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Skeletal anomalies in reared dusky grouper juveniles

Abstract

The first attempts to reproduce dusky grouper (*Epinephelus marginatus*, Lowe 1834) under controlled conditions started in 1995, but the egg and larvae quality was very low. Mass production is still encountering many difficulties, mainly concentrated in the larval period when very high mortality rates are observed, confirming what has been observed in the rearing of other grouper species. The main bottlenecks have been identified as the difficulty to properly nourish the larvae, stress shock syndrome, and the high deformation rates.

We analysed 633 dusky grouper larvae and juveniles (0.2-7.2 cm total length, TL), sampled during two larval rearing cycles carried out in 2001 and 2002 in Italy. The specimens at different development stages were stained *in toto* for bone and cartilage and examined for skeletal anomalies during dusky grouper ontogenesis. The incidence of anomalies in groupers hatched from the same egg batch but reared using two different methods (green waters and semi-intensive rearing) and three stocking densities (8, 16 and 28 larvae/l) was compared, with a view to providing tools for identifying the most appropriate larval rearing method in order to at least limit the onset of skeletal anomalies.

Our results suggest that during development no particular skeletal anomaly patterns (or fate) can be clearly identified as a high variability was observed in malformation typologies and the regions affected. No significant differences in the morphological quality between groupers reared using semi-intensive (LV02 lot) and green water (GW02-01 lot) methodologies were observed, whilst groupers reared at the highest stocking density (28 larvae/l) showed the highest frequency of deformed individuals (75.8%), the highest malformation charge (average of 5.5 anomalies per deformed individual), the largest range of anomaly typologies (38), and the highest incidence of individuals with at least one severe anomaly (30.9%). Whilst in green waters no evident effects of larvae density were observed on survival rates, the survival rate in large volume reared individuals (17.5%) was considerably higher with respect to those reared in green waters (0.2%) at 7-8 larvae/litre. This indicates that the semi-intensive methodology should be considered more effective in enhancing the survival rate of dusky grouper larvae.

Key words:

Epinephelus marginatus, dusky grouper, skeletal anomalies, larvae, juveniles, rearing methodology, survival rate.

1. Introduction

Groupers are fish belonging to the family *Serranidae*, sub-family *Epinephelinae*, a group of 159 species of considerable economic importance in tropical, subtropical and temperate marine waters.

The dusky grouper, *Epinephelus marginatus* (Lowe, 1834), more widely known by its synonym, *E. guaza*, is the most in demand in Europe; it is recreationally important and considered a potential candidate for commercial aquaculture and coastal zone management programs (Glamuzina *et al.* 2000; Marino *et al.*, 2000; 2002a and b; 2003). As a result of the substantial decline in total catches in many fishing areas, *E. marginatus* has been placed on the IUCN Red List and classified as an endangered species since 1996 (Groombridge and Baillie, 1997). The status of the grouper population was recently revised (IUCN, 2008) and is now classified as "EN A2d", that is its current population trend in the wild is decreasing. Given the concern for the conservation of this species, artificial propagation was promoted as a means of producing quality seed for stock enhancement.

Attempts to breed groupers in captivity started about four decades ago and Ukawa et al. (1966) described the successful fertilization and embryonic development of the red grouper *E. akaara*.

Grouper survival from hatching until metamorphosis (varying, according the species, from the 25th to the 50th day post hatching, DPH) is still generally low, ranging from 29.8% at the 35th DPH to 3 - 5% at the 55th DPH. Survival rate at 50 DPH is 3% (Toledo, 2000). During the process of metamorphosis, groupers are known to be extremely sensitive to stress (Doi *et al.* 1991) and high mortality is commonly experienced. Depending on the hatching rate, the initial stocking densities vary from 2 to 36 grouper larvae per litre (Toledo, 2000). No amelioration in larval survival rate was observed on varying stocking

density (Hseu, 2002; Duray et al. 1995; Tookwinas, 1989), whilst the percentage of abnormal larvae (curved and relatively smaller larvae) was significantly higher at incubation densities of 800 and 1,600 eggs/l than at 200 and 400 eggs/l. Hatching rates and percentage of normal larvae are higher at an aeration volume of 100 ml/min than at 500 and 1000 ml/min (Toledo, 2000).

The first attempts to reproduce dusky grouper under controlled conditions started in 1995 (Spedicato et al., 1995). Whilst considerable progress in induced reproduction has been made in recent years (Marino et al., 2001; 2003), large-scale seed production is still encountering many difficulties owing to the high mortality rate, which is mainly concentrated in the larval period, subsequent to the exhaustion of yolk reserves (10th -15th DPH), thus confirming the difficulties encountered in rearing early stages of grouper species. The main causes have been identified as low feeding rates and starvation, particularly before or during exogenous feeding onset (Glamuzina et al., 1998; Spedicato et al., 1995) as reported for the other grouper species (Toledo et al., 2002).

Skeletal malformations and their incidence are one of the most important factors affecting marine larviculture, also of species consolidated for aquaculture (salmon, sea bass, gilthead sea bream, …), with effects on production costs, taking into account that as many as 50-60% of hatchery juveniles present at least one severe, externally detectable skeletal malformation. In the aquaculture industry, losses due to malformations also impact on-growing farms, where malformed market size fish have to be discarded or sold at lower values than market prices. Thus, the reduction of 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 image of aquaculture products in consumers.

Skeletal anomalies are generally associated with a general lowering of performance (i.e. swimming ability, conversion index, growth rate, survival, and susceptibility to stress, pathogens, bacteria) (Hilomen-Garcia, 1997; Koumoundouros et al., 1997; Boglione *et al.*, 2001). The presence of skeletal abnormalities is generally attributed to genetic and epigenetic causes: the differentiation and growth of skeletal elements, in fact, are under the control of the genotype but are also modulated by developmental homeostasis, which acts against environmental and genetic disturbances through

canalisation and developmental stability. So, when extensive environmental and/or genetic stress prevails over developmental homeostasis, then fluctuating asymmetry, variations in meristic counts and skeletal malformations may arise. All these effects may thus be considered as developmental disturbances and indicators of inappropriate rearing conditions. Unnatural environmental conditions (temperature, pH, stocking density, water flow, deficient alimentation, heavy metals, bacteria, parasites, …) that are not optimal for the species can be one of the causes of very high frequencies of malformed juveniles (Afonso *et al.*, 2000; Boglione *et al.*, 2001; 2003; Koumoundouros *et al.*, 2001; 2002; Lewis *et al.*, 2004) in reared fishes.

Therefore, when different developmental anomalies occur in larvae from the same egg batch but reared in diverse conditions, this could be used to identify which of the applied rearing methodologies is more suitable for the considered species. We consequently inspected dusky groupers at different development stages and reared in different stocking densities or under different rearing methodologies (from the same egg batch), with a view to developing tools for understanding skeletal anomaly ontogeny in dusky grouper and identifying which of the tested rearing protocols was the most appropriate for the larval rearing of this species.

2. Materials and Methods

A total of 633 dusky grouper larvae and juveniles were sampled during two larval rearing cycles carried out in 2001 (GW01) and 2002 (GW02 and LV02) (Tab. 1). Samples (n=114, GW01 lot) from the 2001 cycle were used to study skeletal anomaly onset during larval development, and samples (n=519) collected during 2002 were used to evaluate both the effect of rearing methodology (n=220) and rearing densities (n= 397) on the occurrence of skeletal anomalies.

The 2001 larvae used to study skeletal anomaly ontogenesis were all from the same egg batch and reared using the "green waters technique" (GW01 lot) (Saroglia and Ingle, 1992), while the 2002 larvae (all from the same 2002 egg batch), used to study the effects of rearing methodologies, were reared in

semi-intensive conditions (lot LV) (Cataudella *et al.*, 2002) and using the same green waters methodology as in 2001 (lot GW02). The latter lot was again split into three further larval groups and reared at different initial densities (8, 16 and 28 larvae/l) using green waters methodology.

Fertilized eggs were obtained as described in Marino et al. (2003) from one male and three females in 2001 and from two males and two females in 2002. Eggs were obtained by hormonal induction using a polymer-based implant loaded with Gly10 [D-Ala6 Pro9 Net] GnRHa (Marino et al. 2003) at 34.1 µg/kg (body weight). Females and males were stripped and the eggs artificially fertilized. Eggs were rinsed, transferred to a decanter (50l), disinfected in a 0.5% iodine solution and stocked in 1000 litre circular indoor incubators.

The green waters method was applied at the INVE testing center located at Maricoltura Rosignano Solvay (LI, Italy) both in 2001 and 2002. GW larvae, stocked at the three different initial densities, were reared in 6,000 l tanks at 23-25.5°C, salinity 38. Photoperiod was 13L:11D.

In both 2001 and 2002 GW lots, from the 2nd day post hatching (DPH), enriched rotifers (*Brachionus rotundiformis*, DHA Protein Selco for 8 hours), and microalgae (*Isochrysis galbana, Nannochloropsis oculata*) were added to the rearing tanks. Two types of brine shrimp *nauplii*, *Artemia* AF (420 µm) and enriched *Artemia* EG (DC DHA Selco, INVE, 24 h at 26°C), were administered to the larvae from 16th to 45th DPH. Starting from the 20th DPH, commercial micro-particulated extruded diet (Proton, INVE TECHNOLOGIES nv, Belgium, from 80-200 µm to 1200-1400µm) was given for co-feeding and pre-weaning purposes. Weaning and post-weaning were performed with Epac Alfa (1,300-500 µm; INVE, Aquamaks, Italy) and NRD micropellet (INVE, Aquamaks, Italy). Further information is available in De Wolf et al., 2002 and Marino et al., 2002a. The final survival rates for each GW lot is provided in Tab. 1.

Semi-intensive methodology (hereinafter called 'large volumes') was applied at the SMEG farm, located in Latina (Italy). Some 400,000 eggs (from the same GW02 egg batches) were stocked in a circular 60 m^3 tank (diameter 8 m, water height 1.2 m). Optimal hydrodynamic conditions were achieved by suitably regulating custom-built air pumps (air-lifters) that allowed high oxygen concentration and

differentiated current flows in the tank to be maintained, and oily film formation on the water/air interface to be avoided. The tank was filled with sea water and 2 days before stocking the larvae unicellular green algae species (*Chlorella minutissima*, *Isochrysis galbana*, *Nannochloropsis suecica*: 0.02-0.2x10^6 cells/cc) were added to the water tank. Stocking density was 6.6 larvae/l. Water temperature was kept constant at 25°C, salinity at 35, under natural photoperiod, throughout the experiment. Water remained stagnant from day 1 to day 5 post hatching, and was then continuously changed at 20% daily rate until day 20. Water exchange was gradually increased up to 100% per day until 35 DPH. L-type rotifers, *Brachionus plicatilis* (2x10^3 ind./l), were introduced into the tank from the 3rd to the 27th day, twice daily: the first at 8:00 and the second at 14:00. From the 28th day on, *Artemia salina sp. nauplii* were introduced. All live preys were enriched with DHA ("Easy" DHA Selco®'s, INVE, Italy), for 18-24 hours, in 4 administrations, with air and oxygen (10-15 ppm), at a temperature of 20° C.

