OBSERVATION ON SKELETAL DEFORMITY IN HATCHERY-REARED RED SPOTTED GROUPER, *Epinephelus akaara* (Temmick et Schlegel) FROM LARVAL TO JUVENILE STAGE

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ABSTRACT

Skeletal deformity is a significant problem in fish culture. The skeletal deformities in red spotted grouper from yolk-sac to juvenile stages were examined through clearing and staining of the cartilage and bone using Alcian Blue and Alizarin Red S. The overall results showed that the pattern of incidence of deformities showed an increase from preflexion to juvenile stages. The rate of deformities based on ten elements of bone from preflexion to juvenile stages were as follows: vertebral (42.6%—9.0%), dorsal proximal radials (4.8%—25.2%), neural spine (0%—8.4%), haemal spine (0%—6.8%), hypural (1.3%—5.4%), anal proximal radials (0%—5.4%), epural (1.3%—4.9%), parypural (2.0%—4.5%), lower jaw (1.3%—2.5%), and upper jaw (0%). Vertebral and dorsal proximal radials were recognized as the most susceptible parts to deformation. The main types of bone deformity were lordosis, scoliosis, fusion, shortening, branching, supernumerary elements, and saddleback syndrome. Development of saddleback syndrome was detected initially in preflexion stage, which was accompanied by deformity of the neural spines, dorsal proximal radials, and disposition of the distal radials and dorsal spines in later life stages. The skeletal deformity encountered during the larval rearing period could be caused by water surface tension.

KEYWORDS: skeletal deformity, red spotted grouper. *Epinephelus akaara*

INTRODUCTION

Bone deformity is bone that has abnormal form or feature and commonly known as skeletal deformity. The type of skeletal deformity is related to bone element that has abnormal form, such as lordosis that characterized by V shape of vertebrae. Skeletal deformity is one of the most crucial problems in fish culture, since it reduces the growth and survival of affected fishes (Hilomen-Gracia, 1997; Andrades *et al*., 1996; Barahona-Fernandes, 1982). In addition, skeletal deformities affect body form, which is associated with viability, depression of price, and lower market demand for the deformed fish (Setiadi *et al*., 2006; Loy *et al*., 1999; Koumoundouros *et al*., 1997a; Divanach *et al*., 1997; Daoulas *et al*., 1991). The causes of such deformations are poorly understood (Barahona-Fernandes, 1982), but there is some evidence to suggest that deformities can be due to pollutants (Kenedy *et al*., 2002; Spencer *et al*., 2002), genetic (Campbell, 1995), temperature (Wiegand, *et al*., 1989), vitamin (Dedi *et al*., 1995; Takeuchi *et al*., 1995; Haga *et al*., 2002), water current (Divanach *et al*., 1997), disease (Madsen & Dalsgaard, 1999), and rearing (Koumoundouros *et al*., 2001) conditions.

Various studies concerning skeletal deformation have been reported for larval up to juvenile stage of gilthead sea bream, *Sparus aurata* (Koumoundouros *et al*., 1997a,b; Boglione *et al*., 2001), sea bream, Japanese sea bass, and amberjack (Kitajima *et al*., 1994), sea

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bass, *Dicentrachus labrax* (Koumoundouros *et al*., 2002; Divanach *et al*., 1997; Marino *et al*., 1993), Japanese flounder, *Paralichthys olivaceus* (Takeuchi *et al*., 1995), striped trumpeter, *Latris lineata* (Cobcroft *et al*., 2001), and Senegal sole, *Solea senegalensis* (Gavia *et al*., 2002).

More than ten marine species of commercial importance are mass-produced in hatcheries all over Japan for culture as well as for stocking (Kitajima *et al*., 1994). The red spotted grouper, *Epinephelus akaara* is one of the most important species for mariculture. The study on *E. akaara* was focused on spawning behavior and early life history (Okumura *et al*., 2002; Ukawa *et al*., 1966), larval and juvenile development (Mito *et al*., 1967), fin differentiation and squamation (Fukuhara & Fushimi, 1988), the effects of water temperature on embryonic development (Kayano & Oda, 1991), development of mouth parts and feeding (Kayano, 1988), development of the caudal skeleton (Kusaka *et al*., 1994), and early development of the dorsal and pelvic fins (Kusaka *et al*., 2001). However, research focusing on the occurrence skeletal deformation of the red spotted grouper, *E. akaara* from yolk-sac to juvenile stages has not yet been reported. The purpose of present study was to detect bone deformity and determine which part of the skeleton was vulnerable, using clearing and staining methods.

