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Current status of osteological malformations in offspring of Asian seabass *Lates calcarifer*, raised in a Cambodian hatchery

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Abstract — The current status of osteological malformations in the offspring of *Lates calcarifer* raised with the protocol of Cambodian hatchery operation was examined using soft X-ray radiograph and double stained specimens. The occurrence of malformations was observed in various regions of the fish body such as the lower jaw, vertebral centrums and spines. However, no significant difference was observed at the different age of reared fish (p>0.05). The cumulative rate of these malformation compared to normal offspring was 30.67±3.21%, 27.33±3.06%, 31.00±4.36% on 30th and 45th, 60th day after hatching, respectively. The degree of lower jaw malformations was classified into four types, including asymmetric, projected, knobby, and perforated with malformation rates ranging from 12.33±2.31–15.67±2.52%. In vertebrae, three types of malformations namely, compression, fusion, and curvature were identified with rates ranging from 16.33±6.08~19.67±1.52%. For vertebral spines, the types of malformations were mainly observed as supernumerary, fusion, and compression with rates ranging from 19.66±3.21–23.67±6.11%. The current status of osteological malformation with a precise categorization will be useful for clarifying possible causative factors leading to the skeletal malformations of hatchery raised offspring in the further study.

Key words: Osteological malformation, *Lates calcarifer*, Cambodian hatchery

Introduction

Osteological malformation of aquaculture fishes has been reported in various species e.g., rainbow trout *Oncorhynchus mykiss* (Madsen and Dasgaard 1999), red porgy *Pagrus pagrus* (Roo et al. 2010), African catfish *Clarias gariepinus* (Olatunji-Akioye et al. 2010), yellowtail kingfish *Seriola lalandi* (Cobcroft et al. 2004, Cobcroft and Battaglene 2013), seven-band grouper *Epinephelus septemfasciatus* (Nagano et al. 2007), turbot *Scophthalmus maximus* (Aydin et al. 2022), and Asian seabass *Lates calcarifer* (Fraser et al. 2004, Fraser and de Nys 2005, Cobcroft and Battaglene 2013). Fraser et al. (2004) observed spinal development of *L. calcarifer* larvae aging from 2 to 38 days after hatching (DAH), and described spinal malformations in the early stage of development. It is considered that osteological malformation is observed more frequently in hatchery-raised juvenile fish than in wild fish (Boglione et al. 2001, Ma et al. 2014), and it may cause stunting, mortality, and loss of offspring's value as aquaculture products, so that, it should be reduced as little as possible (Boglione et al. 2001, Cobcroft et al. 2001, Haga et al. 2003, Sawayama et al. 2012). Various causes of osteological malformation are considered e.g., low nutritional value of food, unsuitable rearing environment, and inbreeding depression (Barahona-Fernandes 1982, Koumoundouros et al. 1997, Sadler et al. 2001, Tave et al. 2011, Malekpouri et al. 2015). Osteological malformation can be observed in various regions of fish body parts such as jaws, opercula, vertebrae, and fins (Boglione et al. 2001, Fraser et al. 2004, Fraser and de Nys 2005, Tave et al, 2011), but the status and degree of those malformations are not clearly categorized and described. In addition, rearing conditions and the protocols of hatchery operation are not always adequately shown in the previous reports on the malformations in hatchery raised offspring, so that, it is not easily to infer the causes of the malformations from those reports.

Present study shows the status and degree of osteological malformations in *L. calcarifer* offspring raised in a Cambodian hatchery with a categorization of osteological malformations as a basis of the further study for the reduction of offspring's malformations in *L. calcarifer* hatcheries.

Materials and Method

Broodstock used

One female fish weighing 5 kg, and two males weighing 5 kg and 3.5 kg respectively were used as broodstock. The female was grown from a larva bred in the Marine Aquaculture Research and Development Center, Cambodia but the pedigree is unknown. The males were obtained from a net-cage farm in Koh Kong province in Cambodia. Their pedigree is also unknown. Female's maturity was examined by collecting ova from ovary with canulation before use. Male's maturation was confirmed by stripping semen and observing sperm motility under a microscope.