Natural zooplankton, consisting mainly of *nauplii*, juvenile and adult stages of the copepod *Tisbe holoturiae* (about 10 ind./litre), was collected daily from the nearby coastal Lake of Fogliano. The filter concentrated plankton retained on plankton nets of 200 and 500-μm mesh sizes. Thus, two size fractions, 200-500 μm and <200 μm, were collected. Only the <200 μm fraction was fed to the larvae from the 3rd day, while 200-500 μm was used additionally after the 20th day. Zooplankton supplies were added daily at 8:00, at the same time as rotifers and *A. salina nauplii* (0.4-2x10^3 ind./l). Food density in the tank was monitored at 8:00 and 14:00 h and the amount of *B. plicatilis* was adjusted to maintain the density of 6-10 individuals/cc.

At 70 DPH the final survival rate of larvae was 17.5 %. Further information is available in Russo et al., 2009.

Samples of larvae (total length range: 2.0– 57 mm, Tab. 1) were anaesthetized (ethylene glycol-monophenyl ether, Merck, 0.2-0.5 ml l-1), fixed in buffered formalin (4% or 10% in phosphate buffer, 0.1M, pH 7.2, according to the size) and stained *in toto* with Alizarin red for bone and Alcian blue for cartilage (Taylor and Van Dike, 1985). Precocious stages (up to the 30th DPH) were not double stained, in order to avoid cartilage staining (involving the use of acetic acid) demineralised lightly ossified

elements thus masking the beginning of an ossification process. Individuals with TL > 30 mm were first X-rayed (Picker X-Ray 6191 905-E control apparatus by Picker X-Ray Corp., Waite Manufacturing Division. Inc, Cleveland, Ohio, USA; 4min/5mAmp/80Kw, film AGFA Structurix D7 DW Ete) and then double stained.

Observations were performed on both sides of the stained samples under a stereomicroscope (Wild, LEITZ) , and on the left side alone in X-rayed groupers. The length data refer to standard (SL), or total (TL) length, rounded up to the upper 0.5 mm.

The list of anomalies considered is set out in Table 2. Some anomalies displayed different degrees of alteration (see, for example, C3 and C3* in Table 2) and were indicated as distinctive variables.

The anatomical terminology is according to Harder (1975) and Matsuoka (1987), with the exception of terminology for caudal fin structures, which is according to Schultze and Arrantia (1989).

The numerical data set obtained was processed to calculate incidences and to perform a descriptive analysis for each descriptor (anomaly typology) and lot.

Anomaly data for each lot of sampled specimens were converted to binary values (presence or absence of each anomaly type). The binary matrix was then subjected to Correspondence Analysis (CA, Benzécri, 1973), in order to visualize the relationships among lots and the role that each anomaly plays in defining the characteristics of different lots. In order to correctly represent the frequency of specimens without abnormalities during CA vector normalization, a new binary variable (ABS) was used to distinguish between those individuals expressing more than one skeletal anomaly and individuals without anomalies. A unit value (i.e. true) was used for specimens with no anomalies and a null value for specimens with at least one anomaly.

3. Results

A total of 43 out of the 65 malformation typologies considered were observed. Some severe (considered here as those anomalies which deform the external shape of the fish) cephalic anomalies,

such as dislocation of the glossohyal and opercle deformation, were never found, whilst some others, such as vertebral axis deviation or vertebrae fusion, were observed in some body regions and not in others: i.e., vertebrae fusions never occurred in cephalic and hemal vertebrae, and scoliosis was never detected except in the hemal vertebrae.

3.1 Anomalies occurring during development

The first anomalies observed were deformed *trabecula cranii* (Fig. 1.a) and kyphosis in the cranial portion of the notochord (Fig. 1.b) in 2.8 mm and 3 mm (TL) larvae (10 DPH), respectively. Kyphosis was also observed in one 20 day old larva (6 mm TL; Fig. 1.c) and in one 25 day old larva (10 mm TL), affecting the ossifying cranial vertebrae which showed deformed vertebral bodies. The neural arches of the vertebrae involved show abnormal inclination, particularly the anteriormost ones, which take on an S-shape (Fig. 1.c). In all the kyphotic individuals, the swim bladder appeared to be slightly more inflated than in the unaffected (normal) ones (compare figs. 1.a and b).

One larva at 20 DPH (5.5 mm TL) displayed a nasal and premaxilla bone anomaly (Fig. 2.a), a rare deformation which was observed only in one 25 DPH (10 mm TL, Fig. 2.b) and one 60 DPH individual (GW02-2, 23 mm TL, Fig. 2.c).

From the 25th DPH, different degrees of fusion between two caudal vertebrae (anomalies D3 and D3*) began to be observed (Figs. 3-4).

Starting from the 30th DPH, many other anomalies (both in type and incidence) were observed, as shown in Tab. 3 and 4. The 'anomaly charge' (that is the number of anomalies observed/number of individuals affected by at least one anomaly in each age-group) increased with age, with the highest value found in 50 DPH groupers (7 anomalies/individual). From the 50th DPH onward, individuals affected by severe anomalies accounted for about 40% of the total, for each age-group. From the 30th to the 105th day, with the sole exclusion of 50 day-old groupers, anomalies mainly affected the caudal regions, with incidences varying from 51.4 to 81.9% of the malformations observed in each lot (Tab. 4).

3.2 Skeletal anomaly pattern in dusky grouper larvae: effects of rearing methodologies

In this section, a comparison between lots LV02 and GW02-1 was made: the two lots differed in

rearing methodology but the fish density was almost the same (7-8 ind./l).

Twelve out of the 43 above-mentioned anomaly typologies were absent in lots LV02 and GW02: in both lots, no kyphosis or fusion in cephalic vertebrae or lordosis in pre-hemal vertebrae and scoliosis were observed, nor any pre-maxillary and/or maxillary malformations.

No important differences in the morphological quality between groupers reared in large volumes (LV02 lot) and in green waters (GW02-01 lot) emerged (Tab. 5). Larvae reared with the large volumes method (LV-02) showed almost the same percentage of deformed larvae (68% vs 66.3%), anomaly charge (2.9 vs 2.1) and range of anomaly typologies (25 *vs* 21) as larvae reared in green waters. Lot GW02-1 showed a higher incidence of severe anomalies (25.4% vs 21.3) out of the total anomalies observed in this lot. The same severe anomaly charge was found however in both lots (1.4).

Some differences emerge if the different typologies of anomalies occurring in dusky groupers reared with different rearing methodologies are analysed: as many as 9 types of anomalies were observed only in large volumes lot (LV02) and 5 in GW02-01 groupers reared in green waters (Tab. 6). Further, in LV02, no head (14 and 15 typologies) anomalies were observed (Tab. 6), unlike lot GW, where the head anomalies accounted for 8.2% of the total observed anomalies. In both lots, however, the hemal region was the most one affected, accounting for a maximum of 53% of the anomalies observed in lot GW. In this region, dorsal spine anomalies (H8) and those of the respective pterygophores (H11) were predominant, in total representing 27.5 (LV) and 41.8% (GW) of the total anomalies observed in each lot.

Some other differences between lots LV02 and GW021 may be found by examining the localization and the typology of severe anomalies (Tab. 6). In lot LV02, 6.5% of the observed severe anomalies were saddle-back malformations in the cranial or pre-hemal regions (SBa and b, absent in GW021 individuals), no severe malformations were detected in the hemal region and most of the recorded severe anomalies were in the caudal region (53.8% of total severe anomalies). Practically each saddle-back individual also had deformed neural arches of the underlying vertebrae.

The severe anomalies present in GW021, absent in LV02, were: A1 (lordosis in cephalic vertebrae, 2

individuals), C4 (deformed bodies of hemal vertebrae, 5 ind.), 14 (prognathism of dental, 2 ind.) and 15 (reduced dental, 9 ind.), which in total accounted for 13.4% of the total severe anomalies observed in this lot. Further, as many as 32.4% of the total severe anomalies observed in this lot occurred in the head bones (Fig. 5).

3.3 Skeletal anomaly patterns in dusky grouper larvae: effects of rearing density

The analysis was carried out on 60 day old larvae raised in green waters (GW02-01, GW02-02, GW02-03), at three stocking densities (8, 16 and 28 larvae/l).

Groupers reared at the highest stocking density (28 larvae/l, GW02-3) showed the highest frequency of deformed individuals (75.8%, Tab. 7), the highest malformation charge (average of 5.5 anomalies per deformed individual), the largest range of anomaly typologies (38), and the highest incidence of individuals with at least one severe anomaly (30.9%). Conversely, grouper larvae reared at the lowest density (GW02-1) presented the lowest anomaly charge (2.1) and number of anomaly typologies (21). The percentage of severe anomalies was, however, higher in larvae stocked at the lowest stocking density than in the other two lots. Lastly, the severe anomaly charge was practically the same in the three lots.

When the distribution of total anomalies in the different body regions was analyzed (Tab. 8), some differences emerged between the lowest-density-reared lot and the other two. Two typologies were observed exclusively in GW02-2 groupers (A2: kyphosis in cranial vertebrae; SBb: saddle back in pre-hemal region) and as many as 15 only in GW02-3. Among these, we found deformed branchiostegal rays (17*), malformed upper jaw (19), deformed epurals (G10) and anomalies affecting pectoral and pelvic fins (E8, and L8, L11, respectively), which are very rarely observed in reared Teleost fish. Cephalic (head and cranial vertebrae) anomalies were more frequently observed in larvae reared at the highest stocking density, whilst anomalies affecting the hemal region were most frequent in larvae raised at the lowest stocking densities: their incidence decreased with increasing rearing density while in lot GW02-3 the pre-hemal were the most deformed bones. The most frequently observed anomaly in lot GW02-1 was the deformation of the dorsal spines (H11), whilst in the other two lots it was the

deformation of the neural arches of the pre-hemal vertebrae (B5).