MATERIALS AND METHODS

The experiment was conducted from July to October 2001 at the Tamano Station of the Japan Sea-Farming Association, Okayama Prefecture, Japan. Eggs were obtained from natural spawning broodstock which was reared in a concrete 50 m^3 tank. Eggs were collected from a collector using a scoop and held in a bucket prior to be transferred into a 50 L hatching tank. The newly hatched larvae were transferred using a scoop into a 6 x 6 x 1.8 m) larval rearing tank. The enriched SS-type rotifers (*Brachionus* sp.) were offered to larvae from 3 to 6 days after hatching at a density of 10-20 rotifers mL^{-1}. Thereafter enriched S-type rotifers were used from 6 to 40 days after hatching at a density of 5—10 rotifers mL^{-1}. Enrichment was carried out by dipping the rotifers in a super-capsule A-1 solution for 30 minutes. Green water (*Nannochloropsis*) was added into larval rearing tank for rotifers food at a density of 30 x 10^4 cells mL^{-1} day^{-1}. Artificial food fed from 12 to 55 days post hatch. Water exchange started on day 8 (37%) then gradually increased. Water quality variables, such as temperature, pH, DO, salinity, and natural light intensity were monitored daily. The values of the respective factors ranged as follows: 24.0°C—27.9°C, 7.46—8.14, 5.86—8.10 mg L^{-1}, 32 g L^{-1}, and 0–3,000 lux.

One-thousand-six-hundred specimens, from larval to juvenile stage, were collected from rearing tank, with sampling intervals of 0, 2, 4, 6, 8, 10, 12, 14, 20, 25, 30, 40, 45, 60, 80, and 100 days post hatch (n = 100, each). All specimens were anesthetized with MS-222, then transferred into 10% formalin solution diluted with seawater for a day, then transferred into ethanol (75%) solution. All specimens were cleared and stained using Alcian Blue and Alizarin Red S for 0 to 45 day post hatch and 60 to 100 day post hatch using Alizarin Red S only (Potthoff, 1984). They were separated according to ontogenetic stage namely; yolk-sac (TL= 1.75—2.1 mm), preflexion (2.2—5.4 mm), flexion (5.5—10.0 mm), post flexion (10.1—20 mm), transition (20.1—30.9 mm), and juvenile (31.0—88 mm) stages. The deformity of bone was classified into ten elements as follows: (1) upper jaw, (2) lower jaw, (3) neural spine, (4) haemal spine, (5) vertebra, (6) hypural, (7) parhypural, (8) epural, (9) dorsal proximal radial, and (10) anal proximal radial.

RESULTS

The general pattern showed a decreasing rate of normal individuals from yolk-sac to juvenile stage, while the rate of abnormal individuality showed an increase from yolk-sac to juvenile stage (Figure 1). The yolk-sac stage showed that the rate of normal individuals (96.1%) was clearly higher than abnormal (3.9%), but abnormal individuals were higher at preflexion, flexion, post flexion, transition, and juvenile stages; respective to stage, the percentage was 64.5%, 79.1%, 74.5%, 83.1%, and 80.5%. By contrast, the rate of normal individuals became lower from preflexion to juvenile stages; the percentage was 35.5%, 20.9%, 25.5%, 16.9%, and 19.5%, respectively.

Percentage of deformity based on ten bone elements from preflexion to juvenile stages is presented in Figure 2 A-E. Of ten elements of bone deformities examined from preflexion to juvenile stages, the rate of deformation of the vertebral region was the highest (42.6%—

