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Société franco-japonaise d'Océanographie, Tokyo

# Changes in thyroxine ($T_4$) concentrations in larval and juvenile marbled flounder, *Pseudopleuronectes yokohamae*

Rena SHIBATA[1)]*, Kenichi ISHIBASHI[2)], Makoto UEKI[3)], Michihisa ABE[4)] and Takashi YADA[4)]

**Abstract:** There are several reports on the dynamics of thyroid hormones during metamorphosis in flatfish, but there are limited reports on the post-metamorphic juvenile stage. In this study, we investigated changes in thyroxine ($T_4$), a thyroid hormone, from the larval to juvenile stages of the marbled flounder *Pseudopleuronectes yokohamae*. We found that the $T_4$ concentration from 20 days post-hatching (dph) (larval stage) to approximately 120 dph (juvenile stage) substantially increased in the juvenile stage. There was a local maximum $T_4$ concentration in the late developmental stage of juveniles. We also found considerable inter-annual variation in $T_4$ concentrations during this study (2015, 2016, 2018, and 2019). The findings of this study can be used to inform treatment options and management of flatfish seed production to ensure the health and quality of the fish produced

***Keywords :*** *Pseudopleuronectes yokohamae, $T_4$ concentration, development, metamorphosis*

## 1. Introduction

Metamorphosis of flatfish is induced and regulated by two thyroid hormones, triiodothyronine ($T_3$) and thyroxine ($T_4$) (INUI *et al*., 1995; CAMPINHO *et al*., 2012; CAMPINHO, 2019). In many flatfish species, the level of $T_4$ increases considerably during metamorphosis (TAGAWA *et al*., 1990; DE JESUS *et al*., 1991; ARITAKI, 2013; SCHREIBER and SPECKER, 1998). During metamorphosis of flatfish, one eye starts migrating; its

1) Fisheries Technology Institute (Kamisu), Japan Fisheries Research and Education Agency, 7620–7, Hasaki, Kamisu, Ibaraki 314–0408, Japan
2) Futtsu Sea Farming Section, Seed Production Research Laboratory, Chiba Prefectural Fisheries Research Center, 2568–38, Kokubo, Futtsu, Chiba 293–0042, Japan (Choshi Fisheries Office, Chiba Prefectural Government, 2–6385–439, Kawaguchi, Choshi Chiba 288–0001, Japan)
3) Futtsu Sea Farming Section, Seed Production Research Laboratory, Chiba Prefectural Fisheries Research Center, 2568–38, Kokubo, Futtsu, Chiba 293–0042, Japan (Fisheries Resources Division, Chiba Prefectural Government, 1–1, Ichiba, Chuo, Chiba 260–8667, Japan)
4) Fisheries Technology Institute (Nikko), Japan Fisheries Research and Education Agency, 2482–3, Chugushi, Nikko, Tochigi 321–1661, Japan
*Corresponding author: Rena Shibata
Fisheries Technology Institute (Kamisu), Japan Fisheries Research and Education Agency, 7620–7, Hasaki, Kamisu, Ibaraki 314–0408, Japan
Tel: 0479–44–5929
Fax: 0479–44–6221
E-mail: shibata_rena75@fra.go.jp

position becomes asymmetrical relative to the other eye. The skin pigmentation also becomes asymmetrical, with a dark upper side and a white underside. There are many reports on the morphological development of flatfish until the completion of metamorphosis (MINAMI, 1981a; 1981b; 1982). However, the marbled flounder *Pseudopleuronectes yokohamae* continues to develop morphologically even after metamorphosis is completed (I stage), and the juvenile stage is also divided into a J stage (post-metamorphosis) and K stage, according to the levels of skin pigment and lateral line development (FUKUHARA, 1988).

In marbled flounder seed production, juveniles often exhibit loss of fins, particularly the caudal fin, due to nipping. Owing to the fin damage, the fish are likely to contract bacterial infections (SUGIMOTO *et al*., 2007), and their swimming ability might be affected because of the decreased caudal fin area (UEKI *et al*., 2019). This nipping behavior was observed after metamorphosis and tended to increase during the juvenile stage. Salmonids are also known to exhibit nipping behavior, and the plasma $T_4$ level of masu salmon (*Oncorhynchus masou*) is negatively correlated with the frequency of nipping behavior (HUTCHISON and IWATA, 1997). Nipping behavior was reduced by treatment with $T_4$ in brown trout (*Salmo trutta*), steelhead trout (*Oncorhynchus mykiss*), and masu salmon (HUTCHISON and IWATA, 1998). If there is a negative correlation between $T_4$ concentration and nipping behavior, then large fluctuations in $T_4$ during the juvenile stage of the marbled flounder may affect nipping frequency. Understanding the dynamics of $T_4$ concentrations makes it possible to predict when aggression may increase, which is important for improving fish seed production techniques.