This pattern changes slightly if we consider only the incidence of severe anomalies (Fig. 6). The cephalic region showed the highest concentration of severe anomalies in all three lots, followed by the caudal fin and vertebrae in the lots at low and intermediate stocking densities. In GW02-3, 30% of the observed severe anomalies affected the pre-hemal region, which included pre-hemal vertebrae, pectoral and pelvic fins, and predorsal bones.

Three Correspondence Analyses (CA) were carried out on different matrices of skeletal anomaly binary data referring to 2002 groupers. The first data matrix included 519 individuals (4 lots) and 43 variables (42 variables expressing abnormalities, and one variable expressing the absence of abnormalities, ABS). The variables used for CA are those listed in Tab. 8, with the addition of I11 (anomaly of soft dorsal rays) which was observed only in lot LV02 (see Tab. 6). The ordination model on the second (CA2) and third (CA3) correspondence axes (the ordination is shown only in the plane defined by these axes because CA1 accounts only for trivial differences, that is between individuals with and without anomalies) of all the individuals accounted for 4.9% and 4.7% of the overall variance (Figs. 7), whilst the ordination of descriptors is shown in Fig. 9. The mean coordinate values of the individuals in each lot (group centroids) are plotted on a separate graph in order to differentiate more clearly among the different lots (Fig. 8). This plot and that of the descriptor, however, actually share the same reduced space of individual ordination, so that they could be superimposed. In the lot centroid ordination, the axes scale was changed, because group-centroids are much closer to the axis origin than most of the descriptors or individual points.

The semi-intensively reared group (LV02, Fig. 7) is the only one whose centroid is located in an eccentric location on the second and third axis, facing all the GW reared samples that are clustered in the positive semi-plane defined by these axes. Further, LV02 is also the most highly 'grouped' one: the GW individuals actually tend to be more scattered (see Fig. 8). The anomalies that are not too close to the axis origin tend to be associated with GW samples, whereas the remaining ones are more common or independent (Fig. 9). In particular, A2 (kyphosis in cephalic vertebrae), A3* (complete fusion among

cephalic vertebrae) and 19 (malformed pre-maxillary and/or maxillary) anomalies tend to be associated with GW lots reared at intermediate and high densities, whereas 15 (reduced dental), L8 (malformed pelvic basypterygium) and A1 (lordosis in cephalic vertebrae) with the GW lots as a whole.

The second CA was performed on the severe anomaly binary matrix (519 individuals x 19 descriptors) and gave the same ordination (data not shown) and a slightly higher variance (14.7% for the second and the third axes).

Finally, the third CA was performed on a binary data matrix (519 individuals x 26 descriptors), excluding rare anomalies, that is, excluding the anomalies observed in less than 10 individuals (A1, A2, A3, A3*, C4, C5*, D5*, E8, F8, I11, L8, 14, SBa, Sc, 18 and 19). The ordination of all the individuals and the four group centroids in the space defined by the second (CA2) and third (CA3) correspondence axes (the ordination is shown only in the plane defined by these axes as CA1 accounts only for trivial differences between individual with and without anomalies), which constituted 6.9% and 6.5% of the overall variance, is shown in Figs. 10, whereas the ordination of descriptors is shown in Fig. 11. The CA applied to this matrix gave a different ordination with respect to the other two: indeed, by excluding the rare anomalies, a new discrimination emerged between the low and the intermediate/high density lots, as evidenced by the lot-centroid ordination (Fig. 10). The CA2 axis actually discriminated between LV02 and all the GW lots, whilst the CA3 one separated low-density lots from the other GW ones. The G9 and D4 anomalies were associated with GW02-2 and -3, whilst H11, 15, D3* and D6 were associated with LV02 and GW02-1.

4. Discussion

This is the first report on skeletal deformations observed during development in dusky grouper larvae and juveniles, raised under different rearing conditions. Skeletal anomalies were already described in other reared groupers, like the seven-band grouper (*E. septemfasciatus*, Nagano et al*.,* 2007), red (*E. akaara*, Setiadi *et al*., 2006) or orange (*E. coioides*, Toledo, 2000) spotted groupers, but no comparison among incidences in larvae reared with different rearing modalities has been given. Further, some of

these papers dealt exclusively with some peculiar anomalies (saddle-back syndrome and vertebral deformities in red spotted grouper) or did not provide any exhaustive data on skeletal anomaly incidence and typology (orange spotted grouper).

The first skeletal anomaly we observed during the larval development of dusky grouper was kyphosis in the cranial portion of the notochord (2.8 mm SL, 10 DPH), which, along with progressive ossification, became kyphosis of the cephalic vertebrae with deformation of the involved vertebrae. Kyphosis appeared to be associated with an overinflated swim bladder. This is the first time that such precocious kyphosis has been described in groupers. Koumoundouros *et al.* (2002) described the ontogeny and effects of kyphosis in sea bass (*Dicentrarchus labrax*) although they claimed that the notochord was normal in all the fish sampled before the onset of vertebral formation and that kyphosis was not determined before the onset of vertebral deformation. Moreover, they found that this axis malformation was closely associated with branchiostegal ray deformation. The photographs in the article, but not the authors, showed that the centre of kyphosis was located on the vertebrae that overlaid the climax of an asymmetrically inflated swim bladder but no data on the existence of a close association between this anomalous swim bladder and kyphosis were provided by the authors. Conversely, this association was found by Grotmol et al. (2005) in cod (*Gadus morhua*) larvae that exhibited unusually large swim bladders associated with abnormal dorsal curvature of the notochord in the region just behind the cranium. Older larvae showed abnormal neural arches and bodies of the vertebrae involved in the kyphosis. The authors put forward different hypothesis:

1) the overinflated swim bladder plays a role in abnormal notochord curvature by exerting or transmitting an upward mechanical force that deforms the notochord;
2) an increased pressure between the notochord and the swim bladder is the result of the transmission of pressure through the swim bladder from pathologically expanded abdominal organs, such as the overfilled digestive tract observed in the affected larvae;
3) a reduced hydrostatic pressure (turgor) within the notochord may make it prone to deformation.

In this study, we did not observe any appreciable differences among the abdominal organs in kyphotic and normal larvae. Consequently only the first and the third hypothesis may be taken into account for dusky grouper larvae but further, more targeted studies are necessary to identify which of the two hypotheses is the more likely.

In the present study, the analysis of the fate of kyphosis in older groupers indicated that not only did it become rarer and disappear starting from 92 day-old individuals reared in 2001, but that also all the cranial and pre-hemal vertebrae anomalies tended to disappear with age in this lot. In grouper, Nagano et al. (2007) reported that lordosis was the most common deformity observed in the vertebral column, occurring in the caudalmost hemal and first caudal vertebrae. They observed no vertebral deformity in seven-band groupers younger than 57 DPH and the incidence was found to increase with age. Until the 100th DPH, a maximum of 61.9% of individuals were found to have a vertebral deformity. As far as kyphosis is concerned, Koumoundouros *et al.* described an exponential decrease over time of pre-hemal kyphosis in sea bass longer than 17 mm (mean TL), which could be ascribed to the high mortality of the affected fish. In our study, as the oldest individuals were also the least likely to be sampled, further studies on a larger number of individuals are necessary to validate the hypothesis of a selective mortality for kyphotic grouper larvae and postlarvae (or those having larger swim bladders).

Nagano et al. (2007) reported that the first anomalies they observed during seven-band grouper development were jaw deformities (20 DPH, early flexion stage), in particular the lack of supramaxilla or twisted upper and lower jaws. The incidence of these anomalies increased over time (up to 100 DPH), finally affecting a maximum of 98 % of the observed groupers. Also in our observations the first head anomalies were identified in 20 DPH (5.5 mm SL) larvae, but the premaxilla was the affected bone and a dramatic reduction in its occurrence was observed after 30 DPH. In 2001 samples, that is the individuals used to study anomaly development, no other splanchnocranium deformities were noted before or after complete ossification. Different types of jaw deformities developing after the onset of ossification were observed at 14 DPH (6.3 mm SL) in *Seriola lalandi* (Cobcroft et al., 2004), at 18 DPH (ca. 7-8 mm SL) in *Lates calcarifer* (Fraser and de Nys, 2005) and at 44 DPH (>10 mm SL) in

Latris lineata (Cobcroft et al., 2001), but their presence was detected in later specimens only in *S. lalandi.* Conversely, some twisted jaw anomalies similar to the one described by Nagano were observed in some individuals in the 2002 lots, as discussed below.

In 2001 dusky groupers older than 30 DPH, the caudal vertebrae and fin were the most affected elements, with the sole exception of the 50 day lot, in which anomalies mainly occurred in the pre-hemal region. Notochord flexion in dusky grouper is accomplished when larvae are 20 DPH (6 mm long TL) and caudal vertebrae are ossified in 30 DPH (12 mm TL) individuals; the skeletogenetic process is completed (after the juvenile stage has been attained, as scales are first observed in 18 mm TL samples) in dusky groupers longer than 35 mm TL (Boglione, pers. comm.). Therefore, unlike cephalic vertebrae, deformities in caudal vertebrae occurred after ossification had started, and their incidence increased slightly with age in the 2001 lot.

The fact that the 50 day old groupers in 2001 did not show the highest incidence of anomalies in the caudal region could be a consequence of sampling error (different sample sizes), as well as of the larger variability in types and number of anomalies observed in the 50 and 78 DPH lots, as these were the largest samples used to study anomaly development. But, if we analyse the results obtained from observations carried out on the 2002 GW lots (TL ranging from 17 to 35 mm), we find that these lots, which were composed of individuals of comparable age (60 days old), did not display a predominance of anomalies affecting the caudal region again, but rather the hemal (in low and intermediate densities lots, 8 and 16 ind./litre) and the pre-hemal (28 larvae/litre) regions.

As far as the effects of rearing methodology are concerned, if we analyse the overall quality assessment reported in Tab. 5 we find very little difference between the GW02-1 and the LV02 lots. For instance, GW02-1 showed a similar incidence of severe anomalies (25.4% vs 21.3), with a slight difference actually due to a relatively higher incidence of severe anomalies *versus* total anomalies, which is lower than in the LV02 lot: so far, it has been found that fewer GW02-1 individuals (%) are affected by severe anomalies and with the same anomaly charge as in the LV02 lot. The survival rate in large volumes reared individuals (17.5%) was considerably higher than those reared in green waters (0.2%).

This datum indicated that the large volumes method should be considered as more effective for the survival of dusky grouper larvae.