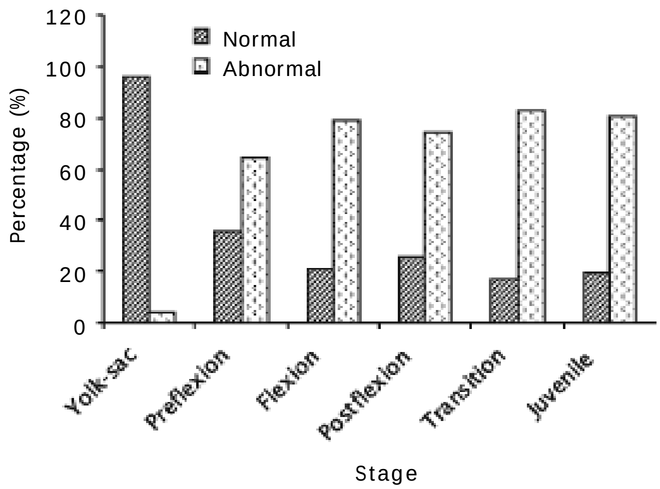


Figure 1. Percentage between normal and abnormal individuals based on developmental stages

89.0%), followed by dorsal proximal radial (4.8%—25.2%), neural spine (0%—8.4%), haemal spine (0%—6.8%), hypural (1.3%—5.4%), anal proximal radial (0%—5.4%), epural (1.3%—4.9%), parhypural (2.0%—4.5%), lower jaw (1.3%—2.5%), and upper jaw (0%).

In general, the highest incidence of deformity occurred at the flexion stage. The rate of deformities at overall elements decreased gradually in the juvenile stage, while that of the dorsal proximal radial, vertebral, neural and haemal spines elements from post flexion to juvenile stages were relatively constant.

The types of skeletal deformity encountered from yolk-sac to juvenile stage are presented in Figure 3 A-N.

The main types of bone deformities observed were as follows:

(1) *Vertebrae*. In several cases, the deformity of the posterior region of the notochord was observed, which was accompanied by the turbulence of the primordial marginal fin-fold (Figure 3A). The most cases of lordosis were found at the yolk sac stage, characterized by axial deviation in the posterior region of the notochord (Figure 3B). Lordosis was the commonest deformity of the vertebrae occurring in the caudal region, which was accompanied by a shortening and fusion of the vertebrae during later life stages. Lordosis was found in all stages, but deformity of the mid vertebral region was observed only in a few specimens. Scoliosis (Figure 3C), characterized by the lateral curvature of the vertebral column was observed rarely. Externally, scoliosis could be recognized by V-shape or S-shape of the body axis from the dorsal view. In several cases a shortened vertebral column, due to deformation of individual vertebra with a triangular shape was observed. The kind of vertebral deformity found mainly in the caudal region was fusion. It was found between vertebrae 21^{st} and 22^{nd} at the highest rate. In most cases, the fusions occurred involving four adjacent vertebrae (Figure 3D). No kyphosis was observed.

(2) *Dorsal and anal proximal radials*. The commonest deformity of the dorsal proximal radials was closely related to saddleback syndrome (SBS). Externally, SBS could easily be recognized because of the depression of dorsal body surface with a V-shape. The depression of the dorsal body was mostly found just behind the head region, which was found at the preflexion stage (ca. 2.26 mm TL) (Figure 3E). This deformity was accompanied by bone deformities of the 1^{st} to 3^{rd} neural spines, the 1^{st} to 3^{rd} dorsal proximal radials, dispositions of the 1^{st} to 3^{rd} dorsal spines and the 1^{st} to 3^{rd} distal radials in later life stage (Figure 3F). In some cases, SBS accompanied by the 4^{th} dorsal proximal

