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Skeletal Formation and Abnormalities in the Caudal Complex of the Japanese Flounder, *Paralichthys olivaceus* (Temminck & Schlegel)

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Abstract : The caudal complex of the Japanese flounder, *Paralichthys olivaceus* (Temminck & Schlegel) was observed to clarify the morphological characteristics in artificial seedlings. During caudal complex development, *P. olivaceus* exhibits a discontinuous zone between Stages D and E, when extensive formation of the pterygiophres, mainly the hypural plates, occurs. The development of the caudal skeleton progresses even after settlement, and is finally completed at about 90 mm TL. In wild fish, bone abnormalities were rarely observed, while in artificial seedlings, such abnormalities as increase of hypural branches, fusion between neighboring spines, and central fusion were observed with high frequency of occurrence. The relationship between bone formation and factors causing bone abnormality is discussed in terms of food nutrition, especially in relation to vitamin A.

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Seedling production techniques have improved remarkably over the last two decades in Japan. These techniques enable the mass production of artificial seedlings for some commercially important fishes such as the Japanese flounder, *Paralichthys olivaceus* (Temminck & Schlegel). However, morphological abnormalities still appear in *P. olivaceus* artificial seedlings at high frequencies, with seedlings of poorer quality than wild fish. Incomplete early development of feeding and swimming organs results in a low survival rate due to organ malfunction at post—larval stages (Hosoya, 1991; Hosoya and Kawamura, 1997). This is an emergent problem, and a solution will promote production of healthy artificial seedlings. In this study, we examine the osteological development of the caudal complex, which is an important swimming organ, and clarify the morphological characteristics of *P. olivaceus* artificial seedlings, comparing them with wild fish. We describe the gross morphology of the caudal complex of *P. olivaceus* , then order artificial seedling bone abnormalities. We discuss the relationship between bone formation and factors causing bone abnormality in terms of nutrition, in particular vitamin A availability.

Materials and Methods

Artificial seedlings of *P. olivaceus* were reared being fed with rotifers, a mix of *Artemia salina* nauplii and artificial diet, from February to June, in 1994 and 1995 at the National Research Institute of Aquaculture (NRIA). Rotifers had been fed beforehand to enrich their nutritional content with the phytoplankton, *Nannochloropsis oculata*. Rearing experiments were conducted in a 500 ℓ fish tank of seawater with water temperature between 15 to 19 ℃. The hatched fry were first reared in stationary water for a week and then transferred to running water (50 ℓ / h). Sampling was done twice a week. Samples were periodically collected in three different fixations, viz., 3% formalin, 70% ethanol and Bouin solution. In order to elucidate the mechanism of bone abnormality expression in relation to nutrition, abnormal fish fed with excessive vitamin A, provided by Tokyo University of Fisheries (TUF), were also analyzed. In addition, juveniles caught off Kasumi in Hyogo Pref., and larvae and juveniles sampled in Maizuru Bay, Kyoto Pref., in May, 1993 were used for wild fish analysis. These samples were provided to the authors due to the cooperative research project with the Department of Fisheries, Faculty of Agriculture, Kyoto University (FAKU). To examine the skeletal system under the binocular microscope, it was stained with either a double— (Kawamura and Hosoya, 1991), or single—staining technique using only Alizarin red S. Ossification was histologically confirmed with the appearance of osteocytes. Anatomical terminology of skeletal system mainly followed Hosoya (1991). Special attention was paid to identify bones based on their histological nature or the manner of formation, viz., dermal–, membrane –, and cartilage–bones according to Patterson (1977).

