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Skeletal Ontogeny and Anomalies in Larval and Juvenile Crimson Snapper, *Lutjanus erythropterus* Bloch, 1790

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DC, ZM and JGQ designed this study. DC, QY and ZM conducted the field work and analyzed the sample. DC, MMH, ZM and JGQ drafted this manuscript.

Key words
Crimson snapper *Lutjanus erythropterus*, Skeleton, Ontogeny, Ossification, Malformations.

ABSTRACT

Skeletal anomalies in farmed fish affect animal welfare and economic return in aquaculture but very limited information exists on skeletal ontogeny and anomalies among species of the family Lutjanidae. This study describes the skeletal ontogeny and anomalies of crimson snapper *Lutjanus erythropterus* larvae and juveniles from hatching to 36 day-post hatching (DPH). Mandible, ceratobranchial, cleithrum and gill arches were the initial skeletal structures appeared at 3 DPH that supported the vital life functions such as feeding and respiration. Ossification of premaxilla and maxilla and dentary started at 3.21 ± 0.25 mm (9 DPH), and completed at 5.91 ± 0.34 mm (18 DPH). The head skeleton formation completed at 22.35 ± 2.26 mm (31 DPH). The axial skeleton development started with the formation of neural arches at 3.64 ± 0.07 mm (10 DPH) and ossification of axial skeleton completed at 11.01 ± 0.88 mm (24 DPH). The fins developed sequentially and the ossification of fins completed at 30.57 ± 2.44 mm (36 DPH). A total of 39.5% fish exhibited anomalies in the present study and the anomalies were: lordosis, vertebral fusion, neural spines bifurcation, connection of adjacent pterygiophores, haemal spine anomaly, neural spines anomaly, anomaly in pterygiophores and supernumerary neural spines. Results from this study add new knowledge to functional morphology of crimson snapper that would be useful to larval aquaculture of marine teleosts.

INTRODUCTION

Skeletal abnormality of farmed fishes is a major drawback in aquaculture. Fish with abnormal mouth, vertebrate or fin shows low feeding or swimming performance, and disease susceptibility (Wittenrich *et al*., 2009; Isaac *et al.,* 2017). Skeletal anomalies cause significant problems for mechanical fish filleting as machines are designed for normal shaped fishes, thus require extra trimming and manual handing. Abnormal fishes intrinsically represent low market value than normal shaped fishes. Some farmers and traders cull out abnormal shaped fishes during marketing to maintain reputation of their business. The economic loss due to body deformity of farmed fish in the European aquaculture industry is estimated to be over €50,000,000 (Boglione *et al*., 2013a).

Since the first publication on body shape anomaly of rainbow trout in 1971 (Aulstad and Kittelsen, 1971), significant progress has been achieved in development of skeletal biology in fish (Boglione *et al*., 2013a, b; Babbucci *et al*., 2016; Azevedo *et al*., 2016). Skeletal anomalies (such as jaw malformation, skull malformation, spine malformation) have been described in many farmed fish species, including European sea bass *Dicentrarchus labrax* (Abdel *et al*., 2004; Georgakopoulou *et al*., 2007), gilthead sea bream *Sparus aurata* (Andrades *et al*., 1996; Georgakopoulou *et al*., 2010), Senegal sole *Solea senegalensis* (Gavaia *et al*., 2002), red sea bream (Kihara *et al*., 2002), yellowtail kingfish *Seriola lalandi* (Cobcroft *et al*., 2004), and golden pompano *Trachinotus ovatus* (Zheng *et al*., 2014). However, very limited information exists on skeletal development and anomalies in the species of the family Lutjanidae except the study on skeletal ontogeny of red snapper (Potthoff *et al*., 1988). A survey in Australian finfish hatcheries has identified skeletal anomalies among snappers and warranted species-

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specific research (Cobcroft and Battaglene, 2013).

The types of skeletal anomalies vary among species, life stages and rearing conditions (Andrades *et al*., 1996; Boglione *et al*., 2013b). The onset of anomalies mostly occurs at the larval and early juvenile stage (Boglione *et al*., 2013a; Estivals *et al*., 2015; Huang *et al*., 2016). In addition, the types of anomalies are extremely diverse and many typologies are difficult to define at the onset of anomalies (Boglione *et al*., 2013b). Setting a reference point for normal skeletal developments is an important step to recognize early stages of skeletal anomalies. Since no information exists on the skeletal ontogeny and anomalies of crimson snappers, the descriptions of skeletal development would minimize this knowledge gap.