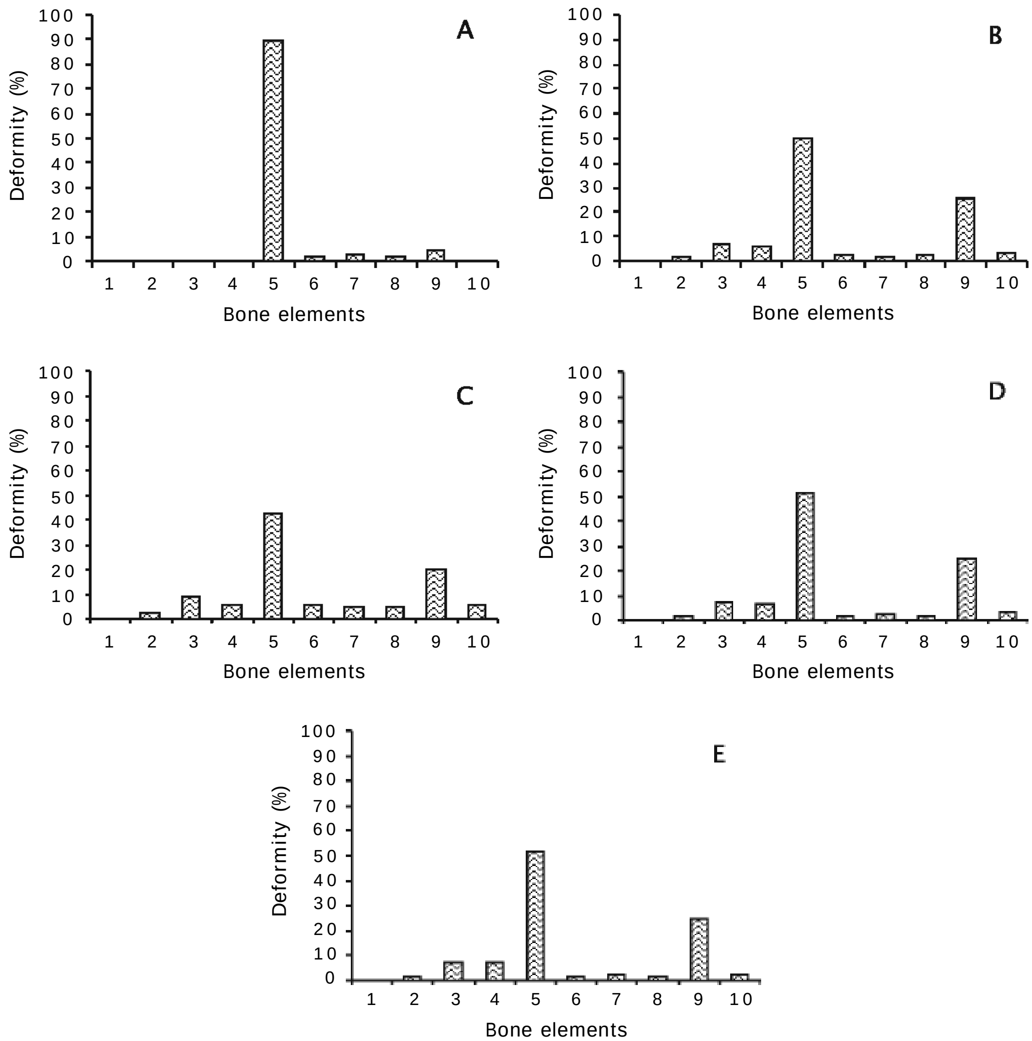
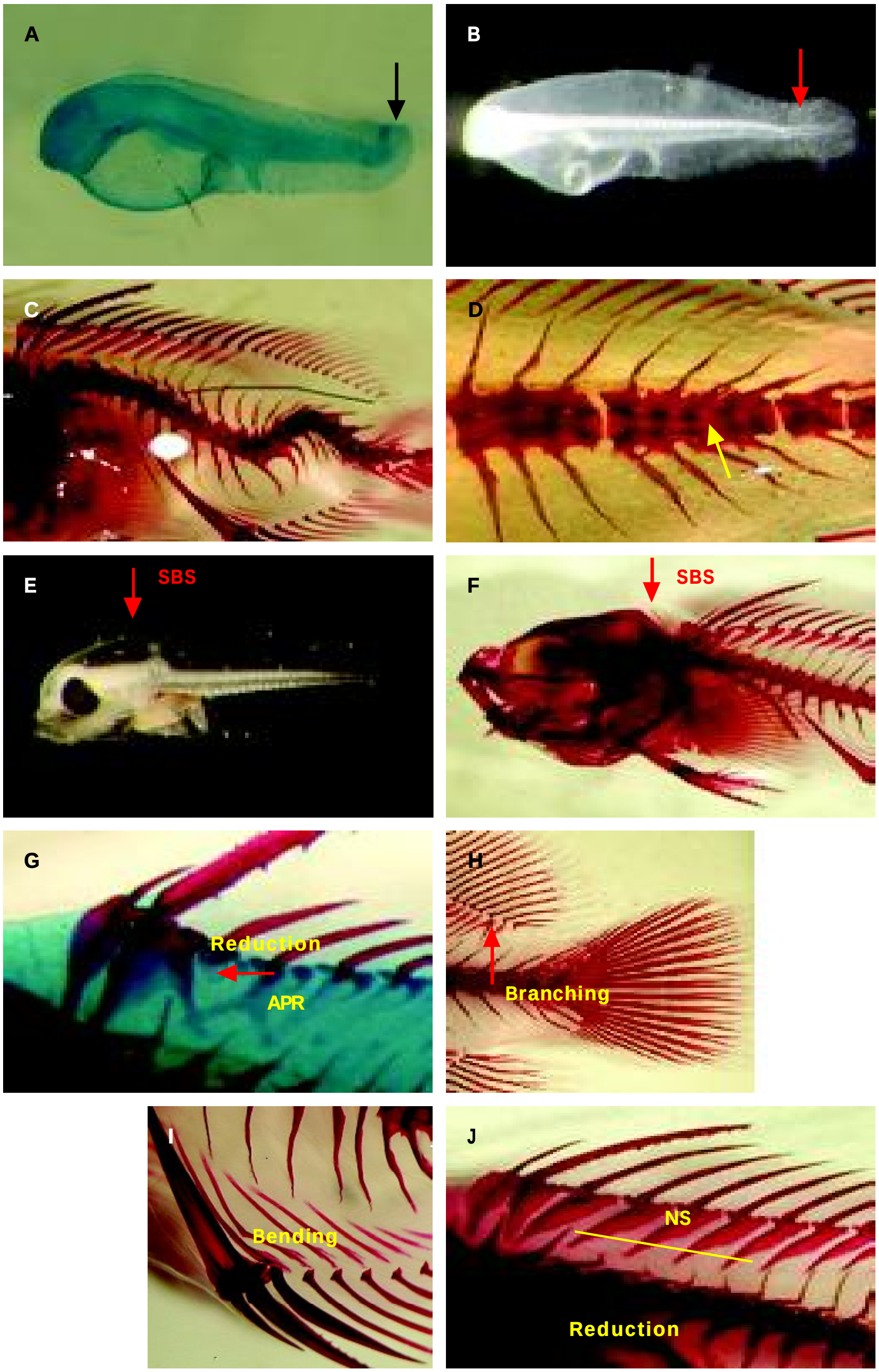