Spawning and egg collection

Semi-artificial breeding was conducted for obtaining fertilized eggs. Human Chorionic Gonadotropin (HCG) was injected for ovulation in the female and spermiation in the males in a rate of 500 IU/kg body weight for female and 250 IU/kg body weight in males. HCG injection was conducted on 26 June 2022 (9:00 pm), and the female and the males were immediately kept together in a circular concrete tank with a capacity of 50 tons. The spawning took place about 48 hours after injection. Fertilized eggs were collected by filtering the water overflown from the tank after about 8 hours from the starting of the spawning. Collected fertilized eggs were immediately transferred to an incubation tank. Most of the eggs hatched out by 3:00 pm of the same day.

Larval rearing

After 48 hours from hatching, larvae were transferred to three indoor circular tanks (600 L each) at a stocking density of 10 individuals/L. Photoperiod was set for 12 hours with fluorescent lights throughout the experiment. Water temperature ranged from 28 to 30°C. Salinity ranged from 29 to 30 PSU. The pH ranged from 8.0 to 8.5. Dissolved oxygen was constantly kept above 3 mg/L by aeration. A monocellular microalga, *Tetraselmis* sp. cultured in advance was introduced into the larval rearing water a density ranges from 30,000 to 50,000 cells/ml from the beginning of larval rearing for the purpose of feeding rotifers, *Brachionus rotundiformis* (S-type rotifer) which are the initial food for the fish larvae from 2nd to 20th day after hatching (DAH). Fish larvae were fed with rotifer twice a day (morning and evening) but the amount of rotifer in a tank was adjusted at a density ranging from 5 to 20 individuals/ml. Rotifers were enriched with DHA (docosahexaenoic acid) protein selco (INVE Aquaculture) for 8 hours before use. *Artemia* nauplii that were incubated in advance, were also used as a food for fish larvae from 11th to 23rd DAH. From 11th to 13th DAH, *Artemia* nauplii were not enriched, and kept the density in a range from 1 to 3 individuals/ml from 14th to 23rd DAH, *Artemia* nauplii were enriched with A1 DHA selco, and used in the density range from 3 to 7 individuals/ml. Formulated diets, Otohime (Marubeni Nisshin Feed Co., LTD.) A1 (75 to 150 μm), Otohime B2 (250 to 360 μm), Otohime S1 (610 to 1410 μm), and Otohime S2 (920 to 1800 μm) were used from the 20th DAH to the end (60th DAH) by progressively increasing the grain size along with the growth of the fish.

Volume of rearing water was 250 L initially, and 50 L was added every day until the volume filled up to 450 L in each rearing tank. Then, the exchange of water was started from 7th DAH. Water exchange rate ranging from 30–500%, was progressively increased along with the increment of fish growth and amount of feed. Cleaning of the tank bottom by siphoning was started from 6th DAH, and was carried out twice a day. Size grading of fish was carried out every 6 days to minimize cannibalism.

Sampling

Sampling was carried out on 30th DAH, 45th DAH, and 60th DAH. One hundred fish were randomly collected from each of the three tanks (totally 300 samples) on each sampling date. Samples were anesthetized with eugenol (clove oil), and fixed in 10% formalin.

Observation and analysis of osteological malformation

X-ray radiographs were taken with SOFTEX-CMB-12 for non-destructive observations of bones of fixed specimens. For detailed observations of malformations, specimens were double stained with the method of Potthoff (1984).

Statistical analyses

All the data present as rates (%) of mean ± standard deviation (SD). The average of occurrence rates of malformation of *L. calcarifer* offspring at different ages were tested by One-way ANOVA, followed by Post-Hoc-Test, Bonferroni Correction ($p<0.05$). Statistical analysis was performed by using Microsoft-Excel.

Results

Malformation rates

Malformations related to abnormal osteological development were observed in the lower jaw, vertebral centrums and spines. Observed malformations ranged from mild to severe levels with simple to multiple types. The total malformation rates at 30th DAH, 45th DAH, and 60th DAH did not show significantly difference ($p>0.05$). The average rates were 30.67±3.21%, 27.33±3.06, and 31.00±4.36%, respectively (Fig. 1).