According to FishBase (http://www.fishbase.org), a total of 67 species of snappers are distributed in tropical and subtropical oceans, and many species are important aquaculture candidate. The crimson snapper *Lutjanus erythropterus*, distributed in the tropical Indo-Pacific (from Gulf of Oman to Southeast Asia, northward to southern Japan and southward to northern Australia), is an important aquaculture candidate in Asia and Australia. Skeletal ontogeny and anomalies provide information on the functional morphology of a species that is important for basic biological perspective and larval aquaculture. Therefore, the objective of this study is to describe the skeletal ontogeny and anomalies of crimson snapper.

MATERIALS AND METHODS

Fishes were maintained according to the recommendation of Chinese Academy of Fishery Sciences Animal Welfare Committee. The protocol, species and number of animals used in this study were approved by the South China Sea Fisheries Research Institute Animal Welfare Committee (Approved Number: 2014YJ01).

Larvae acquisition and rearing

Fertilized eggs were received from Shenzhen Longqizhuang Aquaculture Hatchery, Guangdong Province, P.R. China, and were transported to South China Sea Fisheries Research Institute. Upon arrival, the eggs were hatched in 500-L fiberglass incubators at 27.5°C. On 2 days post-hatch (DPH), larvae were reared into three 2500-L tanks at a density of 60 fish L^{-1}. Each rearing tank was supplied with filtered seawater (5-μm pores) with a daily water exchange rate of 200% of the tank volume. Daily photoperiod of 14 h light and 10 h dark with 2000 lux light intensity was maintained at the water surface. The water temperature and salinity was maintained at 29.0 ± 1.0°C and 33 ± 0.8 ppt, respectively.

Fish were fed with rotifers (*Brachionus rotundiformis*) from 2 DPH to 10 DPH at a density of 10-20 rotifers mL^{-1}. On 9 DPH, *Artemia* nauplii were introduced at 0.1 nauplii mL^{-1}, and increased daily 5 nauplii mL^{-1} until 18 DPH. Afterwards, *Artemia* nauplii were gradually replaced from 19 DPH by inert diets. Fish were fed with inert diets Otohime A1 (~250 μm, Marubeni Nisshin Feed Co. Ltd., Tokyo, Japan) and Huacheng No.5 (850-1100 μm, Haikang Aquatic Biotechnology Co. Ltd., Yantai, China), and the amount of feed was adjusted to apparent satiation. Rotifers and *Artemia* nauplii were enriched with the DHA protein Selco (INVE Aquaculture, Salt Lake City, UT, USA) before adding into the larval rearing tanks. Fish age specific feeding protocols are illustrated in Figure 1.

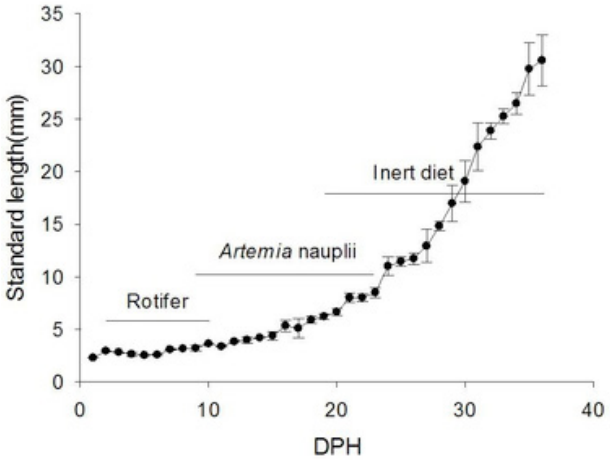

Fig. 1. Standard length of crimson snapper reared under different feeding protocol from 1 to 36 day post hatch (DPH). Feeding protocol was presented above the growth curve.

Staining and visualization

A total of 20 larvae were collected from rearing tanks daily and anaesthetized with Aqui-S (AQUI-S New Zealand Ltd., Lower Hutt, New Zealand). The larvae were initially fixed in 10% neutral buffered formalin, and then stained with alcian blue and alizarin red followed by Taylor and Van Dyke (1985). After staining, samples were photographed under stereomicroscope (Olympus SZ40) equipped with a digital camera (Oneplus A2001). The terminologies of the skeletal elements were adopted from Kihara *et al*. (2002) and Sfakuanakis *et al*. (2004). A total of 450 larvae and juveniles were examined to identify skeletal anomalies. The incidence of anomalies was calculated using the following equation: Incidence of anomalies = (number of larvae with skeletal anomaly/total number of larvae) ×100%.

