $P = 0,001$; Fig. 6c). While the control group had 16.5 ± 1.64% of

345   the individuals with an incomplete operculum, the incidence of this kind of deformity reached

346   39.2 ± 3.05%, in those fish fed R4,500 (ANOVA; $P < 0.05$). Significant statistical differences

347   were also detected in the incidence of deformed operculum depending on the fish side

348   considered, indicating that the expression of this type of deformity was side-dependent. In all

349   experimental groups, the frequency of abnormal operculum was higher in the left than in the

350   right side, independently of the level of dietary VA (t-test, $P < 0.05$; Fig. 6d). However, no

351   statistically significant differences were detected in the frequency of individuals with bilateral or

352   right-sided opercular complex deformities (ANOVA, $P > 0.05$).

353   The number of vertebrae most frequently observed in gilthead sea bream is twenty-four.

354   At the end of the experimental period, statistically significant differences in the mean number of

355   vertebrae were detected between larvae fed R900 and the rest of dietary treatments (ANOVA,

356    $P < 0.001$; Fig. 7). Fish from this experimental group showed a higher frequency of specimens

357   with twenty-five (69.1%) and twenty-six vertebrae (24.2%). Those supranumerary vertebrae

358 were observed in the caudal region, between the urostyle and vertebra number 23, and were

359 significantly correlated to vertebral fusion and compression disorders ($R = 0.959$, $P < 0.0001$).

360   The incidence of specimens with lordotic, kyphotic or scoliotic bends in their vertebral

361 column tended to increase with increasing levels of total VA in enriched rotifers, although this

362 trend was not significant ($R = 0.564$, $P = 0.056$) due to the large variability of replicates.

363 Similarly, no significant differences could be detected between experimental groups (ANOVA,

364 $P > 0.05$; Fig. 8a, 9).

365   The frequencies of deformities in vertebral centrums were significantly affected by the

366 level of VA in the diet (Fig. 8b). In all treatments, the compression of vertebral centrums was

367 more frequent than their fusion with the adjacent ones (Fig, 8c, d). In particular, larvae fed

368 R900 showed the highest incidence of compressed (80%) and fused (40%) vertebral bodies

369 than the rest of the experimental groups (ANOVA, $P < 0.05$). The incidence of both types of

370 deformities in vertebral centrums was 65 and 33% more frequent in the above-mentioned

371 group than in larvae fed rotifers enriched with the control emulsion. Regarding the haemal and

372 neural spines of vertebrae, in the haemal region of the vertebral column, haemal spines were

373 significantly affected by the level of VA in the diet, increasing the incidence of twisted haemal

374 spines with the dose of dietary VA (ANOVA, $P < 0.05$). In contrast, no differences were

375 detected in the incidence of deformities in the neural spine (ANOVA, $P > 0.05$), although high

376 variability between replicates and dietary groups was detected.

377   The incidence of skeletal deformities along the vertebral column in gilthead sea bream

378 juveniles is shown in Figure 10. Independently of the level of VA in the diet, the caudal region

379 was the area most affected by compression and fusion of the vertebral centrums; although the

380 group fed R900 showed the highest percentage of compressed and fused vertebrae in this

381 region; mostly affecting the urostyle (30%) and vertebra number 23 (90%). Vertebrae from the

382 cephalic and prehaemal regions were also affected by the level of VA in the diet, thus juveniles

383 fed rotifers containing high levels of VA during the rotifer-feeding phase showed a higher

384 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

412 **Discussion**

413

414 Generally, marine fish larvae hatch much earlier in their development than other vertebrates,

415 suggesting that the spatiotemporal sequences of the skeletal development in teleosts are quite

416 different from those of higher vertebrates (Haga et al., 2002a). In gilthead sea bream larvae,

417 these developmental processes still continue after hatching, and this particularity facilitates

418 studies of the effects of nutrition on morphogenesis. In particular, several authors have

419 described the morphogenesis and osteogenesis processes in gilthead sea bream (Faustino

420 and Power, 1998, 1999, 2001; Koumoundouros et al., 1997a, b; 2002), while others have

421 focused their objectives in describing and quantifying the typology and incidence of skeletal

422 deformities in this species under different rearing conditions (Koumoundouros et al., 1997b,

423 2002; Chatain, 1994a; Andrades et al., 1996; Boglione et al., 2001). However, none of these

424 studies have evaluated the effect of the diet on skeletogenesis and appearance of skeletal

425 deformities during early ontogeny. In this sense, it has been recently demonstrated that the

426 morphogenesis of marine fish larvae could be perturbed by inappropriate dietary levels of

427 different nutrients (Takeuchi et al., 1998; Haga et al., 2002a, b; Villeneuve et al., 2005a, b;

428 Hernandez et al., 2006; Tarui et al., 2006). Thus, in the present study, we aimed to evaluate

429 the effects of different dietary levels of VA on the incidence of skeletal deformities and larval

430 performance in gilthead sea bream fed rotifers enriched with graded levels of this

431 morphogenetic nutrient.

432   The feeding protocol used in the present study makes difficult to perform accurate

433 nutritional studies, because of the variability of the nutrient content in live prey (Giménez et al.,

434 2007). However, the use of a balanced compound diet for this kind of study, as it has been

435 previously used in European sea bass (Villeneuve et al., 2005a, 2006), was discarded, since a

436 compound microdiet is not completely developed for first feeding gilthead sea bream. Lipid

437 content in rotifers after enrichment with graded levels of total VA was similar in all treatments.

438 This result indicates that the differences observed in total VA content in the live prey were only

439   due to the levels of retinyl palmitate and retinol incorporated into the experimental emulsions

440   and cannot be related to the emulsion preparation and/or the enrichment conditions. Under the

441   present experimental conditions, total VA levels in rotifers increased proportionally to the

442   content of retinyl palmitate in the enriching emulsion. In agreement with Takeuchi et al. (1998)

443   and Giménez et al. (2007), the increase in retinol and retinoic acid, this last form of retinoid not

444   present in the enriching emulsion, indicated that rotifers were able to absorb, digest and

445   metabolize the retinyl palmitate contained in the enriching emulsion. Although retinoic acid is

446   the most active form of VA (Ross et al., 2000), the similar concentration of this retinoid in all

447   batches of enriched rotifers with experimental emulsions suggested that the observed effects of

448   VA on gilthead sea bream larval development were not due to its content in rotifers, rather than

449   the accumulation and transformation of different forms of VA in larvae.

450   At the end of the present experiment, , the different levels of dietary VA used during the

451   rotifer-feeding phase (4-18 dph) significantly affected gilthead sea bream larval growth and

452   survival, indicating that early larval nutrition exerted a strong effect on the further larval

453   performance. Larvae that showed the highest growth in length and dry weight, and survival

454   were those fed rotifers enriched with R450 and R900 emulsions, while higher dietary doses of

455   VA dramatically reduced larval performance. Similar results have been reported in other fish

456   species, such as Japanese flounder (Dedi et al. 1995; Takeuchi et al., 1995, 1998; Haga et al.

