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Investigation of cartilage development in sea bream (*Sparus aurata*) larvae at 22 °C by double staining technique in the first 20 days after hatching

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ABSTRACT

Objective: To investigate the normal cartilage development of the caudal fin system in gilthead sea bream (*Sparus aurata*) larvae under intensive aquaculture conditions was investigated up to age 20 days. This study was carried out because of the increasing incidence of skeletal malformations, especially those related to the columna vertebralis, in bream farming and because the cause and progression of such skeletal malformations are uncertain and there are very few studies on the cartilage-osteological development.

Materials and Methods: Approximately 400 *Sparus aurata* larvae were used to determine the development. The materials were stained with Alcian Blue for cartilage staining and Alizarin Red for bone staining. 300 mg alcian blue and 100 ml 70% ethanol solution were mixed with 100 mg alizarin red and 100 mg 95% ethanol solution. A solution was prepared by adding 100 ml of glacial acetic acid and 1700 ml of 70% ethanol to this mixture. The larvae were kept in this solution in the oven at the appropriate temperature for 4 days and washed in running water for 2 hours after being removed from the oven. The prepared specimens were examined under stereo microscope.

Results: It was observed that the cartilage formation in the caudal fin of the larvae started at 13 days of age. In 13-day-old larvae, hypural 1 (ventral extensions of the vertebrae) was measured as 0.035 mm on average. At 20-day old larvae, hypural 1, hypural 2, hypural 3, hypural 4, hp parhypural and hs (heamal spine) were detected and measured. Hypural 1 and hypural 4 were found to be quite thickened. no ossification was observed in 20-day-old material. In this study, the development up to 20 days was analysed since cartilage development was especially considered.

Conclusion: The results of skeletal development in *Sparus aurata* larvae showed similarities when compared with the findings in other fish species. Additionally, cartilage anomalies were observed at a rate of 13%. The observed anomalies were detected as tail curl.

Keywords: *Larval rearing, Cartilaginous development, Caudal fin, Sparus aurata*

INTRODUCTION

No osteological structure was detected on the spine of bream larvae at hatching. In the study, cartilage formation in the caudal fin was not observed in 1-12 days old bream larvae. The first structure formed in the caudal tail on the spine was Hy1 in the ventral region with an average length of 0.035 mm in 13-day-old larvae (Figure 2). This marks the beginning of cartilage development in the caudal fin of the larvae. During the 14–20-day period, hy 1, hy2, hy3, hy4, hp and hs structures gradually developed and increased in size. While the lengths increased, transverse enlargement and thickening were

observed on the 16th and 20th days depending on the days. hy2 was observed for the first time in 15-day-old larvae (Figure 3). Thickening was observed to be more effective on hyl and hy4. In 16-day-old larvae, hy3 was observed for the first time, while hyl and hy2 continued their development. It was found that hy 1, which was larger than the others from the 16th day onwards, continued to grow transversely from the 18th day onwards, and the other protrusions took the shape of a rectangle rather than a transverse rod. In 17 days, old sea bream larvae (Figure 4), no new cartilage was observed, growth continued. On the 18th day, hs cartilage was detected. On the 19th day (Figure 5), hyl, hy2, hy3, hy4, hy4, hy5, prh and hs were observed. On the 20th day (Figure 6), the development continued. These structures increased with the increase in the age of the fish, the increase in number was completed and the increase in length and width continued. On the 20th day, it was determined that the caudo ventral end of hy4 expanded more and resembled a triangular shape.

In all periods of development of the caudal fin complex, especially in cartilage formation, no changes were observed between parhypural, hypural 1, 2, 3 and 4 in the form of cartilage fusion and then separation.

In study, fifty-two larvae with abnormal tails were observed. Cartilage Anomalies were observed with a rate of 13%. These anomalies were observed as tail curling. Figure 7 shows the tail anomaly pictures of the 13th, 15th, and 16th days.

MATERIALS and METHODS

This study was carried out with sea bream larvae obtained dead from the hatchery of a private aquaculture facility in Muğla and reared at 22°C.

Larval Production and Investigation Group Organization

In larval production, black coloured polyester tanks were preferred and each tank had a volume of 1 m^3. When incubation was completed, 80 prelarvae/litre were stocked in the tanks. After 3 days of prelarval stage in dark environment, larvae passed to postlarval stage on the 4th day with mouth opening. In the tanks where they were kept, water ph:7.25 and temperature values varied between 20-24°C on average. Larvae of known age were used by the producer.

In the study, an average of 20 dead larvae were sampled from day 1 to day 20 from a fixed tank containing sea bream larvae. The samples were preserved in 10% formaldehyde.