Results

Characteristics of the caudal complex in *P. olivaceus* Accurate information on the skeletal system of normal fish from wild populations is indispensable to elucidate the morphological characteristics of artificial seedlings with bone abnormalities. Although *P. olivaceus* is a commercially important fish species in Japan, information on its skeletal system is scarce except for conclusive descriptions by Amaoka (1969) and Sakamoto (1984), and general descriptions of the anatomy of *P. olivaceus by* Shiomitsu and Naito (1994). Otherwise, flounder osteology has been only partially inspected so far (*i.e.* branchial apparatus by Hosoya and Kawamura, 1993). Also, Balart (1985) has already disclosed the formation of the caudal skeleton of *P. olivaceus* based on standard length. However, it was not referred to the developmental staging (*i.e.*, that of Minami, 1982) which enables discussion and comparison with the accumulated information on flatfish aquaculture. These circumstances lead us to redescribe the standard structure and formation process of the caudal complex in *P. olivaceus* below, as basic and neccesarry knowledge to specify the factors to bring about its skeletal abnormalities.

1. *General morphology* The caudal complex of *P. olivaceus* is strikingly compressed. The maximum height of the total pterygiophores, articulating with the second (**Fig. 1, A: PU2**) and latter preural centra, reaches about 1.6 times the maximum length of the pterygiophores. The articulation of the caudal complex with fin rays extends from the posterior end of the hypurals to the neural and haemal spines on the second preural centrum. The caudal centrum is fused with the first preural centrum to form the urostyle. No free uroneurals are present. There are two epurals. The second epural (EP2) is markedly smaller than the first epural (EP1). However, "the second epural" identified in this paper according to Balart (1985), was regarded as "the first uroneural" by Amaoka (1969) or "the stegural" by Sakamoto (1984). The homology of this bone needs to be anatomically scrutinized in the future. The first (HY1) and second (HY2) hypurals are fused to each other at the anterior base to form the lower hypural plate, keeping the tendency of branching at the posterior end. Likewise, the third (HY3) and fourth (HY4) hypurals are fused to each other to form the upper hypural plate as well. The lower hypural plate is articulated with the urostyle at the anterior base, while the upper hypural plate is completely fused with the urostyle. Both the fifth hypural (HY5), and the parhypural (PH) which lacks the parhypural zygapophyses, are completely separated from each counter-centrum. There are five