Also the rearing densities tested did not seem to have a clear-cut effect on overall quality in dusky grouper juveniles or on the survival rates (which were nevertheless very low, never exceeding 1.1%). Indeed, the incidence of (even severely) deformed individuals was lower at the intermediate density tested (16 ind./litre, GW02-2 lot), whilst the relative abundance of severe anomalies was higher at the lower density (8 ind./litre). However, the hypothesis that 28 larvae/litre could be considered too stressful a density for dusky grouper larvae seems to be supported by the fact that the highest scores for the average anomaly charge, the number of observed anomaly types, the frequencies of deformed individuals (Tab. 7) and the lowest survival rate (0.1%) were observed in the GW02-3 lot. Further, in the GW02-1 lot the anomalies were mainly concentrated in the hemal region, whilst in groupers reared at higher densities, deformities tended to occur in the pre-hemal region. In particular, pre-hemal scoliosis and lordosis, and the deformation of pectoral and pelvic fins make their appearance only in intermediate and high density-reared individuals. Increased activity of these fins due to a stronger competition in more crowded tanks could be hypothesized as the causative factor for the pre-hemal vertebrae malformations. The pectoral fin enables fish to maintain their station in vortex streets, in yawing turns, braking, hovering, in generating locomotor forces and in benthic station holding (Lauder & Drucker 2004). Conversely, very little is known about the function of the pelvic fins: from recent analyses of turning and manoeuvring in fish it is clear that fish actively use their pelvic fins as control surfaces during manoeuvres (Drucker and Lauder, 2003). However, all these activities presumably increase in high density tanks.

One interesting and original result obtained in this study is that the tested rearing conditions do not appear to have a strong effect on skeletal anomalies in dusky groupers. Some slight effects of rearing methodology on skeletal patterns emerged from the CA results: the results obtained from the application of this analysis actually show that severe anomalies were the main discriminating factor between 'large volumes' and 'green waters' reared groups, whilst the most frequent anomaly typologies

(that is, excluding rare anomalies and considering only those anomalies that were observed in more than 10 individuals) allowed a degree of discrimination among all the lots. However, the differences in the morphological quality in the different reared lots is very low compared with that observed in juveniles of sea bass (*D. labrax*) and gilthead sea bream (*Sparus aurata*) siblings reared using intensive *vs* semi-intensive methodologies (Boglione, 2005; Boglione *et al.*, 2003; Cataudella *et al.*, 2002; 2006; Koumoundouros *et al.*, 1996).

Consequently, our results show that during the development of dusky grouper no unambiguous pattern of skeletal anomalies emerged, as a high variability was observed in malformation typologies and the regions affected. The overall morphological quality did not seem to be affected by reducing rearing densities from 16 to 8 larvae/litre or by using different rearing methodologies. Only survival rates were found to be modulated by rearing conditions, reaching the highest value (17.5%) in semi-intensive (large volumes) methodology. Some authors identified the final larval stages (metamorphosis from pterygiolarva to juvenile stage) as the crucial phase in which major efforts should be made to improve the survival rate (but no data are provided on skeletal anomalies): attempts have been made to intervene in the hormone regulation of metamorphosis in *E. coioides* larvae, using T3 or T4 thyroid hormones. The percentage of metamorphosed larvae (Tay et al. 1994) and the survival rate (Toledo, 2000) increased significantly. However, the best results they obtained consisted of larval survival from hatching to day 35 ranging from 5.4% to 29.8% and a survival rate at harvest (day 55, about two inches) of 3% (Toledo 2000). Our data on dusky grouper evidenced the highest values (17.5%) at an advanced stage (70 DPH) in large volume conditions. Therefore other causal factors need to be investigated in order to ameliorate the overall morphological quality of reared dusky grouper juveniles, i.e. the breeders' rearing conditions or hormone treatments (for sex reversion): inappropriate feeding, unnatural physiological treatment, or stressful rearing conditions could affect egg and, consequently, larval quality.

5. Acknowledgements

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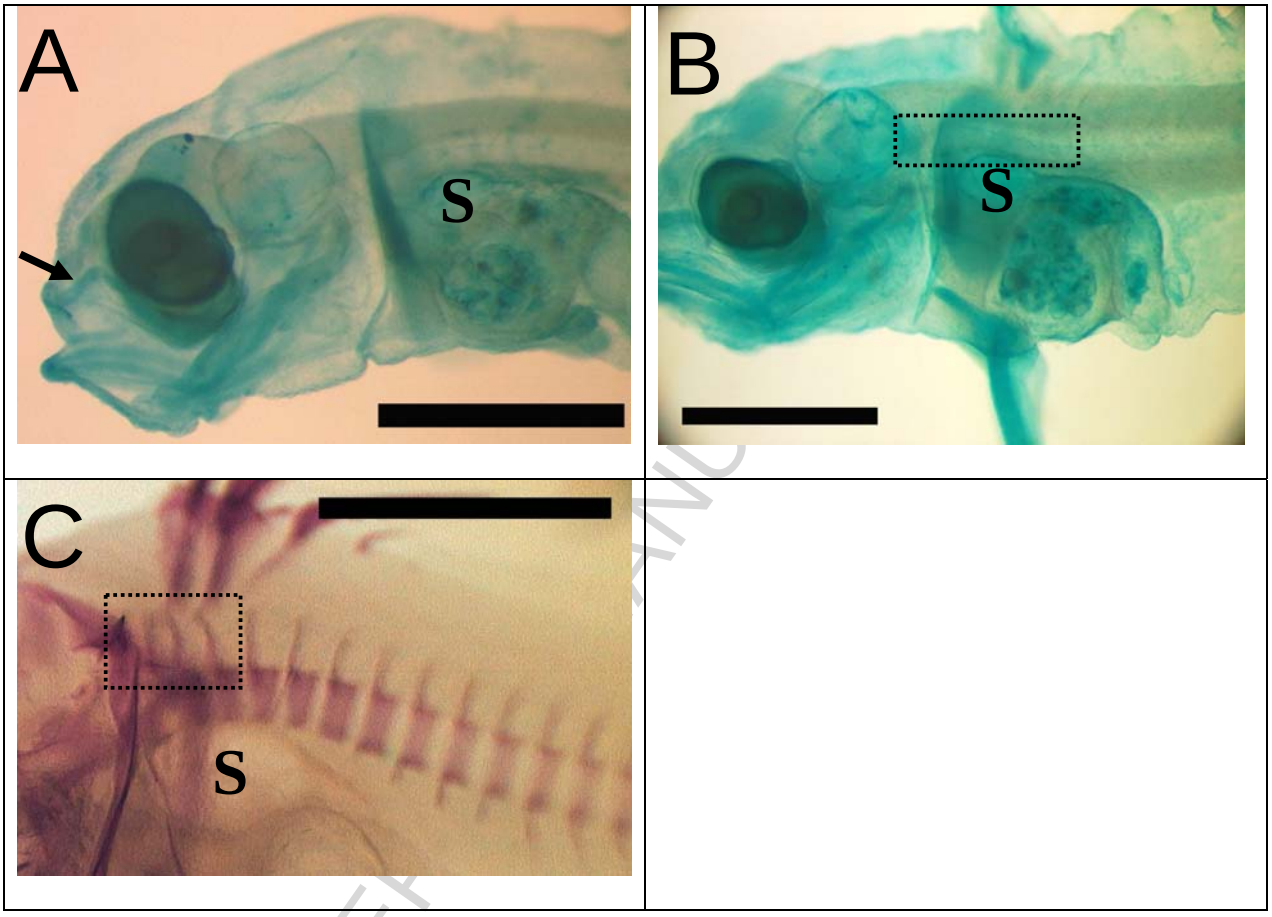
Fig 1



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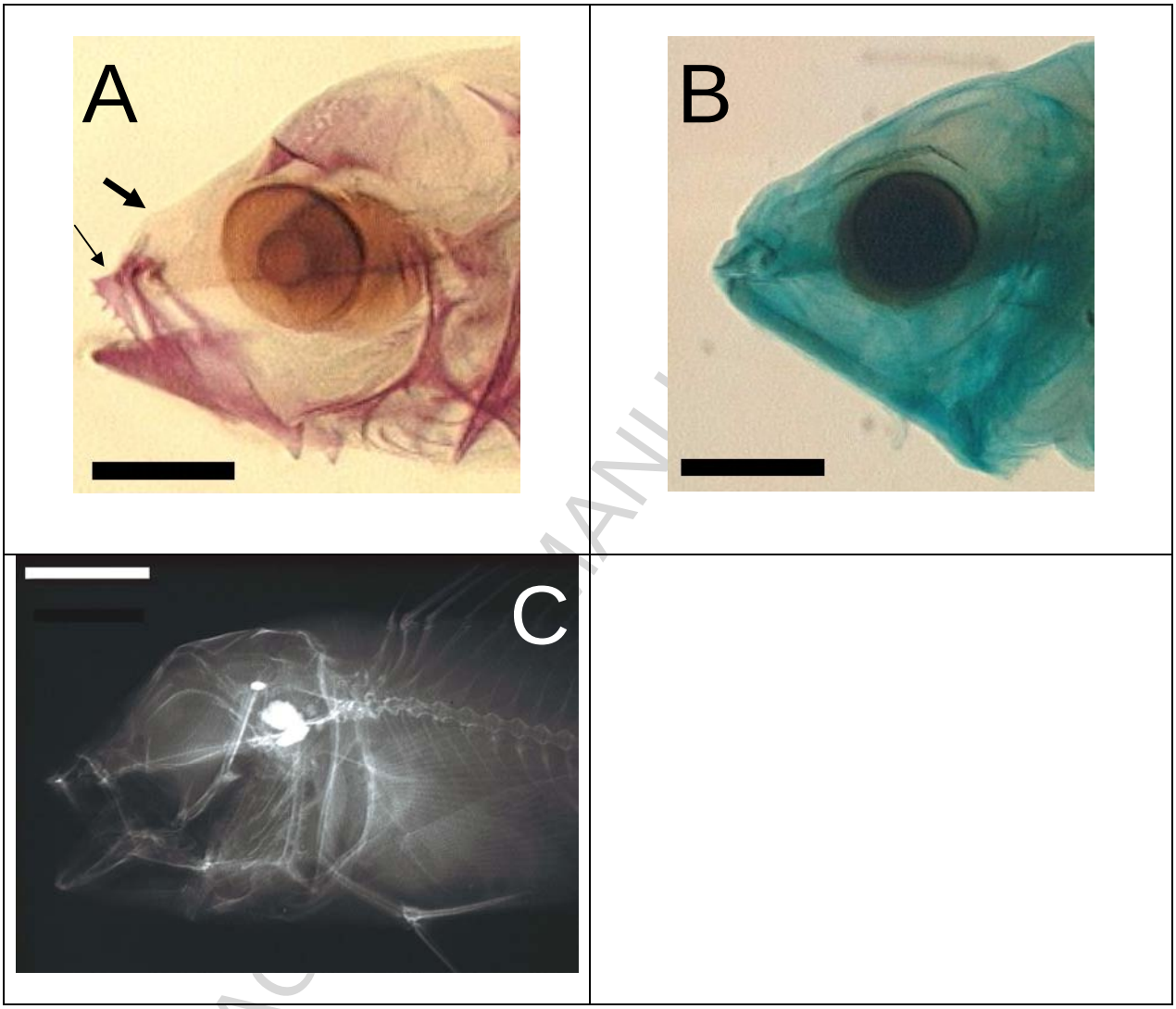