Figure 2. Percentage of deformity based on ten bone elements at preflexion (A), flexion (B), post flexion (C), transition (D), and Juvenile (E) stage

radial and spine deformities was also observed. A slight degree of saddleback syndrome in the middle part of dorsal region was also found. Other types of deformity observed at the dorsal proximal radials were fusion, branch, and shortening (Figure 3G). In a few cases, the fusion was caused by the joining of the neighboring two dorsal proximal radials due to the lack of one of the distal radial (Figure 3H). The most frequently observed deformity of the anal proximal radials was found at the 2nd anal proximal radial (Figure 3I). The branch and fusion of the anal proximal radials were observed in several cases, but no shortening of the anal proximal radial was observed. All the types of deformity observed in the dorsal and anal proximal radials appeared from the preflexion to juvenile stages and from the flexion to juvenile stages, respectively.

(3) *Upper and lower jaws.* No deformity was detected in the upper jaw. The deformity occurring in highest frequency was that of the lower jaw which was shortened. Severe deformity of shortened lower jaw was caused by a reduction in the size of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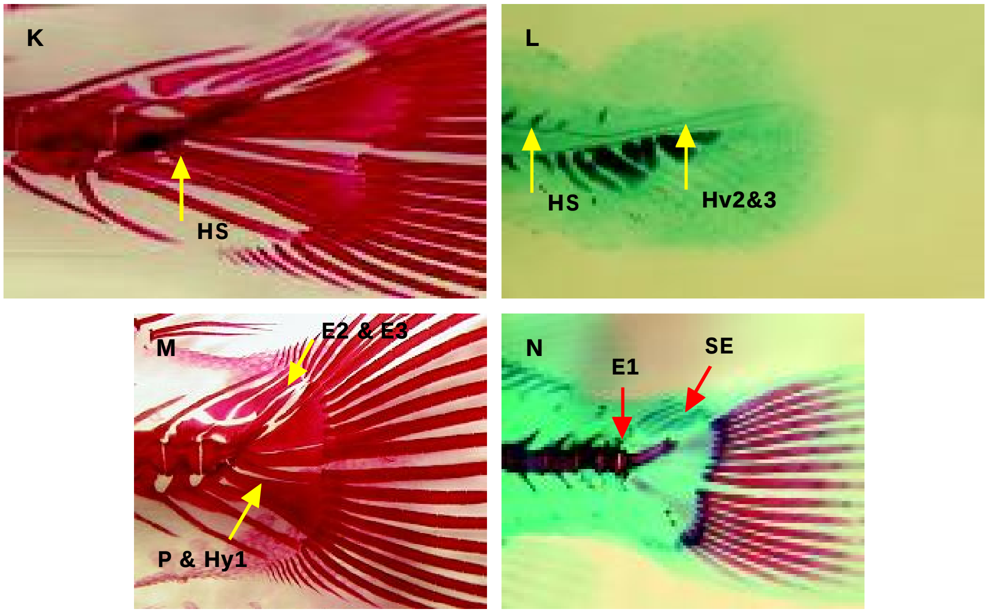

