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本学は、次の者に博士（学術）の学位を授与したので、高知大学学位規則第14条に基づき、その論文の内容の要旨及び論文審査の結果の要旨を公表する。

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# 高知大学学報

本学は、次の者に博士（学術）の学位を授与したので、学位規則（昭和28年文部省令第9号）第8条の規定に基づき、その論文の内容の要旨及び論文審査の結果の要旨を公表する。

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氏名(本籍)	TRAN DUC HAU (ベトナム)
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論文の内容の要旨

The ayu, *Plecoglossus altivelis* (Osmeridae), is an amphidromous fish with an annual life cycle, spawning in the lower reaches of rivers in autumn. Newly hatched larvae are immediately swept downstream to the sea, where they spend the winter months until ascending rivers as juveniles in spring. This species is distributed from the Panke Pond, Hokkaido (ca. 45°N) to the Ryukyu Islands (ca. 26°N) in the Japanese Archipelago, and from Chongjin, northern Korea (ca. 42°N), round the Korean Peninsula, along Chinese coasts (Liaoning, Hebei, Shandong, Zhejiang, Fujian, Guangxi and Taiwan), to the Kalong River (the Bac-Lon River in Chinese), northern Vietnam (ca. 21°30'N) in the continent. It should be determined whether the distributional regions of this species are the most latitudinally expanded of all amphidromous fishes.

The *P. altivelis* distributed in the Japanese Archipelago had been divided into two subspecies, i.e., the ayu *Plecoglossus altivelis altivelis* from Hokkaido to the Yakushima Island and the Ryukyu-ayu *Plecoglossus altivelis ryukyuensis* in the Ryukyu Islands. On the other hand, althou

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Although the Chinese ayu was recently nominated as a subspecies (*Plecoglossus altivelis chinensis*), differing from either *P. a. altivelis* or *P. a. ryukyuensis*, on the basis of morphology, this differentiation is problematic because of having too poor characters to compare among the ayu populations. Thus, the status of continental populations is uncertain.

In contrast to a great attention of molecular studies on the ayu in the Japan-Ryukyu Archipelago, a limited number of works have applied genetic analyses to the continental amphidromous population. It was showed that the Korean populations have been found genetically quite similar to *P. a. altivelis* in the Japan Islands, but different to *P. a. ryukyuensis* in the Ryukyu Islands. However, these works examined only the Korean population as continental ones so that little information exists for other continental populations.

In Japan, it was found that ayu larvae appeared abundantly in the surf zone of sandy beaches, and subsequently it was shown that a proportion of ayu larvae and juveniles remain and grow within the estuary. However, little is known about the early life history in the continental ayu.

Recently, the ayu population has decreased greatly in Japan, and it was hypothesized that this phenomenon can be attributed to an increase in sea water temperatures, and more frequent larval mortality due to higher temperatures because of the global warming. Is this true? I fortunately was able to collect ayu larvae in the Kalong River, which is probably their southernmost distributional area. Therefore, I could ask how ayu lead their early life history in tropical waters.

In the present study, to clarify the early life history of ayu distributed in northern Vietnam, I tried to examine and compare genetically, morphologically and ecologically the Vietnamese ayu with those collected from different areas of Japan.

## 1. Early life history of the Vietnamese ayu

### Water conditions of the Kalong River system

The Kalong River system was vertically well-mixed and received tidal exchange of water, except for a stratification in temperature in late February. The temperatures and salinities when the larvae were collected ranged from 12.1 (middle January) to 21.3°C (December), and from 3.5 (December) to 29.8 psu (middle January), respectively. The temperatures were lower in the sea than those in the river and estuary. Seasonal relationship between the temperature and salinity revealed a reciprocal pattern.

### Distribution

The early life history of ayu (*P. altivelis*) was investigated in the Kalong and Tien Yen River systems, northern Vietnam, which is probably the most southern distribution locality for this species, during the period of November 2010 to February 2011. A total of 248 larvae were captured in the Kalong, and none were collected in the Tien Yen.

***In the central current:*** A total of 227 larvae of ayu were collected by a larva net (1 m mouth-diameter, 0.5 mm mesh-aperture) from December to February, with a peak abundance in early January. They were preflexion to flexion larvae (chiefly preflexion with yolk), ranging from 5.2 to 12.9 mm in size. Larvae less than 8 mm were predominant, and only 30 larvae were larger than 8 mm. The larvae were collected only at the river stations from December to early January, and gradually expanded to be distributed toward the estuary until middle February; subsequently few appeared in late February

***In the bank waters:*** A total of 21 larvae were collected by a small seine net (1 × 4 m, 1 mm mesh-aperture) in the bank waters in January and February 2011. They were composed of flexion and postflexion larvae (mostly postflexion), ranging from 14.1 to 23.8 mm, with a mode at 18–20 mm. The CPUE increased seasonally until late February, except for no larvae

in middle January, then subsequently tended to continue to rise after February. The larvae occurred only at the estuarine stations in January and started to be found at the river stations in February. No larvae were found at the sea stations. The size and ages of ayu larvae ranged from 14–16 and 14–18, 20–24 and 30–41, and 16–21 mm BL and 24–40 days old, respectively; distributed in early January, middle February and late February, respectively. The hatch-date distributions never had overlapped over the occurrence period.

### **Description of the Vietnamese larvae**

Flexion larvae were identified to the species based on the myomere counts (62–65), distinctive pigmentation on the dorsal margin of the caudal peduncle, and formation of dorsal and anal fins. Identification of larvae at a stage earlier than flexion was verified by melanophore patterns traced back from the flexion-stage larvae. Additionally, my larvae could be distinguished from salangid fishes, whose larvae morphologically and meristically resemble ayu larvae, but with closely located dorsal and anal fins.