457   2003), European sea bass (Villeneuve et al., 2005a), red sea bream (Hernández et al., 2006)

458   and Atlantic salmon (Ørnsrud et al., 2002) where high dietary doses of VA led to a lower growth

459   and survival. However, the results from the above-mentioned studies are not directly

460   comparable due to different experimental dietary levels of VA, feeding protocols and diets (live

461   prey and inert diets). Nevertheless, survival and growth results observed in gilthead sea bream

462   larvae fed the control diet were similar to those obtained in commercial hatcheries (Tandler et

463   al., 1995; Başaran et al., 2004), ensuring that valid physiological and nutritional observations

464   could be drawn from this study.

465   Correct maturation of the larval digestive system allows larvae digesting and

466   assimilating the ingested diet, incorporating the needed amount of nutrient required for normal

467   growth and harmonious development. Pancreatic and brush border intestinal enzyme activities

468   have been widely used in nutritional studies as markers of larval fish development (Zambonino-

469   Infante and Cahu, 2001). In this study, low trypsin specific activity at 18 dph in gilthead sea

470   bream larvae fed high levels of VA, might be indicative of a delay in the maturational process of

471   the exocrine pancreas, as Villeneuve et al. (2005a) already reported in European sea bass fed

472   inert diets containing different levels of dietary VA. However, dietary effects of VA

473   administrated during the rotifer feeding phase (4-18 dph) were not evident after larval weaning

474   at 60 dph, which might indicate that larvae fed high levels of VA were able to recover the

475   normal digestive status regarding the pancreatic enzymes, once the excess of VA was

476   eliminated from their diet (*Artemia* feeding and inert diet phases). In contrast to the results

477   reported by Villeneuve et al. (2006) with European sea bass fed high levels of VA, the specific

478   activity of amylase in gilthead sea bream was not affected by the dietary levels of VA, although

479   numeric values tended to decrease with dietary doses of VA, but they were not significant due

480   to large variability between replicates and experimental treatments.

481   The specific activity of brush border intestinal enzymes was also affected by the dietary

482   dose of VA. Alkaline phosphatase is considered to serve as a marker for the maturation of the

483   brush border of enterocytes: the greater its activity, the better the level of intestinal maturation

484   (Zambonino-Infante and Cahu, 2001). However, high recorded activities of this brush border

485   enzyme at 18 dph in fish fed the highest dose of VA (R4500) in comparison to the rest of

486   dietary treatments might be attributed to an increase in cell proliferation (hyperplasia) induced

487   by dietary VA (Reifen et al., 1998; Uni et al., 2000), rather than a more advanced stage of

488   maturation of the intestinal mucosa, as aminopeptidase N specific activities indicated (three

489   times lower than in the other groups). At the end of the study, the low brush border enzyme

490   activity (alkaline phosphatase and aminopeptidase N) found in larvae from R2250 and R4500

491   groups, indicated dietary VA interfered with the normal development of the intestinal mucosa

492   and consequently, this might have impaired normal larval growth and further development. It

493   has been reported in different vertebrate species that VA influences enterocyte proliferation

494   and maturation, and decreases brush border enzyme-specific activity (Reifen et al., 1998; Uni

495   et al., 2000; Villeneuve et al., 2005a).

496   Dietary VA also affected the normal process of bone formation and skeletogenesis in

497   gilthead sea bream. The skeletal structures most affected by high levels of dietary VA were

498   those from the cranial skeleton (splanchnocranium), vertebral centrums and caudal fin

499   complex. Many authors have reported that the operculum complex, premaxilla, maxilla and

500   dentary bones were the cranial structures mostly affected by skeletal deformities (Barahona-

501   Fernandes, 1982; Chatain, 1994b; Andrades et al., 1996; Francescon et al., 1988; Boglione et

502   al., 2001; Faustino and Power, 2001; Villeneuve et al., 2005). In this study, the premaxilla,

503   maxilla and dentary bones were the cranial structures affected by dietary VA levels, resulting in

504   a large incidence of animals with compressed snout. This kind of deformity is quite common in

505   gilhead sea bream intensive larval rearing conditions (Andrades et al., 1996; Loy et al., 1999;

506   Boglione et al., 2001) and it has been also reported in other finfish species (Barahona-

507   Fernandes, 1982; Haga et al., 2003; Villeneuve et al., 2005a, 2006). The high incidence of this

508   kind of deformity under current experimental conditions might be linked to the ontogeny of the

509   splanchnocranium formation in gilthead sea bream, since the maxillar, premaxillar and dentary

510   are some of the first skeletal structures to appear and ossify due to their important functional

511   roles (Faustino and Power, 2001), although the frequency of the detection might be influenced

512   by the fatal nature of these kinds of deformities (Barahona-Fernandes, 1982).

513   Under intensive rearing conditions, opercular abnormalities in gilthead sea bream can

514   affect up to 80% of the population, seriously compromising both fish morphology and biological

515   performance (Koumoundouros et al., 1997b). Considering that defects in the opercular

516   complex are frequent and have been reported in many different fish species (Barahona-

517   Fernandes, 1982; Beraldo et al., 2003; Fraser and Nys, 2005), this structure seems to be

518   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

600   accelerated the normal differentiation pattern of vertebral centrums of these regions that

601   appear chronologically earlier (Faustino and Power, 1998).

602   The caudal fin complex was the most affected region of the gilthead sea bream skeleton

603   affected by dietary treatments as seen by the high incidence of skeletal deformities in fish fed

604   different doses of dietary VA. Deformities affected all skeletal elements composing the caudal

605   fin, although the most affected were, in order of importance, the epurals, hypurals, parahypural,

606   neural arch and uroneurals. The skeletal elements that compose the caudal fin (urostyle and fin

607   elements) are formed either by endochondral or by intramembranous ossification (Gavaia et

608   al., 2002). The first group included the parahypural, hypurals, epurals, uroneurals and the

609   specialized neural arch; and the second group included the vertebra 23, the urostyle and the

610   caudal fin rays. The present results indicate that independently of the ossification process type

611   that takes place in the different skeletal elements that compose the caudal fin, it seems that the

612   effect of dietary VA is the same, and might be more related to the intense ossification process

613   induced by the VA that disrupted the normal and harmonious development of the above-

614   mentioned skeletal elements. Differences in sensitivity to dietary VA amongst caudal fin

615   skeletal elements might be due to their differential ontogenetic development, and to differences

616   in the exposure time to VA. Thus, the skeletal structures more sensitive to dietary VA were

617   those that differentiated earlier, such as epurals, hypurals and parahypurals, whose cartilages

618   appeared between 4.4 - 5.1 mm Ls and were not completely ossified until 14.7 – 16.0 mm Ls

619   (Faustino and Power 1998). In contrast, the uroneurals, which appeared at larger sizes (10.4 –

620   16.0 mm Ls, Faustino and Power 1998) when all the elements of the caudal complex were

621   already ossified, were less affected by dietary treatments (<5%) due to its late ossification

622   process (Koumoundouros et al. 1997a; Faustino and Power 1998).

