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RESEARCHES ON THE ESTABLISHMENT OF PUFFERFISH FOOD CULTURE IN VIETNAM

ベトナムのフグ食文化形成に関する研究

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ABBREVIATIONS

L. spadiceus:	*Lagocephalus spadiceus*
L. cheesemanii:	*Lagocephalus cheesemani*
L. lunaris:	*Lagocephalus lunaris*
L. inermis:	*Lagocephalus inermis*
S:	Spawning season
NS:	Non-Spawning season
I:	Intestine
G:	Gonads
L:	Liver
S:	Skin
M:	Muscle
TTX:	Tetrodotoxin
TTXs:	Tetrodotoxin and it's analogs
ELISA	Enzyme linked immunosorbent assay
HPLC	High performance liquid chromatography
FLD	Fluorescent detection
LC-MS	Liquid chromatography-Mass spectrometry

SUMMARY IN ENGLISH

In Japan, pufferfish has been developed as a delicious high-class food culture, although it is toxic. On the other hand, Vietnam has a long coastline with abundant pufferfish, and it could be a potential fishery resource but the pufferfish food culture is not developed. This applicant conducted research on the idea that introducing a Japanese-style pufferfish food culture in Vietnam could contribute to food safety and security, economic effects, nutrition, and so on. This thesis consists of the following four chapters. The studies that make up Chapters 2 and 3 are original studies and have already been published in English-language academic journals.

Chapter 1 Issues and solutions for utilizing pufferfish resources in Vietnam

Chapter 2 (Study 1) Acceptability evaluation by Vietnamese about non-toxic cultured pufferfish in comparison with grouper and mackerel

Chapter 3 (Study 2) Tissue distribution of tetrodotoxin and its analogs in *Lagocephalus* pufferfish collected in Vietnam

Chapter 4 Birth and development of pufferfish food culture in Vietnam

Chapter 1 describes the research background with a literature review and the purpose of this research as a whole. In particular, the knowledge, legal measures / systems, and measures required to establish a new culture of eating pufferfish in Vietnam are discussed. As a basis for this, the applicant stated that she worked on Study 1 (whether pufferfish is allowed to be eaten by Vietnamese experts on Japan) and Study 2 (tetrodotoxin in tissue of pufferfish collected in Vietnam). Furthermore, the conclusion of an exchange agreement between two Japanese-based and two Vietnam-based organizations, which is a prerequisite for advancing these two studies, is also described. More specifically, regarding Chapter 1.

There are more than 100 species of pufferfish (scientific term: *Tetraodontidae,* Japanese term: fugu) that inhabit coastal and freshwater areas around the world, and there are large numbers in subtropical and tropical waters, including in Vietnam. Pufferfish have delicious white meat, but some species possess a strong neurotoxin that has often killed people. Death from pufferfish toxin is frequent in Vietnam so that the capture, marketing, and ingestion of pufferfish are currently prohibited by law. On the other hand, pufferfish are used as edible fish in Japan, and in particular, *Takifugu rubripes* Torafugu is treated as a high-class fish, but there is almost no poisoning due to this.

The toxicity of pufferfish varies depending on the habitat, species, season, gender, organs and so on. The toxic substances in pufferfish are tetrodotoxin (TTX) and analogs (TTXs). High concentrations of TTXs have been confirmed in some amphibians, arthropods, toxic mollusca, and flatworms. The mechanisms of how these organisms become toxic have not been known clearly until now. The TTXs of pufferfish are thought to be derived from their food sources because cultured Torafugu grown on artificially compounded feed is non-toxic. In Japan, the species and parts of edible pufferfish are legally stipulated, and their processing is limited to specialists such as pufferfish processing chefs who have been certified by each prefecture government. This system works well, and currently there are very few deaths from eating pufferfish in Japan each year. Most of these death cases are due to non-licensed preparation.

Based on the successful model system in Japan, to establish a pufferfish food culture in Vietnam, an agreement to establish a pufferfish food culture in Vietnam was signed by Jumonji University, Mitsui Suisan Co., Ltd., the Vietnam National Institute of Nutrition, and the Vietnam Research Institute of Marine Fisheries. The agreement will be continuously renewed every five years unless otherwise noted. The main contents are related to the establishment of a pufferfish toxicity test system and a pufferfish processing system in Vietnam.

In Chapter 2, I conducted Study 1 on a comparison of the taste of dishes using cultured pufferfish from Japan and grouper and mackerel from Vietnam to see whether the taste of Japanese pufferfish dishes would be accepted by Vietnamese. Japanese pufferfish processing license-holder chefs prepared these three kinds of fishes in fried and hot pot dishes. In addition, Japanese-style fried fish, hot pot, sashimi (dishes with raw fish), hirezake (Japanese sake with grilled pufferfish fin in it), nikogori (a kind of jelly made from pufferfish skin), skin mix (boiled fugu skin sliced and mixed with chili, miso, and vinegar), tataki (fish grilled on the outside and kept raw inside), and shirako tofu (a kind of jelly made with pufferfish shirako) were served and evaluated for their taste. The number of panelists selected was 53 in the capital city of Hanoi and 54 in the city of Da Nang, where one of the largest fishing ports of Vietnam is located. The panelists were nutrition and marine science researchers, marine products company staff and government employees who are instrumental in establishing laws. The results show that when comparing dishes using Japanese cultured Torafugu and Vietnamese high-quality fish, more than 90% of panelists answered that "fugu was tastier than Vietnamese high-class fish". Acceptability was scored by the 5-point method. The average score for various pufferfish dishes was over 4.40, which was very high. On the questionnaire whether they would like to eat more pufferfish, introduce it to their acquaintances, or make pufferfish a new food culture, more than 80% of the panelists answered "yes". In conclusion, Vietnamese panelists evaluated pufferfish as delicious and can become a new food culture.

In Chapter 3, I conducted Study 2 on the measurement of the content of tissue-specific TTX and TTXs in *Lagocephalus* pufferfish (Sabafugu) collected in Vietnam. There are several species belonging to Sabafugu, which inhabit widely from temperate to tropical seas. Some of them are considered to be almost non-toxic in Japan. As mentioned above, the toxicity of pufferfish is said to vary greatly depending on the habitat, and the toxicity of pufferfish from Vietnam has not been elucidated. In this study, we analyzed the TTXs content of each part of the genus Sabafugu collected in each region of Vietnam. When the pufferfish was mixed with the main catch, we asked the fishermen's associations in each region to freeze it and make it available to us. From 2017 to 2019, a total of 108 Sabafugu (*L. spadiceus* Shirosabafugu, *L. cheesemanii* Kurosabafugu, *L. lunaris* Dokusabafugu, *L. inermis* Kanafugu) were used as samples; they were collected in the waters off Hai Phong, Nghe An in the north, Thanh Hoa, Da Nang in central, Vung Tau and Kien Giang in southern Vietnam. These frozen samples were delivered to Japan by air, dissected in a semi-thawed state, and each organ was extracted with diluted acetic acid in a hot immersion using conventional method used to prepare the test solution. The test solution was diluted with a neutral phosphate buffer solution, and the TTXs content was analyzed by the ELISA kit using a novel anti-TTX polyclonal antibody newly

developed at Kitasato University. For samples in which high levels of TTXs were detected were analyzed by HPLC fluorescence (HPLC-FLD) to determine the content of TTX - a highly toxic component. In addition, for some of the samples, the presence or absence of deoxy analogs of TTX that could not be detected by HPLC-FLD was analyzed by the LC-MS method. The results show that almost no TTX and only almost non-toxic 5,6,11-trideoxy TTX (TDT) were detected in all organs of Shirosabafugu. In Kurosabafugu, trace amounts of TTX were detected in the gonads and intestines, but no TTX was confirmed in the muscles. High concentrations of TTXs were detected in Kanafugu by the ELISA method. In Dokusabafugu, trace amounts of TTX were confirmed in muscles and high concentrations in other organs. The results of this study suggest that Vietnamese Kurosabafugu and Shirosabafugu muscles are safe for food.

In Chapter 4, we considered what is necessary for the birth and development of a pufferfish food culture in Vietnam. The main elements are the expectations of the Vietnamese government, the establishment of a pufferfish poison analysis laboratory, the establishment of a pufferfish processing license system, and the development of the pufferfish industry.