The larvae are very elongate and have 60–65 myomeres (40–45 + 19–21). The straight, long gut reaches to 76–78% BL, and the anus hardly migrates throughout the larval period. Most of larvae smaller than 8 mm remain some the yolk, which is completely consumed by about 9 mm. Larvae less than 9 mm have only the pectoral fin placed low on the body. Incipient pectoral rays are present at ca. 24 mm. The caudal anlage begins to develop at ca. 9 mm, pushing up the notochord tip during 14–15 mm, with notochord flexion and the rays being completed at ca. 16 mm. The anal and dorsal anlagen are present at ca. 12 and 14 mm, respectively; their incipient rays start to differentiate in larvae smaller than 16 mm, and the full complements of these fin rays are present at ca. 20 mm. The pelvic bud appears slightly anterior to the dorsal-fin origin (myomere 20–21) at ca. 21 mm.

The operculum does not cover the posterior-most gill filaments at least until ca. 24 mm.

The nasal pit is differentiated by ca. 14 mm, and starts to divide at 20 mm.

Initially melanophores are distributed behind the cleithral symphysis, dorsal to the anus, along the dorsal and ventral margins of tail posterior to the anus; a single row of melanophores is found along ventral midline of the gut; and two rows of melanophores at long intervals dorso-laterally on the fore- and midgut. The latter rows are gradually denser with growth.

## 2. Comparison of the early life history of the ayu between Vietnam and Japan

**Genetic:** The genetic results based on the coding region *ND4-tRNA<sup>Ser</sup>* genes in the mitochondrial DNA of seven populations of the ayu from Vietnam, the Japan Islands and the Ryukyu Islands indicate that no haplotype was shared geographically among the populations. In the *ND4-tRNA<sup>Ser</sup>* gene region, one fixed substitution was detected between the Vietnamese ayu and *P. a. altivelis*, and none of fixed substitutions between the Vietnamese ayu and *P. a. ryukyuensis*. The Vietnamese ayu has been found genetically quite similar to the *P. a. altivelis* in the Japan Islands, but not at all to the *P. a. ryukyuensis* in the Ryukyu Islands. This discrepancy can be resolved by paleogeographic considerations. This study also reveals the genetic variability of the Vietnamese population to be very low, in contrast to that of populations in the Japan Islands.

**Ontogeny:** There was little difference in morphology and pigmentation during developmental sequence between the Vietnamese ayu, *P. a. altivelis* and *P. a. ryukyuensis*. Furthermore, comparing development in proportions to the body length of the larvae ranging 5–30 mm BL in size between the Vietnamese ayu and *P. a. altivelis* from the Shimanto estuary facing the Pacific, Muko estuary facing Seto Inland Sea and Niigata coast facing the Japan Sea, there also were few differentiations in morphometrics through the

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ontogeny among the four sites. The turning point of the proportional changes in the Vietnamese ayu is at ca. 12 mm BL, and in *P. a. altivelis* at ca. 10 mm BL. Additionally, the ratio of snout length to body length has a little difference in growth between the Vietnamese larvae and the Japanese larvae. The snout length increases with growth in the Vietnamese ayu, whereas this value increases markedly from ca. 1 to 4%, until ca. 10 mm BL, subsequently becoming constant up to 30 mm BL in the larvae of *P. a. altivelis*.

**Distribution:** The larval occurrence in the Kalong River was 1–2 months later than for *P. a. altivelis* in Japan and *P. a. ryukyuensis* in the Ryukyu Islands probably because of the delay until a reasonable photoperiod for the start of spawning in the lower latitudinal region. Considering the delay occurrence, it is conceivable that the Vietnamese larvae aggregate in the bank waters in March and April like the *P. a. altivelis* in Japan. The larvae were never collected from the sea, where the temperatures were lower than in the river and estuary in January and February, unlike in Japan.

**Growth:** Comparing larval growth among Vietnam, the Shimanto estuary, Muko estuary and Niigata coast, it was highest in Niigata coast, being located most northerly, and there was little difference among the other three sites. The higher growth in Niigata coast was due to larvae born in October, when the water temperature was higher and the ayu larvae were seldom born in the other sites.

In conclusion, it is important that temperatures of the river and estuary were much higher in the Kalong River than in any of the Japanese rivers during early life period of the ayu, and the present study could reveal that they are still breeding in the tropical region. The results from comparison of the ayu larvae between Vietnam and Japan suggest that the early



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life history of ayu is externally not so valuable, but internally different among latitudinal locations. High variability in genetic and reproduction would be potentially associated with increasing ability to cope with environmental change during the ayu evolution. Accordingly, the present study demonstrates convincingly why the distributional regions of the ayu are the most latitudinally expanded of all amphidromous fishes in the world.