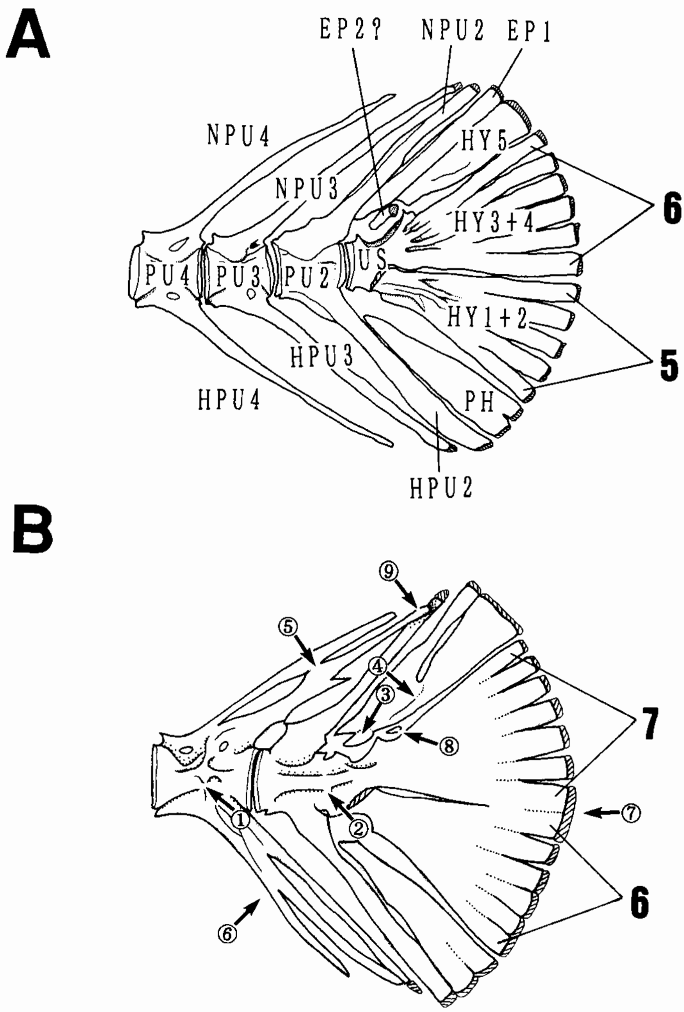

Fig. 1. Caudal complex of *Paralichthys olivaceus*.
A, normal wild fish; **B**, summary for the bone abnormality in artificial seedlings. ①, central fusion between PU4 and PU3; ②, central fusion between PU2 and US; ③, fusion between EP1 and EP2; ④, medial fusion between EP1 and HY5; ⑤, bridge between NPU4 and NPU3; ⑥, bridge between HPU4 and HPU3; ⑦, distal fusion between HY3 and HY2; ⑧, excessive ossicle; ⑨, distal crossing between NPU3 and NPU2. EP, epural; HPU, haemal spine of preural centrum; HY, hypural; NPU, neural spine of preural centrum; PH, parhypural; PU, preural centrum; US, urostyle. Numerals relevant to the posterior margin of each hypural plate indicate the number of branches.

branches at the posterior end of the hypural plates in the first and second hypurals, six in the third and fourth hypurals, one in the fifth hypural, and two in the parhypural. The total number of caudal fin rays is eighteen. A single ray is articulated with the first epural, three with the fifth epural, six with the third and fourth hypurals, five with the first and second hypurals, two with the parhypural, and one with the second haemal spine of the preural centrum (HPU2).

Compared with other flatfishes which have no free epural such as species belonging to the genera *Pseudorhombus* and *Tarphops*, *Paralichthys olivaceus* exhibits primitive features with the presence of two free epurals (sensu Balart, 1985); they include one identical with "the first uroneural" of Amaoka (1969). The fusion of the first and the second hypurals, and that of the third and the fourth hypurals, are also more derivative than other flatfishes with independent hypurals such as *Psettodes, Citharoides and Lepidoblepharon* (See Amaoka, 1969).

2. *Developmental process of the caudal complex* The developmental process of the caudal complex of *P. olivaceus* is described below (**Fig. 2**), following the stages of Minami (1982). The description of stages in the planktonic period was based on artificial seedlings, with post-settlement stages on wild fish.

Stages A–B (**Fig. 2–B**). TL (Total Length) 5.01–5.09 mm. Although the skeletal system was undeveloped, the notochord supported the whole caudal region. The notochord sheath, which is composed of connective tissue, has begun to segment from the ventral surface of the posterior end of the notochord.

Stage C (**Fig. 2–C**). TL 5.56 mm. Two neural spine cartilages and two ray–like structures appeared dorsally, while segments in the notochord sheath have been transformed into cartilage primordial.

Stage D (**Fig. 2–D, D'**). TL 5.95–5.97 mm. Three neural spine cartilages were clearly recognized. The notochord began to bend dorsad at its posterior end. Segmentation in the notochord sheath proceeded all over the caudal region. The ventro–posterior end segment has become three or four visible cartilaginous primordials, meeting three or four fin rays soon.

Stage E (**Fig. 2–E**). TL 8.38 mm. Formation of pterygiophore cartilages, mainly hypural cartilages, has rapidly occurred; the outline of the upper and lower hypural plates has appeared. In addition to the parhypural cartilage and two epural cartilages, the neural and haemal spine cartilages have become more visible in shape.

Stages F–G (**Fig. 2–G**). TL 9.85–11.61 mm. Around this stage, bones have appeared finally. The neural and haemal spine cartilages have begun to ossify. As the first appearing membrane bone, centra have started to surround the