Lower jaw malformation

Lower jaw malformation was evaluated externally and

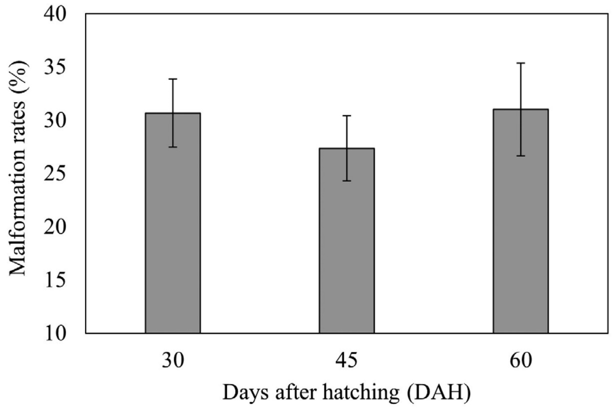

Fig. 1. Malformation rates (%) at different ages. No significant difference at different ages of reared fish ($p > 0.05$, one-way ANOVA, Post-Hoc test, Bonferroni Correction).

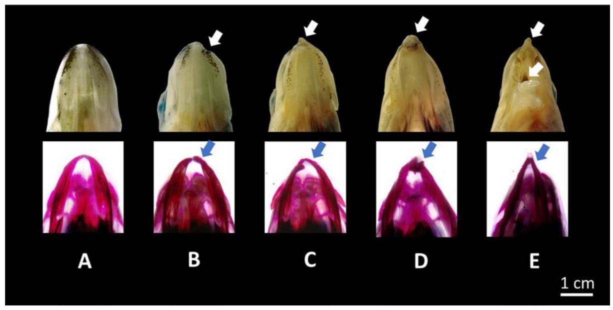

Fig. 2. Ventral views of lower jaw of *Lates calcarifer* offspring raised in the Cambodian hatchery. (A) shows a normal status. Types of lower jaw malformation are classified into four as; (B) Asymmetric, (C) Projected, (D) Knobby, (E) Perforated. The arrows indicate the positions of malformation.

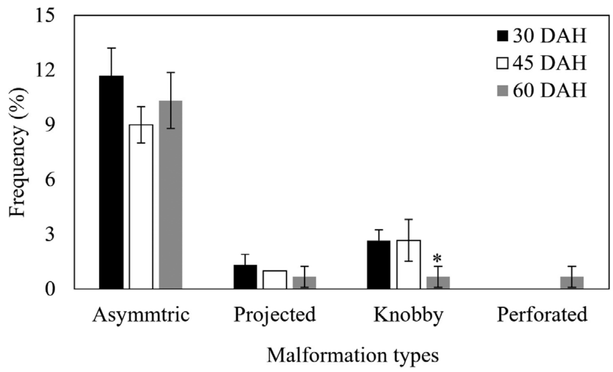

Fig. 3. Lower jaw malformation rates (%) in *Lates calcarifer* offspring raised in the Cambodian hatchery. Asterisk (*) indicates significant difference at different ages of reared fish, ($p < 0.05$, one-way ANOVA, Post-Hoc test, Bonferroni Correction).

internally under a microscope. The degrees of the lower jaw malformation were classified into four types, namely, asymmetric, projected, knobby, and perforated (Fig. 2B–E) from the ventral view. Normal specimen has a symmetric rounded outline of the lower jaw tip and well arched dentary (Fig. 2A). 'Asymmetric' has an asymmetric and often concave outline of the mandible with a dentary bone that is twisted near the symphysis on the one side (Fig. 2B). 'Projected' has a projection at the tip of the mandible which was resulted from the thinning of the dentary bone near the symphysis (Fig. 2C). 'Knobby' has an enlarged "knob" at the tip of lower jaw which was resulted from the misjoining and crossover of the dentary bone at the symphysis (Fig. 2D). 'Perforated' has a hole on the isthmus resulted from thinned dentary bone and sharpened dental arch (Fig. 2E).