## 論文審査の結果の要旨

申請者・テウラン デック ハウ君の学位論文は、世界で最も緯度的分布が広い魚類：アユの初期生活史を、分布の南限であるベトナム北部で明らかにし、さらに本邦の3地域（土佐湾、瀬戸内海、日本海）のものらと比較することによって、そのたぐいまれな多様性を論じた世界的発見を含む画期的な論文である。その審査結果を以下に要約する。

近年、両側回遊魚でかつ年魚であるアユの本邦での資源量は、減少の一途を辿り、その中でも、本邦有数の漁獲量を誇った高知県の凋落は著しく、特に日本最大であった四万十川のアユは壊滅状態にあると言ってもいい。この原因として、温暖化が挙げられることが多いが、本当にそうなのか？ アユは前述したように南北に長く分布し、日本列島では北海道北部から沖縄まで、大陸では朝鮮半島北部からベトナム北部まで、すなわち両者とも亜寒帯から熱帯までの分布し、その距離は緯度で約20度に達する。このように、亜寒帯から熱帯まで分布する魚種が、温暖化によって致命的な影響を受けるだろうか？ その真の原因は、アユの多様性を探ることによって、見出されると考えられた。この命題に迫るため、申請者はベトナムでのアユ仔稚魚の発見から始まった。なぜなら、アユの初期生活史の研究は、本邦では盛んに行われ、その全貌が各地で明らかになっているが、大陸ではほとんど全く調査・研究がなされていなかったからである。

本論文は、基本的には次の三章からなる。

第1章：アユのベトナム北部での初期生活史

第2章：ベトナムおよび日本間での比較

第3章：総合論議

第1章では、約4ヶ月間、ベトナムで滞在し、月1-2回、北部のカロン川およびティエン・エン川で調査を行った結果、アユの仔稚魚を発見し、さらに数か月に渡ってまとまった数の採取に成功した。これは、アユという特異な生物を考えた場合、世界的な発見と言っていい。アユ採集時には、物理的環境も可能な限り把握し、ベトナムにおける初期生活史の全貌をほぼ明らかにできた。さらに、アユの繁殖は、水温と日長時間に影響を受けると言われていたが、ほとんど日長時間だけに左右されることが判った。

第2章では、ベトナムのアユを、本邦での異なった三地域のものらと比較することによって、アユの本質を明らかにすることを目的とした。比較した項目の以下の通りである。

- ①集団遺伝学
- ②形態
- ③発育
- ④成長

まず、mt-DNA で解析した結果、遺伝的にはベトナム産および日本産の間には、違いは見出せず、ほとんど同じ集団という驚くべき事実が明らかになった。すなわち、同種でありながら、亜寒帯から熱帯まで分布し、何らかの遺伝的交流が今だに続いていることになる。個体発生的

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には、ベトナム産と日本産（土佐湾、瀬戸内海、日本海）の間には、顕著な違いがみられなかった。ところが、成長をみると、四者間の中で、最も平均日間成長の高かったのは、最高緯度である日本海（新潟県）のアユで、最低緯度であるベトナムがそれに続いた。水温が比較的高い土佐湾（四万十川）および瀬戸内海（兵庫県・武庫川）産のアユ仔稚魚の日間成長は低く、ほぼ同程度であった。これらのことは、アユの本質を穿っており、初期成長にとって必ずしも水温が重要でないことを示唆している。

第3章では、第1・2章の結果および考察を踏まえ、アユの長大な分布域と本種が持っている戦略的多様性の関係を論議した。

以上、これらの成果を、申請者は平成24年7月25日に開催された公開審査会で効果的に発表し、質問へも適切に対応できた。また、申請者は、本論文内容の一部から、国際雑誌（Ichthyological Research）に、2012年4月に1報を公表している。

以上から、黒潮圏を代表とする魚と言っても過言ではないアユの生活史を扱った申請者の提出論文は博士論文としての申し分なく、近年で最も逸出した論文と言ってもいい。

ふりがな 氏名（本籍） 学位の種類 学位記番号 学位授与の要件 学位授与年月日 学位論文題目  発表誌名	フェブリツァ ドウィランティ FEBRIZA DWIRANTI（インドネシア） 博士（学術） 甲総黒博第7号 学位規則第4条第1項該当 平成24年9月30日 <b>Effects of gametophytes of <i>Ecklonia kurome</i> on the regulation of blood glucose and lipid in mice</b> (マウスにおけるクロメ配偶体の血糖と脂質の制御への影響)  Febriza Dwiranti, Masanori Hiraoka, Takahiro Taguchi, Yuko Konishi, Mari Tominaga and Akira Tominaga. Effects of Gametophytes of <i>Ecklonia kurome</i> on the levels of Glucose and Triacylglycerol in <i>db/db</i> , Prediabetic C57BL/6J and IFN- $\gamma$ KO mice. International Journal of Biomedical Science. 8:64-75. 2012.  -  <div style="text-align: right;">           審査委員 主査 教授 富永 明                              副査 教授 溝渕 俊二                              副査 准教授 田中 尚弘         </div>
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論文の内容の要旨

**[Purpose]**

Modernization of lifestyle, people adopt a modern western diet (such as; fast and instant food) for saving time. The problems of these foods have quantitative and qualitative effects, for example: a high proportion of fats, high calorie density and high glucose intake. Unbalanced food intake, mainly total calories contribute to obesity and type2 diabetes mellitus (T2DM: type 2 diabetes). Type 2 diabetes, T2DM is a complex genetic disease comprised of many metabolic disorders with a common phenotype of glucose tolerance or disorder of dysregulated energy metabolism because of failure to produce sufficient insulin and insulin resistant. Effective management of type 2 diabetes cannot be achieved without proper attention to activities and nutrition. Here, I used edible algae to ameliorate type 2 diabetes.

This dissertation highlights the studies of gametophytes of *Ecklonia kurome* on the regulation of blood glucose and lipids in mice. In Japan, *Ecklonia* species have been believed to improve the circulation of blood. Indeed, bio-active compounds from edible algae have been reported not only in the prevention of hypertension but also in anti-diabetic activities.