SUMMARY IN JAPANESE
要旨

日本では、有毒であるが美味な高級魚としてフグを食す文化が形成されている。一方、ベトナムは長い海岸線を有し、フグの種類また生息数が豊富であり、水産資源として期待できるが、フグ食文化は発達していない。本論文は、これらの事実に着目し、日本方式を参考とするフグ食文化の形成が、ベトナムにおける食の安全安心、経済効果、食文化の醸成質的量的、栄養、多方面に資するとの観点から取り組んだ研究成果を取りまとめたものである。本論文は英文で執筆され次の4章からなる。なお第２章および第３章を構成する研究はオリジナルな研究であり、それらの成果の一部は既に英文学術雑誌において公表されている。

第1章　研究背景・文献レビュー及び日越専門家の交流協定締結
第2章（研究1）養殖トラフグ、ハタ及びサワラ料理のベトナム人における官能評価の比較
第3章（研究2）ベトナムで採集されたサバフグの組織別テトロドトキシンとその関連成分の含有量測定
第4章　ベトナムにおけるフグ食文化の誕生と発展

第1章では文献レビューによる研究背景および本研究全体の目的が述べられ、とくにベトナムでの食経験が乏しいフグを食すという新しい文化をベトナムに根付かせるために求められる知見、法的措置・制度、方策など等が検討されている。そのための基礎として、申請者は研究1（フグの喫食がベトナムの専門家に許容されるか）および研究2（ベトナムで採集されたフグの組織別テトロドトキシン類含有状況）に取り組んだとしている。さらに、この二つの研究を進めるための前提となる日越関連４機関による交流協定の締結についても記載されている。第1章に関し、より具体的には次のとおりである。フグ科魚類は世界各地の沿岸や淡水域に100種以上生息し、熱帯・亜熱帯海域では漁獲量も多い。フグは白身魚であり、その肉は美味である反面、強力な神経毒を保有するものがあり、しばしば死亡例を含む食中毒を引き起こしてきた。ベトナムではフグ中毒が多発しており、現在、フグ科魚類の捕獲、販売、摂取が法律で禁止されている。一方、日本ではフグ類は食用魚として利用されており、特にトラフグは高級魚として扱われ、これによる食中毒はほとんど起きていない。フグの毒性は、生息海域、種類、季節、性別、臓器などによって異なる。フグ毒の本体はテトロドトキシン(TTX)とその誘導体(TTXs)である。これら有毒生物の毒化機構は依然として不明である。配合飼料で育成した養殖トラフグが無毒であること、無毒フグに毒餌を与えると毒化することから、フグのTTXsは餌に由来すると考えられている。日本では食用可能なフグの種類と部位が法的に定められており、その取り扱いは都道府県ごとに認定したフグ処理師等の専門家に限定されている。この対策は有効に機能し、現在日本でのフグの喫食による死者は毎年数名となっており、その大部分は素人処理によるものである。

交流協定は、十文字学園女子大学、ミツイ水産株式会社、ベトナム国立栄養研究所、ベトナム海洋漁業研究所のあいだで締結した。協定は、特に意見がなれ、5年ごとに継続的に更新される。主な内容は、ベトナムのフグの毒性試験制度、フグ処理師制

度の設立などに関するものである。

第2章では、研究1として、養殖トラフグ、ハタおよびサワラ料理のベトナム人における官能試験結果の比較を行った。日本のフグ料理の味がベトナム人に受け入れられるかどうかを調べるために、日本の養殖トラフグとベトナムの高級魚（ハタ、サワラ）で比較を行った。日本のフグ処理師免許保持者が、これら3種類の魚の揚げ物と鍋料理を作った。さらに日本で一般的なトラフグの調理法、すなわち唐揚げ、鍋料理、刺身、ヒレ酒、煮こごり、皮の和え物、たたき、雑炊、白子豆腐を作り、官能評価を行った。選ばれたパネリストは107人で、栄養学及び海洋生命学分野の研究者、海産物会社員やフグに関する法律作成に関与する政府職員とした。その結果、日本の養殖トラフグとベトナムの高級魚を使った料理の比較では、90%以上が「フグはベトナムの高級魚よりもおいしい」と回答した。5点満点法の平均点では、各種フグ料理の平均スコアは4.40超と非常に高い評価を得た。質問表で、フグをもっと食べたいか、知人に紹介したいか、フグを新しい食文化にしたいかについて聞いた結果、80%以上のパネリストが「はい」と答えた。以上の結論として、ベトナムのパネリスト達の大部分から「フグは美味しくて新しい食材になる」という評価を得た。

第3章では、研究2として行われたでは、ベトナムで採集されたサバフグの組織別TTXsの含有量を測定した。フグ類の毒性は産地や季節により大きくことなるとされており、ベトナム産サバフグ類の毒性については解明されていない。フグの収集は、漁師が捕獲した魚に混ざっていた場合、冷凍保存して提供していただくよう各地の漁業組合に依頼して行った。2017年から2019年までの期間に、ベトナムの長い沿岸で、北から南までの6漁港に水揚げされた合計108匹のシロサバフグ、クロサバフグを試料とした。技術の確認のために、有毒であることがわかっているドクサバフグ及びカナフグ数匹についても分析した。これら試料を凍結状態で日本に空輸し、半解凍状態で解剖して定法により希酢酸で熱浸抽出して検液を調製した。検液を中性リン酸緩衝液で希釈し、北里大学で新たに開発した新規抗TTXポリクローナル抗体を用いるELISAキットに付して含まれるTTXs含量を分析した。高レベルのTTXsが検出された試料については、検液をHPLC蛍光法(HPLC-FLD)で分析し、強毒成分であるTTXの含有量を調べた。さらに一部の検液については、HPLC-FLDでは検出できないTTXのデオキシ誘導体の有無をLC-MS法で分析した。その結果、シロサバフグの全ての部位でTTXはほとんど検出されず、ほぼ無毒の5,6,11-トリデオキシTTX（TDT）のみが検出された。クロサバフグは生殖腺と腸に微量のTTXが検出されたものの、筋肉にTTXは確認されなかった。カナフグにはELISA法でTTXsが高濃度に検出された。ドクサバフグでは、筋肉に微量、他臓器には高濃度のTTXが確認された。以上の結果、ベトナムのシロサバフグとクロサバフグの筋肉は食料として安全であることが示唆された。

第４章では、ベトナムにフグ食文化誕生と発展のために必要なことについて考察した。主な内容は、ベトナム国政府の期待すること、フグ毒分析研究所の設立、フグ処理師免許制度の創設およびフグ産業の発展である。

CHAPTER 1. ISSUES AND SOLUTIONS FOR UTILIZING PUFFERFISH RESOURCES IN VIETNAM

1. Issues for utilizing pufferfish resources in Vietnam

Around the world, there are more than 100 species of the pufferfish *Tetraodontidae* inhabit in coastal and freshwater areas, and there are a large amount in tropical and subtropical waters including in Vietnam. The latest survey by Vietnamese Seafood Research Institute in 2004 have identified 49 pufferfish species belonging to 18 varieties in 4 families [1]. Total estimating catch amount is about 37,000 ton/year [2, 3]. In this total amount the ratio of the Tetraodontidae family accounts for about 84.7% [2, 3]. Further, in this family include *L. spadiceus* and *L. cheesemanii* [2, 3].

Pufferfish is white meat fish and delicious. In Japan, it is treated as a high-class fish, and food safety is controlled very well; only a few deaths per year are caused by eating pufferfish [4]. However, in Vietnam pufferfish poisoning has occurred frequently [5]. Some pufferfish species are safe for food but some contain a strong neurotoxin; thus they frequently cause illness, even death, because people usually lack sufficient knowledge and misidentify the safe and toxic species [6–13]. The Vietnamese government has not been able to control accidental pufferfish poisonings effectively. On December 22nd 2003, the Ministry of Fisheries of Vietnam (06/2003/CT-BTS) issued the law to prohibit catching, transporting, storing, processing, selling, consuming, etc [14]. Since that time, large amounts of pufferfish have been taken in nets [1, 2] but all must be thrown because of the law. In order to take advantage of the abundant pufferfish resources and create a new marine culinary culture in Vietnam, first of all, it is essential to learn what the Japanese did to create safe management systems for pufferfish food culture.

Research on Vietnamese pufferfish is still limited. Up to now, there has not been a study on the species and toxicity of all pufferfish in Vietnam [1, 2]. In addition, the development of analytical techniques or research extension for pufferfish also faces many technical difficulties [1, 2]. Vietnamese must learn necessary studies from Japanese specialists.

2. Success of Japanese pufferfish food culture and industry

2.1. History and development until the lifting of the ban on pufferfish

The teeth of pufferfish were discovered in the dumpsites of villages in the Jomon period (14000-300BCE) thus pufferfish was eaten at least 6 thousand years ago in Japan [4, 15, 16]. Records of regular consumption of pufferfish date to the Nara period (710-794 AD), followed by the Heian period (794-1185) [15]. It is reported that many samurai warriors from different parts of Japan who had gathered in Shimonoseki to invade the Korean Peninsula under Toyotomi Hideyoshi died by eating the internal organs of pufferfish [16].

Then, in the 19th century, a law prohibiting the consumption of pufferfish was passed in response to an outbreak of deaths due to the fish's toxins. Ito Hirobumi, the prime minister at the time, praised the taste of pufferfish, and the prohibition law was repealed [16].