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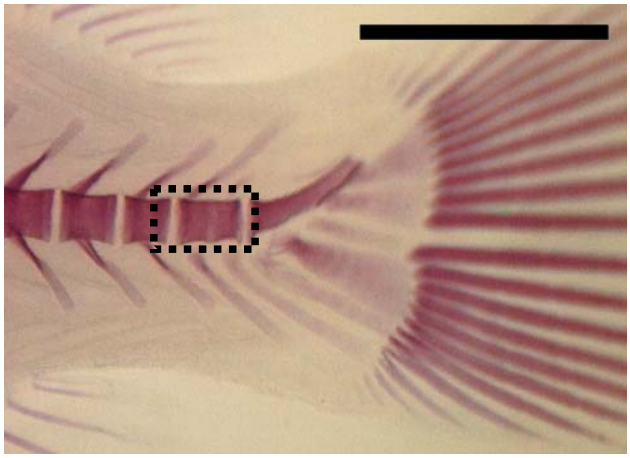

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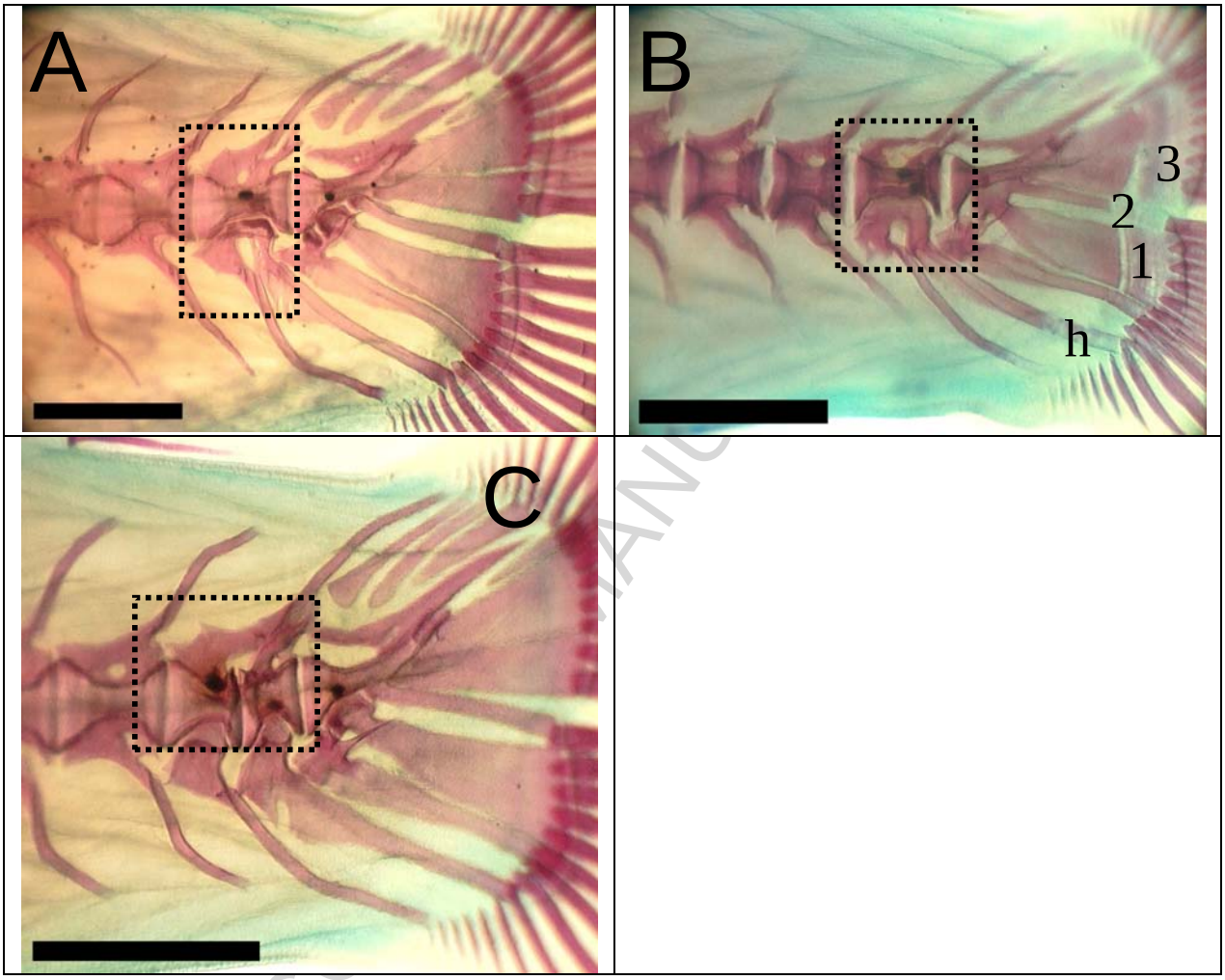

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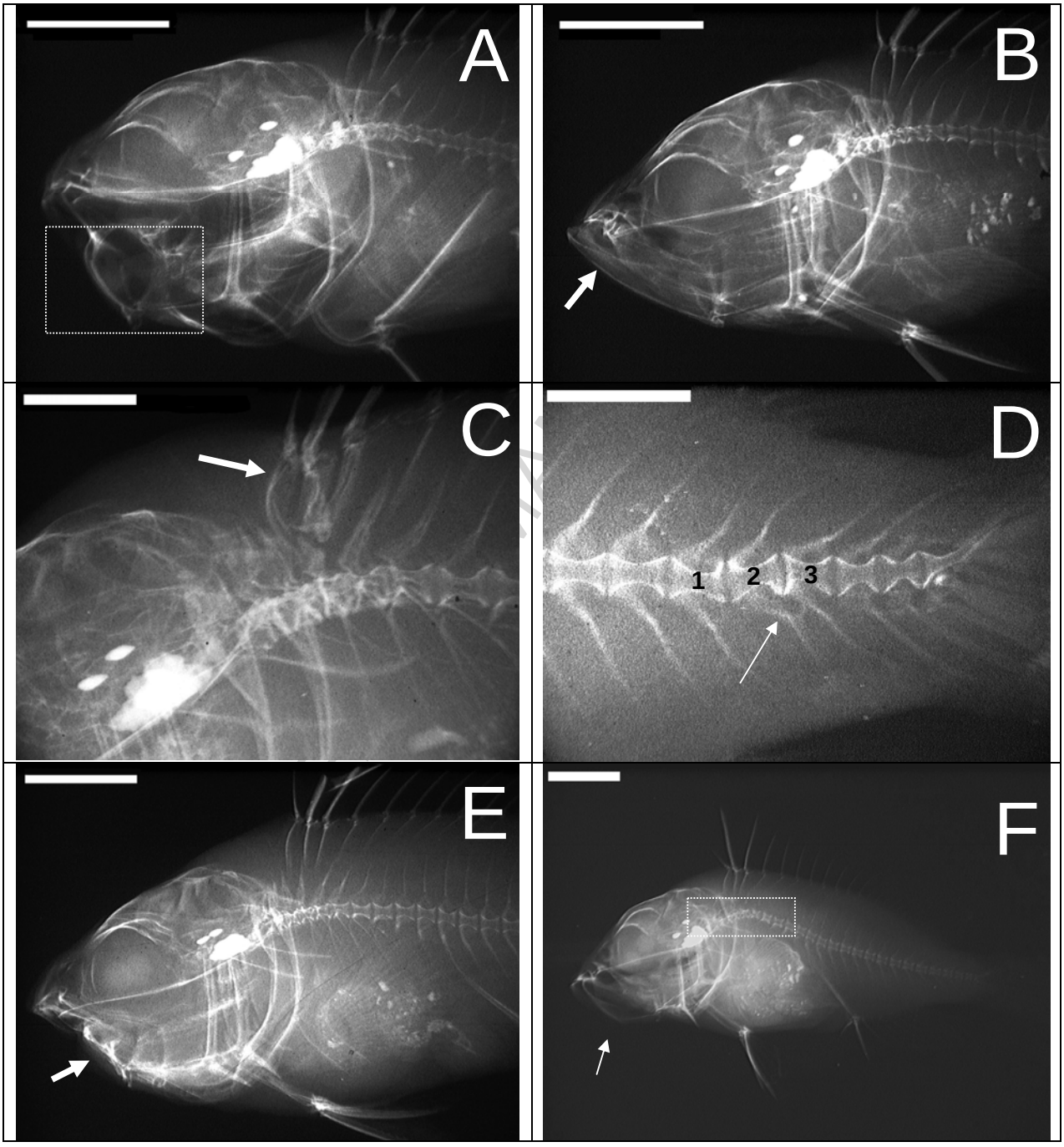

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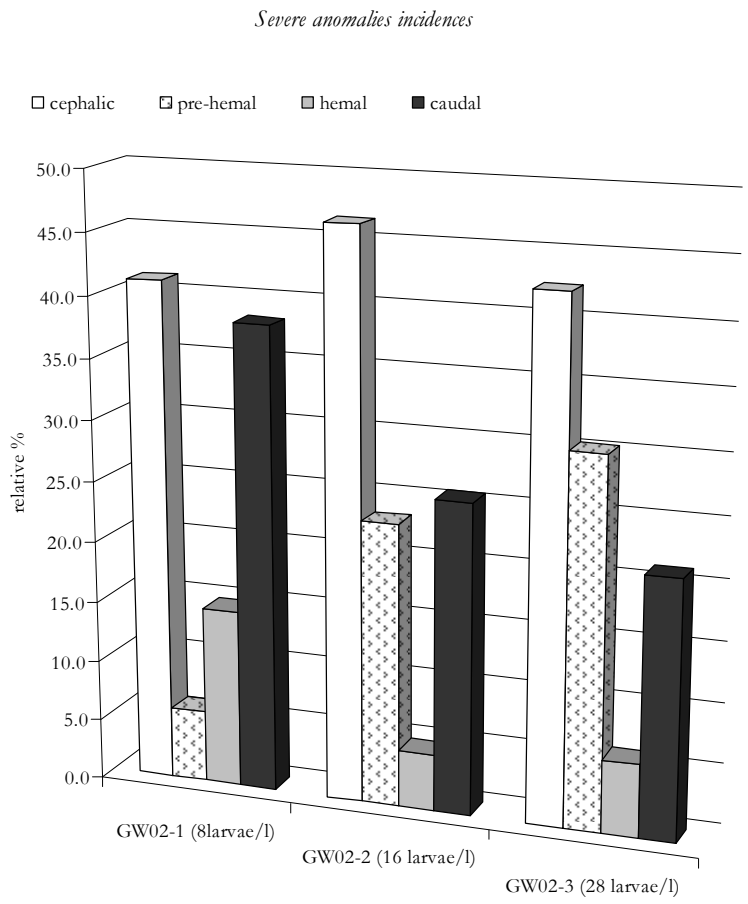