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**[Material and Methods]**

On this experiment, two type of mice models, wild type mice (C57BL/6J), and IFN- $\gamma$  KO mice (C57BL/6J-*Ifng<sup>tm1T5</sup>*) were employed. On the other hands, the *db/db* mice model was used for comparison with the results of this experiment. The *db/db* mice are leptin receptor deficient and are used as the model of type 2 diabetes. C57BL/6J mouse is common inbred strain that is susceptible to diet-induced obesity, type 2 diabetes and atherosclerosis, in a manner analogous to most cases of type 2 diabetes in humans. IFN- $\gamma$  KO mice have a genetic background of C57BL/6J mouse with disrupted Interferon- $\gamma$  (IFN- $\gamma$ ) genes. IFN- $\gamma$  is a cytokine involved in inflammatory responses that induces fever and enhances thermogenesis.

Three marine algae consisting of two brown algae, *Ecklonia kurome* (*E. kurome*) and *Cladosiphon okamuranus* (*C. okamuranus*), and a red alga *Poryphyra* were used in this experiment. Especially, gametophytes (n) and sporophytes (2n) of *E. kurome* were compared in terms of their activity to ameliorate prediabetic condition of C57BL/6J mice. The *E. kurome* and a red alga *Porphyr*a species (*Porphyr*a sp.) were isolated as strains and have been kept as culture collections in Usa Marine Biological Institute, Kochi University. The thalli of these algae were cultured using the “germing cluster” method and deep seawater supply developed by Hiraoka and Oka (2008).

The micro gametophytes of *E. kurome* were cultured in a glass flask containing 500 mL ES medium developed by Provasoli (1968).

The schedules of the experiments are described in Table 1.

**Table1. Schedules of administration of diets and algae**

Group No. Strain	Stage 1 (8 weeks)	Stage 2 (4 weeks)	Stage 3 (3 weeks)	Stage 4 (3 weeks)
Serum				↓
Flow cyto.				⊗
OGTT	↓	↓	↓	
1 (C57BL/6J)	ND (CE-2)	ND	ND	ND
2 (C57BL/6J)	HFD (HFD32)	HFD + DW	ND + DW	
HFD + DW				
3 (C57BL/6J)	HFD (HFD32)	HFD + <i>E. kurome</i> G	ND + <i>E. kurome</i> G	HFD + <i>E. kurome</i> G
4 (C57BL/6J)	HFD (HFD32)	HFD + <i>E. kurome</i> S	ND + <i>E. kurome</i> S	HFD + <i>E. kurome</i> S

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5 (C57BL/6J)	HFD (HFD32)	HFD + <i>C. okamuranus</i>	ND + <i>C. okamuranus</i>	HFD + <i>C. okamuranus</i>
6 (C57BL/6J)	HFD (HFD32)	HFD + <i>Porphyra</i> sp.	ND + <i>Porphyra</i> sp.	HFD + <i>Porphyra</i> sp.
7 (IFN- $\gamma$ KO) ND (CE-2)	ND (CE-2)	ND (CE-2)	ND (CE-2)	
8 (IFN- $\gamma$ KO) HFD + DW	HFD (HFD32)	HFD + DW	ND + DW	
9 (IFN- $\gamma$ KO)	HFD (HFD32)	HFD + <i>E. kurome</i> G	ND + <i>E. kurome</i> G	HFD + <i>E. kurome</i> G

■: Bleeding for serum collection; ☒ : Bleeding for Flowcyto. (Flow cytometry); □ : OGTT was performed at the end of each stage as indicated. To prepare sera, 100  $\mu$ L blood was collected from orbital sinus of each mouse eleven days before being sacrificed. Lymphocytes for flow cytometry were collected one day before being sacrificed and stained as described in Materials and methods. *E. kurome* G: gametophytes of *Ecklonia kurome*, *E. kurome* S: sporophytes of *E. kurome*, *C. okamuranus*: *Cladosiphon okamuranus*, *Porphyra* sp: species belonging to genus *Porphyra*. In groups 1, 7, 8, and 9, each group consists of 5 female mice. In groups from 2 to 6, each group consists of 8 female mice. From group 1 to 6, eight weeks old female C57BL/6J mice were used. IFN- $\gamma$  KO mice were 8 to 15 weeks old female mice. All algae homogenate suspended in distilled water (DW) was administered using capillaries as described in Materials and methods.

Normal diet (ND: CE-2) and High Fat Diet 32 (HFD: HFD 32) were purchased from CLEA Japan. CE-2 contains 4.8% crude fat and HFD 32 contains 7.1% saturated fatty acids (4% palmitic acid and 2.4% stearic acid), 21.2% monounsaturated fatty acids (20.5% oleic acid), 3.3% polyunsaturated fatty acid (3.2% linoleic acid). HFD 32 contains 27.4% carbohydrates and CE-2 contains 54.6% carbohydrates. For Oral Glucose Tolerance Test (OGTT), one drop of blood was collected from a tail vein of each mouse hourly (0, 1, 2, and 3 hours) after oral administration of glucose (2 g/kg body weight). Blood glucose was measured with Accu-Chek (Roche Diagnostics K.K., Tokyo, Japan) in all experiments. Interpretation of OGTT status was defined according to recommended diagnostic criteria by World Health Organization and International Diabetes Federation (2006)

Serum was prepared from the blood collected from orbital sinus of each mouse using a glass capillary. Serum levels of glucose, triacylglycerol, and cholesterol were measured by Hitachi Clinical Analyzer S40 (Hitachi, Ltd., Tokyo, Japan) using S-Test Cartridges for glucose, triacylglycerol, and cholesterol (Alfa Wassermann, Inc., West Caldwell, NJ). Serum levels of insulin and leptin were measured according to the manufacturer's protocol. TMB (3, 3', 5, 5'-tetramethylbenzidine) was used as a chromogen. The levels of both insulin and leptin in the sera were evaluated by measuring the absorbance at 450 nm using a microplate reader ThermoMax ROM v102 (Molecular Devices, Sunnyvale, CA).