Since then, further efforts were made to ensure the safe consumption of pufferfish, and in 1930, the Tokyo Pufferfish Ryori Renmei (Cooking Alliance) was formed. They publicized safe handling methods and lectures, which contributed to the spread of pufferfish consumption. In order to remember their efforts, there is a monument memorializing pufferfish in Ueno Onshi Park in Ueno, Tokyo [15, 16]. According to the statistics of the Ministry of Agriculture, Forestry and Fisheries, in 2000, the amount of cultivated Torafugu was about 5,000 tons and the amount of catch in natural was about 11,000 tons. The cultivated pufferfish amount is increasing when the natural pufferfish is decreasing rapidly [4].

Noguchi et al. [17] verified the hypothesis that non-toxic pufferfish can be produced if they are cultured with TTX-free diets in net cages at sea or in aquaria on land, where the invasion of TTX-bearing organisms is completely shut off. To confirm this hypothesis, more than 5,000 specimens of Torafugu cultured in this manner for 1-3 years were collected from several locations in Japan during 2001-2004, and toxicity of their livers and some other parts was examined according to the Japanese official mouse assay method for TTX. In addition, some specimens were submitted to LC/MS analysis. The results showed that all the livers and other parts tested were non-toxic in both the mouse assay (less than 2 MU/g) and LC/MS analysis (less than 0.1 MU/g) [17]. Thus, it is undoubtedly confirmed that pufferfish are rendered toxic through the food chain, and non-toxic pufferfish can be successfully produced by culture in net cage at sea or aquaria on land [17]. But until now, the government has not allowed the use of cultivated fugu-kimo (fugu liver) as food material [4, 18].

2.2. The Japanese pufferfish food safety management system

In the waters near Japan there are 61 species [4, 18]. Only 21 of these species have been shown to be non-toxic or edible with proper treatment by the Ministry of Health Labor and Welfare, and are landed for fishing [4, 18]. Depending on the species of pufferfish the edible part, and even in the same species the toxic level of each part, varies greatly [4, 18]. Therefore, only those with enough knowledge are allowed to process pufferfish. To receive a license, one must have more than 2 years of culinary experience (these are mainly holders of a Japanese chef's license), and pass the pufferfish processing license examination [15].

The Japanese Ministry of Health, Labor and Welfare sets and regulates food safety standard values by using a unit called the Mouse Unit (MU), which is the poison measurement standard. In the definition of pufferfish poison 1MU means that the amount of the toxin is sufficient to kill a ddY male mouse weighing 20g within 30 minutes [15].

In practice, since the amount of pufferfish consumed at one time would not be more than 1,000g, it would not be fatal at 10MU toxicity or less. Furthermore, it seems that a toxic amount of 5 MU or less is actually non-toxic. Base on this, toxicity of under 5 MU is treated as safe,

under 10 MU is treated as non-toxic; 10 MU or more is treated as weak-toxic; 100 MU or more is treated as strong-toxic; 1,000 MU or more is treated as extremely strong-toxic. The definitions of non-toxic, weak-toxic, strong-toxic, and extremely strong-toxic proposed by Dr.Iwao Tani are shown as follows [15].

Non-toxic	: under 10 MU/g (in other words, intake of less than 1,000g it would not be fatal)
Weak-toxic	: 10 - under 100 MU/g (in other words, an intake of 100g to 1kg is a lethal dose)
Strong-toxic	: 100 - under 1,000 MU/g (in other words, an intake of 10g to 100g is a lethal dose)
Extremely strong-toxic	: from 1,000 MU/g (in other words, an intake of under 10g is a lethal dose)

Other analytical methods are valid in research; however, for the safety of pufferfish for food use, only the Japan Food Analysis Center is authorized, and the method is specified as a biological assay using mice.

2.3. Pufferfish toxin analysis technology development

The pufferfish toxin is tetrodotoxin (TTX) and analogs (TTXs). On the other hands, in some amphibian, arthropod, toxic mollusca, and flatworms high concentrations of TTXs have been confirmed [15]. The mechanism of poisoning of these toxic organisms remains unclear. It is thought that the TTXs of pufferfish are derived from the diet because the cultured tiger pufferfish grown on the compound feed is non-toxic and poisonous when the non-toxic pufferfish is fed with poisonous bait [4]. Therefore, in order to determine the toxicity of pufferfish in a country, it is necessary to obtain data by analyzing the amount and composition of TTXs for each species [4, 16].

In addition to the mouse test, many qualitative and quantitative methods for TTX have been developed. Several methods include mouse bioassay, high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS), which are commonly used for qualitative and quantitative TTX detection, but other methods include gas chromatography-mass spectrometry (GC-MS), infrared spectroscopy (IR) and nuclear magnetic resonance spectroscopy (1H-NMR) [6, 19–23]. Among these, HPLC has a minimum detection level of 0.03 μg [24]. LC-MS, a minimum detection level of 0.005 μg [25], is the most powerful and sensitive tool for qualitative and quantitative TTX detection. TTX can also be determined by thin layer chromatography (TLC) or electrophoresis [26]. Although these methods are not analytical tools, they are quite simple and practical.

The above methods all need machines and sample preparation techniques, so they are quite costly as well as time-consuming; the latest methodologies, KIT ELISA for TTXs and SPS,

have been developed to overcome these shortcomings [27, 28]. The polyclonal antibody reacts well with TTX and its analogs (TTXs), including 4-epiTTX, 11-oxoTTX and 5,6,11-trideoxy TTX (TDT), except 4,9-anhydro TTX [27, 28]. An enzyme-linked immunoassay system (ELISA) using this antibody has also been developed. This new polyclonal antibody, developed in tandem with analytical procedures using one-step direct Elisa (TTX-Elisa Kit), has been shown to be useful for detecting TTXs in toxic organisms with a defined range of 0.005μg ~ 0.5 μg [13, 27, 28].This antigen is sensitive to most TTXs, detecting much broader toxin-related substances than HPLC.

With a long history of the world's leading in the art and safety culinary culture, along with the development of technology and techniques from toxin analysis, food processing, and fish farming, it can be said that Japan are successfully symbols in building pufferfish food culture.

3. Japan-Vietnam exchange agreement for the development of pufferfish food culture in Vietnam

As described above, pufferfish is delicious and expensive in Japan; the resource is abundant in Vietnam. I am making an effort to establish pufferfish food culture in Vietnam. To this end, we signed an agreement on cooperation. The Vietnam National Institute of Nutrition (NIN), the National Institute of Marine Fisheries (RIMF), Mitsui Suisan Co., Ltd. of Japan, and the Asian Nutrition and Food Culture Research Center (ANFCRC) of Jumonji University signed the research cooperation agreement in September 2017 (shown below). This agreement shall come into effect upon signing by all the parties and will be valid for a period of 5 years, and may be extended for subsequent 5 year periods beyond its original date of expiration if no proposal for amendment and/or termination is made by at least one party. The four parties will work together to lift the ban on pufferfish as food and establish a pufferfish food culture in Vietnam, as is currently underway.

4. Preliminary research for lifting the ban on pufferfish in Vietnam

In order to lift the law which prohibits harvesting and using Vietnamese pufferfish, it is necessary to carry out a toxicity test and clarify the type and parts of pufferfish which can be eaten. Even if a toxicity test, which is difficult, is carried out, the effort would be pointless unless pufferfish is accepted as a part of food culture by Vietnamese people. Therefore, I first conducted a sensory test (Chapter 2). After confirming the favorable results of the sensory test, I learned to apply analysis methods such as ELISA test, HPLC-FLD test and LS-MS test, using pufferfish collected from throughout the country (Chapter 3)

NATIONAL INSTITUTE OF NUTRITION | JUMONJI UNIVERSITY | MITSUI MARINE PRODUCTS, INC | RESEARCH INSTITUTE FOR MARINE FISHERIES

AGREEMENT OF THE SCIENTIFIC COOPERATION ON PUFFER FISH STUDY IN VIET NAM

The Research Institute for Marine Fisheries (RIMF) and the National Institute of Nutrition (NIN) in Vietnam and Mitsui Marine Product, Inc (MMP), and The Asian Nutrition and Food Culture Research Center, Jumonji University (ANFCRC) in Japan (here by Party/Parties) conclude this *"Agreement of the Scientific Cooperation on Puffer fish Study in Viet Nam"* upon the principles of equality and reciprocity among the Parties, to lift the puffer fish (Fugu) ban law and establish the culture of Fugu as food in Vietnam. For the above purpose, the Parties will develop cooperative research activities, as follow (Appendix):

1. Importation of Japanese cultured Fugu to Vietnam
   a. Japanese cultured Fugu will be brought to Vietnam and chefs holding a Japanese Fugu license will cook and serve Fugu dishes to prominent Vietnamese.
   b. Based on the result of the above study, the two Vietnamese institutes will apply to the government to make it legally possible to import Japanese cultured Fugu to Vietnam.
2. Safety study of Vietnam Fugu
   a. The two Vietnamese institutes will collect Fugu and send them to Japan.
   b. The Japanese institutes will conduct safety tests of Fugu organs and tissues on mice.
3. Establishment of a Fugu chef license in Vietnam
   a. The Vietnamese institutes will endeavor to establish a Fugu chef license
   b. Until the establishment of a Fugu license system in Vietnam, only chefs with a Japanese Fugu license/will be permitted to prepare Fugu dishes in Vietnam.
4. Establishment of Fugu culture techniques.
5. Study of the nutritional value of Fugu.
6. Apply for various funds: All the Parties will try to obtain various funds for the above activities.