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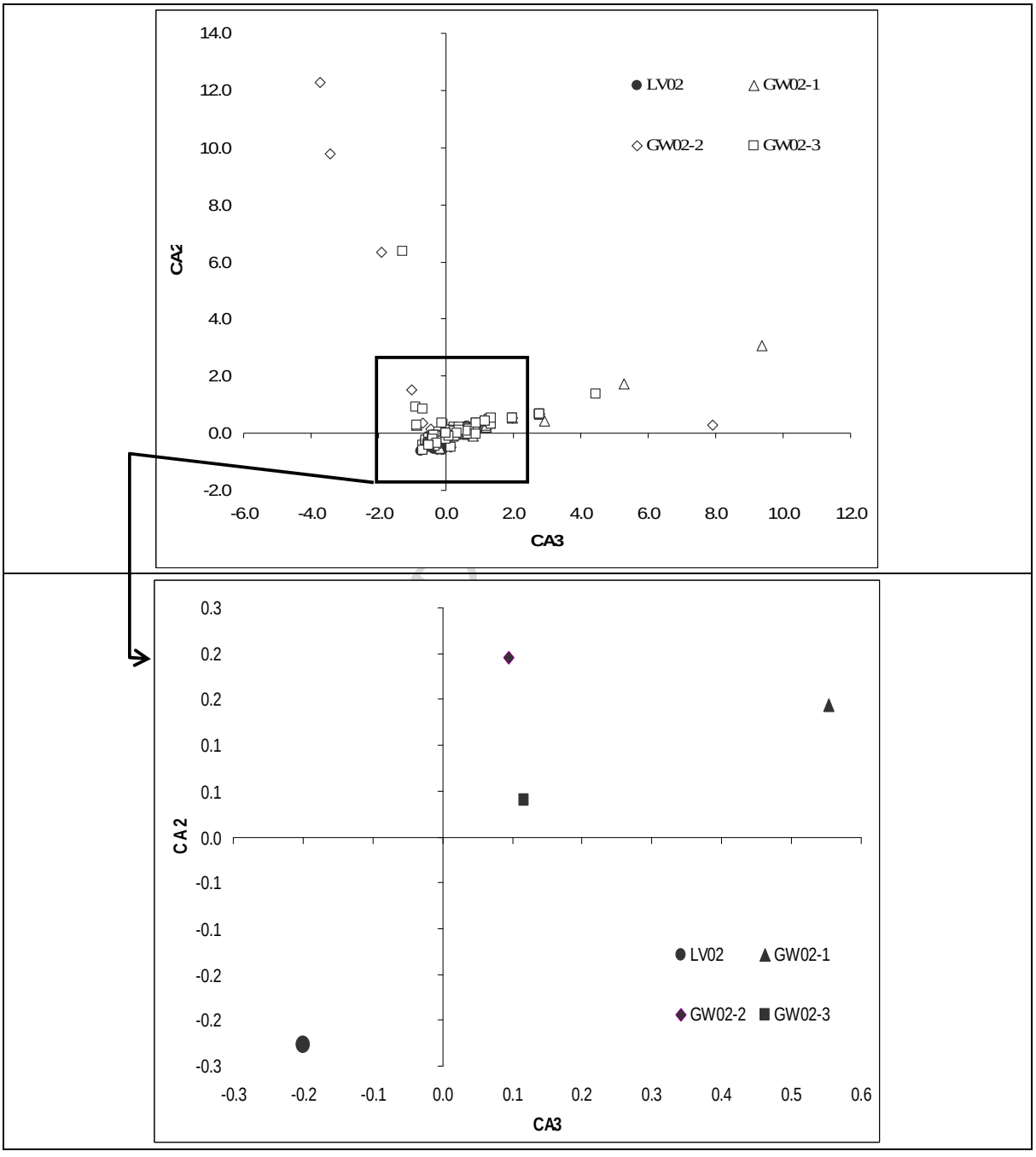

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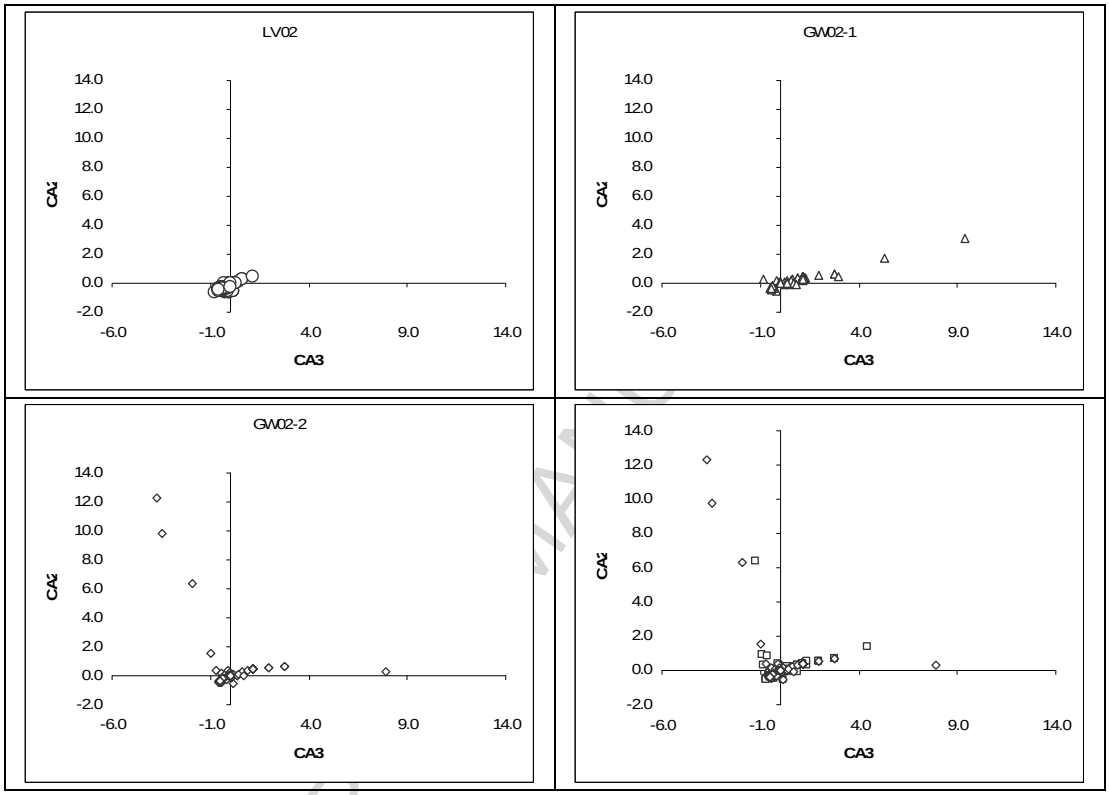

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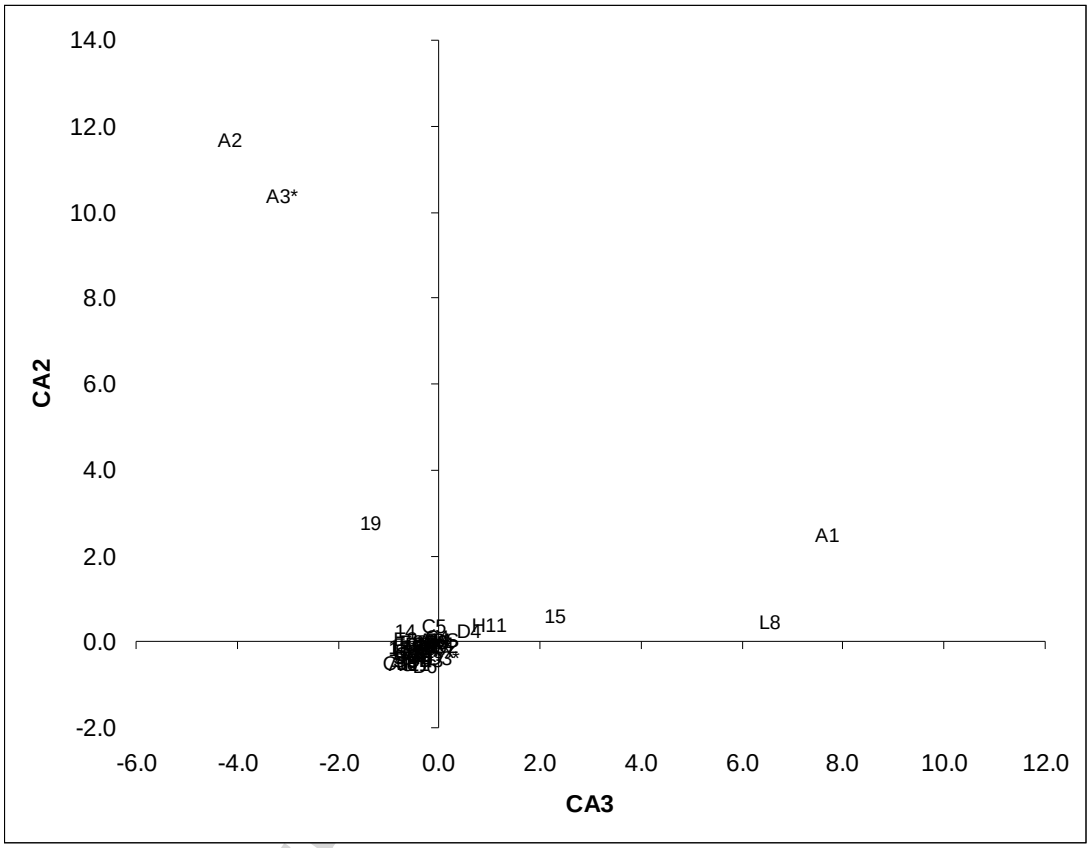
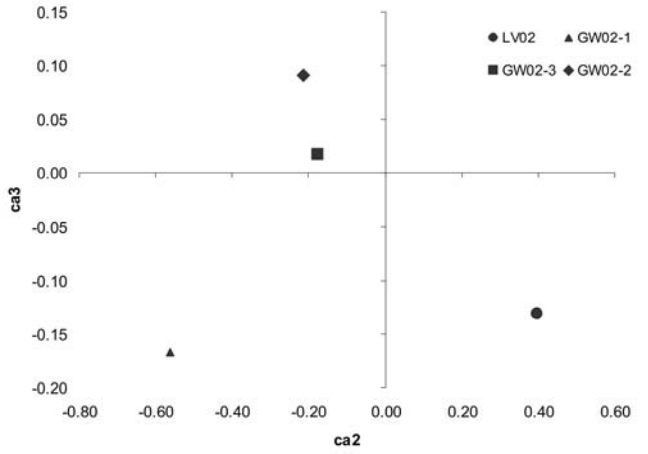