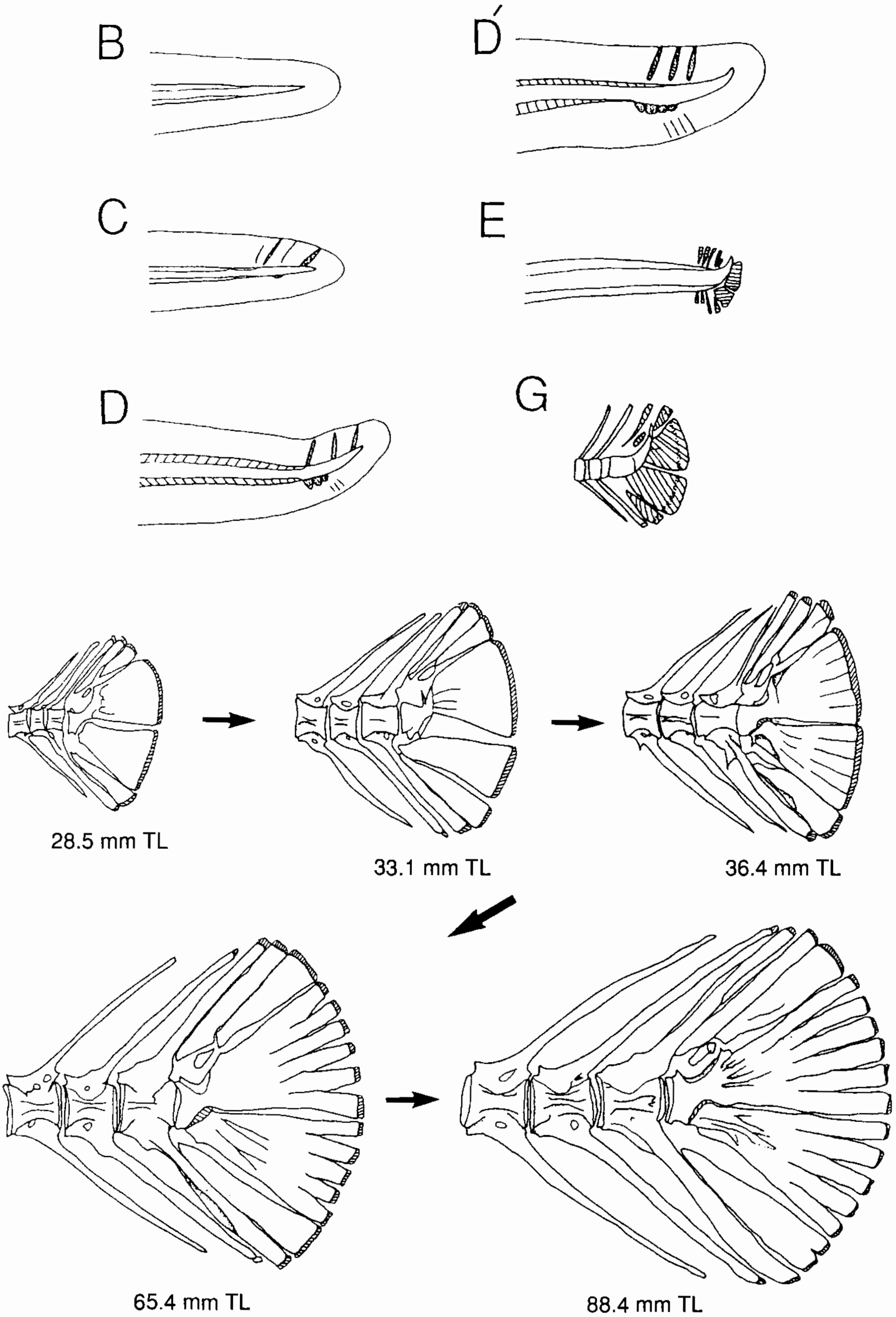

Fig. 2. Osteological development of the caudal complex in *Paralichthys olivaceus*.

Upper half (**B**–**G**), planktonic larvae with stages defined by Minami (1982); lower half, settling juveniles and young with total length.

notochord partly. Between the first and second preural centra, however, a large unossified region (See Hosoya and Kawamura, 1997) is still present. The lower hypural plate has continued to develop and expand. The fifth hypural cartilage has appeared. Two epural cartilages have already differentiated into ossicles of different size.

TL 28.5 mm. After settlement, ossification has progressed all over the caudal region. In the hypural plates, however, not only the upper but also the lower plate still remain in a plate. Finally, the unossified region between the first and the second preural centra has been completely covered by membrane bone. The hypural plates have become fused with the urostyle.