623   Although dietary VA affected the incidence of skeletal deformities in the caudal fin

624   complex, these abnormalities were not lethal but seriously affected the external appearance

625   and quality of larvae and juveniles, confirming the data reported by Koumoundouros et al.

626   (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

654 dietary VA levels should incline us to test lower doses of VA in live preys enrichments during

655 early larval stages and higher doses afterwards.

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840

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)		Survival (%)
	18dph	60dph	18dph	60dph	
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
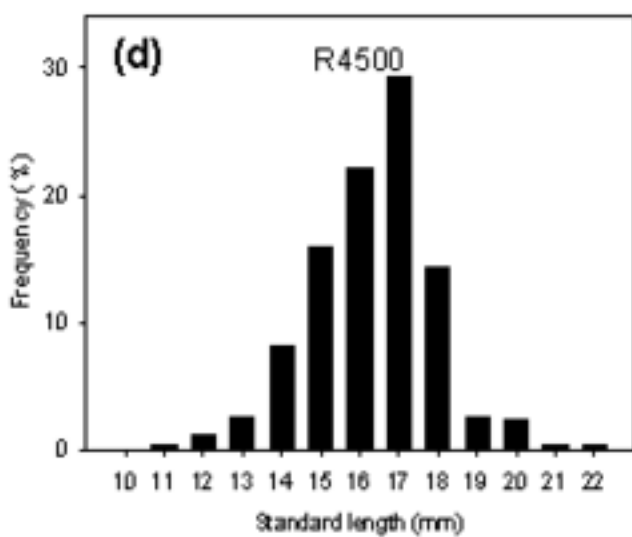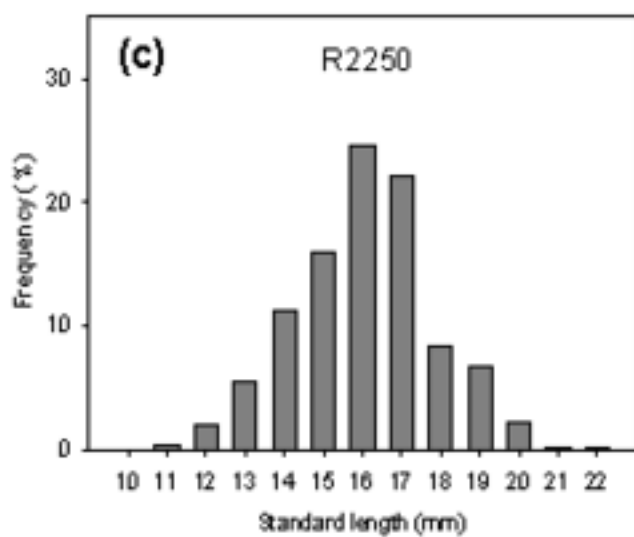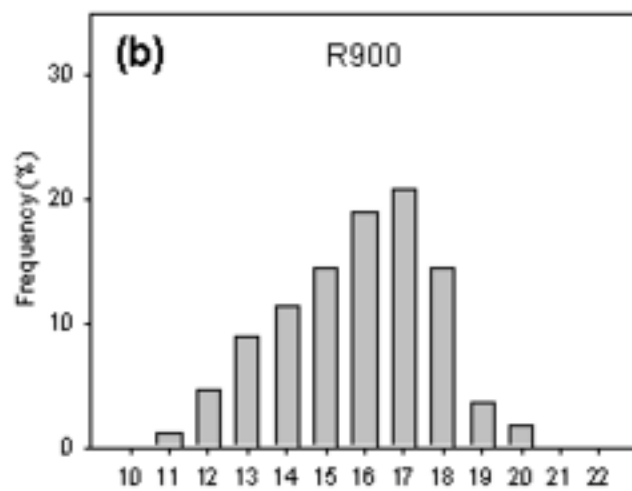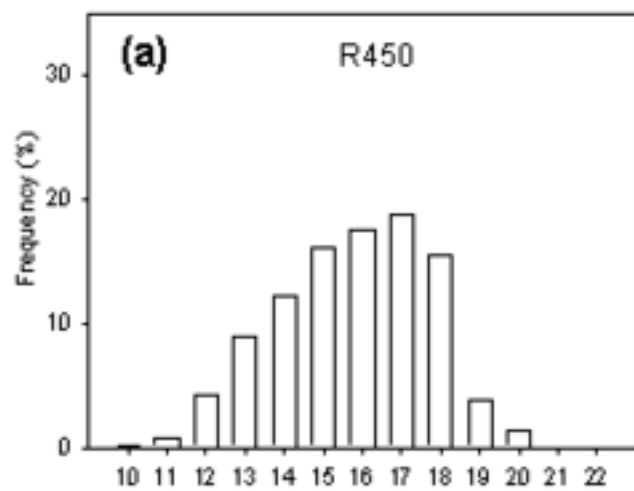