This agreement shall come into effect upon signing by all the Parities and shall be valid for a period of 5 years from the date of its signing. This agreement may be extended for subsequent 5

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year periods beyond its original date of expiration if no proposal for amendment and/or termination is made by at least one Party. Amendment and/or termination of this agreement should be made by written notice. Any Party may modify and/or terminate this agreement by giving 60 day prior written notice to the other Parties. Contradictions upon the interpretation of this agreement should be negotiated and solved through efforts made by all the Parties. This agreement has been made in three languages (Japanese, Vietnamese, and English) to confirm the content and signed by the representative of each Party.

Director, Vietnam National Institute of Nutrition

Prof. Le Danh Tuyen August, 2017

Director, Vietnam Research Institute for Marine Fisheries

Dr. Nguyen Quang Hung August, 2017

President of Mitsui Marine Fisheries Co.

Mr. Yoshinari Ito August, 2017

Director of Asian Nutrition and Food Culture Research Center, Jumonji University

Prof. Shigeru Yamamoto August, 2017

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Appendix

DETAILS PLAN FOR THE SCIENTIFIC COOPERATION ON PUFFER FISH STUDY IN VIET NAM

The Research Institute for Marine Fisheries (RIMF) and the National Institute of Nutrition (NIN) in Vietnam and Mitsui Marine Product, Inc (MMP), and The Asian Nutrition and Food Culture Research Center, Jumonji University(ANFCRC) in Japan (here by Party/Parties) conclude this *"Agreement of the Scientific Cooperation on Puffer fish Study in Viet Nam"* upon the principles of equality and reciprocity among Parties, to lift the puffer fish (Fugu) ban law and establish culture of Fugu as food in Vietnam. The following paragraphs show the detail of the agreement.

1. Importation of Japanese cultured Fugu to Vietnam

a. Japanese cultured Fugu will be brought to Vietnam and chefs holding a Japanese Fugu license will cook and serve Fugu dishes to prominent Vietnamese. MMP will support all the expenses for this (purchase of Fugu in Japan, sending [shipment of] Fugu to Vietnam, transportation, accomodations and salary for chefs). NIN and RIMF will invite prominent Vietnamese, and prepare rooms for the lecture[s], cooking, cooking demonstrations and taste tests.

b. Based on the results of the above study, NIN and RIMF will apply to the government promptly to make it legally possible to import Japanese cultured Fugu to Vietnam. All costs for this will be covered by NIN and RIMF.

2. Safety study of Vietnam Fugu

a. NIN and RIMF will collect Fugu and send them to Japan. They will choose 2 or 3 types of Fugu and collect from 5 to 10 places covering from north to south of the whole country in two seasons (spawning and non-spawning seasons) and send them to MMP, Japan. The costs for this will be covered by NIN, and RIMF.

b. The MMP will conduct safety tests of collected Fugu organs and tissues tissues on mice. Costs for this will be covered by MMP.

c. Tetrodotoxin measurement in tissues that show toxicity in mouse examination will be conducted for a few years in Japan. The details will be discussed in the future.

3. Establishment of a Fugu chef license in Vietnam

NIN and RIMF will endeavor to establish a Fugu chef license. By [With] the establishment of a Fugu license system in Vietnam, only chefs with a Japanese Fugu license will be able to/will be permitted to prepare Fugu dishes in Vietnam. MMP will help Vietnamese chefs to get the Japanese Fugu chef license. NIN and RIMF try to establish the license system by the time safety test of Fugu is completed.

4. Establishment of Fugu culture techniques.

MMP will teach the techniques of Fugu culture to Vietnamese. All four Parties will conduct

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discussion on the establishment of a culture facility at a later date.

5. Study of the nutritional value of Fugu.

NIN will analyze the nutritional value of Fugu, using its own funds.

6. Apply for various fund

All the Parties will try to obtain/secure various funds for the above activities. For example, all the Parties will apply for JICA; NIN and RIMF groups will apply for the Vietnamese government funds, etc.

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CHAPTER 2. ACCEPTABILITY EVALUATION BY VIETNAMESE ABOUT NON-TOXIC CULTURED PUFFERFISH IN COMPARISON WITH GROUPER AND MACKEREL (STUDY 1)

ABSTRACT

Background and purpose. World-wide there are few countries in which pufferfish (fugu) is eaten as in Japan, it is banned in Vietnam. Consequently, there is a huge amount of pufferfish inhabiting in Vietnamese water, but whenever accidentally catching it, we have to throw or use as fertilizer. To utilize it as a resource in Vietnam, at first it is essential to determine whether Vietnamese can accept fugu as a food, so we did this sensory study. *Methods.* We compared the sensory reaction to Japanese cultured *Takifugu rubripes* and fish from Vietnamese waters: grouper and mackerel. The 107 panelists were Vietnamese volunteers working in the field of nutrition, employees of marine companies, or government officials who could influence the relevant laws. Each panelist tried 10 dishes which were prepared by chefs holding a Japanese pufferfish processing license. After eating, they were asked to fill out the sensory questionnaire, used a five-point scoring method, with 5 as the highest score, and reaction rating questionnaire. *Results.* The average score for the various puffer dishes was 4.40 in the good rank flavor was rated best. For questions such as "would you like to eat more fugu, do you want to introduce it to others, do you want fugu to become a new food culture?", Over 90% of the panels answered "Yes". *Conclusion.* Fugu's acceptable flavor was confirmed for Vietnamese when it was prepared properly by fugu-licensed Japanese chefs.

Key words: pufferfish, fugu-licensed, sensory test, food culture, *Takifugu rubripes*, Vietnam, Japan

1. INTRODUCTION

Worldwide, only Japan is well known for a special pufferfish (fugu) food culture. Japanese Ministry of Health and Welfare enacted a guideline of edible species, in that only 22 species of fugu are allowed, and only the people who has pufferfish processing license can cook [1–3]. Marine fugu are believed to accumulate *tetrodotoxin* (TTX) but non-toxic fugu can be produced if they are raised with TTX-free diets in net cages at sea or aquaria on land, where the invasion of TTX-bearing organisms is completely shut off [4]. By cultured fugu, some studies showed that it was delicious and rich in nutrients [5,6] . Fugu is high-class fish and expensive in Japan and now Japanese are trying to import fugu from the other countries such as China or Korea.

According to a report by the Research Institute for Marine Fisheries, Vietnam has about 60 species, resources are over 37,000 ton/year [8, 9]. There are hundreds of food poisoning related to toxic fugu per years [10] so that the Ministry of Fisheries (now the Ministry of Agriculture and Rural Development) has implemented a ban on fugu since 2003 [11]. In order to lift the law which prohibits the harvesting and using Vietnamese fugu, it is necessary to carry out a toxicity test and clarify the type and parts of fugu which can be eaten. Even if a toxicity test, which is difficult, is carried out, the effort would be pointless unless fugu is accepted as a part of food culture by Vietnamese people. Therefore, first of all, in this research, two sensory tests were carried out with various dishes of Japan cultured Torafugu that was confirmed to be safe and got permission of Vietnam government.

2. METHODS

Study design: We evaluated Japanese fugu dishes and Vietnamese luxury fish dishes of mackerel and grouper, by using a sensory test two times in June and December of 2017. The first study was conducted at the Vietnam National Institute of Nutrition in Hanoi and the second study at a hotel in Da Nang. After trying each dish, the panel were asked to score their reaction on the response sheets. We used 5-point scale (extremely good:5, good:4, neither good or bad:3, bad:2, extremely bad:1) to evaluate the flavor of each dish at 3 items: overall taste, aroma and texture. The panel were free to eat all dishes randomly and in unrestricted quantities. After that, panelists were asked to fill in the questionnaire of sensory test and acceptance of fugu dishes (Picture 3).

Ethical committee and customs clearance permission of Japanese fugu: Currently the use of pufferfish is banned in Vietnam, so it cannot use Vietnamese pufferfish because the safety has not been guaranteed. To conducting research in addition to ethical permission, it was often necessary to get approval from the Vietnamese Ministry of Health. Japanese cultured pufferfish is a high-class fish, which was tested safety in Japan and passed the customs clearance, so it was allowed to bring into Vietnam.