TL 33.1 mm. The fifth hypural and the parhypural have grown to have a close connection with the first epural and the second haemal spine of the preural centrum.

TL 36.4 mm. Some vertical cracks have developed in the posterior part of the hypural plates and the fifth hypural, which show the beginning of branching caudad in the hypurals.

TL 65.4 mm. The branching in the posterior part of the hypural plates has advanced. There are six branches in the upper hypural plate and five in the lower. In the fifth hypural, however, a crack has atrophied to convert it into the original mono -structure.

TL 88.4 mm. It is not until two branches have developed markedly in the parhypural that the formation of the caudal complex is completed.

Compared with other skeletal systems, *i.e.,* feeding organs, organogenesis in the caudal complex is so late that it is finally completed at about 90 mm TL. A markedly discontinuous zone is present between Stages D and E. In the formation of the hypural plates, fusion of cartilage elements precedes the secondary branching in the posterior part of the plates. In the formation of caudal centra, an unossified region between the first and second preural centra remains till the post developmental periods as has been previously reported (Hosoya and Kawamura, 1997). Among the above characteristics, the secondary branching in the hypural plates strongly contrasts with fusion of the hypurals in standard perciform fishes, giving rise to hypural plates.

Abnormality in the caudal complex Bone abnormality is known to appear frequently in artificial seedlings of many fish species. Abnormality in the caudal complex was observed in this study as well, in 30 to 60 % of the artificial seedlings of *P. olivaceus*, reared in 1994 and 1995. Many of the symptoms were due to heterochronical "acceleration", in which organogenesis is accelerated compared to the normal condition, while the original body size is maintained (Gould, 1977).

1. *Numerical abnormality* The most typical symptom of numerical abnormalities was observed in the number of branches in the posterior part of the hypural plates.

In general, in normal *P. olivaceus*, there are six branches in the upper hypural plate and five in the lower. In wild fish, not only from Kasumi but also from Maizuru Bay, the constitution of hypural plates was 6 + 5 without exception (**Fig. 1, A ; Fig. 3, A**). In contrast, artificial seedlings tended to show increased numbers of branches. Seven branches were seen in the upper hypural plate with six in the lower (**Fig. 1, B**). In addition, the constitution of hypural plates varied greatly. In artificial seedlings from the NRIA, the normal type (6 + 5) and the three types of abnormalities (7 + 6, 7 + 5, 6 + 6) were recognized. These abnormal types (19 or 20 in total) always have one or two more caudal fin rays than the normal type (18), because the increase of branches in the hypural plates is directly reflected in the number of caudal fin rays.

2. *Qualitative abnormality* The most typical qualitative abnormality is exemplified by fusion of bone elements. In wild fish, qualitative abnormalities are hardly observed. In this study, only one sample fish of 28.5 mm TL from Maizuru Bay showed fusion between the first and the second haemal spines from the preural centra (**Fig. 2**). In contrast, fusions between neighboring bone elements were not rare in the haemal or neural spines of artificial seedlings. Both the seedlings from the NRIA and those involved in the TUF nutrition experiment with excessive dosages of Vitamin A showed accelerated fusion in the above bones, while β-carotene did not (See Takeuchi *et al.,* 1995). We could reconfirm that the fusions of two or three centra, *i.e.,* the urostyle and the second to third preural centra, represent a central abnormality (**Fig. 3, B・C・D**). Central fusion was ascertained to be accelerated by excessive dosages of Vitamin A in the nutrition experiment. The frequency of caudal centrum-fusion differs with region. In some 50 artificial seedlings from the NRIA, there were two individuals having a central fusion of the fourth and fifth preural centra (the 24th and 23rd caudal vertebrae), and seven individuals having that of the second and third preural centra (the 26th and 25th caudal vertebrae). In addition, the following qualitative abnormalities were observed in artificial seedlings: fusion between the first and second epurals and the fifth hypural, partial connection of neighboring bone elements in the haemal, neural spines and hypurals, and appearance of an extra-ossicle (**Fig. 1, B ; 3, C・E・F**).