Most studies on the changes in $T_4$ concentration in flatfish are based on the developmental stages until metamorphosis, and there are few studies on changes in $T_4$ levels during the juvenile stage. As a preliminary approach to determine whether there are fluctuations in $T_4$ concentrations during the juvenile period, when nipping behavior increases, we examined the dynamics of $T_4$ from the larval to juvenile stages of marbled flounders. The findings will be used to inform treatment options and the management of flatfish seed production, to ensure the health and quality of fish produced.

## 2. Materials and methods

Marbled flounders that had been artificially fertilized and reared between 2015 and 2019 (except in 2017) at the Seed Production Research Laboratory, Futtsu Sea Farming Section, Chiba Prefectural Fisheries Research Center, Japan, were used as experimental animals. The fish were reared at a slightly fluctuating temperature (13–16 ℃) in 20-kL water tanks up to 50 days post-hatching (dph), and in 50-kL water tanks thereafter (after 50 dph).

In 2015, to characterize metamorphic progression, larvae were classified using the staging criteria listed in Table 1 (FUKUHARA, 1988). We sampled fish at different developmental stages: F (24 dph; pelagic larvae), G-H (24 dph; settling larvae), I (35 dph), J (48 dph), and K (122 dph). After 2016, samples were differentiated based on dph at intervals of 10 days for 20–70 dph, and at 10–30 days for 80–120 dph. The total number of fish sampled in each of the four years was 30, 83, 84 and 82, respectively.

Whole-body $T_4$ concentrations in larvae and juveniles were measured and analyzed at the Nikko Field Station, Fisheries Technology Institute, Japan. The $T_4$ contained in the frozen fish was extracted and measured mainly following the method described by KOBUKE *et al.*

**Table 1.** External features of *Pseudopleuronectes yokohamae* at different larval and juvenile stages: stage F-I, modified from FUKUHARA (1988); stages J and K, as described by FUKUHARA (1988).

| Stage | Criteria |
|---|---|
| F | Eyes asymmetrical but not visible from the right |
| G | Eyes asymmetrical, left eye visible from right side but pupil not visible |
| H | Pupil of left eye visible from right side, but half of pupil not beyond ridge of head |
| I | More than half of pupil beyond ridge of head, completing metamorphosis |
| J | Body surface heavily covered with tiny melanophores in metamorphosed fish |
| K | Lateral line discerned clearly in 70-d old larvae, ocellated pigment patterns dispersed when juvenile is more than 20 mm standard length |

(1987) and TAGAWA and HIRANO (1987). The frozen fish were minced, and then ice-cold 100% ethanol was added to the sample according to the size and homogenized with a homogenizer. The homogenate was decanted into an Eppendorf tube, and the tube and blade used for homogenization were washed with ice-cold ethanol. These rinses were added to the original homogenate. After centrifugation at 5,000 rpm for 10 min, the precipitate was re-extracted with ice-cold ethanol. Supernatants were combined and vacuum dried at 37 ℃. $T_4$ levels were measured using an enzyme immunoassay kit (Gen Way Biotech, San Diego, CA, USA). Optical density was measured with a microplate reader (SpectraMax 190, Nihon Molecular Devices, Tokyo, Japan). The measuring range of the standard was 3–250 ng/ml. The mean of the measured data was calculated and is hereafter referred to as the $T_4$ concentration.

## 3. Results

The changes in mean whole-body $T_4$ concentrations during the larval and juvenile stages are shown in Fig. 1. In 2015, the mean $T_4$ concentrations at stages F, G-H, I, J, and K were 0.71, 0.85, 1.01, 1.42, and 0.18 ng/g, respectively. The maximum $T_4$ concentration was observed in stage J. After 2016, the $T_4$ concentrations showed a considerable increase during the juvenile stage, increasing the most at 70 dph (2016) and 110 dph (2019) between 20 and 120 dph. Although the year-to-year fluctuation was large (Fig. 1), the maximum $T_4$ concentration in marbled flounders was observed after 70 dph, corresponding to the late juvenile stage (K stage) (FUKUHARA, 1988).