The lower jaw malformation rate in the samples of 30th DAH (n=300) was 15.67±2.52% whose breakdown was i.e., 11.67±1.53% Asymmetric, 1.33±0.58% Projected, and 2.67±058% Knobby; in 45th DAH (n=300) was 12.67±1.53% whose breakdown was i.e., 9.00±0.00% Asymmetric, 1.00±0.00% Projected, and 2.67±1.15% Knobby; in 60th DAH (n=300) was 12.33±2.31% whose breakdown was 10.33±1.35% Asymmetric, 0.67±0.58% Projected, 0.67±0.58% Knobby, and 0.67±0.58% Perforated. The total lower jaw malformation rate was not significantly different at the different ages of reared fish ($p > 0.05$). Among four types of lower jaw malformation, only knobby showed a significant difference over the time changing. The significantly lowest rate of this malformation type was observed on 60th DAH (Fig. 3).

Vertebral and spinal malformations

Vertebral and spinal malformations were observed and evaluated from both soft X-ray radiographs and double stained specimens (Figs. 5 and 6). Types of vertebral malformation were classified into three conditions, namely Compression, Fusion, and Curvature. Based on microscopic examination of stained specimens 'Compression' was found to have shortened vertebrae, 'Fusion' fused vertebrae and 'Curvature' shows a bent backbone. Vertebral spine malformations were mainly three types, supernumerary (a greater number than usual), fusion, and compression in both neural spines and haemal spines.

The total vertebral malformation rates were 18.33±4.93% at 30th DAH, 19.67±1.52% at 45th DAH, and 16.33±6.08% at 60th DAH. Statistically, there was no significant difference among the different ages ($p > 0.05$). Among three types of vertebral malformations, 'Curvature' type did not show significant difference ($p > 0.05$) among the three different ages, while the 'compression' and the 'fusion' types showed significant differences among different ages ($p < 0.05$). For the compression type, the significantly highest rate was observed at 45th DAH, while fusion type was observed between 30th DAH and 60th DAH. Vertebral malformation rate in 30 DAH samples (n=300) was 18.33±4.93% including 0.67±0.58% Compression, 11.67±2.08% Fusion, and 10.33±3.21% Curvature; in 45th DAH samples (n=300) was 19.67±1.52% including 2.33±0.58% Compression,

Fig. 4. A double stained specimen of *Lates calcarifer* juvenile with normal condition of vertebrae.

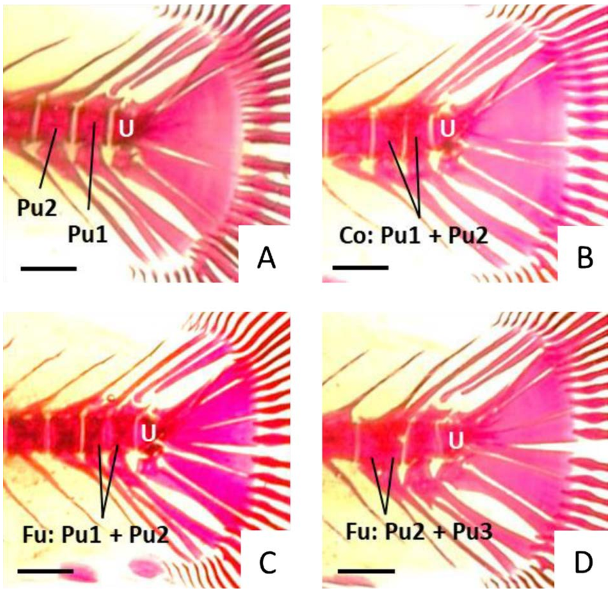

Fig. 5. Mild malformation of caudal vertebrae in *Lates calcarifer* offspring. A: Normal condition of caudal vertebrae with well-defined urostyle (U), and preural vertebra 1 and 2 (Pu1, Pu2). B: Compression (Co) of Pu1 and Pu2. C: Total fusion of Pu1 with Pu2. D: Total fusion of Pu2 with Pu3. All scale bars are 1 mm.