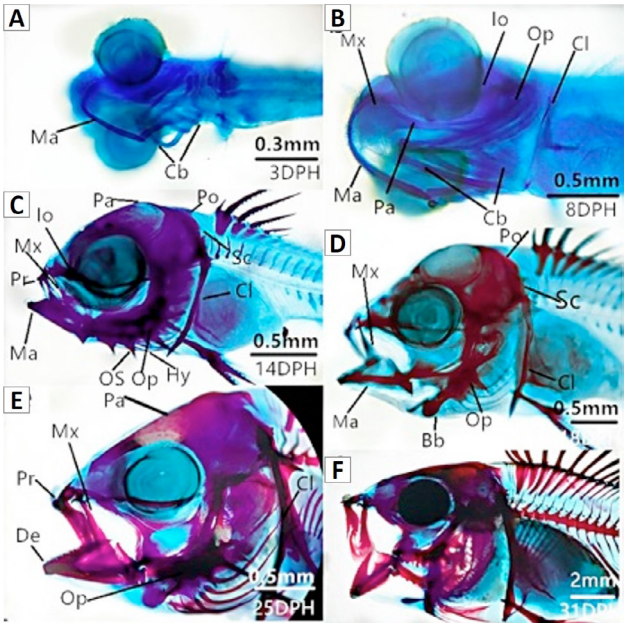

Fig. 2. Formation and ossification of the skull in larval crimson snapper. **A**, Larvae on 3 DPH show developing mandible (Ma) and ceratobranchial (Cb); **B**, Larvae on 8 DPH show developing maxillary (Mx), cleithrum (Cl), operculum (Op), parietal (Pa), infraorbital (Io) and cartilaginous mandible and ceratobranchial; **C**, Larvae on 14 DPH show developing premaxillary (Pr), posttemporal (Po), supracleithrum (Sc), hyoid (Hy), opercular spine (OS) and cartilaginous maxillary, parietal, infraobital, cleithrum; **D**, Larvae on 18 DPH show cartilaginous basibranchial and ossification of mandible, operculum, cleithrum; **E**, Larvae on 25 DPH show ossification of dentary, maxillary, parietal; **F**, Larvae on 31 DPH show ossification of neurocranium.

RESULTS

Head skeleton

In crimson snapper larvae, the ceratobranchial, basibranchial and hypobranchial cartilages were attached to the gill arch. The first four gill arches formed at 2.83 ± 0.01 mm (standard length ± SD, 3 DPH, Fig. 2A). In front of the anterior end of the hypobranchial cartilage, the hypohyal cartilage formed, and symmetrically connected to a pair of ceratohyal-epihyal cartilage. The Meckel's cartilage stretched out forward, surpassing the ventral area of the eyes. The head of quadrate cartilages bound to the Meckel's cartilage and the hyomandibular-sympletic cartilage. The neurocranium, trabeculae cartilage and ethmoid cartilage formed at this stage (Fig. 2B). The tranvecula cartilages, connecting with the posterior end of the ethmoid cartilages, stretched through the midcourt line of the two eyes. Figure 2B, C, D and E shows formation of different jaw elements. Ossification of jaw elements such as premaxilla and maxilla and dentary started at 3.21 ± 0.25 mm (9 DPH), and completed at 5.91 ± 0.34 mm (18 DPH, Fig. 2D). The skull formation completed at 22.35 ± 2.26 mm (31 DPH, Fig. 2F).