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The percentage of Treg cells among lymphocytes of each group was evaluated from the pooled peripheral blood. Ten million lymphocytes were collected from pooled blood from each group by using Lympholyte-M (Cedarlane, Hornby, Ontario, Canada). Treg cells were stained using Mouse peridinin chlorophyll protein (PerCP) conjugated anti-CD4 antibodies (PerCP anti-CD4 Ab) and fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated anti-CD25 antibodies (FITC-anti-CD25 Ab or PE-anti-CD25 Ab) according to the manufacturer's protocol (Bio Legend, San Diego, CA). Five million stained cells were analyzed with a FACSCalibur (Becton Dickinson and Co., Mountain View, CA), using Quadra Stats of BD Cell Quest™ Pro Version 5.2.

For the OGTT, analysis of variance (ANOVA) was performed and significance was determined using Tukey-Kramer's post hoc test. For serum glucose, triacylglycerol, cholesterol, leptin, insulin, adipose tissues, body weights, and food intakes, one way ANOVA was used to evaluate the statistical significance. A  $P$  value of  $< 0.05$  was considered statistically significant.

### [Results and Discussion]

#### Effect of gametophytes of *E. kurome* on Oral Glucose Tolerance Test (OGTT)

The glucose tolerance test is usually used to differentiate individuals with impaired glucose tolerance and those with type 2 diabetes from normal ones. Overnight fasting is useful for studies where the focus is on glucose utilization in mice, because it nearly depletes glycogen stores in the mouse. For these studies, C57BL/6J and IFN- $\gamma$  KO mice are fasted for 18 hours as recommended by Ayala *et al* (2010).

At stage 1, mice were administered with either ND or HFD for 8 weeks. The results of OGTT of IFN- $\gamma$  KO mice at stage 1 (Figure 1b) showed different results from those of C57BL/6J mice (Figure 1a). During the administration of ND, the OGTT showed a higher level of blood glucose in IFN- $\gamma$  KO mice, compared with that of C57BL/6J mice. Although HFD induced a higher level of blood glucose compared with ND group in the OGTT of C57BL/6J mice, this effect of HFD was not observed clearly at first but appeared three hours after glucose administration in IFN- $\gamma$  KO mice.

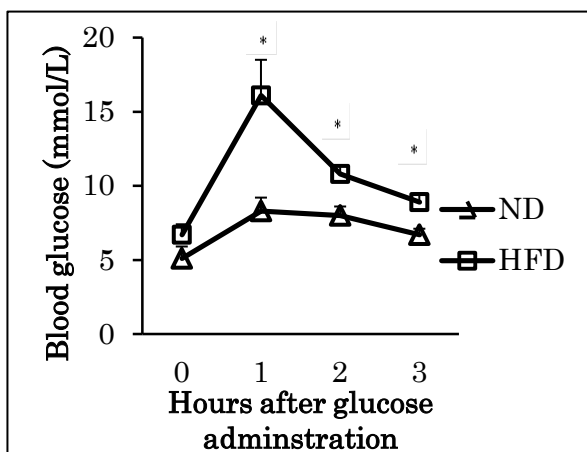


Figure 1a. Effects of HFD on the OGTT in C57BL/6J mice at stage 1. \* indicates that there is a significant difference at each point between groups (ANOVA, Tukey-Kramer's post hoc test,  $P < 0.05$ ).

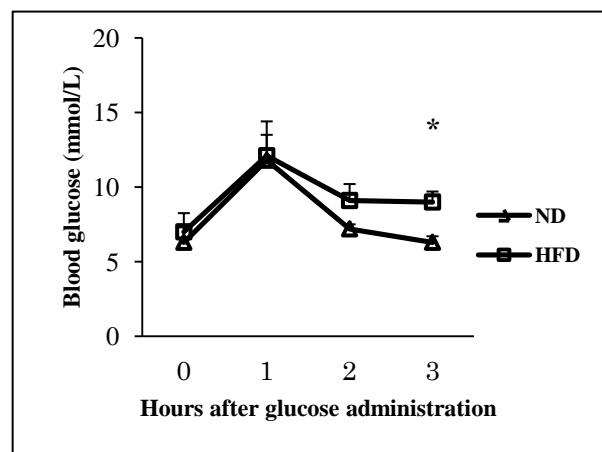


Figure 1b. Effects of HFD on the OGTT in IFN- $\gamma$  KO mice at stage 1. \* indicates that there is a significant difference at each point between groups (ANOVA, Tukey-Kramer's post hoc test,  $P < 0.05$ ).

## 論文の内容の要旨

These results indicate that IFN- $\gamma$  is involved in the regulation of blood glucose.