Fig 10



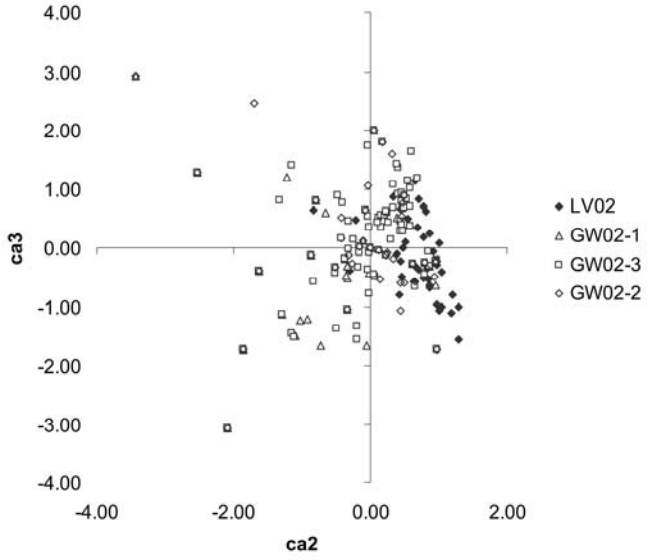


Fig 11

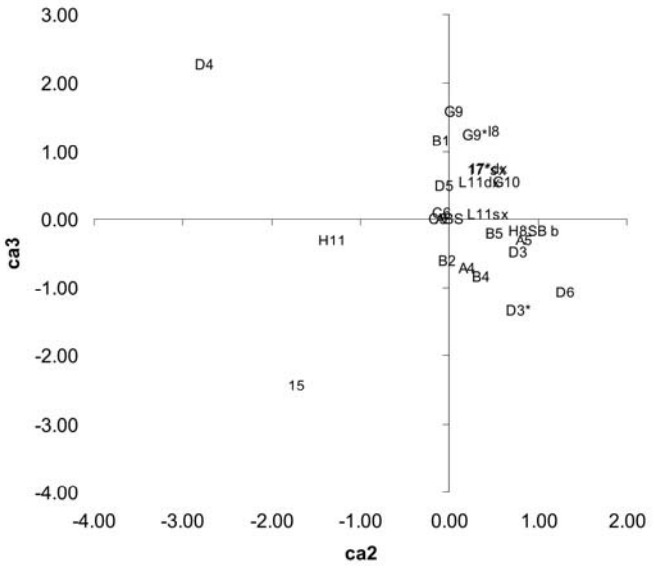


Fig. 1: Precocious anomalies in dusky grouper larvae

A: 10 DPH dusky grouper (2.8 mm TL). Normal notochord, deformed *trabecula cranii* (arrow). S: normally-inflated swim bladder. Alcian blue. Bar = 0.5mm.

B: 10 DPH dusky grouper (3 mm TL). Kyphosis (rectangular area) in the cephalic region of notochord. S: over-inflated swimbladder. Alcian blue. Bar = 0.5 mm.

C: 20 DPH (6 mm TL) dusky grouper. Over-inflated swim bladder (S) and kyphosis in the above vertebrae (region of cephalic vertebrae). At this stage, the ossifying kyphotic vertebrae show deformed vertebral bodies. The neural arches of the involved vertebrae show also abnormal inclination, particularly the anteriormost which adopt an S-shape (rectangle). Alizarin red. Bar = 1 mm.

Fig. 2: Premaxilla anomalies in dusky grouper larvae

A: 20 DPH dusky grouper (5.5 mm TL). Premaxilla (thin arrow) and nasal (large arrow) malformations. Alizarin red. Bar = 0.5mm. B: 25 DPH dusky grouper (10 mm TL). Premaxilla and nasal malformations. Alcian blue. Bar = 1 mm. C: 60 DPH dusky grouper (GW02-2; 16 ind./l) (SL: 1.9 cm; TL: 2.3 cm). Premaxilla anomaly. Bar = 1 mm.

Fig. 3: 25 DPH dusky grouper (11 mm TL). Fusion between two caudal vertebrae (box). Alizarin red. Bar = 1 mm.

Fig. 4. Different types of caudal region anomalies in 50 DPH dusky grouper.

A: (2 mm SL; 27 mm TL). Fusion between two caudal vertebrae in 50 DPH dusky grouper., as evidenced by the presence of two neural and hemal arches on the same vertebral body (rectangle). The vertebral body is deformed. Alcian blue and alizarin red. Bar = 1 mm.

B: (13 mm SL; 17 mm TL). Fusion between two caudal vertebrae (rectangle) and bone decalcifications and fractures in hypurals (1, 2, 3) and modified hemal spine (h) in 50 DPH dusky grouper.. Alcian blue and alizarin red. Bar = 1 mm.

C: (17 mm SL; 22 mm TL). Deformed bodies and fused neural arches of two caudal vertebrae in 50 DPH dusky grouper (rectangle).. Alcian blue and alizarin red. Bar = 1 mm.

Fig. 5. Different types of anomalies in 60 DPH dusky grouper.

A: GW02-1dusky grouper (8 ind./l; TL: 1.6 cm; SL: 2.1 cm). Rectangle: deformed dental. Bar = 1 mm.

B: GW02-1dusky grouper (8 ind./l; TL: 1.5 cm; SL: 2.0 cm). Arrow: prognathism of dental. Bar = 1 mm.

C: GW02-1 dusky grouper (8 ind./l; TL: 2.2 cm; SL: 2.9 cm). Arrow: malformations of pterygophores of dorsal spines . Bar = 0.5 mm.

D: GW02-1 dusky grouper (8 ind./l; TL: 1.7 cm; SL: 2.4 cm). 1-3: Deformed hemal vertebrae and fusion occurring between hemal arches of 2nd and 3rd involved vertebrae (arrow). Bar = 0.5 mm.

E: GW02-1dusky grouper (8 ind./l; TL: 1.8 cm; SL: 2.4 cm). Arrow points at a reduced dental. Bar = 1 mm.

F: GW02-3 dusky grouper (28 ind./l; TL: 1.5 cm; SL: 2 cm). Deformed dental (arrow) and kyphosis in the cranial and pre-hemal vertebrae (rectangle). Bar = 1 mm.

Fig. 6: In the graph on the left, the distribution of severe anomalies in the different body regions in 'green waters' reared groupers at different initial densities is shown. Cephalic: includes head and cranial vertebrae; Pre-hemal: pre-hemal vertebrae, pectoral and pelvic fins, predorsal bones; Hemal: hemal vertebrae, dorsal and anal fins; Caudal: caudal vertebrae and fin.

Fig. 7. CA applied on all anomalies binary matrix: ordination of all the individuals (on top) and of lot centroids (on bottom). See text for explanations.

Fig. 8. CA applied on all anomalies binary matrix: ordination of single lots is presented on different graphs.

Fig. 9. CA applied on all anomalies binary matrix: Ordination model of descriptor points (see text for explanations).

Fig. 10. CA applied on 519x26 binary matrix: ordination of all the individuals (on the top) and lot centroids (on the bottom). See text for explanations.

Fig. 11. CA applied on 519x26 binary matrix: ordination model of descriptor points (see text for explanations).

Tab. 1: Origin, age and rearing techniques of dusky grouper larvae and juveniles samples.
Density: initial density (ind/litre); DPH: days post hatching; TL: total length; SL: standard length.

Case study		Rearing techniques (year)	Density	DPH	Valid no.	code	Survival rate % (DPH)	TL_{range} (cm)	SL_{range} (cm)
Skeletal anomaly development (total n=114)		"Green waters" (2001)	8.5	10	12	**GW01**	7.9 (75 DPH)	0.2-0.4	
				15	5			0.4-0.5	
				20	5			0.5-0.6	
				25	5			1-1.1	
				30	5			1.1-1.3	0.8-1
				40	6			1.1-2.2	0.8-1.6
				50	18			1.7-2.7	1.3-2.0
			21	78	48		4.6 (75 DPH)	3.4-7.2	2.1-5.6
				92	5			3-4	2.3-3.1
				106	5			6.2-7	4.8-5.7
Density effects (total n=397)	***Rearing effects (total n=220)***	"Large volumes" (2002)	7	70	122	**LV02**	17.5 (70 DPH)	2.6-6.7	1.9-5.2
		"Green waters" at three stocking densities (2002)	8	60	98	**GW02-1**	0.2 (60 DPH)	1.9-3.4	1.4-2.6
			16	60	150	**GW02-2**	1.1 (60 DPH)	1.8-3.5	1.3-2.9
			28	60	149	**GW02-3**	0.1 (60 DPH)	1.7-3.4	1-2.7

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Tab. 2: List of the considered anomalies. Bold letters evidence heavy anomalies (affecting the external shape of larvae and juveniles). Malformation typologies indicating different modalities of the same anomaly (see, for example, 3 and 3) are shown as distinctive variables.*