Cartilage Structure Analyses

Alizarin red and Alcian blue staining method was applied for the developmental analysis of caudal fin cartilage structure of *Sparus aurata* larvae. *Sparus aurata* larvae stored in 10% formol were washed with distilled water and placed in containers containing 95% ethanol. 300 mg alcian blue and 100 ml 70% ethanol solution and 100 mg alizarin red and 100 mg 95% ethanol solution were mixed. A solution was prepared by adding 100 ml of glacial acetic acid and 1700 ml of 70% ethanol to this mixture. The larvae were kept in this solution in the oven at the appropriate temperature for 4 days and washed in running water for 2 hours after being removed from the oven. The prepared specimens were examined under stereo microscope.

The procedures applied in this study were approved by Balıkesir University Experimental Animals Application and Research Center with the ethics committee report numbered 2024/6 at the meeting dated 04.07.2024.

RESULTS

Dead fish samples collected from the same tanks on different days were preserved under appropriate conditions and after staining. Hypural (hy 1, hy2, hy3 and hy4), paryhypural (prh) and heamal spine (hs) cartilage development in caudal fin were examined under stereo microscope (Figure 2, 3). *Sparus aurata* larvae were examined from the first day (Figure 1) until the twentieth day by taking 20 different samples from each. No bone development was observed in any fish during the twenty-day period, but cartilage development was measured. No cartilage formation was observed in the caudal fin of the larvae analysed from one day to twelve days. It was observed that cartilage formation in the caudal fin of the larvae started at thirteen days (Figure 2).

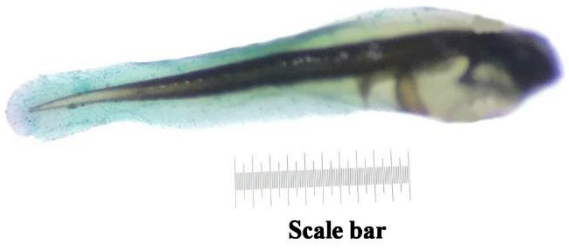

Figure 1. One-day-old sea bream larva. (Scale bar 1 mm).

Table 1. Hs, PrH (Parhipural), Hy1, Hy2, Hy3 and Hy4 measurements in *Sparus aurata* larvae.

	HS	PrH, Parhipural, mm	Hy1, mm	Hy2, mm	Hy3, mm	Hy4, mm
Day 13	-	-	0.03	-	-	-
Day 14	-	-	0.055	-	-	-
Day 15	-	0.02 (only in 2 items)	0.11	0.07	-	-
Day 16	-	0.06	0.125	0.09	0.035	-
Day 17	-	0.075	0.14	0.11	0.08	-
Day 18	-	0.15	0.21	0.15	0.14	0.09
Day 19	0.14-0.11	0.21	0.24	0.17	0.15	0.12
Day 20	0.23	0.3	0.28	0.19	0.17	0.15

Development measurements of caudal fin in *Sparus aurata* larvae are described in Table 1.

No osteological structure was detected on the spine of bream larvae at hatching. In the study, cartilage formation in the caudal fin was not observed in 1-12 days old bream larvae. The first structure formed in the caudal tail on the spine was Hy1 in the ventral region with an average length of 0.035 mm in 13-day-old larvae (Figure 2).

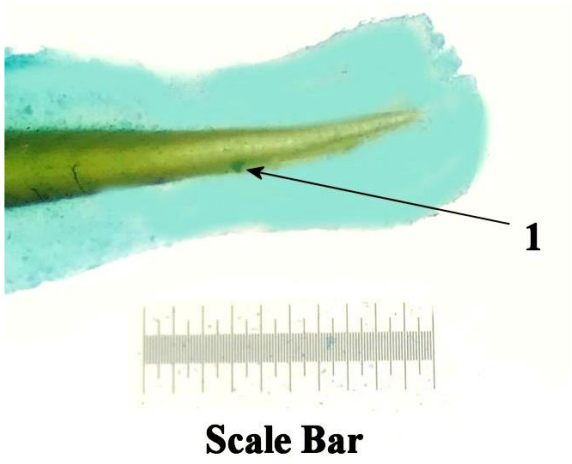

Figure 2. 13-day-old sea bream larva. 1- Hy1 (Scale bar 1 mm).

This marks the beginning of cartilage development in the caudal fin of the larvae. During the 14–20-day period, hy 1, hy2, hy3, hy4, hp and hs structures gradually developed and increased in size. While the lengths increased, transverse enlargement and thickening were observed on the 16th and 20th days depending on the days. hy2 was observed for the first time in 15-day-old larvae (Figure 3).