Materials Preparation: Cultured *Takifugu rubripes* Torafugu for the study were purchased at an aquaculture company in Japan and viscera were removed and then the fish was kept in a freezer under −20°C. The fugu was delivered from Miyazaki, Japan to Hanoi and Da Nang, Vietnam by air. We used cold gel to keep the fish under −20°C during shipping. In Vietnam, the fugu was kept in a freezer under −20°C. One day prior to the experiment, both mackerel and grouper were purchased at a market in Vietnam, viscera were removed and the fish were kept in refrigerators under 4°C. The frozen fugu was moved to a refrigerator and defrosted at about 4°C in more than 10 hours. Other materials were chosen and prepared under the control of Japanese pufferfish processing license holders (Picture1). We used the same recipe for fugu, mackerel and grouper in the hotpot dish and Japanese style fried dish to make the comparison among them.

Prior to the main study, in a pilot study (n=10), various Japanese fugu recipes [2] were evaluated. Based on that pilot, the menu items for the main study were determined: Japanese style fried dish, hot pot, sashimi (dishes with raw fish), hirezake (Japanese sake with grilled fugu fin in it), nikogori (a kind of jelly made from fugu skin), skin mix (boiled fugu skin was sliced and mixed with chili, miso, and vinegar), tataki (fish grilled on the outside and kept raw inside), and shirako tofu (a kind of mousse made with fugu milt) (Picture 2). Total the amount of fugu for 1 serving set of sensory studies with 8 fugu dishes was 400g (50g per dish × 8 dishes = 400g) so at least 42.8 kg needed to be cooked. In case something happened, we

brought about 90kg of frozen Japanese fugu to Vietnam. By same way, we calculate the amount of grouper and mackerel which were used for taste test. All the dishes were made as close as the time of the test at lunch time that why most of work had to be done since previous night.

Panel selection: To evaluate Vietnamese acceptance of fugu dishes and to lift the law banning fugu in Vietnam in future, all 107 panelists were Vietnamese who may have influence on the law concerning fugu. In addition, they had experience with the sensory evaluation of food, especially fish, particularly the staff of National Institute of Nutrition, the Research Institute for Marine Fisheries and the government officials. They were adults, in good health, had no allergies to any materials used in this test, were not anomic, were not ageusic, had normal colour vison and able to detect anomalies in the appearance of fish and fish food in a consistent manner, and were able to rely on sensory perceptions and to report them appropriately. The panel were asked to avoid eating or drinking too much the day before the experiment, to get enough sleep, and to eat their usual breakfast on the day of the experiment. Informed consent was obtained from participants. We requested participants to report any adverse reactions during and after eating the food items.

Statistical Analysis: Data were statistically analyzed by Student *t*-test, and Tukey test, $p<0.05$ was significant different.

Picture 1. Fugu preparation by Japanese pufferfish processing license holding chef

Picture 2-1. Fugu sashimi

Picture 2-2. Fugu tataki

Picture 2-3. Japanese style fried fugu

Picture 2-4. Left: Nikogori; middle: Skin mix; right: Shirako tofu

Picture 2. Fugu dishes for sensory test

Date Place

Age Sex: 1 Male 2 Female

1. Evaluate the items below to the gradual increase of the following characteristics on a scale from 1 to 5 and please check (✓) in the box

No	Dish		Item	5	4	3	2	1
1	Fugu sashimi		Overall taste					
			Texture					
			Aroma					
2	Hirezake		Overall taste					
			Aroma					
3	Hot pot	Fugu	Overall taste					
			Texture					
			Aroma					
		Mackerel	Overall taste					
			Texture					
			Aroma					
		Grouper	Overall taste					
			Texture					
			Aroma					
4	Japanese style fried	Fugu	Overall taste					
			Texture					
			Aroma					
		Mackerel	Overall taste					
			Texture					
			Aroma					
		Grouper	Overall taste					
			Texture					
			Aroma					
5	Nikogori		Overall taste					
			Texture					
			Aroma					
6	Skin mix		Overall taste					
			Texture					
			Aroma					
7	Tataki		Overall taste					
			Texture					
			Aroma					
8	Shirako tofu		Overall taste					
			Texture					
			Aroma					

2. Questionnaire on acceptance of Fugu dishes

Please circle the number of the answer that matches your opinion

2.1 Would you want to eat the fugu dishes again?

1. Yes 2. No 3. No each other

2.2 If we do other sensory investigations, would you be willing to participate in other taste tests?

1. Yes 2. No 3. No each other

2.3 Would you recommend Fugu dishes to your friends?

1. Yes 2. No 3. No each other

2.4 Would you introduce Fugu dishes to your family?

1. Yes 2. No 3. No each other

2.5 Do you think Fugu dishes can become a new food in Vietnam?

1. Yes 2. No 3. No each other

Picture 3. Questionnaire of sensory test and acceptance of fugu dishes

3. RESULTS

The sensory evaluation panelists (aged 28 - 38) consisted of 61 males and 46 females, more than 80% of panel liked the fish dishes, mackerel or grouper dishes (Table 1)

Table 1. Characteristics of Panelists (N=107)

	Panelists	
	n	%
Like fish dishes	98	92
Like mackerel dishes	93	87
Like grouper dishes	103	96

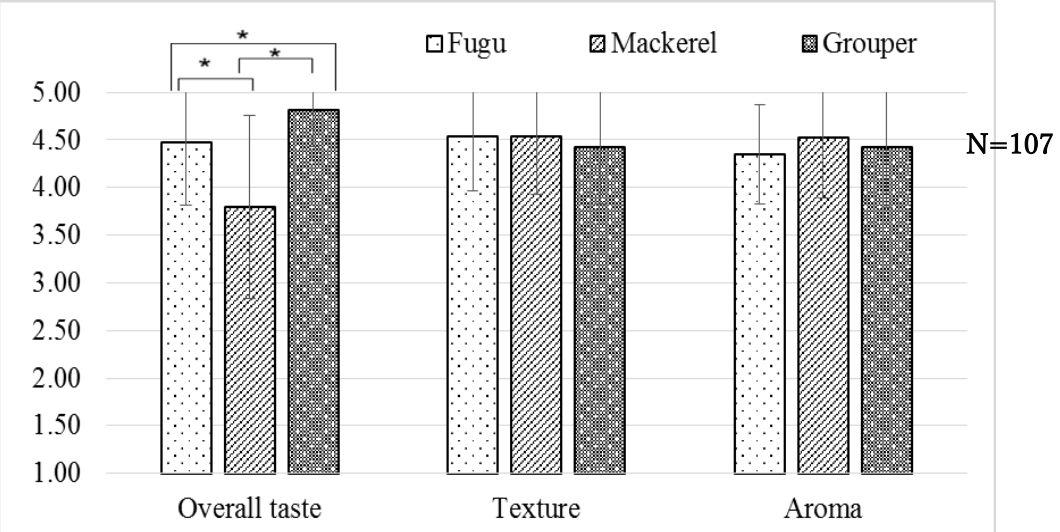

Figure 1. Comparison of taste evaluation among fugu, mackerel and grouper in Japanese style fried dish (*: p<0.05, by Tukey)

Figure 1 showed the results of Japanese style fried dish, the overall taste of these three fish showed significant difference ($p<0.05$). When compared to grouper, the texture of fugu was the same, aroma was little lower but still in the good rank flavor was rated best; the overall taste of both was very good and even in the hotpot dish the score for fugu was higher in overall taste.

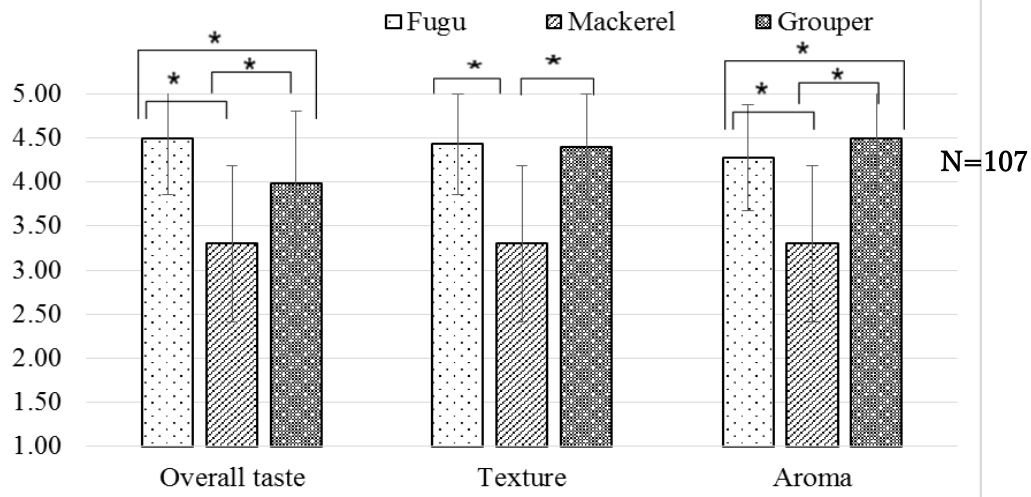

Figure 2. Comparison of taste evaluation among fugu, mackerel and grouper in hot pot dish (*:p<0.05, by Tukey)

Figure 2 showed the hot pot dish, overall taste of these three fish was significantly different ($p<0.05$) and fugu got the highest score; texture of fugu compared to grouper or mackerel had a significant difference. In comparison with mackerel, fugu was more delicious, with no fish smell as with mackerel in the hotpot dish, and texture was the same in the fried dish and better in the hotpot dish. In flavor, fugu was not only good but also better than these 2 high-class fish.