Discussion

Developmental stage In the results, the developmental stages of the caudal

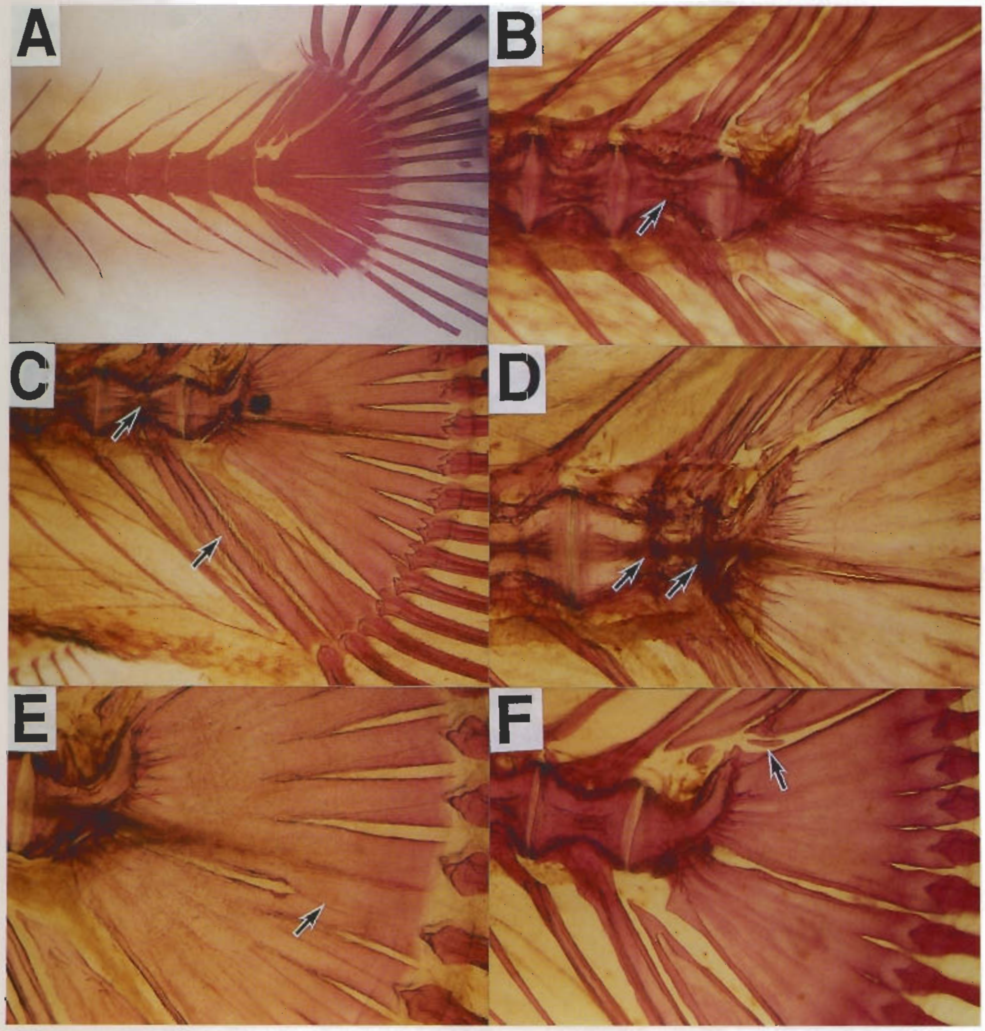

Fig. 3. Symptoms of osteological abnormalities in artificial seedlings of *Paralichthys olivaceus*. **A**, normal wild fish; **B**, central fusion between PU3 and PU2; **C**, amalgamation of central fusion between PU3 and PU2, and partial fusion between HPU3 and HPU2; **D**, central fusions among PU3, PU2 and US; **E**, distal fusion between HY3 and HY2; **F**, excessive ossicle.

complex in *P. olivaceus* were described, following the developmental stages of Minami (1982). The formation of the caudal complex is not gradual but progresses intermittently from Stage A to Stage I, a stage soon after settlement. For example, a gentle discontinuous zone was recognized between Stages D and F. The rapid formation of pterygiophores, mainly the hypural plates, may be responsible for this discontinuity.