Thickening was observed to be more effective on hyl and hy4. In 16-day-old larvae, hy3 was observed for the first time, while hyl and hy2 continued their development. It was found that hy 1, which was larger than the others from the 16th day onwards, continued to grow transversely from the 18th day onwards, and the other protrusions took the shape of a rectangle rather than a transverse rod. In 17 days, old sea bream larvae (Figure 4), no new cartilage was observed, growth continued.

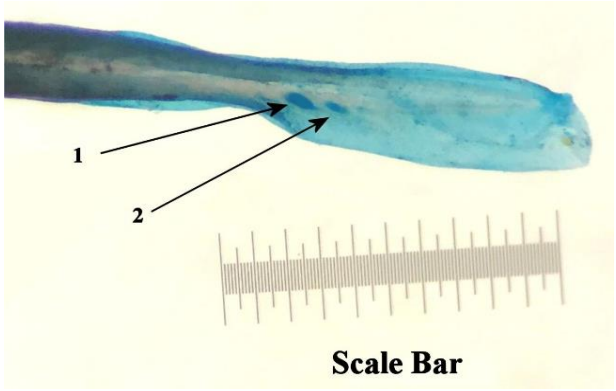

Figure 3. 15-day-old sea bream larva. 1- Hy1, 2- Hy2 (Scale bar 1 mm).

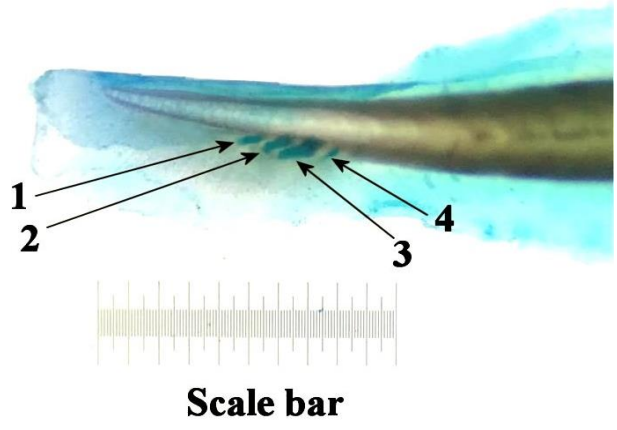

Figure 4. 17-day-old sea bream larva. 1- Hy 3, 2- Hy 2, 3- Hy 1, 4-Prh (Scale bar 1 mm).

On the 18th day, hs cartilage was detected. On the 19th day (Figure 5), hyl, hy2, hy3, hy4, hy4, hy5, prh and hs were observed. On the 20th day (Figure 6), the development continued. These structures increased with the increase in the age of the fish, the

increase in number was completed and the increase in length and width continued. On the 20th day, it was determined that the caudo ventral end of hy4 expanded more and resembled a triangular shape.

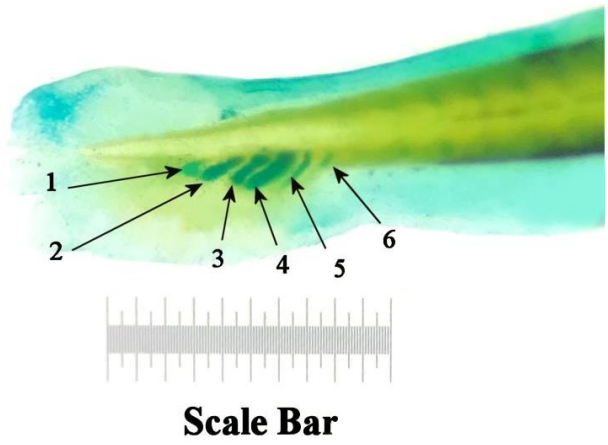

Figure 5. 19-day-old sea bream larva, 1-Hy4, 2-Hy3, 3-Hy2, 4-Hy1, 5- Prh,6- Hs (Scale bar 1 mm).

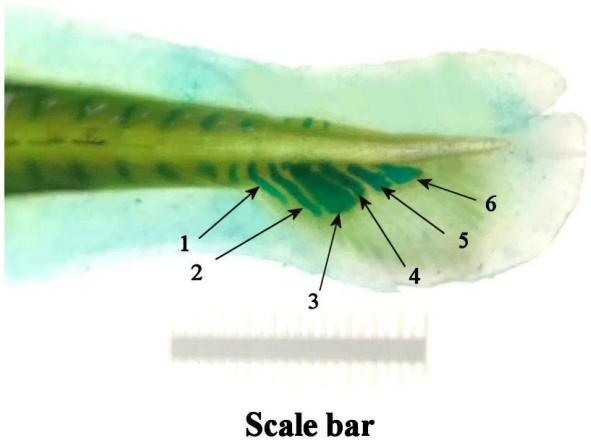

Figure 6. 20-day-old sea bream larva, 1-Hs, 2-PrH, 3-Hy1, 4-Hy2, 5-Hy3, 6-Hy4 (Scale bar 1 mm).