Figure 3. The types of skeletal deformities encountered from yolk-sac to juvenile stages: (A) Primordial fin-fold deformity, (B) notochord curvature (lordosis), (C) scoliosis, (D) fusion of four adjacent vertebrae, (E) saddleback syndrome (SBS), (F) saddleback syndrome (SBS) and lower jaw deformity, (G) fusion between the second and third of proximal radials, shortening of the fourth proximal radial, and branching of the fifth dorsal proximal radial, (H) fusion of two proximal radial due to lack of one of distal radial, (I) bend of the second anal proximal radial (APR), (J) reduction of neural spine (NS) from number 4 to 8, (K) bend of haemal spine (HS), (L) bend of haemal spine (HS) and fusion between the second and third hypurals (Hy2&3), (M) fusion between the second and the third epurals (E2&3), parhypural (P) and the first hypural (Hy1), and (N) E1 is the first epural, and supernumerary epural (SE)

the dentary (Figure 3F). Mild deformity of shortened lower jaws was also observed. Lower jaw deformity occurred from the flexion to juvenile stages.

(4) *Neural and haemal spines*. Deformities such as branching, shortening, and fusion in both neural and haemal spines were observed. In some cases, severe deformity of shortening of neural spines was found from the 4th to 8th vertebra (Figure 3J), and the fusion of neighboring neural spines was also observed. The commonest deformity of the haemal spine was found in the caudal region caused by its bending (Figure 3K). Deformities exemplified by shortened, fused and/or branched haemal spines were also observed in some cases. All type deformities found in either neural or haemal spines appeared from the flexion to juvenile stages.

(5) *Hypural, parhypural, and epural*. Deformation of the hypural was caused by its fusion, generally occurring between the 1st and 2nd and the fourth and fifth *hypurals*. Fusion was also found between the 2nd and 3rd ones in several cases (Figure 3L). A supernumerary hypural was rarely found between the 2nd and 3rd hypurals. The most frequently observed deformity of the parhypural was the fusion between the parhypural and first hypural (Figure 3M). In the pre-flexion stage, the parhypural and first hypural was originally fused in cartilaginous condition, with both normally conditioning separations in the flexion stage. A supernumerary parhypural were observed in a few cases. The most frequently observed deformity of the epural was the fusions between the 2nd and 3rd epurals (Figure 3M). A supernumerary

eupural was found behind the 3[rd] epural (Figure 3N). The shortening and branching of the epural were also observed. Most types of deformity found in the hypural, parhypural, and epural were not heavy and appeared from preflexion to juvenile stages.

DISCUSSION

The incidence of various types of the skeletal deformity on several bony elements in cultured fish has been reported. Shimizu & Takeuchi (2002) observed that bone deformities of blue fin tuna, *Thunnus orientalis*, occurred most frequently in the dorsal (83.8%) and anal (12.5%) pterygiophore elements. In Senegal sole, *S. senegalensis*, bone deformation was commonly found in the caudal vertebra (28%) and neural spine (11%) elements (Gavia *et al*., 2002). In hatchery-reared gilthead sea bream, *Sparus aurata*, the deformities were concentrated on the caudal complex and caudal vertebral elements and the rates of abnormal individuals were 28.9% and 19.1%, respectively (Boglione *et al*., 2001). In reared red sea bream, *P. major*, the commonest bone abnormalities were found in the dorsal proximal radial and centrum elements (Matsuoka, 1987). The results of present study, based on ten bone elements, showed that the most frequent deformities were observed mainly at the vertebral and dorsal proximal elements (Figure 2A-E). These conditions indicated that in the red spotted grouper, the caudal vertebral and dorsal proximal radial elements were most susceptible to deformations. When the results of present study are compared to other species the incidence of bone deformities in the red spotted grouper are differed from that of other species in the bone elements. This study assumes that the characteristic bony elements of the deformities observed in the red spotted grouper are probably related to its unique death in a mass at the water surface (water surface tension-related deaths). According to Yamaoka *et al*. (2000), the lateral side of the body and caudal part were frequently caught by the water surface and then the fish struggled to detach themselves from the water surface. Furthermore, they observed that while swimming they detached their dorsal fin at the water surface in many cases. These hard actions to detach themselves from the water surface surely affect the ontogenetic development of the vertebral column and dorsal proximal elements.