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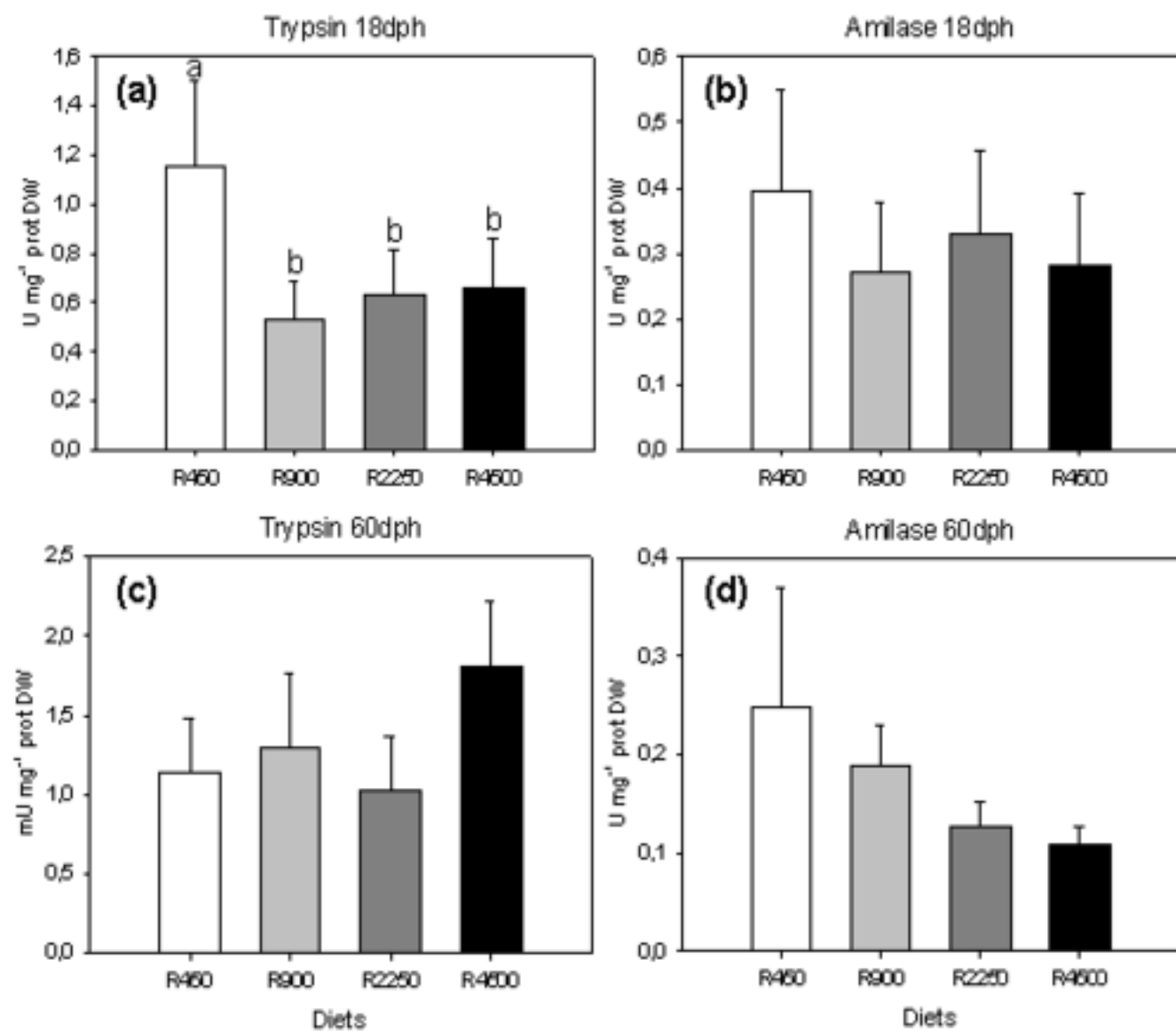
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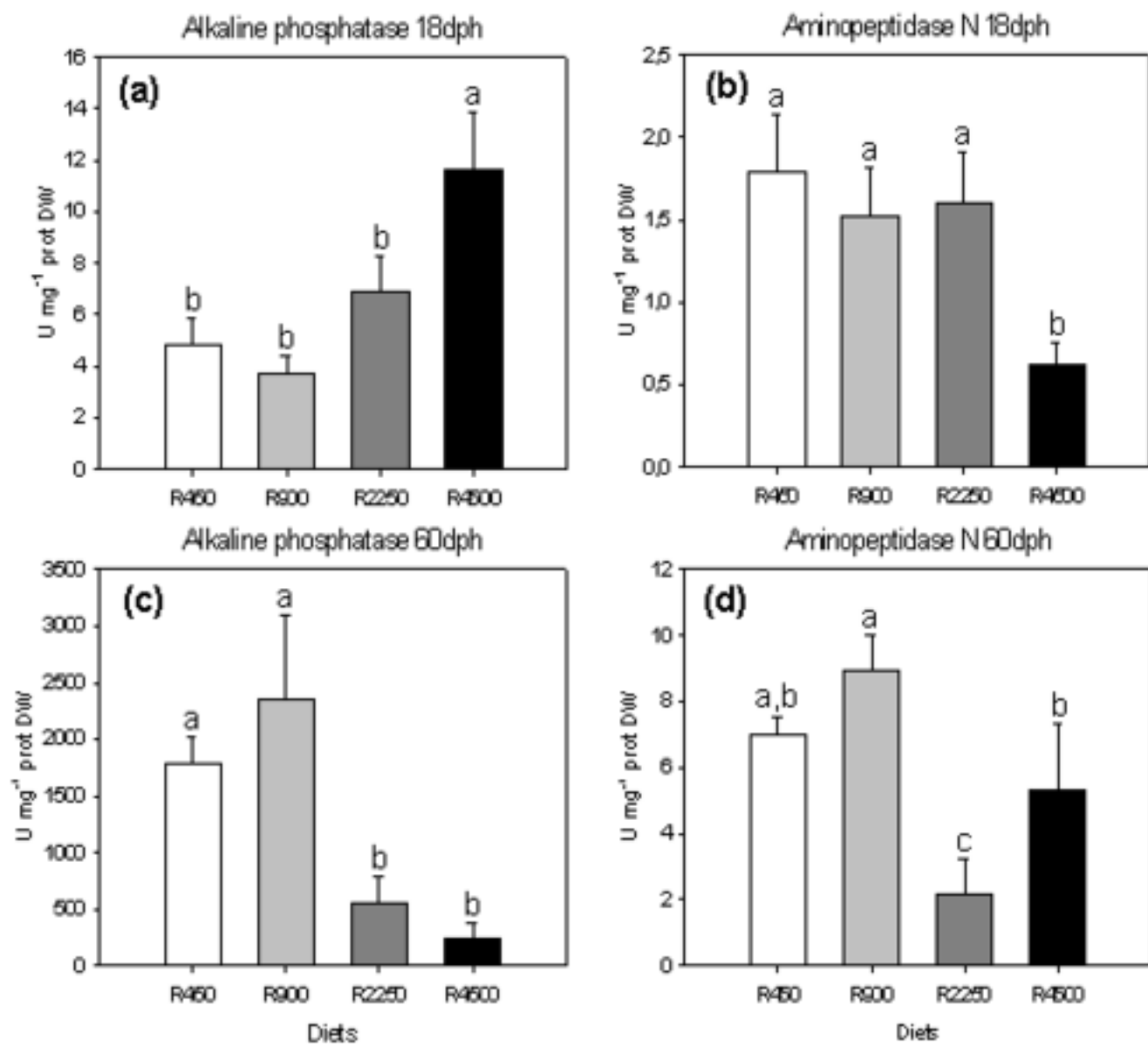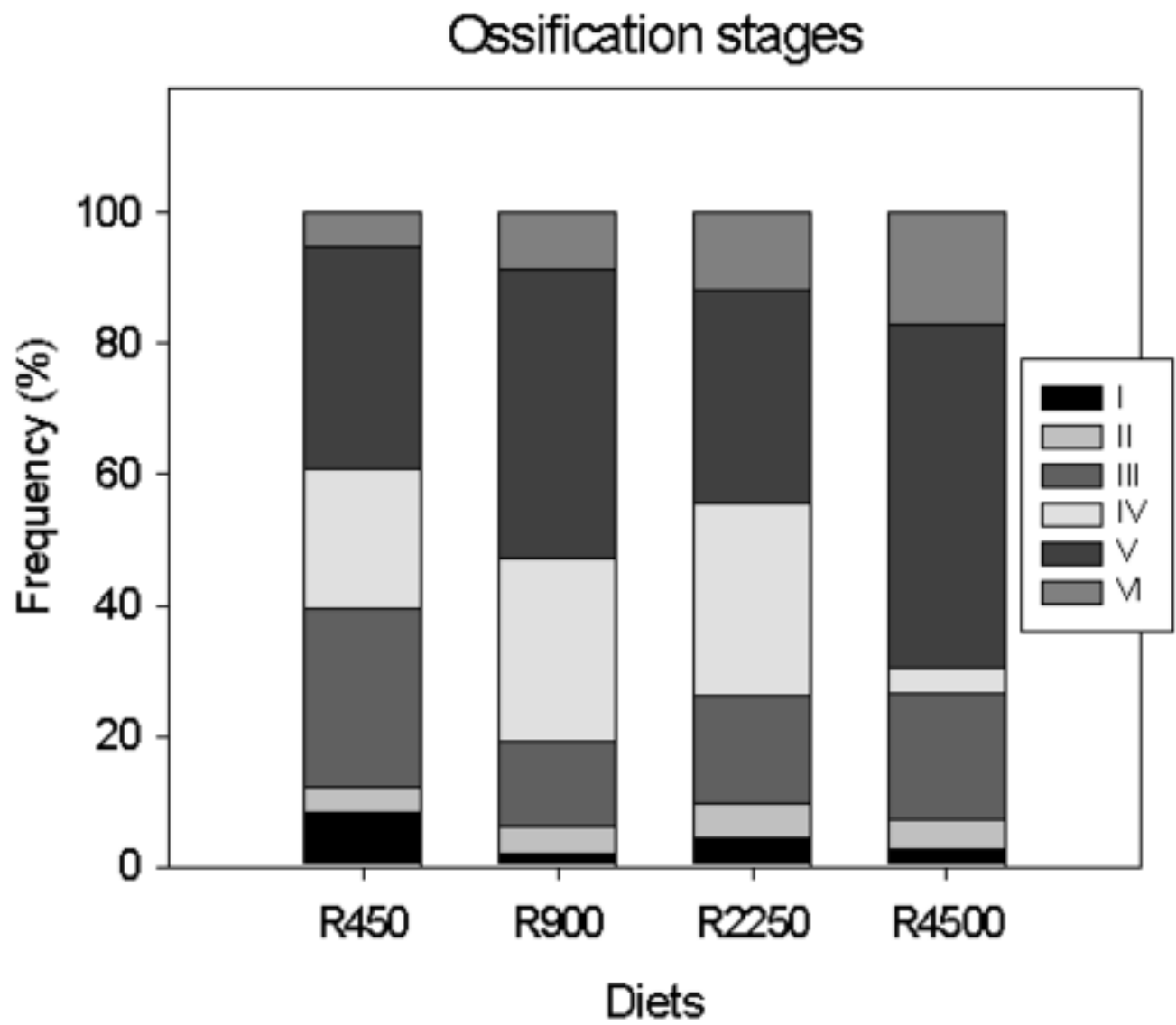
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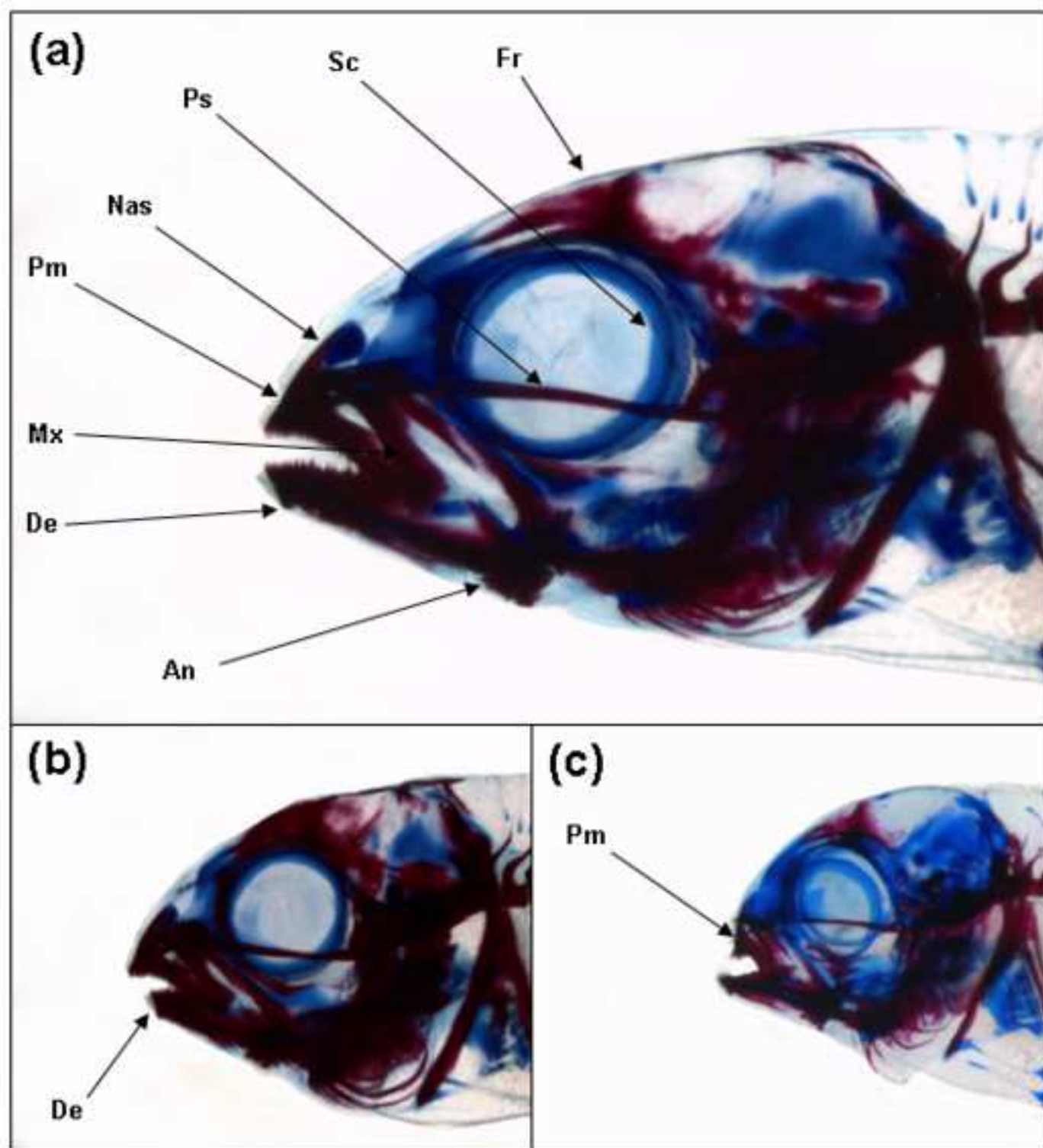