The gradation in the early development of *P. olivaceus* has already been ascertained with morphometric analysis, with two points of drastic change present at around 7 mm and 10 mm TL respectively (Kawamura and Hosoya, 1997). The first point lies between Stages D and E, while the second between Stages F and G. During formation of the cranial skeleton, there was a discontinuous zone in ossification, mainly in the membrane bone, between Stages D and E (Hosoya and Kawamura, 1997). During osteological development, the cranial and caudal skeletons share a discontinuous zone between Stages D and E, which are characterized by growth of cartilage in the cranial skeleton, and ossification of the membrane bone in the caudal complex, respectively. Accordingly, this discontinuous zone may be regarded as a drastic point of change in the formation of an internal skeleton. From the overall results of the preceding studies (Hosoya and Kawamura, 1997; Kawamura and Hosoya, 1997), the developmental stages of *P. olivaceus* may be grouped into three phases (A−D, E−F and G−I) based on changes in external organs, or two phases (A−D, E−I) based on changes in internal organs. Osteological development is very characteristic in that qualitative changes continue to the last developmental stages even after settlement, despite no obvious discontinuous zone after Stage E.

Background of bone abnormality As one approach to clarify the morphological characteristics of artificial seedlings, the caudal complex of both wild fish and artificial seedlings of *P. olivaceus* were compared. Some bone abnormalities were recognized in about half of the artificial seedlings used in this study, while such abnormalities were rarely seen in wild fish. The symptoms of bone abnormality were an increase of branches in the hypural plates, central fusion, connection of neighboring bone elements in the neural and haemal spines, and appearance of an extra−ossicle. All abnormalities originated from accelerated development. According to Matsusato (1986), these symptoms could be classified as bone abnormality caused by abnormal chondrogenesis and osteogenesis. The induction of bone abnormalities and the amount of calcium present in the environmental water, especially the amount of hydroxyapatite, has been shown to be closely related. Calcium assimilation is controlled by tyroid hormone and calcitonin, which is secreted from the corpuscles of STANNIUS. High doses of Vitamin A have also been shown to induce several kinds of bone abnormalities (Takeuchi *et al.*, 1995; Dedi *et* *al.*, 1997). In some cases, the symptom of bone abnormalities in the caudal complex was abnormalities in the membrane bones, such as central fusion. However, other symptoms always include abnormalities in the cartilage bones. With regards to the mechanism responsible for the formation of membrane bone abnormalities, the fact that caudal centrum–fusion occurs frequently in the parts to be formed last in osteological development is highly suggestive (Hosoya and Kawamura, 1997). In contrast, it is well known that Vitamin A changes into retinal phosphoric acid ester, one of the mucopolysaccharides, among which acid mucopolysaccharide is one of the main constituent of cartilaginous substances, "chondrin" (Hosoya, 1991; Kawamura and Hosoya, 1991). It was reported that larval flounder do not have the ability to convert β-carotene to Vitamin A (Takeuchi *et al.*, 1995). Overdosages of Vitamin A may induce accelerated development of cartilage or cartilage bones, if calcium concentration and hormone balance conditions are suitable. In the early development of *P. olivaceus*, cartilages grow rapidly between Stages D and E. Therefore, in order to prevent bone abnormalities in the caudal complex, attention must be paid to nutrition constituents of the food during these stages.

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References

Amaoka K., 1969: Studies on the sinistral flounders in the waters around Japan–Taxonomy, anatomy and phylogeny. *J. Shimonoseki Univ. Fish.*, **18**, 65–340.

Balart E. F., 1985: Development of median and paired fin skeleton of *Paralichthys olivaceus* (Pleuronectiformes: Paralichthyidae). *Jpn. J. Ichthyol.*, **31**, 389–410.