Jaw deformity has been observed in some cultured fish, such as sea bass, red sea bream, gilthead sea bream, striped trumpeter, and Japanese flounder (Haga *et al*., 2002; Cobcroft *et al*., 2001; Boglione *et al.,* 2001; Daoulas *et al*., 1991; Matsuoka 1984; Barahona-Fernandes, 1982). In most of these cases, the rate of jaw abnormality is very low compared to that of other bony elements. Jaw deformity in general trends to decrease in later life stages (Barahona-Fernandes, 1982). Present study indicated that jaw abnormality rate decreased in the juvenile stages, suggesting that fish with deformed jaws had an impaired ability to ingest food either in the form of live prey (rotifers) or artificial diet. Thus, the fish with deformed jaws may have a reduced survival rate. Haga *et al*. (2002) revealed that severe jaw deformity could lead to difficulties in feeding due to a masticator disorder. In addition, in reared European sea bass, *D. labrax*, the progressive reduction in the prevalence of larval mouth abnormalities from day 6 to day 40, strongly suggests their lethal character (Barahona-Fernandes, 1982).

Skeletal deformity in the caudal complex such as parhypural, hypural, and epural in red sea bream, gilthead sea bream, blue fin tuna, *Dentex dentex*, and Senegal sole have already been reported (Gavia *et al*., 2002; Koumoundouros *et al.*, 2001; Shimizu &Takeuchi, 2001; Koumoundouros *et al*., 1997a; Matsuoka, 1984). In most cases, the type of deformity was the fusion between the parhypural and the first hypural. This is in accord with results of present study, but did not find severe deformity of the caudal complex such as in gilthead sea bream, *S. aurata* and *D. dentex* described by Koumoundouros *et al*. (1997a; 2001).

Many types of saddleback syndrome (SBS) have been reported in reared tilapia, *Oreochromis aureus* (Tave *et al*., 1983), gilthead sea bream, *S. aurata* (Koumoundouros *et al*., 2001), and white sea bream (Sfakianakis *et al.*, 2003). The degree of SBS deformity was quite variable and exhibited a continuous distribution ranging from that lacking only the first spine to that lacking the dorsal fin (Koumoundouros *et al.*, 2001; Tave *et al.*, 1983). In reared white sea bream, SBS affected the dorsal fin and vertebral column, where some of the pterygiophores were fused with its underlying neural process, but SBS was not associated with the lack of dorsal fin elements

(Sfakianakis *et al*., 2003). In contrast, according to observation in this study showed that SBS was not related to the lack of the dorsal distal radials, proximal radials, and fin elements. SBS appeared before the development of dorsal proximal radial, neural spine, dorsal spine and dorsal distal radial. Thus, SBS affects the deformities of those parts. Tave *et al.* (1983) reported that SBS was related to genetic factors (heredity). In contrast, SBS has also been reported to be correlated with rearing conditions (Koumoundouros *et al.,* 2001). The observation of present study indicated that SBS occurred in the preflexion stage. Thus, genetic factor cannot be included as a caused agent of SBS. This study suggested that SBS is plausibly due to water surface attachment.