Table 2. Average score of sensory evaluation of 8 fugu dishes (N=107)

	Fugu sashimi	Hirezake	Fugu hot pot	Fugu fried	Skin mix	Nikogori	Shirako tofu	Tataki
Overall taste	4.82±0.39	4.42±0.69	4.50±0.64	4.48±0.66	4.56±0.62	4.56±0.62	4.41±0.71	4.59±0.50
Texture	4.46±0.54		4.50±0.57	3.84±0.96	4.48±0.54	4.48±0.54	4.44±0.57	4.48±0.57
Aroma	4.49±0.54	4.49±0.67	4.28±0.60	4.82±0.39	4.64±0.48	4.64±0.48	4.56±0.57	4.53±0.54

Values are mean ±SD

Table 2 showed the sensory evaluation of the 8 fugu dishes, in average point, the overall taste was higher than 4.42, the texture was higher than 3.84, the aroma was higher than 4.49. All of the points were at the good rank. The overall taste of sashimi was 4.82 the highest score.

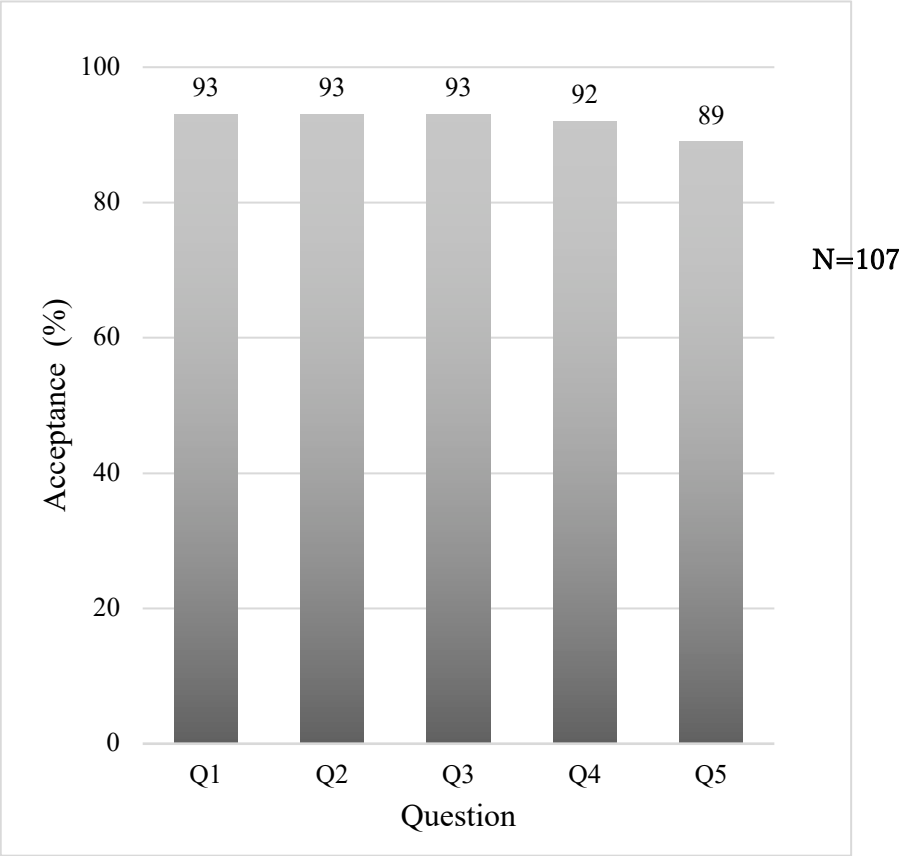

Figure 3. Percent of panelist answered "Yes" about questions on acceptance of fugu dishes (%) (Q1: Would you want to eat the fugu dishes again?; Q2: If we do other sensory investigations, would you be willing to participatein other sensory test?; Q3: Would you recommend fugu dishes to your friends?; Q4: Would you introduce fugu dishes to your family?; Q5: Do you think fugu dishes can become a new food culture in Vietnam)

Figure 3 showed how the panelists accept fugu dishes: more than 92% of the panel wanted to eat them again and would recommend them to other people, 89% panel thought that fugu could become a new food in Vietnam.

4. DISCUSSION

In Vietnam, the Government strictly prohibits processing, trading, and using of pufferfish; therefore, nobody knows the flavor of fugu dishes. However, with this sensory test, more than 90% of the panelists scored fugu dishes as high or best flavor, 80% said they would like to eat fugu again and hoped it would become a new food culture in Vietnam.

Eating and cooking Vietnamese fugu is illegal in Vietnam; therefore, when conducting these researches, we used Japanese fugu which was confirmed as safe in Japan. Every time when we did these researches, we needed the permission of Vietnam government; all cooking period was under the control of National Research Institutes.

On the other hand, only Japan has a law for fugu culture as well as a pufferfish processing license system, that why this study followed Japanese cooking methods and all dishes were cooked by professional fugu chefs.

Grouper and mackerel are two of the most esteemed fish in Vietnam, considered to be excellent in overall taste, aroma, and texture. Three fish are white meat fish, the texture of these are considered as similar. Vietnamese usually use the muscle of fish to cook some dishes, such as: hot pot, fried; to compare among 3 kind of fishes we chose these 2 dishes only.

In this study, we invited 107 adults from Hanoi and Da Nang, the number of the panelists were sufficient for this study. According to “Sensory evaluation - guide of food practice”, at ranking test the minimum size of panel participating in the test was 12 [12]. According to the guideline number JIS Z 9080:2004 of Japan Industrial Standards Committee about sensory evaluation, in ranking test them minimum sample size are 7 if they are professional, 20 if they have experiences, 30 if they have no experience [13]. Moreover as similar previous studies about sensory of sushi or food from liver of Japanese cultured fugu [14, 15] in this study we tried to collect more than 50 panel per test.

There are many method to evaluate sensory, for example hedonic scaling - 9 point scale, magnitude estimation, category-ratio scales [16] and 5 point scale. Each method has its own feature. We used the 5 point scale which suitable with the untrained panel and situation of this study as shown in similar studies [5, 6, 17].

In Vietnamese food culture state, most traditional dishes are cooked well. Recently, food safety is a major problem in Vietnam; every year there are 250 - 500 cases of food poisoning: 7,000 -10,000 victims and 100 - 200 deaths, 33 - 49% from microorganisms [18–20]. Vietnamese do not eat raw food much, especially raw fish. Fish intake in

Vietnam in 2010 was only 59g (15.8% of protein intake) [21] lower than in Japan: 73g (20.7% of protein intake) [22]. Fish in Vietnam is a luxury item and many times more expensive than meat or eggs. This may be the reason for low fish intake. In this study, the Japanese fugu dishes were very highly evaluated and appreciated by Vietnamese panel. Furthermore, fugu has no small bones, usually has no fishy smell, is delicious and is easy for even the elderly or children to consume.

In particular, sashimi got the highest evaluation even though Vietnamese are not familiar with raw fish; in future we hope that fugu will become a part of Vietnamese food culture, and by Japanese experiences and cooking methods, Vietnamese can develop the new fish food culture.

The study results also helped the government and National Research Institutes acknowledge that "it was possible to make delicious and safe Vietnamese fugu food, moreover it could become a popular food culture in Vietnam". As a result, the National Research Institutes will send the government proposals for promptly legalizing safe fugu and propagating Japanese fugu culture in Vietnam.

Previous study showed that fugu consume tetrodotoxin (TTX) holdings organisms finally become toxic fish. If fugu are prevented from TTX holdings organisms, fugu will be nontoxic (≦10MU) [1, 3, 23]. Japan have developed technique to cultivate nontoxic fugu [5, 6] and strictly management fugu food safety system. These should be established in Vietnam in parallel with fugu new food culture.

In the future, we will analyze toxic in tissue and clarify the types of pufferfish in Vietnam can be used as food. From those results, safe species of fugu could be chosen to expand the new food culture in Vietnam. We hope these will not only be a new food but will also play a part in "international humanity and cultural understanding", "economic development", "the knowledge society", "technology knowledge" and "practicing globalization".