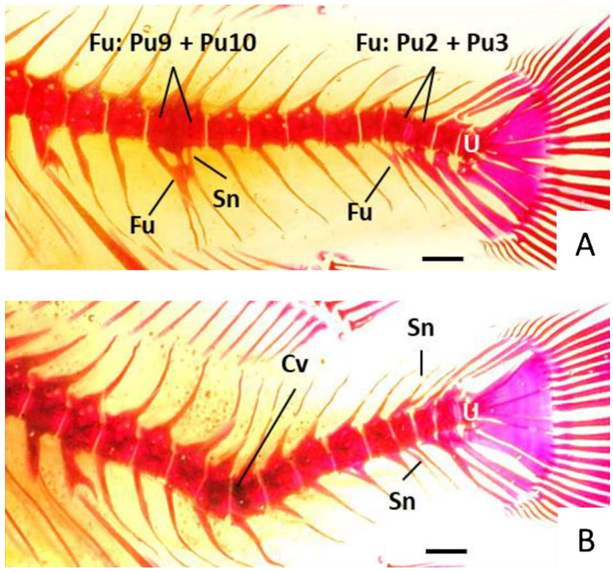

Fig. 6. Severe malformation of caudal vertebrae in *Lates calcarifer* offspring. A: Total fusion at multiple sites between Pu9-Pu10, and Pu2-Pu3. Supernumerary (Sn) of haemal spine on Pu9. B: Curvature (Cv) at the joint between Pu8 and Pu9. Supernumerary of neural and haemal spines on Pu3. All scale bars are 1 mm.

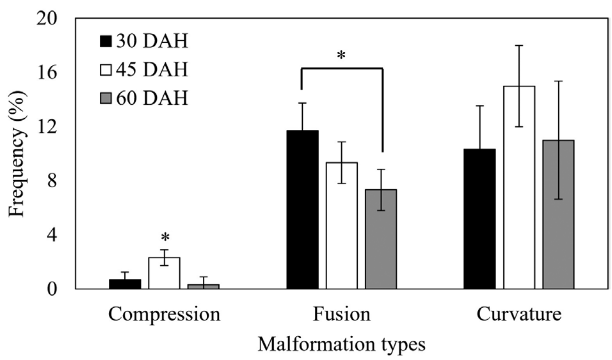

Fig. 7. Vertebral malformation rates (%) in *Lates calcarifer* offspring raised in the Cambodian hatchery Asterisk (*) indicates significant difference at different ages of reared fish ($p<0.05$, one-way ANOVA, Post-Hoc test, Bonferroni Correction).

9.33±1.53% Fusion and 15.00±3.00% Curvature; in 60th DAH samples (n=300) was 16.33±6.80% including 0.33±0.58% Compression, 7.33±1.53% Fusion, 11.00±4.36% Curvature (Fig. 7). Degree (severity) of curvature varied ranging from 177 to 140 degree in angle.

The total spinal malformation rates were 19.66±3.21% at 30th DAH, 21.67±3.51% at 45th DAH, and 23.67±6.11% at 60th DAH, and the differences among different ages were not significant ($p>0.05$). However, among six types of vertebral spines malformation, five types showed a significant difference ($p<0.05$) except Fusion of neural spines ($p>0.05$). Vertebral spine malformation rate in 30th DAH samples (n=300) was 19.66±3.21% including 6.67±1.52% neural spine fusion, 5.33±1.15% neural spine supernumerary, 10.67±1.52% haemal spine fusion, 6.33±1.52% haemal spine supernumerary; in 45-day-old samples (n=300) was 21.67±3.51% including 6.33±1.52% neural spine fusion, 5.33±2.30% neural spine supernumerary, 9.67±2.08% neural spine compression, 17.33±2.52% haemal spine fusion, 3.67±1.15% haemal spine supernumerary, 6.67±1.52% haemal spine compression; in 60-day-old samples (n=300) was 23.67±6.11% including 3.33±1.52% neural spine fusion, 0.33±0.58% neural spine supernumerary, 6.33±0.58% neural spine compression, 20.67±4.93% haemal spine fusion, 1.00±0.00% haemal spine supernumerary, 5.67±1.52% haemal spine compression (Fig. 8).