Lordosis is a common deformity of the vertebral column in many species (Divanach *et al*., 1997; Andrades *et al*., 1996; Chatain, 1994; Kitajima *et al*., 1994). In reared gilthead sea bream, lordosis was found in the yolk-sac stage in which the most frequent deformation was caused by axial deviation of the notochord due to the lack of the vertebral column (Andrades *et al*., 1996). In hatchery-reared red sea bream, Japanese sea bass, and amberjack, the abnormality of swimbladder was caused by preventing the larvae from gulping air, which was accompanied by the lordosis (Kitajima *et al*., 1994). Similar result had been provided for sea bass and sea bream both of which had an abnormal swim bladder in the early stage of development, leading to lordosis in later life stage (Chatain, 1994). However, based on observation of present study showed that lordosis in the yolk-sac stage, in which the deformity was concentrated nearly at the posterior caudal region, was not associated with swimbladder inflation. Lordosis was accompanied by shortening and fusion of the caudal vertebrae in later stages, while it occurred at the middle part of vertebral column being caused by abnormality of the swimbladder.

In the most severe case of vertebral fusion, shortening of the body has been reported (Divanach *et al*., 1997; Dedi *et al*., 1995). In reared red sea bream, the fusion was mostly observed at the posterior caudal vertebrae (Matsuoka, 1987). In reared ayu, the rate of occurrences of vertebral fusion of the posterior caudal vertebrae, mainly on the penultimate vertebra, was higher than at other locations (Komada, 1980). Gill & Fisk (1966) reported that the location of highest frequency of vertebral fusion between sockeye salmon and pink salmon was different; the highest incidence of fusion (47.0%) being observed in the caudal region in sockeye salmon, while in pink salmon (48.7%) it was found in the abdominal region. In Japanese flounder, the highest rate of the vertebral fusion occurred in the abdominal region (Takeuchi *et al.*, 1998; Takeuchi *et al*., 1995 Dedi *et al*., 1995). In red spotted grouper, the highest rate of vertebra fusion occurred primarily in the caudal region, the highest being found in the vertebra numbers 21 and 22. This study suspects that there seems to be a relationship between the specific behavior of this species and locations of deformities. Yamaoka *et al*. (2000) reported that in the pre-larval stage of red spotted grouper, the larvae swam up toward the water surface and then the larvae were trapped at the water surface by mucus on their body. Thus, even if the larvae could escape from the surface trap after hard body action, it would give the caudal part of the notochord heavy damage resulting in the occurrence of vertebral fusion in later stages.

The factors causing deformities in fishes include temperature (Polo *et al.,* 1991; Kayano & Oda, 1991; Wiegand *et al.*, 1989; Devauchelle *et al*., 1986), disease (Bucke, 1974), genetics (Campbell, 1995; Piron, 1978), nutrition (Watanabe *et al*., 1983), vitamin C and A (Madsen & Dalsgaard, 1999; Gapasin *et al*., 1998; Takeuchi *et al*., 1995; Dedi *et al*., 1995; Wimberger, 1993), pollutants (Kennedy *et al*., 2002; Spencer *et al*., 2002; McCann & Jasper, 1972) and rearing conditions (Koumoundouros *et al.*, 2001; Boglione *et al.*, 2001; Divanach *et al*., 1997; Matsuoka, 1984). Based on observations of present study could be characterized as follows; (1) deformities appeared in yolk-sac stage, (2) the rate of abnormal individuals increased in later life stages, and (3) the highest incidence of skeletal deformities occurred at the flexion stage. Considering the three characteristics, this study indicates that critical stage for deformations is from the yolk-sac to flexion stages. Water surface attachment, which occurred during early stages, might be related to such deformities.

Different factors in rearing conditions may be related to the occurrence of different types of abnormal bone formation (Matsuoka, 1984). Among four reared parental stocks of sea bass from hatching to 100 days of age, different rate of each bone deformation were observed (Barahona-Fernandes, 1982). Water temperature also acts as one factor causing deformity in the red spotted grouper as shown by Kayano & Oda (1991). However, water temperature cannot be considered as a unique causative factor for deformation in the yolk-sac stage. Although water temperature was within the optimum range of 24.0°C—27.9°C during the experiment, abnormal individuals with notochord distortions were present. This study suspected that surface attachment in larval stages can contribute to abnormalities at the yolk-sac stage. Koumoudouros *et al*. (1997) revealed that notochord distortion in the yolk-sac stage cannot be used as the only criterion for genesis of this abnormality, since handling stress in some cases can lead to artifact notochord distortions. The rate of deformity occurrence of vertebral column between water surface trapped individuals and non-trapped ones should be compared in further study.

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