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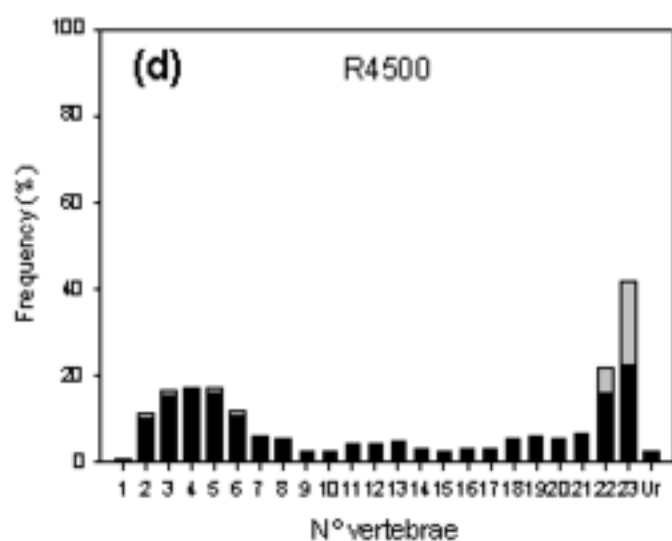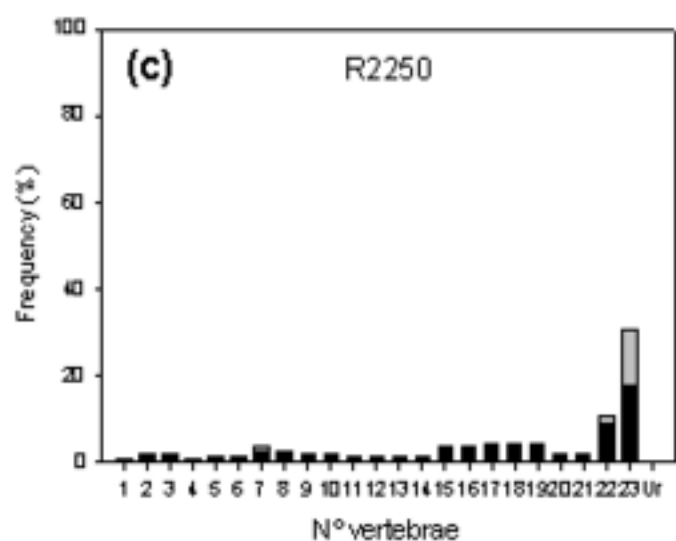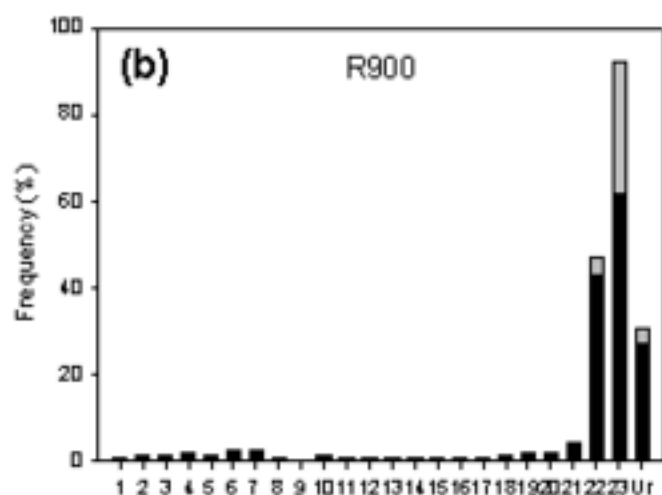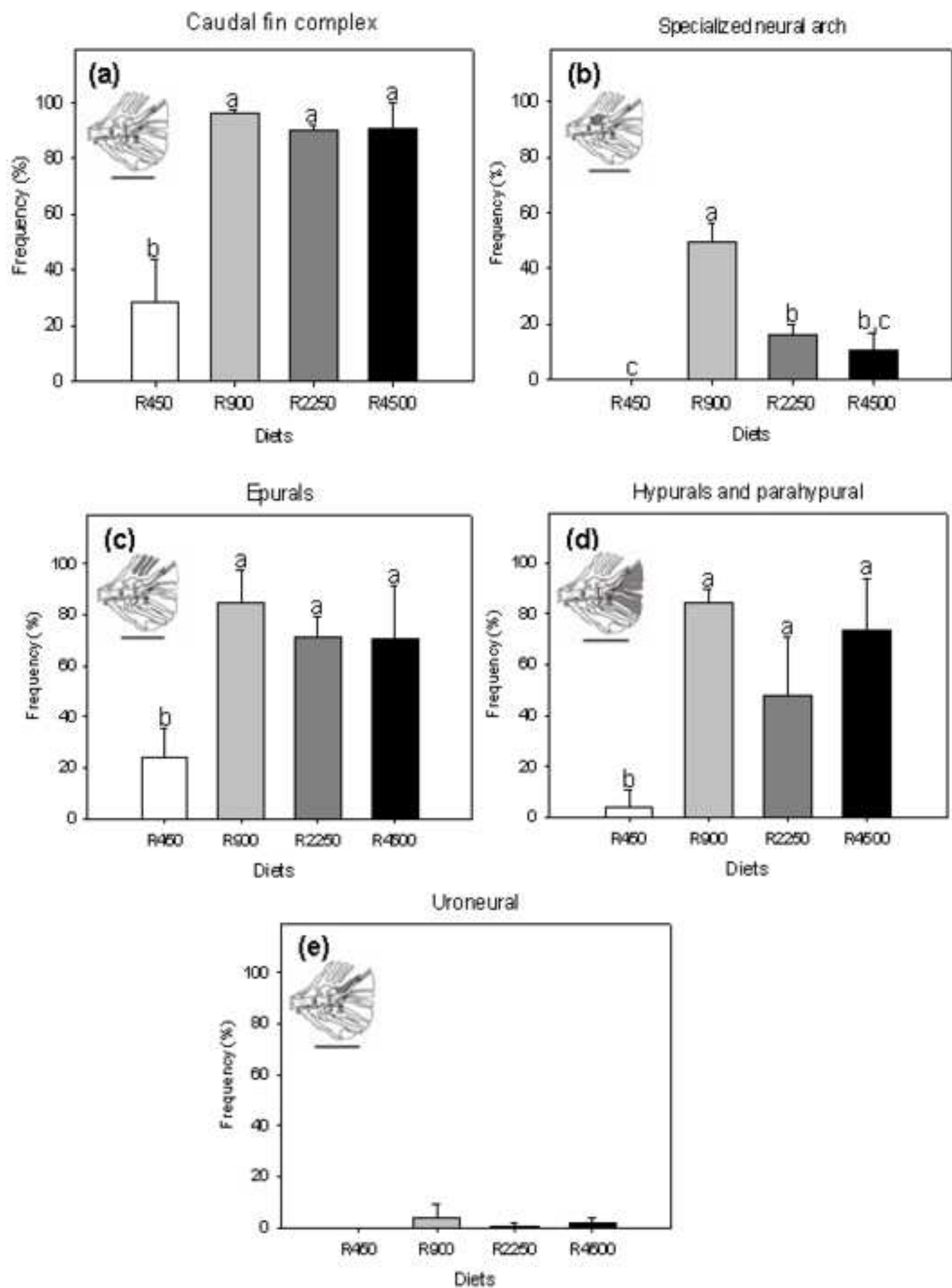Percentage of individuals with diferent number of vertebra
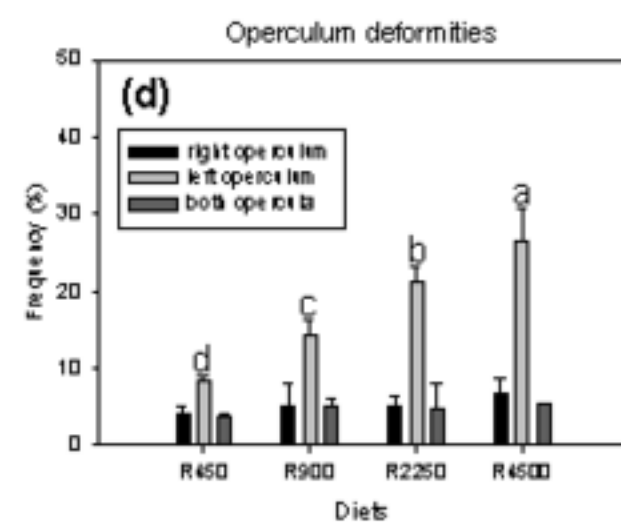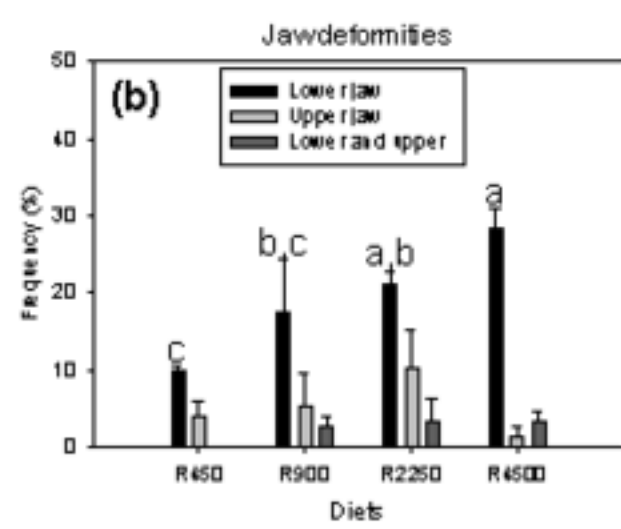