Discussion

Total malformation rate

To a greater or lesser degree, some kind of malformation

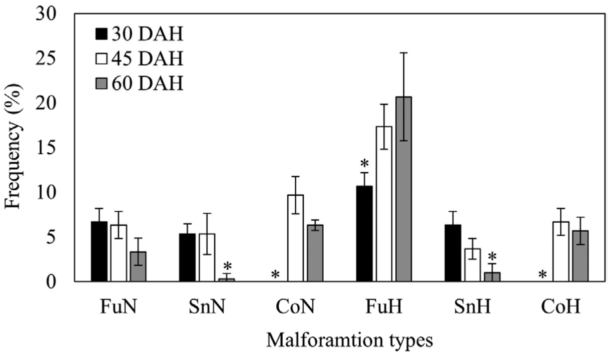

Fig. 8. Vertebral spine malformation rates (%) in *Lates calcarifer* offspring raised in the Cambodian hatchery. Asterisk (*) indicates significant difference at different ages of reared fish ($p<0.05$, one-way ANOVA, Post-Hoc test, Bonferroni Correction). Abbreviation: FuN=Fusion neural spine, SnN=Supernumerary neural spine, CoN=Compression neural spine, FuH=Fusion haemal spine, SnH=Supernumerary haemal spine, and CoH=Compression haemal spine.

was observed about 30% of the samples of three different ages in the present study. Cobcroft et al. (2004) reported the malformation rate of *S. lalandi* ranging from 6.7 to 37.9%. Fraser and de Nys (2005) reported that *L. calcarife*r had malformation rate ranging from 4.24 to 35.71%. Total malformation rate in the present study was near to the highest value in those studies. Probably, it is because they were produced from hormonized broodstock that compulsorily ovulated premature ova, and they did not grow up under the non-pressure of 'natural selection'.

Lower jaw malformation

In the breakdown of lower jaw malformation, 'Asymmetric' type was almost stably seen in each age (9.00–11.67%). So, the 'Asymmetric' type is not a lethal malformation at least by 60 DAH. In contrast, other types including 'Projected', 'Knobby' and 'Perforated' were rated low (0.00–2.67%), so that those types of malformation may affect survival of the fish in the population. In fact, individuals with those malformations were relatively smaller than normal individuals, so they can be the prey of larger individuals. Several expressions for jaw malformations such as pinched, twisted, elongated, and split have used in the literature for *L. calcarife*r (Fraser and de Nys 2005). Only four types of lower jaw malformations, namely elongated, moderate split, severe split, and pinched, were reported in hatchery-reared *L. calcarife*r, (Cobcroft and Battaglene, 2013). By comparing malformation types, only asymmetric was similar to lower jaw malformation types which reported by Fraser and de Nys (2005) and Cobcroft and Battaglene (2013), while projected, knobby, and perforated relatively were new finding in the present study.

Vertebral malformation

Malformation rates of vertebrae in the present study ranged from 16.33±6.08–19.67±1.52%, including three types i.e., 'Compression', 'Fusion', and 'Curvature'. Compression seems to be the prior stage of Fusion because fused vertebral columns are always shorter than normal columns. Vertebral curvature is a rather severe malformation, where it was found in 31 cases in 30-DAH samples concurrently only with 12 cases of Fusion; 45 cases in 45-DAH samples concurrently with 14 cases of Fusion, 33 cases in 60-DAH samples concurrently with 9 cases of Fusion. Number of cases without Fusion are much more than that of cases with fusion in three different ages. The reason of this is considered that the curvature relieves the pressure that compresses and fuses the vertebrae. Previous studies on vertebral malformation in fish used the categories of curvature common in human beings, i.e., Kyphosis, Lordosis and Scoliosis, however, those types of malformation occur only in abdominal vertebrae. In case of fish, the vertebral curvature occurs mostly in caudal vertebrae, so that, those categories were not adopted in the present study. Curvature in the present study mostly observed at the position between 8th and 10th preural vertebra, and between 1st and 3rd preural vertebra. Those positions may be the points that the bending force is acting on during larval development. Among three types of vertebral malformation in this study, fusion and curvature overlapped with the vertebral malformation types of *L. calcarifer* reported by Fraser et al. (2004) and Cobcroft and Battaglene, (2013), while compression was a new finding of vertebral malformation in this study.