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APPENDIXES

Supplementary Picture S1. Presentation of Mr. Ito Yoshinari and I at Fugu Food Culture Conference in Hanoi

Supplementary Picture S2. The view of Fugu Food Culture Conference in Hanoi

Supplementary Picture S3. Presentation of Mr. Ito Yoshinari at Fugu Food Culture Conference in Hanoi

Supplementary Picture S4. The chefs hold pufferfish processing license from the left Mr. Ito Yoshinari, Ms. Maki Kai and Mr. Yanagi at Fugu Food Culture Conference in Hanoi

Supplementary Picture S5. The dishes for sensor test at Fugu Food Culture Conference in Hanoi

Supplementary Picture S6. The attendees at Fugu Food Culture Conference in Hanoi

Supplementary Picture S7. The view of Fugu Food Culture and Safety Conference in Da Nang

Supplementary Picture S8. The view of Fugu Food Culture and Safety Conference in Da Nang

CHAPTER 3. TISSUE DISTRIBUTION OF TETRODOTOXIN AND ITS ANALOGS IN LAGOCEPHALUS PUFFERFISH COLLECTED IN VIETNAM (STUDY 2)

ABSTRACT

Pufferfish belonging to *Lagocephalus* are composed of several species, some of which have been recognized non-toxic. Although fish belonging to this genus inhabit widely from temperate to tropical seawaters, toxin distribution has remained unclear. The present study was conducted to thoroughly survey the presence of tetrodotoxin (TTX) and its analogs (TTXs) in the extracts from various tissues of *Lagocephalus* pufferfish including *L. spadiceus*, *L. cheesemanii, L. lunaris*, and *L. inermis* collected in Vietnam by using the TTX enzyme-linked immunosorbent assay (ELISA) kit. Analyses using the TTX-ELISA kit demonstrated the presence of a considerable amount of TTXs in the extracts prepared from various tissues and organs of *Lagocephalus* pufferfish. TTX was detected in gonads and intestine of *L. cheesemanii* as well as *L. lunaris* by high performance liquid chromatography with fluorescence detection, but not in tissues and organs of *L. spadiceus*. The extract from muscle of *L. spadiceus* was further subjected to liquid chromatography-mass spectrometry analyses to investigate toxin components, revealing the presence of a high amount of 5,6,11-trideoxyTTX (TDT), but no TTX. Since the toxicity of TDT is very weak, *L. spadiceus* muscle was regarded to be non-toxic as far as samples collected in the present study are concerned.

Keywords: ELISA, Food safety, *Lagocephalus spadiceus*, Pufferfish, Tetrodotoxin analogs, Toxicity

1. INTRODUCTION

Tetrodotoxin (TTX), one of the most potent natural neurotoxins (Narahashi et al. 1967; Kao 1982), has been first detected in pufferfish (Takahashi and Inoko 1889 a, b) and later determined for its unique structure by Tsuda et al. (1964), Woodward (1964) and Goto et al. (1965). Subsequently, TTX was found in various organisms, including vertebrates and invertebrates (Mosher et al. 1964; Noguchi and Hashimoto 1973; Kim et al. 1975; Noguchi and Arakawa 2008) and even bacteria (Yasumoto et al. 1986; Noguchi et al. 1987; Biessy et al. 2019).

Pufferfish belonging to *Lagocephalus* are composed of several species, including half-smooth golden pufferfish *L. wheeleri*, brown-backed toadfish *L. gloveri*, and lunartail puffer *L. lunaris*, which are all captured near Japanese coastal lines (Hashimoto et al. 1984). Recently, *L. wheeleri* has been found to be a junior synonym of *L. spadiceus* (Matsuura 2010), whereas *L. gloveri* to be that of *L. cheesemanii* (Matsuura and Satoh 2017). Two species, *L. spadiceus* and *L. cheesemanii* are usually non-toxic when caught in Japan, whereas *L. lunaris*, usually caught near Taiwan or in tropical areas, is often found to be toxic (Harada 1979).

While the genus *Lagocephalus* is well recognized, classification at the species level was somewhat difficult and had not been studied in depth until recently. Matsuura and his colleagues have recently clarified synonym relationships for a few species including those described above (Matsuura 2010; Matsuura et al. 2011; Matsuura and Satoh 2017). Not small quantities of *Lagocephalus* pufferfish caught in the South China Sea are carried back or exported to Japan and used as materials for seafood processing (Tabeta and Kumagai 1980). *L. spadiceus* and *L. cheesemanii* caught in Japan are non-toxic and their muscle part commonly used as materials with reasonable prices for seafood products typically treated by drying in Japan. On the other hand, it is now totally prohibited to distribute any pufferfish species including those of *Lagocephalus* to the market in Vietnam (Vietnam Fishery Bureau, Ministry of Agriculture and Rural Development, 2003), although many incidences caused by the intake of toxic pufferfish have been reported (Nguyen 2005; Vu et al. 2009). However, it has not been known whether or not the same fish species of *L. spadiceus* and *L. cheesemanii* caught in the South China Sea are non-toxic as in the case of those caught near Japan. Meanwhile, it has been reported that some individuals of *L. spadiceus* were toxic when carried back or imported from the South China Sea off Taiwan (Harada 1979) and Vietnam (Tabeta and Kumagai 1980). Furthermore, Hwang et al. (1992) reported that *L. spadiceus* and *L. cheesemanii* collected from seawaters of Taiwan were toxic, though weak. However, Brillantes et al. (2003)

claimed that *L. spadiceus* caught in the sea of Indonesia and landed in Thailand was non-toxic, whereas many samples of *L. lunaris* were toxic. Therefore, it is now worth trying to survey toxicity of these *Lagocephalus* pufferfish inhabiting the sea area in southeastern Asia in more details following species identification again in viewpoint of food safety and food hygiene.

We have recently developed a novel polyclonal antibody against TTX using its haptenic antigen (Sato et al. 2019). This polyclonal antibody reacted well with TTX and its analogs (TTXs), including 4-*epi*TTX, 11-oxoTTX and 5,6,11-trideoxyTTX (TDT), excepting 4,9-anhydroTTX. The enzyme-linked immunosorbent assay (ELISA) system using this specific antibody was also developed. This newly developed polyclonal antibody with analytical procedures using direct one-step ELISA (TTX-ELISA kit) has been demonstrated to be useful to detect TTXs in toxic organisms.

The objective of the present study was to thoroughly survey the toxicity of *Lagocephalus* pufferfish including *L. spadiceus* and *L. cheesemanii* caught from various coastal and ocean areas off Vietnam by using the above mentioned TTX-ELISA kit. The toxin extracts from some specimens were also subjected to high performance-liquid chromatography with fluorescence detection (HPLC-FLD) and liquid chromatography-mass spectrometry (LC-MS) analyses to identify toxin components.

2. MATERIALS AND METHODS

2.1. Sampling and tissue collection

Totally 108 samples of *L. spadiceus, L. cheesemanii, L. lunaris* and *L. inermis* were collected from fishing ports near various coastal and ocean areas off Vietnam, including the South China Sea off Hai Phong in the north and off Vung Tau in the south as well as the Gulf of Thailand off Kien Giang, from 2017 to 2019 (Figure 1 and Table 1). These pufferfish were stored frozen at -20˚C and transported to the laboratory of School of Marine Biosciences, Kitasato University, Japan.

Toxic pufferfish *Takifugu pardalis, T. snyderi, T. flavipterius* (former *T. poecilonotus*) and *T. porphyreus* were collected from a fish market in Ofunato, Iwate Prefecture, Japan, from April to July in 2016, and their visceral parts were used to prepare authentic TTXs as the standard for analytical procedures.

2.2. Preparation of the extract and assay procedures for TTXs by ELISA

All specimens were species-identified by their morphological characters and measured for their weight and total length, and then dissected into intestine, liver, muscle,

gonads, and skin on site. Two grams homogenate of each organ was extracted with 0.1 % acetic acid, according to the method for TTX described in Standard Methods of Analysis in Food Safety, Japan (Sato and Kodama 2015).