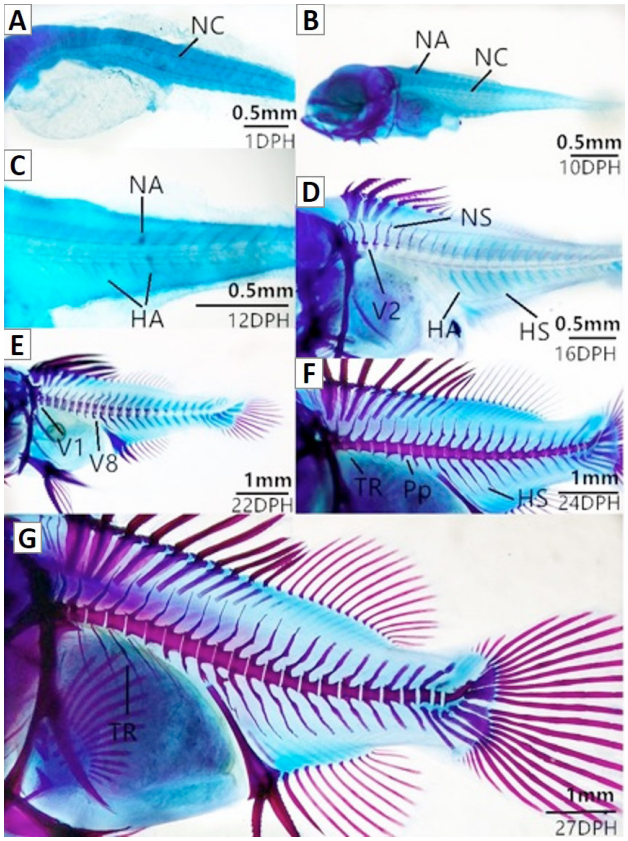

Fig. 3. Formation and ossification of vertebral column in larval crimson snapper. **A**, Larvae on 1 DPH show developing notochord (NC); **B**, Larvae on 10 DPH show developing neural arch (NA); **C**, Larvae on 12 DPH show developing haemal arch (HA); **D**, Larvae on 16 DPH show developing neural spine, vertebral centra, 1st vertebra to 5th vertebra (V1 to V5) and haemal spine (HS); **E**, Larvae on 22 DPH show cartilaginous of vertebra centra, (V1 to V16). F) Larvae on 24 DPH show developing parapophysis (Pp), thoracic rib (TR) and ossification of vertebral centra, haemal spine; **F**, Larvae on 27 DPH show ossification of vertebral column.

Vertebral column and fins

The axial skeleton consisted of the vertebrae and epineurals, and the median fin supports, including the proximal and distal pterygiophores. Notochord was the only axial structure at the lengths between 2.23 mm to 3.21 mm (1-9 DPH, Fig. 3A). The neural arches were the first element of the vertebral column that developed at

3.64 ± 0.07 mm (10 DPH, Fig. 3B). The haemal arches developed subsequently at 3.78 ± 0.08 mm (11-12 DPH, Fig. 3C). The buds elongated ventrally and joined together forming the haemal arch and then the spine appeared and elongated ventrally. The neural arches to ossified at 5.28 ± 0.57 mm (15 DPH), and the haemal arches ossified at 6.66 ± 0.31mm (20 DPH). Ossifications of neural arches, neural spines, haemal arches and haemal spines completed at 11.01 ± 0.88 mm (24 DPH, Fig. 3F).

Vertebral column developed at 5.35 ± 0.67 mm (16 DPH, Fig. 3D). Vertebral centra (V1-V8) ossified at 6.66 ± 0.31 mm (20 DPH), and the ossification of centrum proceeded from cephalic to caudal region (Fig. 3E). Ossification of vertebral centra completed at 12.93 ± 1.58 mm (27 DPH, Fig. 3G). Parapophysies, the process of plural rib formation, started at 11.01 ± 0.88 mm (24 DPH, Fig. 3F) with the formation of first thoracic rib. The ossification of thoracic ribs completed between 12.92 mm to 19.09 mm (27-30 DPH).

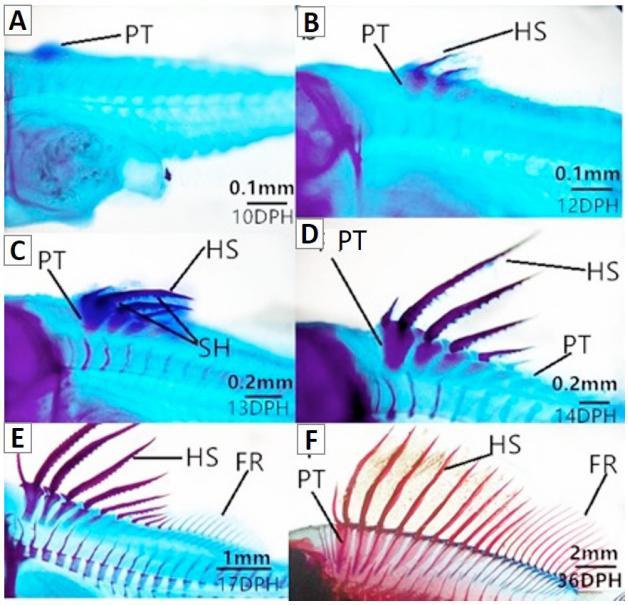

Fig. 4. Formation and ossification of dorsal fins in larval crimson snapper. **A** and **B**, Larvae on 10 DPH show developing dorsal pterygiophores (PT); **C**, Larvae on 12 DPH show developing hard spine (HS) and cartilaginous of pterygiophores; **D**, Larvae on 13 DPH show developing sharp hooks (SH); **E**, Larvae on 14 DPH show first five hard spine and cartilaginous 6th spine; **F**, Larvae on 17 DPH show ossification of fin rays and pterygiphores.

The formation of anterior dorsal pterygiophores started at 3.64 ± 0.07 mm (10 DPH, Fig. 4A) toward the caudal end. The spines of anterior dorsal pterygiophores formed at 3.84 ± 0.11 mm (12 DPH, Fig. 4B). Sharp hooks of the dorsal fin formed at 3.97 ± 0.13 mm (13 DPH, Fig. 4C). First five hard spines and 6th cartilaginous spine developed at 4.13 ± 0.12 mm (14 DPH, Fig. 4D). Ossification of anterior dorsal pterygiophores completed at 5.12 ± 0.92 mm (17 DPH, Fig. 4E). The dorsal fin rays developed at 5.34 ± 0.96 mm (16 DPH). The dorsal pterygiophores, hard spines and fin rays ossified completely between 26.48 and 30.7 mm (34-36 DPH, Fig. 4F).