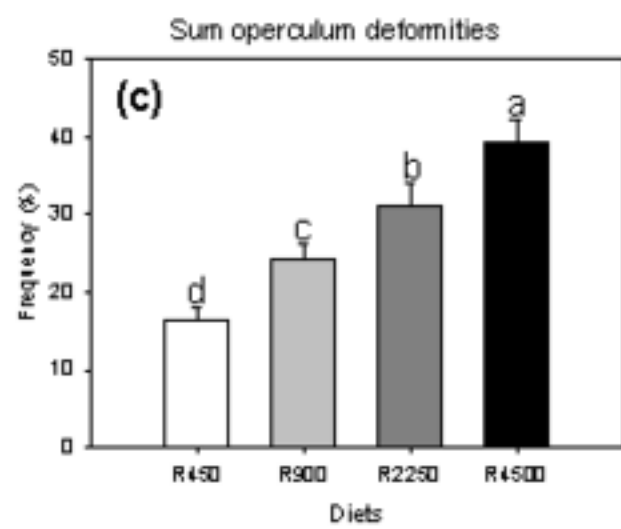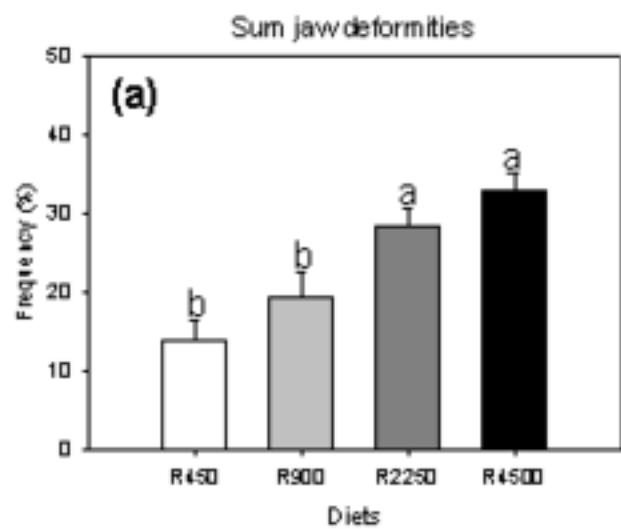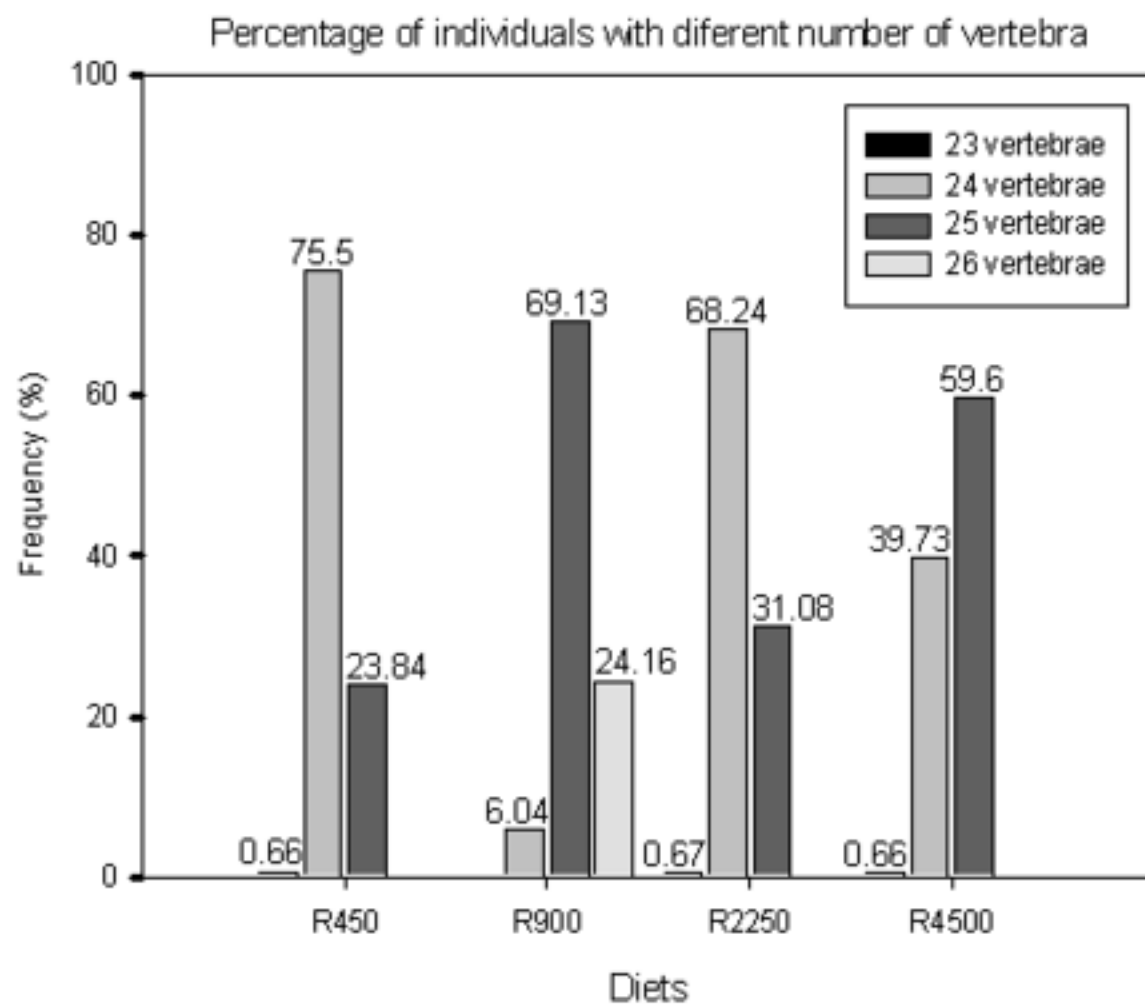
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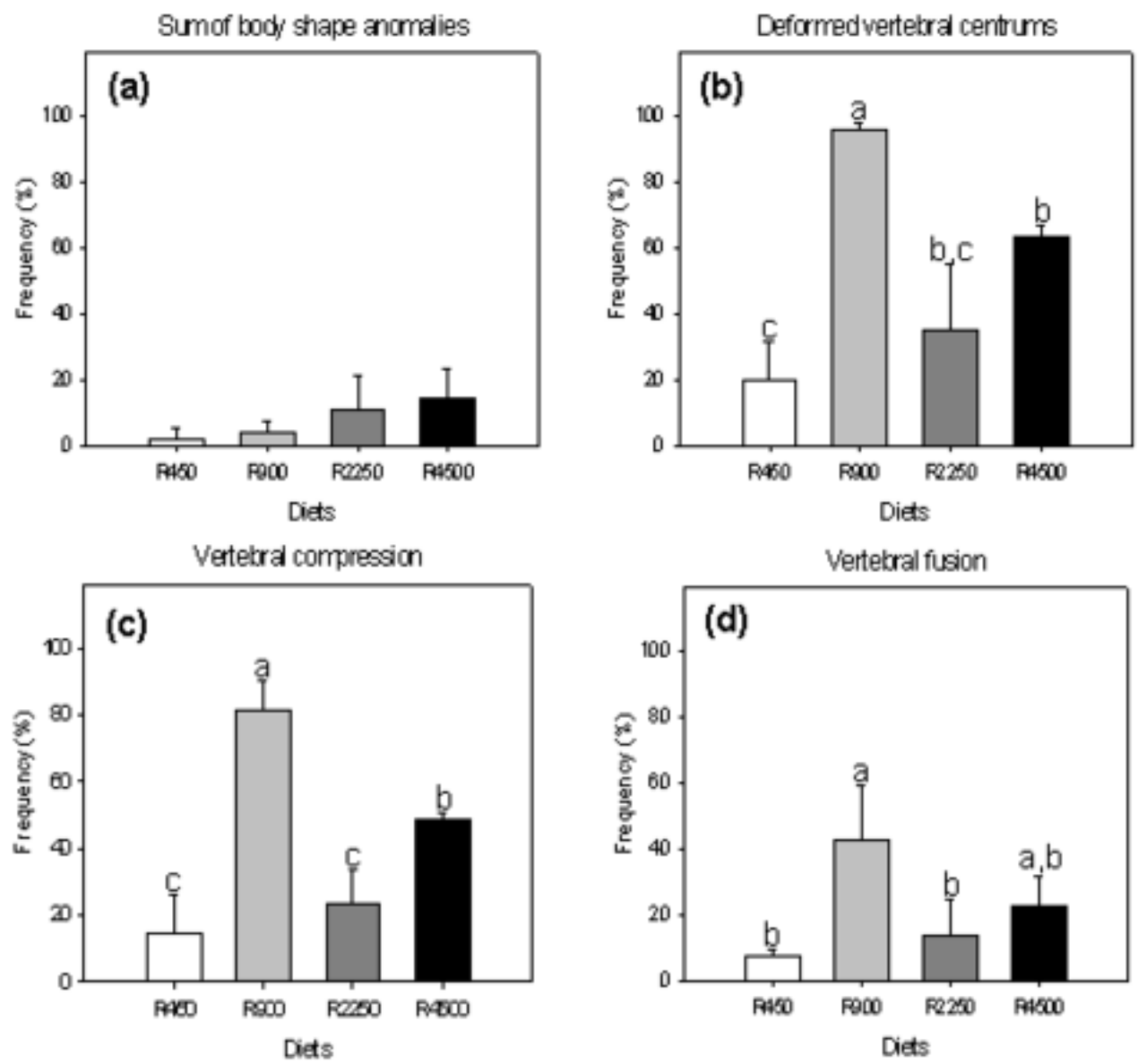
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Figure 11

Figure 12