Gametophytes of *E. kurome* were administered orally to prediabetic C57BL/6J and IFN- $\gamma$  KO mice and OGTTs were performed to evaluate the effects of the algae. The two prediabetic strains of mice were administered with gametophytes of *E. kurome* together with HFD for four weeks and the administration of gametophytes of *E. kurome* did not have any effects on the OGTT in either C57BL/6J or IFN- $\gamma$  KO mice at stage 2. At stage 3, gametophytes of *E. kurome* down-regulated the level of blood glucose in OGTTs of both C57BL/6J and IFN- $\gamma$  KO mice, when ND was administered after making them prediabetic (Figure 2a and b). Therefore, gametophytes of *E. kurome* can be used to improve glucose tolerance.

### Effect of gametophytes of *E. kurome* on the regulation of blood glucose and lipids

The next step was to determine the effects of administering marine algae on the glucose and lipids when mice were fed with HFD. At stage 4, serum levels of glucose, triacylglycerol, cholesterol, leptin and insulin were measured.

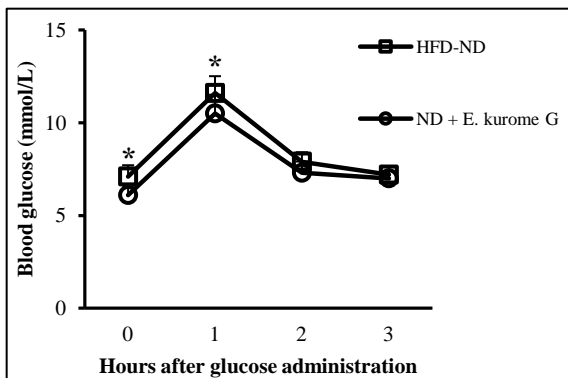


Figure 2a. Effects of gametophytes of *E. kurome* on the OGTT in C57BL/6J mice at stage3. \* indicates that there is a significant difference at each point between groups (ANOVA, Tukey-Kramer's post hoc test,  $P < 0.05$ ).

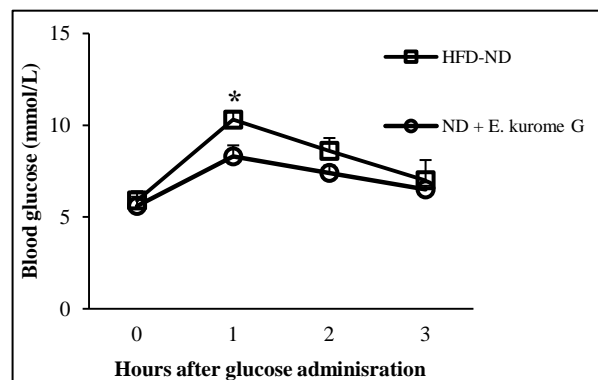


Figure 2 b. Effects of gametophytes of *E. Kurome* on the OGTT in IFN- $\gamma$  KO mice at stage 3. \* indicates that there is a significant difference at each point between groups (ANOVA, Tukey-Kramer's post hoc test,  $P < 0.05$ ).

There were significant differences between the group administered with gametophytes of *E. kurome* and that with sporophytes of *E. kurome*, or with *C. okamuranus* at the serum glucose level in C57BL/6J mice. Only gametophytes of *E. kurome* lowered serum level of glucose to normal level in C57BL/6J mice at stage 4. This effect of gametophytes of *E. kurome* was not observed in IFN- $\gamma$  KO mice.



## 論文の内容の要旨

The serum levels of triacylglycerol in control HFD group and sporophytes of *E. kurome*-treated group were  $29.6 \pm 6.5$  mg/dL and  $34.6 \pm 9.7$  mg/dL, respectively. These values were lower than the level of ND group ( $67.5 \pm 10.4$  mg/dL). The serum level of triacylglycerol in the group administered with gametophytes of *E. kurome* was higher ( $51.8 \pm 17.1$  mg/dL) than the level of HFD group in C57BL/6J mice. In contrast, gametophytes *E. kurome* did not have any effect on the serum triacylglycerol in IFN- $\gamma$  KO mice.

It is possible that gametophytes accumulate triacylglycerol or carbohydrates by converting glucose as energy sources before fertilization, because it is common for plants to accumulate energy sources at a certain stage of the lifecycle such as when producing seeds. This hypothesis may be supported by the fact that gametophytes but not sporophytes of *E. kurome* up-regulated the serum level of triacylglycerol to the level of ND group in C57BL/6J mice. This effect of gametophytes of *E. kurome* was not observed in IFN- $\gamma$  KO mice, suggesting that IFN- $\gamma$  may be involved in the regulation of the metabolism of glucose and lipid.

At the serum levels of total cholesterol, there were also significant differences between ND group and HFD groups only in C57BL/6J mice. Gametophytes of *E. kurome* did not have significant effect on it in either strain of mice.

At the end of stage 4, fat tissue around uterus was measured. Fat tissue accumulated in the groups of HFD-fed mice in both C57BL/6J and IFN- $\gamma$  KO mice. Obesity is the natural result of a diet rich in fat. Administering gametophytes *E. kurome* together with HFD significantly decreased the amount of fat tissue around uterus in IFN- $\gamma$  KO but not C57BL/6J mice.

There were significant differences at serum levels of leptin between ND group and HFD group in both strains of mice. The weight of fat tissue around uterus was correlated with serum level of leptin in both strains of mice. This is consistent with other studies that leptin is produced in adipose tissue and circulating levels of leptin directly correlate with adipose tissue mass. Fat tissue around uterus in IFN- $\gamma$  KO mice was higher than that of C57BL/6J mice.