Cleithrum in pectoral fin started to develop at 2.62 ± 0.09 mm (3 DPH, Fig. 5A), and clearly visible at 3.25 ± 0.12 (10 DPH, Fig. 5B). Pectoral fin plate and the fin rays developed at 6.22 ± 0.28 mm (19 DPH, Fig. 5C). The proximal pterygiophore developed at 11.45 ± 0.42 mm (25 DPH, Fig. 5D). The coracoid, scapula, proximal pterygiophore developed at 22.48 ± 0.94 mm (32 DPH, Fig 5E). Pectoral fin rays started to ossify at 11.01± 0.88 mm (24 DPH), and ossification completed at 30.47 ± 2.14 mm (36 DPH, Fig. 5F).

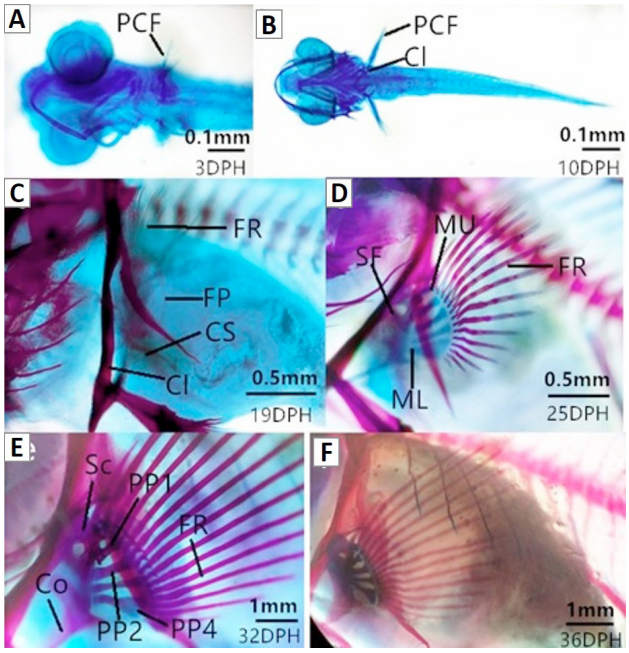

Fig. 5. Formation and ossification of pectoral fins in larval crimson snapper. **A**, Larvae on 3 DPH show developing pectoral fins (PCF); **B**, Larvae on 10 DPH show pectoral fins and cleithrum; **C**, Larvae on 19 DPH show developing fin rays (FR), fin plate (FP), coracoid-scapula (CS) and ossification of cleithrum; **D**, Larvae on 25 DPH show developing scapular foramen (SF), metacleithrum lower (ML), metacleithrum upper (MU) and ossification of fin rays; **E**, Larvae on 32 DPH show cartilaginous of coracoid (Co), scapula (Sc), proximal pterygiophore (PP), 1st proximal pterygiophore to 4th proximal pterygiophore (PP1-PP4); **F**, Larvae on 36 DPH show ossification of pectoral fins.

The basipterygium appeared at 3.61 to 3.64 mm (9-11 DPH) as a small cartilaginous element that gradually elongated to 3.84 mm (11-12 DPH, Fig. 6A, B). The hard spine developed at 3.99 ± 0.28 mm (13 DPH, Fig. 6C). Pelvic fin rays developed at 5.91 ± 0.34 mm (18 DPH, Fig. 6D). The hard spines ossified at 11.01 ± 0.88 mm (24 DPH, Fig. 6E), and the fin rays ossified at 30.57 ± 2.44 mm (36 DPH, Fig. 6F).