Figure 7. Examples of tail anomalies on the 13th, 15th and 16th days (Scale bar 1 mm).

In all periods of development of the caudal fin complex, especially in cartilage formation, no changes were observed between parhypural, hypural 1, 2, 3 and 4 in the form of cartilage fusion and then separation.

In study, fifty-two larvae with abnormal tails were observed. Cartilage Anomalies were observed with a rate of 13%. These anomalies were observed as tail curling. Figure 7 shows the tail anomaly pictures of the 13th, 15th, and 16th days.

DISCUSSION

This study is one of the rare studies in which cartilage development in cultured larvae of *Sparus aurata* was stained with alizarin red and alcian blue and real pictures were given periodically as development.

Normal and abnormal cartilage and osteological development of the caudal fin in *Sparus aurata* larvae is important in fish farming. Because this level of development affects the morphology, growth and survival of the fish and changes the quality of the fish produced

Normal and abnormal cartilage and osteological development of the caudal fin in *Sparus aurata* larvae is important in fish farming. Because this level of development affects the morphology, growth and survival of the fish and changes the quality of the fish produced (Koumoundouros et al., 1997). These developmental disorders are mostly caused by larval and juvenile stages as a result of abiotic conditions. Examples of abiotic factors in aquaculture include water, sunlight, tides, pH, temperature, minerals, volcanic eruptions and storms. (Bolla and Holmeord, 1988; Wiegand et al., 1989).

Early in the development of the caudal fin complex, no cartilaginous fusion between the parhypural, hypural 1 and 2 has been reported to be observed, which is consistent with our study (Faustino and Power, 1998). Later in development, when the larva is about 5.1 mm in size, hypural 1 and 2 have been reported to be joined proximally by a temporary cartilaginous bridge and then fused with the parhypural at about 7.1 mm in size (Faustino and Power, 1998). These fusions were not seen in our study. Since only cartilage development up to day 20 was found in our study, it was predicted that such fusion might occur at later periods. It was reported by the same author that when ossification was completed, all hypurals were once again separated from each other and from the parhypural. This kind of separation was not observed in our study.

It has been reported that some cartilaginous centres in the caudal fin were reduced in size or triangular in shape and the associated arches and spines were observed as double, twisted, fused or broken without any specific abnormality, and an extra hypoplasmic fin was found on the dorsal part (Koumoundouros et al, 1997). In our study, such a double, fused or broken cartilaginous formation was not observed. Only a small proportion showed a bent tail as shown in the picture (Figure 7). It was reported to be caused by lateral bending of the plate formed by deformed and fused Hy elements. However, despite these anomalies, it was reported that the swimming behaviour of the specimens did not change, only the cartilage development was different (Koumoundouros et al., 1997). In our study, such bending was detected.

Barahona-Femandes (1982) reported that abnormalities occurring in *Dicentriochus labrax* in the first 40 days after hatching are mostly lethal, but those occurring after 40 days (post larval stage) are not lethal.

In *Sparus auruta*, Papema (1978) described a curvature of the caudal peduncle. Malformations of the caudal skeleton have also been reported in other species such as Pagrus major and *Dicentrzothus labrax* under rearing conditions (Matsuoka, 1985; Daoulas et al., 1991; Boglione et al., 1993; Marino et al., 1993). In cultured specimens of *Dicentrurchus labrax*, a higher rate (34%) of bone abnormality was observed in the caudal fin (Marino et al., 1993). In our study, cartilage anomalies were observed at a rate of 13%. The observed anomalies were detected as tail curling. In previous studies (Matsuoka, 1985; Daoulas et al., 1991; Boglione et al., 1993; Marino et al., 1993), tail anomalies were also observed as curling. However, the rate in our study is quite low. It is thought that this low rate is not related to the rearing of larvae at 22°C.

Determination of growth stages in terms of cartilage development is important in terms of species breeding and fisheries biology (Koumoundouros et al., 1999). However, knowing the normal development of the species is very important for the successful production of that species (Çoban et al., 2009). Especially in commercial production enterprises, deformations cause economic losses up to 100% and 60% of these deformations occur on the spine (Koumoundouros et al., 1997).

CONCLUSION

Cartilage and osteological development studies are of great importance in preventing deformations under aquaculture conditions, eliminating them in advance and removing them from the system. In future studies, the genetic and environmental effects on the development of bone and cartilage structures should be studied.

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