Spinal malformation

Spinal malformation rates in this study ranged from 19.66 to 23.26%, including supernumerary, fusion, and compression at neural and haemal spines. Various types of vertebral and spinal deformities often co-occurred. Fraser et al. (2004) dealt hatchery raised *L. calcarifer* aging from 2 to 38 DAH, and expressed several types of spinal deformity, but was not a categorization and a definition of terminologies. Present study dealt *L. calcarifer* aged from 30 to 60 DAH, so that chronologically more advanced status than Fraser et al. (2004) was shown. Regarding to spinal malformation, Fraser et al. (2004) reported several types, namely paired heamal arch neural spine fragment, broken neural spine, and paired haemal spine in hatchery-reared *L. calcarifer*. However, only supernumerary haemal spine, observed in this study, is similar to *L. calcarifer*, reported by Fraser et al. (2004), while remained vertebral spines malformation types were new finding in the present study.

Causes of osteological malformation

Present study aimed to show the basis of studies on osteological malformation in *L. calcarifer*, and is not able to give the answer for the causes of the malformations. Previous authors considered various causes of malformations, e.g., 24-hour-lighting and white light in European seabass *Dicentrarchus labrax* (Villamizar et al.,2009), colors of rearing tank wall in striped trumpeter *Latris lineata* (Cobcroft and Battaglene 2009), high stocking density in dusky grouper *Epinephelus marginatus* (Boglione et al. 2009). Nutritional factors are also considered e.g., excessive use of vitamin A for the nutritional enrichment of rotifer to gilthead sea bream *Sparus aurata* larvae (Fernández et al., 2008); deficiency of vitamin C in *L. calcarifer* (Fraser and de Nys 2011), and deficiency of certain minerals in Atlantic salmon *Salmo salar* (Fjelldal et al. 2009), deficiency of dietary phospholipid in ayu *Plecoglossus altivelis* larvae (Kanazawa et al., 1981). Physical factors also considered e.g., unusual water flow in red sea bream *Pagrus major* (Kihara et al. 2002) and *D. labrax* (Sfakianakis et al. 2006), unusual water temperature during early development in *D. labrax* (Sfakianakis et al. 2006). Triploidy is known as a genetic factor of skeletal malformations in *S. salar* (Sadler et al. 2001).

Regarding to malformation rate, currently, no any standardized to be employed to access it in the hatcheries, since its variability is different by species and the rearing protocol of each hatchery. The severity of malformation can affect the survival rate of the fish. Generally, the batch with a high frequency of severe skeletal malformation in early stage may results with a higher mortality, and the survived larvae or juvenile at the harvesting stage may present as a lower incidence of malformation (Brorahona-Fernanses 1982). In contrast, the individual with minor affected may exhibit high survival rate the end of rearing period, associated with a higher incidence malformation (Cobcroft and Battaglene 2009). However, the lethal skeletal malformation of *L. calcarifer* in early stage currently is unclear; thus, dead fish from rearing tanks should be included.

In conclusion, the present study provides the first detail description insight into the current status of osteological malformations in the offspring of *L. calcarifer* raised using the protocol of Cambodian hatchery operations. Additionally, it contributes the precise categorization analysis of skeletal malformation. Skeletal malformation may occur due to environmental, nutritional, and genetic factors, but confirmation of the causative factor(s) is not easy to determine unless the experimental conditions are controlled. In reality, setting a good experimental system in hatcheries which have their own mandate of offspring production is not easy to do without disturbance. The current status of osteological malformation with a precise categorization will be useful for clarifying possible causative factors leading to the skeletal malformations of hatchery raised offspring in the further study.

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