Insulin resistance was observed in prediabetic HFD-fed mice in overnight-fasted C57BL/6J but not IFN- $\gamma$  KO mice. Gametophytes of *E. kurome*, sporophytes of *E. kurome* and *C. okamuranus* had a tendency to ameliorate the insulin resistance in C57BL/6J mice. Serum insulin level in IFN- $\gamma$  KO mice fed with HFD was lower ( $1.0 \pm 0.8$  ng/mL) than that in C57BL/6J mice ( $1.4 \pm 0.3$  ng/mL).

Gametophytes of *E. kurome* had a tendency to reduce the Treg cells compared with control group in both *db/db* and IFN- $\gamma$  KO mice. De Rosa *et al.* (2007) reported that leptin down-regulates the Treg cells and stimulates the proliferation of IFN- $\gamma$  secreting Th1 cells. This may suggest that gametophytes of *E. kurome* may replace the function of leptin, in part, in regulation of Treg cells.

There was no significant difference in body weights among HFD groups either in C57BL/6J or IFN- $\gamma$  KO mice, although groups of HFD-fed mice had higher body weights in average compared with those of ND-fed mice in both strains of mice.

These results suggest that gametophytes of *E. kurome* have bioactive molecules which may function to reduce the blood glucose and white adipose tissue by regulating immune system and endocrine system associated with life cycle and cell differentiation.

## 論文の内容の要旨

Gametophytes of *E. kurome* are useful in down-regulating blood glucose and are good candidates to be used as an alternative medicine, because they are one stage of an edible alga.

## 論文審査の結果の要旨

アジアでは、近年、工業化と食事の欧米化により糖尿病等の生活習慣病が急速に増加している。この病気の予防には適度な運動と過剰な炭水化物や脂肪の摂取を控えることが重要と考えられている。本研究の目的は野生型マウスC57BL/6Jに高脂肪食を投与し、2型糖尿病の前段階の状態を作製し、その耐糖能異常を改善できる食品を海藻から選択し、その糖・脂質代謝への影響を解析することである。インターフェロン- $\gamma$  (IFN- $\gamma$ ) 遺伝子欠損 (IFN- $\gamma$  KO) マウス, C57BL/6J-*Irfngtm1Ts*を用いた理由は、このマウスが親系統であるC57BL/6Jに比べて成長が悪くエネルギー代謝に欠陥があると考えたからである。また、食欲抑制性ホルモンであるレプチンは免疫抑制性の制御性T細胞を減少させ、インターフェロン- $\gamma$  (IFN- $\gamma$ ) 産生性タイプ1ヘルパーT細胞を増殖させることが報告されている。このレプチン受容体に欠損があり、このホルモンのシグナルが伝達されない*db/db*マウスは過食で、運動不足になり、2型糖尿病を発症する。

本研究で使用した *Ecklonia kurome* (クロメ) 配偶体がこの2型糖尿病モデル、*db/db* マウスで血糖を低下させる効果を有することを参考論文で報告している。また、同時にクロメ配偶体は *db/db* マウスの制御性T細胞を減少させることを報告している。

餌として4.8%の脂質を含む通常食CE-2と32%の脂質を含む高脂肪食HFD32を用いた。食用海藻としてクロメ配偶体、クロメ孢子体、*Cladosiphon okamuranus* (オキナワモズク)、*Porphyra species* (アマノリの1種)を蒸留水中でポリトロンにて破砕してホメジェネートとして80度 $^{\circ}$ Cで1時間処理を2回繰り返して10mg/0.2mL/mouseでキャピラリーを用いて1日おきに経口投与した。

まず、高脂肪食HFD32を8週間投与して前糖尿病状態(耐糖能異常)をC57BL/6Jおよび、IFN- $\gamma$  KOマウスで作製した。前糖尿病状態は18時間絶食した後、経口糖負荷試験で確認した。前糖尿病の状態はWorld Health Organization(WHO)の2006年基準を利用した。Impaired Glucose Tolerance (耐糖能異常): 絶食時グルコース: $<7.0$  mmol/L and 糖負荷後2時間の血漿グルコース: $\geq 7.8$  and  $<11.1$  mmol/L。ここでは糖負荷は2g/kg body weightである。野生型のC57BL/6Jマウスに通常食を投与した場合は18時間絶食後、糖負荷を行ったところ、血糖値は0h:5.1; 1h:8.3; 2h:8.0; 3h:6.7 (単位mmol/L、8匹の平均値)であった。高脂肪食を8週間投与した場合、絶食後の血糖値はそれぞれ0h:5.9; 1h:16.1; 2h:10.8; 3h:8.9 (単位mmol/L、8匹の平均値)であった。これに対して、IFN- $\gamma$  KOマウスでは通常食で血糖値はそれぞれ0h:6.3; 1h:11.8; 2h:7.2; 3h:6.3 (単位mmol/L、5匹の平均値)であった。興味深いことに高脂肪食を投与したときもこの値は0h:6.9; 1h:12.2; 2h:9.1; 3h:8.9(単位mmol/L、5匹の平均値)でほとんど変化しない。しかし、親系統野生型C57BL/6Jマウスと対照的に、糖負荷3時間後でもこの値はほとんど低下せず、このとき初めて通常食に比べて有意に高い血糖値を示した。このことはIFN- $\gamma$ が血糖の消費に重要な役割を果たしていることを示している。また、高脂肪食投与後の経口糖負荷試験で糖負荷1時間後に血糖値が上昇することにIFN- $\gamma$ が関与していることを示唆している。