Dedi J., Takeuchi T., Seikai T., Watanabe T. and Hosoya K., 1997: Hypervitaminosis A during vertebral morphogenesis in larval Japanese flounder. *Fish. Sci.*, **63**, 466–473.

Gould S. J., 1977: Ontogeny and phylogeny. Harvard Univ. Press, Cambridge, pp. 501.

Hosoya K., 1991: Osteological development, and development of feeding and swimming abilities in fish. A course to master the basic theory in the practice of fish farming technology in 1991, the series of development in larval and juvenile periods. **3**, pp. 1–18 (In Japanese).

Hosoya K. and Kawamura K., 1993: Skeletal structure of the branchial arches in *Paralichthys olivaceus* (Temminck et Schlegel). *Bull. Res. Inst. Aquacult.*, **22**, 1–10.

Hosoya K. and Kawamura K., 1997: Osteological evaluation in artificial seedlings of *Paralichthys olivaceus* (Temminck and Schlegel). *UJNR Technical Report*, **24**, 107–114.

Kawamura K. and Hosoya K., 1991: A modified double staining technique for making a transparent fish – skeletal specimen. *Bull. Natl. Res. Inst. Aquacult.*, **20**, 11–18 (In Japanese with English abstract).

Kawamura K. and Hosoya K., 1997: Larval morphometry of the Japanese flounder, *Paralichthys olivaceus. Ichthyol. Res.*, **44**, 389–398.

Matsusato T., 1986: Studies on the skeletal anomaly of fishes. *Bull. Natl. Inst. Aquacult.*, **10**, 57–179 (In Japanese with English abstract).

Minami T., 1982: The early life history of a flounder, *Paralichthys olivaceus. Bull. Jpn. Soc. Sci. Fish.*, **48**, 1581–1588. (In Japanese with English summary)

Patterson C., 1977: Cartilage bones, dermal bones and membrane bones, or the exoskeleton versus the endoskeleton. In "Problems in vertebrate evolution" ed. by S. M. Andrews, R. S. Miles and A. D. Walker, Academic Press, London, pp. 77–121.

Sakamoto K., 1984: Interrelationships of the family Pleuronectidae (Pisces: Pleuronectiformes). *Mem. Fac. Fish. Hokkaido Univ.*, **31**, 95–215.

Shiomitsu K. and Naito K., 1994: The Japanese flounder. In "Glossy atlas of fish anatomy (hard cover)" ed. by A. Ochiai, Midori Shobo, Tokyo, pp. 162-163 for explanation, 255–258 for atlas (In Japanese).

Takeuchi T., Dedi J., Ebisawa C., Watanabe T., Seikai T., Hosoya K. and Nakazoe J., 1995: The effect of β-carotene and vitamin A enriched *Artemia* nauplii on the malformation and color abnormality of larval Japanese flounder. *Fish. Sci.*, **61**, 141–148.

ヒラメ尾部骨格の形成と異常

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摘　要

ヒラメ人工種苗魚の骨異常発現機構を明らかにする一環として、遊泳器官として重要な尾部骨格について調べた。ヒラメ尾部骨格は、2個の独立した上尾骨を備え、第1と第2下尾骨、および第3と第4下尾骨がそれぞれ基底において癒合することで特徴づけられた。また、尾部骨格の形成過程を南（1982）の発育段階区分に従い追求したところ、発育段階DとEの間に不連続帯を認め、この時期に下尾骨板を中心とした担鰭骨の形成が急激に起こることを明らかにした。尾部骨格の形成は着底後の稚魚期にまで及び、最終的には全長約90mmになって完成した。尾部骨格における異常は天然魚ではほとんど見られないが、人工種苗魚では下尾骨板後縁の分枝数の増加、神経棘・血管棘・椎体における隣接要素の癒合などの骨異常が高頻度で認められた。これらの症状の多くは、骨形成が過度に促進される形成促進（acceleration）によるものと考えられた。さらに、骨異常を生じる機構について、尾部骨格形成の不連続期に起こる軟骨形成の不全を中心に考察した。