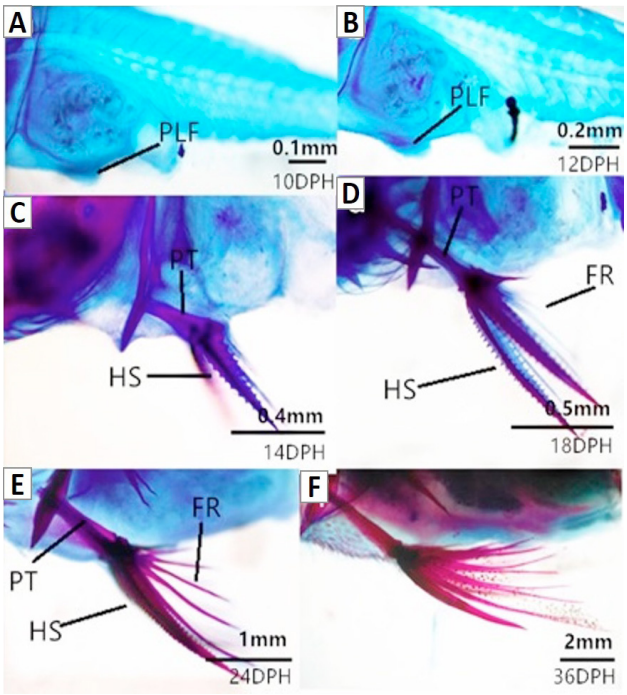

Fig. 6. Formation and ossification of pelvic fins in larval crimson snapper. **A** and **B**, Larvae on 10-12 DPH show developing pelvic fins (PLF); **C**, Larvae on 14 DPH show cartilaginous of pterygiophores (PT), hard spine (HS) and sharp hooks; **D**, Larvae on 18 DPH show developing fin rays (FR); **E**, Larvae on 24 DPH show ossification of pterygiophores and hard fins; **F**, Larvae on 36 DPH show ossification of pelvic fins.

The anal proximal pterygiophore developed as small cartilages between 3.99 mm to 4.02 mm (13-14 DPH, Fig. 7A, B). Subsequently, a hard spine formed before anal fin rays between 5.12 mm to 5.35 mm (15-16 DPH) from the cephalic to caudal direction (Fig. 7C). Pterygiophores, hard spine and fin rays started to ossify at 11.01 ± 0.88 mm (22-24 DPH, Fig. 7D, E) and ossification completed at 26.48 ± 1.04 mm (34 DPH, Fig. 7F).

Formation and ossification of caudal complex

The caudal complex in crimson snapper consisted of the epurals, hypurals, preural arches, modified spines, urostyle, preural centra and caudal fin rays.

Hypurals 1-3 developed first as a small cartilage at 4.20 ± 0.01 mm (14 DPH, Fig. 8A). Afterwards hypurals 3-5, the neural spines, haemal, epural and caudal fin rays developed between 5.34 mm to 5.91 mm (16-18 DPH, Fig. 8B, C). Upward flexion of the urostyle developed at 11.45 ± 0.42 mm (25 DPH, Fig. 8D). The urostyle and fin rays ossified between 18.35-21.21 mm (29-30 DPH, Fig. 8E, F). The hypurals, modified neural spines and modified haemal spines ossified at 26.48 ± 1.04 mm (34 DPH, Fig. 8G). Except epural, ossification of the caudal complex completed at 30.57 ± 2.44 mm (36 DPH, Fig. 8H).

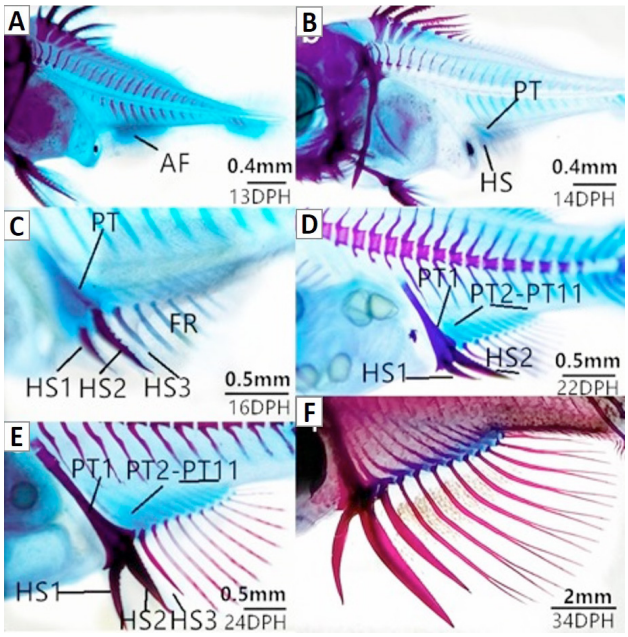

Fig. 7. Formation and ossification of anal fins in larval crimson snapper. **A**, Larvae on 13 DPH show developing anal fins (AF); **B**, Larvae on 14 DPH show developing pterygiophores (PT) and hard spine (HS); **C**, Larvae on 16 DPH show cartilaginous of pterygiophores, three hard spine and fin rays; **D**, Larvae at 22 DPH show 2nd to 11th pterygiophores; **E**, Larvae on 24 DPH show ossification of pterygiophores, hard spine and fin rays; **F**, Larvae on 34 DPH show ossification of anal fins.