高脂肪食を投与しながら海藻ホメジェネートを4週間投与しても効果は認められなかった。次に、高脂肪食から通常食に餌を変え3週間海藻ホメジェネートを投与した。この食餌の変化にかかわらず、前糖尿病状態は両系統のマウスでまだ維持されていた。この時、C57BL/6Jで同時に海藻ホメジェネートを投与したところ、経口糖負荷試験で糖負荷1時間後にクロメ配偶体投与群は孢子体投与群に比べて血糖値が低かった。さらに3時間後、有意差は認められなかったが海藻ホメジェネートの中、クロメ配偶体投与群が唯一7mmol/L以下の血糖値を示した。この結果はクロメ配偶体投与が前糖尿病状態の症状の改善に有効である可能性を示している。IFN- $\gamma$  KOマウスでもクロメ配偶体ホメジェネートは、対照群(高脂肪食 $\rightarrow$ 通常食、DW投与)に比して前糖尿病状態の症状を改善させている。

## 論文審査の結果の要旨

前糖尿病状態を維持するため、再度、通常食から高脂肪食にもどした。この間、3週間海藻ホモジェネートを投与した。最後に採血にして血糖値、トリアシルグリセロール、総コレステロール、体脂肪、レプチン、インスリンを測定した。C57BL/6Jでは、クロメ配偶体投与群のみで血糖値の有意な低下が認められ、クロメ胞子体および他の海藻ホモジェネート投与では同様な効果は認められなかった。このクロメ配偶体による血糖値の低下はIFN- $\gamma$  KOマウスでは認められなかったため、IFN- $\gamma$ がクロメ配偶体の血糖値低下には必要であると考えられる。体脂肪としては、子宮近傍の脂肪組織の重量を測定し、体重100gあたりの脂肪組織重量を比較検討した。

IFN- $\gamma$  KOマウスではクロメ配偶体は有意に体脂肪を抑制していた。しかし、C57BL/6Jではクロメ配偶体の脂肪組織重量抑制効果は認められなかった。レプチンもIFN- $\gamma$  KOマウスではクロメ配偶体投与群で有意に減少していた。IFN- $\gamma$ が存在しない方がクロメ配偶体の効果が出やすいことはIFN- $\gamma$ が脂肪蓄積の際の増悪因子となっていることを示唆しているのかもしれない。

C57BL/6Jではトリアシルグリセロールは対照群（高脂肪食→通常食→高脂肪食、DW投与）で通常食群より低下しており、クロメ配偶体投与群ではこれが通常食群に近くなっている。この変化はクロメ胞子体投与群では認められなかった。また、このクロメ配偶体の効果はIFN- $\gamma$  KOでは認められなかった。このことは、高脂肪食が投与された場合、クロメ配偶体がグルコースからトリアシルグリセロールへの代謝を促進している可能性を示唆しているが、結論を得るにはさらなる研究が必要である。総コレステロールはC57BL/6Jマウスでは通常食より有意に高かったが、IFN- $\gamma$  KOマウスではこの差は認められなかった。両系統マウスで、いずれの海藻ホモジェネート投与も総コレステロール量を変化させなかった。

絶食時のインスリン濃度はC57BL/6Jマウスでは高脂肪食投与対照群（高脂肪食→通常食→高脂肪食、DW投与）で有意に高くなっていた。*Prophyra* sp.以外すべての海藻ホモジェネート投与群ではインスリン濃度が低下傾向にあり、*Prophyra* sp.以外の海藻ホモジェネートはインスリン抵抗性の改善に有効である可能性がある。この傾向はIFN- $\gamma$  KOマウスでは認められなかった。IFN- $\gamma$ がインスリン抵抗性の緩和に必要であることを示唆している。

最後に、炎症を抑制し、炎症性サイトカインであるIFN- $\gamma$ の産生細胞と拮抗的に働く制御性T細胞（Treg）の割合をクロメ配偶体が低下させるかどうかを検討した。野生型のC57BL/6Jマウスではクロメの配偶体だけでなく胞子体にも弱いながらTreg細胞を減少させる効果がみられた。したがって、血糖値を低下させることとTreg細胞は直接は関係ないと思われる。IFN- $\gamma$  KOマウスにおいては、クロメ配偶体投与群でTreg細胞数の著しい低下がみられたことから、クロメ配偶体がこの点に関してはレプチン様の作用を示すことが考えられる。しかし、摂餌量、体重に有意差がないことからクロメ配偶体が強い食欲抑制作用を持つことは考えられない。IFN- $\gamma$  KOマウスにおいてクロメ胞子体も同じ作用を持つかどうか検討する必要がある。IFN- $\gamma$ が存在しない場合に、Treg細胞が増加しているため、クロメ配偶体の効果が見やすくなっているのかもしれない。

本研究は黒潮圏で近年増加の一途をたどる生活習慣病の一つ、2型糖尿病の前段階の状態の人が糖尿病になる前に黒潮圏で生育する食用海藻を利用してその発症を予防する可能性を考え、食用藻類の糖・脂質代謝制御作用を解析したものである。食品による疾病の予防は時間のかかる方法ではあるが、生活習慣を改善して、この病気の発症を遅らせる本質的な方法でもある。医薬品を使わずに、最大限土地の食材を使って生活を改善することによる病気の予防は黒潮圏科学の持続型社会の考えに合致するものである。グルコース、脂肪、コレステロール、レプチン、IFN- $\gamma$ 、インスリン、制御性T細胞などの多くの指標を考慮に入れた点が評価されるが、相互の関連はいまだ明瞭にはなっていない。しかし、今後の研究の手掛かりを提供した点と食生活の改善で生活習慣病の予防に貢献できる材料を提案したことが評価できる。