REGION	A	Cephalic vertebrae (carrying epipleural ribs)
	B	Pre-hemal vertebrae (carrying epipleural and pleural ribs and with open hemal arch, without hemal spine)
	C	Hemal vertebrae (with hemal arch closed by a hemal spine)
	D	Caudal vertebrae (with hemal and neural arches closed by modified spines)
	E	Pectoral fin
	F	Anal fin
	G	Caudal fin
	H	Dorsal spines
	I	Dorsal soft rays
	L	Pelvic fin
TYPES	**S**	**Scoliosis**
	SB	**Saddle-back**
	1	**Lordosis**
	2	**Kyphosis**
	3	**Incomplete vertebral fusion**
	3*	**Complete vertebral fusion**
	4	**Malformed vertebral body**
	5	Malformed neural arch and/or spine
	5*	Extra-ossification in the neural region
	6	Malformed hemal arch and/or spine
	6*	Extra-ossification in the hemal region
	7	Deformed pleural rib
	7*	Extra-ossification of pleural ribs
	8	Malformed pterygophore (deformed, absent, fused, supernumerary)
	9	Malformed hypural (deformed, absent, fused, supernumerary)
	9*	Malformed parahypural (deformed, fused, reduced)
	10	Malformed epural (deformed, absent, fused, supernumerary)
	11	Malformed ray (deformed, absent, fused, supernumerary)
	12	**Swim-bladder anomaly**
	13	**Presence of calculi in the terminal tract of the urinary ducts**
	14	**Prognatism of dental**
	15	**Reduced dental**
	16	**Dislocation of glossohyal**
	17sx	**Deformed or reduced left opercle**
	17dx	**Deformed or reduced right opercle**
	17*sx	Deformed or reduced left branchiostegal ray
	17*dx	Deformed or reduced right branchiostegal ray
	18	Malformed predorsal bones
	19	**Malformed pre-maxillary and/or maxillary**

Tab. 3: General data on deformed individuals, incidences and typologies of skeletal anomalies observed during the dusky grouper development. Bold letters evidence some of the highest observed values. DPH: days post-hatching.

	30 DPH	**40 DPH**	**50 DPH**	**78 DPH**	**92 DPH**	**106DPH**
Observed individuals	5	6	18	48	5	5
Malformed individuals (n) [a]	1	5	18	38	5	2
Frequency of deformed individuals (%)	20.0	83.3	**100.0**	79.2	**100.0**	40.0
Number of observed anomalies	1	12	132	146	13	11
Average anomalies charge [b]	1.0	2.4	**7.3**	3.8	2.6	5.5
Number of observed anomaly typologies	1	8	15	**17**	6	8
Severe anomalies (n) [c]	1	10	9	40	5	2
Severe anomalies (%)[d]	**100.0**	83.3	6.8	27.4	38.5	18.2
Individuals with at least one severe anomaly (n)	1	5	7	22	2	2
Individuals with at least one severe anomaly (%)[e]	20.0	**83.3**	38.9	45.8	40.0	40.0
Severe anomalies charge [f]	1.0	2.0	1.3	1.8	**2.5**	1.0

DPH: days post hatching

[a] Number of individuals with at least one anomaly

[b] Number of total anomalies/number of malformed individuals

[c] Number of severe malformations

[d] Number of severe anomalies/number of total anomalies.

[e] Frequency (%) of individuals (on the total individual in each lot) with anomalies which may affect the external shape of fish (i.e. cephalic anomalies or scoliosis, kyphosis, lordosis, etc.).

[f] Number of severe anomalies/number of individuals with severe anomalies.

Tab. 4: Relative frequencies (%) of each observed anomaly in dusky grouper juveniles reared with the green waters technique (lot GW01; density for 0-30 DPH larvae: 8.5 larvae/litre; for 78-106 DPH: 21 larvae/litre). Anomalies are arranged according the body region. The highest value for each age-group is evidenced with bold figures, while square figures indicates anomalies observed only in one age-group. Bolds codes indicate severe anomalies. DPH: days post-hatching. For anomalies code see Tab. 2.

Body region	Anomaly code	30 DPH (n=5)	40 DPH (n=6)	50 DPH (n=18)	78 DPH (n=48)	92 DPH (n=5)	106 DPH (n=5)
cephalic	**A2**				0.7		
	A3*				0.7		
	A5		8.3	3	13.7		9.1
	total	*0*	*8.3*	*3.0*	*15.1*	*0*	*9.1*
pre-hemal	**B1**				0.7		
	B2			1.5	5.5		
	B3				0.7		
	B4				1.4		
	B5		16.7	**52.3**	**21.2**	**30.8**	9.1
	B5*			0.8			
	SB(b)			0.8			
	total		*16.7*	*55.4*	*29.5*	*30.8*	*9.1*
hemal	**C4**				2.7		
	C5			26.5			
	C6			2.3			
	H7				0.7		
	H8			2.3			
	I8				0.7		
	total	*0*	*0*	*31.1*	*4.1*	*0*	*0*
caudal	**D3**	**100**	8.3		7.5		
	D3*		8.3	1.5	7.5	7.7	**18.2**
	D4		**25**	3		23.1	
	D5		8.3	2.3	11	7.7	9.1
	D5*			0.8			
	D6		16.7	1.5	14.4	15.4	**18.2**
	D6*						9.1
	G9		8.3	0.8	6.2	15.4	
	G9*				4.8		**18.2**
	G10			0.8			9.1
	total	*100*	*74.9*	*10.7*	*51.4*	*69.3*	*81.9*

Tab. 5: Quality comparison between dusky grouper juveniles reared with the 'large volumes' (LV02; 7 larvae/litre) and the 'green waters' (GW02-1; 8 larvae/litre) methodologies. Bold letters evidence the highest observed values.

	LV02	**GW02-1**
Observed individuals	122	98
Malformed individuals (n) [a]	83	65
Frequency of deformed individuals (%)	**68.0**	66.3
Number of observed anomalies	244	134
Average anomalies charge[b]	**2.9**	2.1
Number of observed anomaly typologies	25	21
Severe anomalies (n) [c]	52	34
Severe anomalies (%)[d]	21.3	**25.4**
Individuals with at least one severe anomaly (n)	37	25
Individuals with at least one severe anomaly (%)[e]	**30.3**	25.5
Severe anomalies charge[f]	1.4	1.4

[a] Number of individuals with at least one anomaly

[b] Number of total anomalies/number of malformed individuals

[c] Number of severe malformations

[d] Number of severe anomalies/number of total anomalies.

[e] Frequency (%) of individuals (on the total individual in each lot) with anomalies which may affect the external shape of fish (i.e. cephalic anomalies or scoliosis, kyphosis, lordosis, etc.).

[f] Number of severe anomalies/number of individuals with severe anomalies.

Tab. 6: Relative frequencies (%) of each observed anomaly in dusky grouper juveniles reared with the 'large volumes' (LV02; 7 larvae/litre) and the green waters (GW02-1; 8 larvae/litre) methodologies. Anomalies are arranged according the body region. The highest value in each lot is evidenced with bold figures. Square highlights anomalies occurring only in one lot. Bolds codes indicate severe anomalies.

Body region	Anomaly code	LV02	GW02-1
cephalic	**14**		1.5
	15		6.7
	A1		1.5
	A4	0.4	0.7
	A5	8.2	3.0
	total	*8.6*	*13.4*
pre-hemal	**SB a**	1.2	
	B2	1.6	0.7
	B4	1.2	0.7
	B5	13.5	16.4
	SB b	5.3	
	L11sx	1.6	
	L11dx	0.8	
	L8		0.7
	18	2.9	
	total	*28.3*	*18.7*
hemal	**C4**		3.7
	C5	0.4	1.5
	C6	3.3	3.7
	F8	1.2	2.2
	H11	2.9	**29.9**
	H8	**24.6**	11.9
	I11	1.6	
	I8	2.0	
	total	*36.1*	*53.0*
caudal	**D3**	1.6	3.0
	D3*	7.4	1.5
	D4	2.5	5.2
	D5	0.4	0.7
	D6	4.1	
	G9	3.7	3.0
	G9*	0.4	1.5
	G10	7.0	
	total	*27.0*	*14.9*

Tab. 7: Quality assessment of dusky grouper juveniles reared with the "green waters" methodology at different larval densities. The highest values are highlighted with bold script. See Tab. 5 for further explanations.

	GW02-1 8 ind./l	**GW02-2** 16 ind./l	**GW02-3** 28 ind./l
Observed individuals	98	150	149
Malformed individuals (n)	65	93	113
Frequency of deformed individuals (%)	66.3	62.0	**75.8**
Number of observed anomalies	134	244	626
Average anomalies charge	2.1	2.6	**5.5**
Number of observed anomaly typologies	21	24	**38**
Severe anomalies (n)	34	43	66
Severe anomalies (%)	**25.4**	17.6	10.5
Individuals with at least one severe anomaly (n)	25	28	46
Individuals with at least one severe anomaly (%)	25.5	18.7	**30.9**
Severe anomalies charge	1.4	**1.5**	1.4

Tab. 8: Relative incidence (%) of each anomaly observed in dusky grouper juveniles reared with the "green waters" technique at different larval density. Anomalies are arranged according the body region. Bold codes indicate severe anomalies. The highest value in each lot is evidenced with bold figures. Square highlights anomalies occurring only in one lot.

Body region	Anomaly code	GW02-1 (8 ind./l)	GW02-2 (16 ind./l)	GW02-3 (28 ind./l)
Cephalic	**14**	1.5	1.2	0.2
	15	6.7	1.6	2.2
	17*sx			7.2
	17*dx			7.3
	19		1.2	0.5
	SB a		0.4	0.3
	A1	1.5		0.2
	A2		0.4	
	A3			0.2
	A3*		1.2	0.2
	A4	0.7	2.4	1.1
	A5	3.0	5.2	1.8
	total	*13.4*	*13.7*	*21.1*
pre-hemal	**SB b**		0.8	
	B1		0.4	1.4
	B2	0.7	0.8	0.6
	B4	0.7	1.6	0.8
	B5	16.4	**27.7**	**18.4**
	18			0.2
	E8sx			3.7
	E8dx			3.4
	L11sx			2.2
	L11dx			3.0
	L8	0.7	0.4	
	total	*18.7*	*31.7*	*33.7*
Hemal	**S c**			0.2
	C4	3.7	0.8	0.5
	C5	1.5	14.1	14.2
	C5*			0.2
	C6	3.7	3.6	5.3
	F8	2.2		0.3
	H11	**29.9**	12.4	7.7
	H8	11.9	8.0	3.4
	I8			1.8
	total	*53.0*	*39.0*	*33.4*
Caudal	**D3**	3.0		0.3
	D3*	1.5	0.8	0.8
	D4	5.2	3.6	1.1
	D5	0.7	0.8	1.4
	D5*			0.2
	D6			0.2
	G9	3.0	8.8	3.2
	G9*	1.5	1.6	1.4
	G10			3.2

	total	*14.9*	*15.7*	*11.8*