Skeletal anomalies

In the present study, skeletal anomalies were observed from the yolk sac stage to the juvenile stage. At 36 DPH, a total of 39.5% juvenile had anomalies of which 12.5% had one type of anomaly while 27% had multiple anomalies. The rate of each type of anomaly was: lordosis (10.5%) characterized by a V-shaped dorsoventral curvature of the vertebral trunk (Fig. 9A, B), vertebral fusion (2%, Fig. 9D), bifurcated neural spines (1%, Fig. 9E), connection of

adjacent pterygiophores (4%, Fig. 9A), malformed haemal spines (32%, Fig. 9C), malformed neural spines (32%, Fig. 9B), malformed pterygiophores (2%, Fig. 9C) and supernumerary neural spines (6%, Fig. 9F). However, no anomalies observed in the skull, dorsal fins, pectoral fins, pelvic fins and anal fins during larval development up to 36 DPH.

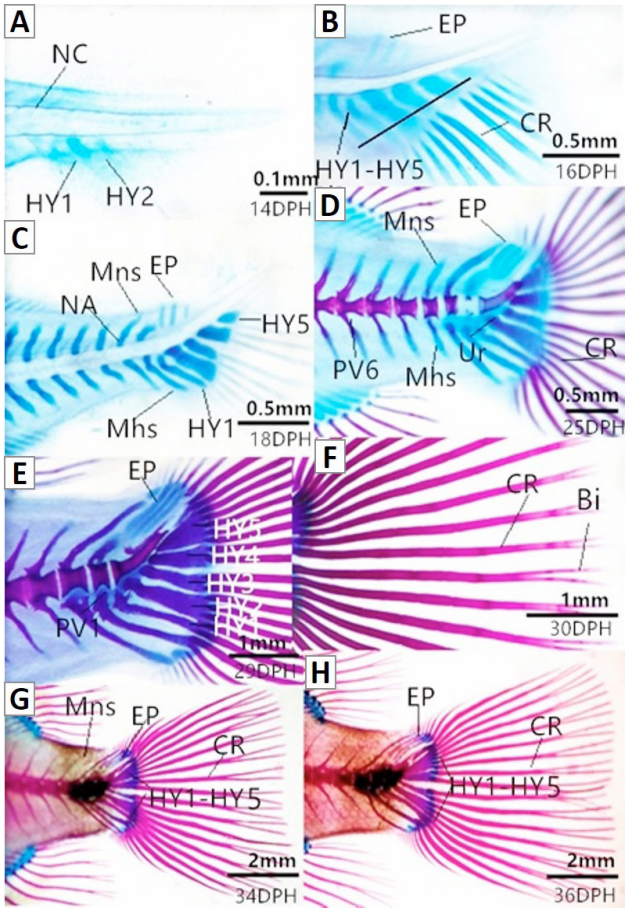

Fig. 8. Formation and ossification of caudal complex in larval crimson snapper. **A**, Larvae on 13 DPH show two cartilaginous hypurals (HY) and Notochord (NC); **B**, Larvae on 16 DPH show developing caudal fin rays (CR), three epurals (EP), five hypurals, 1st hypural to 5th hypural (HY1 to HY5); **C**, Larvae on 18 DPH show five cartilaginous hypurals, three cartilaginous epurals, neural arch (NA), modified neural spines (Mns) and modified haemal spines; **D**, Larvae on 25 DPH show cartilaginous urostyle (Ur) and preural vertebra (PV); **E**, Larvae on 29 DPH show ossification of urostyle; **F**, Larvae on 30 DPH show ossification of fin rays and fin rays end bifucated (Bi); **G**, Larvae on 34 DPH show ossification of hypurals, modified neural spines and modified neural spines; **H**, Larvae on 36 DPH show ossification of caudal complex.

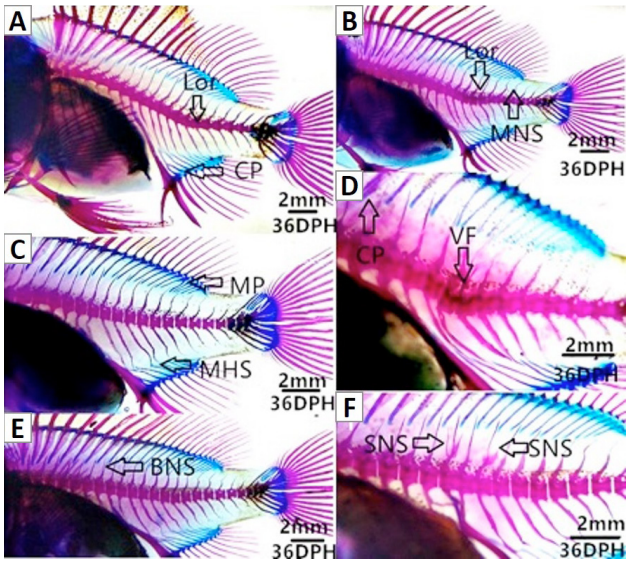

Fig. 9. Skeletal anomalies in crimson snapper at 36 DPH. **A**, Lor, lordosis. CP, connection of adjacent pterygiophores; **B**, MNS, malformed neural spines; **C**, MHS, malformed haemal spines. MP, malformed pterygiophores; **D**, VF, vertebral fusion; **E**, BNS, bifurcated neural spines; **F**, SNS, supernumerary of neural spines.