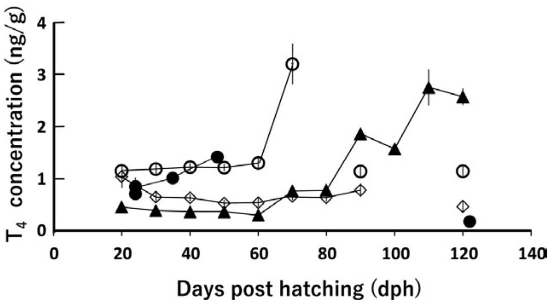


**Fig. 1** Changes in the mean whole-body $T_4$ concentrations from larval to juvenile stages of the marbled flounder *Pseudopleuronectes yokohamae* (●, 2015; ○, 2016; ◇, 2018; ▲, 2019). Sample sizes of each stage or days post hatching (dph) were 6–12 individuals. Error bars indicate SEM.

## 4. Discussion

Based on the changes in $T_4$ concentrations for each stage (F-K) in 2015, the post-metamorphosis J stage had the highest $T_4$ concentration. Based on the $T_4$ concentration of the marbled flounder up to 45 dph in a study by TAGAWA and KIMURA (1991), it was inferred that the $T_4$ concentration peaked at the post-metamorphosis stage. Therefore, we expected there to be a $T_4$ peak at the same stage during metamorphosis in this study. Many studies have reported that the peaks in $T_4$ concentration of other flatfish occurred before metamorphosis was complete (TAGAWA *et al.*, 1990; DE JESUS *et al.*, 1991; ARITAKI, 2013; SCHREIBER and SPECKER, 1998; EINARSDÓTTIR *et al.*, 2006; KLAREN *et al.*, 2008). However, the marbled flounder in our study showed a different trend to other flatfish, where $T_4$ concentrations increased in the post-metamorphic J stage but not in the metamorphosis completion stage (stage I).

The peak $T_4$ concentration in this study was 1.42 ng/g, which was lower than that reported in previous studies (e. g., Japanese flounder *Paralichthys olivaceus,* 12.2 ng/g (DE JESUS *et al.,* 1991) or 15 ng/g (TAGAWA *et al.,* 1990); brown sole *Pseudopleuronectes herzensteini,* 16.1–40.6 ng/g (ARITAKI, 2013); spotted halibut *Verasper variegatus* 2.3–15.9 ng/g (ARITAKI, 2013)). On the one hand, the difference between our results and those of past reports might be due to the difference in the measurement method used. The previous studies mainly used radioimmunoassay (RIA) for measurement, while non-radioactive enzyme immunoassay (ELISA) was used in the present study. On the other hand, this may also be due to interspecific differences, particularly differences in the thyroid to body weight ratios that are associated with different body types.

$T_4$ concentrations in 2016 were different from those in 2018 and 2019. The intra- and inter-assay errors in the measurement system used in this study were both within 10%, and a common sample was used as a reference to confirm the continuity of the measured values. However, the high value in 2016 exceeded the measurement error and was a continuous value. We considered that $T_4$ concentrations in 2016 compared to those of 2018 and 2019 were certain to represent a different trend.

In marbled flounder seed production, juveniles often exhibited caudal fin loss. Damage begins with a slight loss of the caudal fin, which then increases until the caudal fin area of some fish is reduced by more than half. In this study, we defined these as "seriously damaged fish." These fish are considered to exhibit reduced swimming performance (UEKI *et al.*, 2019). In a small-scale experiment conducted in 2018, 100% of the fish that were reared in a 100-L tank at the same stocking density as this study (2,500 individuals/$m^2$ bottom area) exhibited caudal fin loss by 70 dph, and 15% of these were seriously damaged (UEKI *et al.*, 2019). Furthermore, under the same rearing conditions as this study, the proportion of seriously damaged fish increased to approximately 39% after 102 dph (UEKI, 2020). These results indicate that nipping behavior increases during the juvenile stage. It is likely that nipping behavior increases when the $T_4$ concentration decreases after it peaks, which was reported in a previous study on masu salmon (HUTCHISON and IWATA, 1997). Future studies should investigate when nipping behavior increases, and whether the frequency of nipping changes after $T_4$ treatment.

In this study, we found that there was a local maximum $T_4$ concentration in the late developmental stages of juvenile marbled flounder, and that the annual fluctuations in $T_4$ concentrations in the larval and juvenile stages were large. To fully understand these fluctuations, future studies should increase the number of samples per test and shorten the sampling intervals, allowing for fine-scale changes in $T_4$ concentrations to be detected. Additionally, studies should aim to determine the concentrations before and after metamorphosis in other flatfish species, including the measurement of $T_3$ concentrations and their effects on fish physiology and behavior.

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