DISCUSSION

This is the first description of the skeletal ontogeny and anomalies of the larval and juvenile crimson snapper *L. erythropterus*. This study adds new knowledge to skeletal ontogeny and anomalies in crimson snapper that would be useful from basic biological perspective and larval aquaculture of marine teleosts.

Marine teleosts have a very shorter embryonic period (compared to freshwater species), and the larvae hatch out without the development fins, mouth and anus. The initial anatomical regions with osteological development were mandible, pectoral fin (cleithrum), ceratobranchial and gill arches at 3 DPH in crimson snapper. The Mandible directly involved in feeding and cleithrum supports the sternohyoideus muscle that is involved in mouth movement (Wagemans *et al.*, 1998), therefore development of mandible and cleithrum supported feeding during early larval stages. The development of ceratobranchial and gill arches supports respiration. A similar early osteological development to support feeding and respiration has been observed in numerous marine fish species (Gluckmann *et al.*, 1999; Koumoundouros *et al.*, 2000, 2001; Çoban *et al.*, 2009).

In crimson snapper, the fins developed sequentially as - pectoral, anal, dorsal, caudal and pelvic. The cartilaginous pterygiophore and basipterygium developed before

fin rays. The ossification of fins completed at different lengths, but ossification completed at a similar rate in all the fins. This pattern of development and ossification rate of pterygiophore and basipterygium among different fins are similar among other species in Perciformes (Matsuoka, 1985; Faustino and Power, 1998). However, the sequence of fins development was different in *L. erythropterus* from *Diplodus puntazzo* although both species belongs to Perciformes. In *Diplodus puntazzo* larvae, the fins development sequence was-pectoral, caudal, dorsal, anal and pelvic fin (Sfakianaki *et al*., 2005).

In addition to the variability of the developmental sequence, the most remarkable variability exists is developmental duration of skeletal structures. This variability is mainly attributed by the environmental and physiological conditions of each species. For example, fins development completed at 16.0 mm (SL) in *Sparus aurata* (Faustino and Power, 1998), and 15.5 mm TL in *Pagrus pagrus* (Coban *et al*., 2009), whereas fins development completed at a much higher lengths of 30.57 mm (SL) in this species. In fact, it is extremely difficult to compare the developmental duration of different species since the culture conditions of different species are not similar.

Certain skeletal structures are species specific among marine teleosts. For example, most species have five hypurals in the caudal region (Gavaia *et al*., 2002; Sfakianakis *et al*., 2004; Wang *et al*., 2010) whereas other species have six hypurals (Chen *et al*., 2011). Variations also exist in the number of epurals among different species. For example, majority of species have two or more epurals (Kohno, 1997; Laggis *et al*., 2014) whereas a few species have only one epurals in the caudal complex (Koumoundouros *et al*., 1999). We observed five hypurals and three epurals in crimson snapper which is conserved in most species.

Osteological development in fish larvae begins with cartilage formation prior to ossification (Faustino and Power, 1998). In this study, vertebral column ossification started at the cephalic region and proceeded in the caudal region. In addition, the ontogeny of the vertebral centra proceeds from the front to the end. Among the species in Lutjanidae, ossification of the vertebral centra proceeds caudally up to the preural centra (Potthoff *et al*., 1988), whereas the first centrum ossify after the formation of the second centrum in Sparidae (Faustino and Power, 1998; Koumoundouros *et al*., 1999, 2001; Sfakianakis *et al*., 2004).

The development of skeletal anomalies is linked to both environmental and biotic factors. However, the specific aetiologies for skeletal anomalies in crimson snapper could not be confirmed. In this study, vertebral anomalies particularly malformed neural spine and malformed haemal spines were most frequently observed. Similar to this species vertebral anomalies were the most common type in many other species, such as gilthead sea bream *Sparus aurata* (Boglione *et al*., 2001), Pandora *Pagellus erythrinus* (Sfakianakis *et al*., 2004), zebrafish *Danio rerio* (Ferreri *et al*., 2000), European sea bass *Dicentrarchus labrax* (Barahona-Fernandes, 1982) and striped trumpeter *Latris lineata* (Negm *et al*., 2014).

CONCLUSIONS

A comprehensive understanding of the skeletal development facilitates hatchery management and larval rearing of a species. This study has explored the skeletal ontogeny and identified some skeletal anomalies in crimson snapper. Results from the present study would be useful to understand functional morphology and larval aquaculture of marine teleosts.

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Statement of conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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