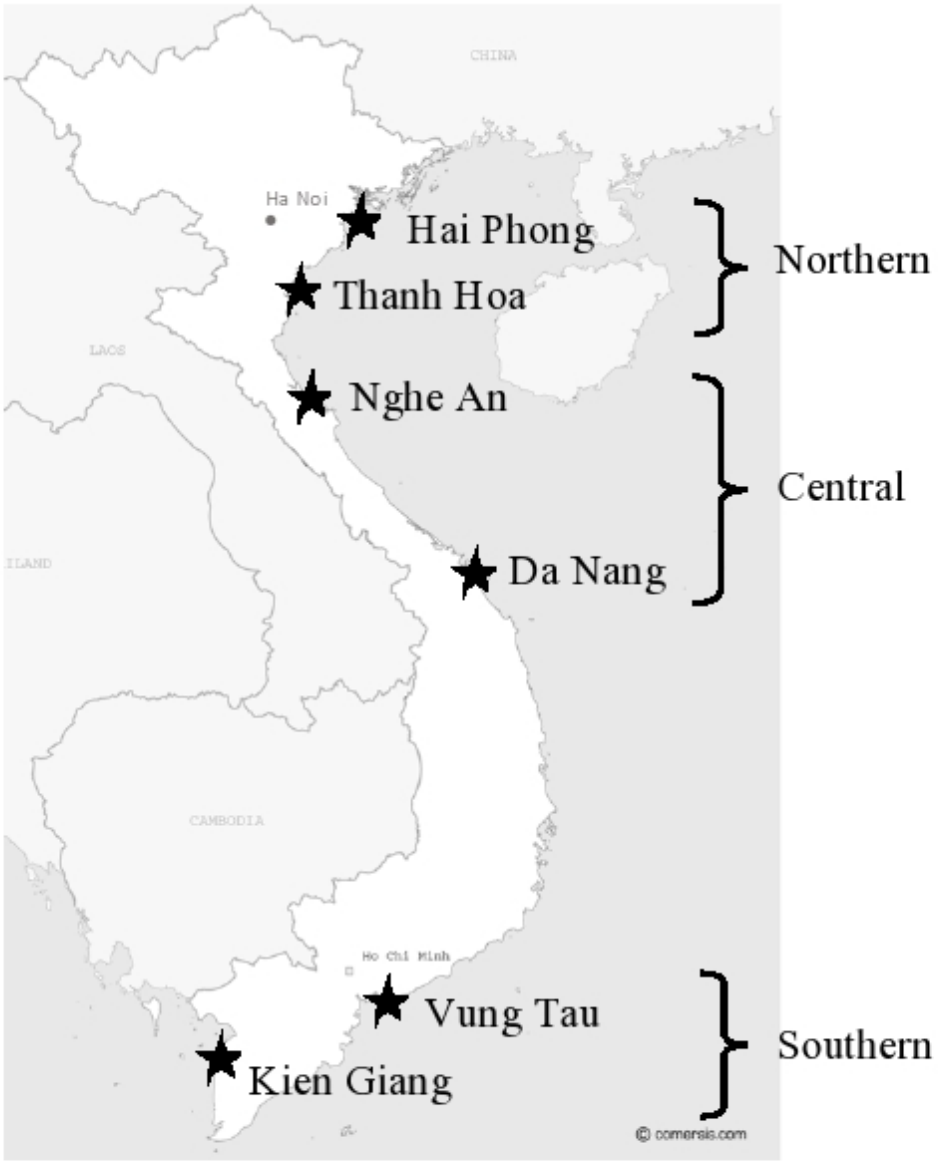


Figure 1. Six coastal locations for collecting *Lagocephalus* pufferfish in Vietnam *(Image of map obtained from comersis.com)*

Figure 1 showed six coastal locations for collecting *Lagocephalus* pufferfish in Vietnam: Hai Phong and Thanh Hoa in the northern region, Nghe An and Da Nang in the central region, and Vung Tau and Kien Giang in the southern region.

The total concentrations of TTXs in *Lagocephalus* tissue extracts were determined by ELISA essentially according to Sato et al. (2019). Briefly, the polyclonal antibody prepared by Sato et al. (2019) was diluted 100 times with 0.9% (*w*/*v*) NaCl in 0.01 M Tris-HCl buffer (pH 8.2), and 100 μL portions were added to each well of a 96-well ELISA plate (Maxisorp, Thermo Fisher Scientific, Waltham, MA, USA). After stirring at 5°C overnight, the plate was washed with phosphate-buffered saline free of Ca^{2+} and Mg^{2+} [PBS(-)], added with 350 μL Block Ace (4% *w/v* in water; KAC Co., Ltd., Kyoto, Japan) solution to each well, and left at 5°C overnight. The plate was washed with PBS(-) containing 0.05% (*v/v*) Tween 20 (PBST) twice. Then, the sample extract was diluted 10-1,000 folds with 0.1 M sodium phosphate buffer (pH 7.4) and 50 μL solution were added to each well of the ELISA plate coated with the antibody specific to TTXs. Next, 50 μL biotin-TTX (2 nM, diluted with the same buffer) was added to each well and the plate was incubated at 37°C for 15 min. After the solution was discarded, the wells were washed with PBST and added with 50 μL horseradish peroxidase streptavidin solution [2,000 times dilution with PBS(-); Funakoshi, Tokyo, Japan]. The plate was incubated at 37°C for 15 min and the solution was discarded. After washing the wells three times with PBST, 100 μL Sigma-Fast tablets (OPD-H_2O_2, Sigma-Aldrich, St. Louis, MO, USA, dissolved in 10 mL water) solution were added to each well. The plate was incubated at 37°C for 5 min, and to the wells were added 100 μL HCl (2 M). Finally, each well was subjected to measurement of absorbance at 490 nm with a plate reader (iMark, Bio-Rad, Hercules, CA, USA). The toxin solution samples were analyzed in triplicate. As reported previously, detection limit and working range for TTX were around 3 nM、and 10 to 300 nM of test solution, respectively (Sato et al., 2019).

2.3. Analytical procedures of TTXs by HPLC-FLD and LC-MS

Extracts were prepared from various tissues of four *Lagocephalus* pufferfish with 0.1% acetic acid by heating in a boiling water bath for 5 min as mentioned in section 2.2 and directly subjected to HPLC-FLD. For LC-MS analysis, the extracts prepared as above were further subjected to successive column chromatography on activated charcoal (for chromatography; Wako, Osaka, Japan), Bio-Gel P-2 (200-400 mesh; Bio-Rad), and Bio-Rex 70 (200-400 mesh; Bio-Rad) according to the method described by Sato et al. (2019) to partially purify TTXs. Authentic TTX and its analogs were prepared from toxic *Takifugu* pufferfish collected in Japan as described in section 2.1 essentially according to the above-mentioned procedures.

TTX, 4-*epi*TTX and 4,9-anhydroTTX were quantified by HPLC-FLD using post-column derivatization developed by Yotsu et al. (1989) with minor modifications as

follows: HPLC column, J-Pak Symphonia C18 (5 μm, 4.6 × 150 mm; Jasco, Tokyo, Japan); mobile phase, 0.06 M heptafluorobutyric acid (HFBA, 98%; Sigma-Aldrich)/0.05 M ammonium acetate buffer (pH 5.0), 0.4 mL/min; reaction reagent, 4 M sodium hydroxide, 0.4 mL/min; detector, FP-2020 Plus (Jasco, Ex 365 nm, Em 510 nm, Gain ×1000); pump for mobile phase, PU-2080 Plus (Jasco); pump for reaction reagent, PU-2080 Plus (Jasco); reaction coil, i.d.0.5 mm × 200 cm (stainless, 120°C in dry oven); integrator, Chromatocorder 21 (SIC, Tokyo, Japan); injection volume, 10 μL (overload injection).

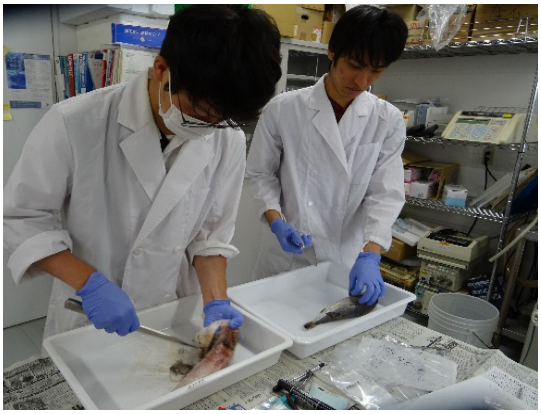
TTXs were also analyzed by LC-MS using a LC-20AD solvent delivery system (Shimadzu, Tokyo, Japan) and an amaZon SL mass spectrometer (Bruker, Bremen, Germany), with an ESI source. LC was performed on a TSK-GEL Amide-80 column (i.d. 2.0 mm × 150 mm, 3 μm, Tosoh, Tokyo, Japan) with 16 mM ammomium formate in water/acetonitrile/formic acid (30:70:0.002, *v/v*) at the flow rate of 0.2 mL/min at 25°C (Yotsu-Yamashita et al. 2011).

Picture 1a. Front side of fish

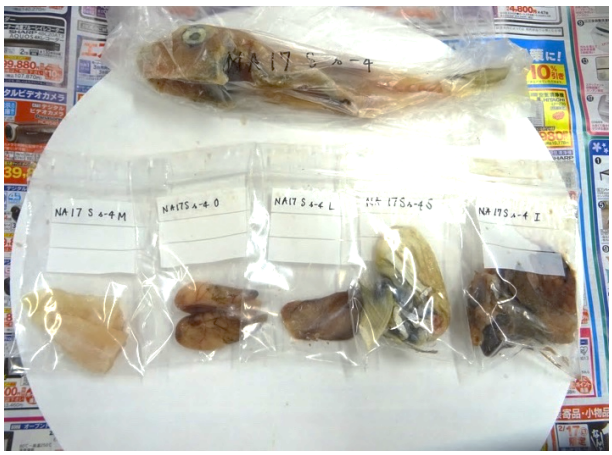
Picture 1b. Back side of fish

Picture 1. How to measure pufferfish sample size

Picture 2a. Preparing sample



Picture 2b. Isolating internal organs



Picture 2c. Hall body (up side) and organs after isolated
(from right to left: muscle, ovary, liver, skin, intestine)

Picture 2. How to isolate the pufferfish organs

Picture 3a. Scale exactly 2g of each organ

Picture 3b. Puree each sample and extract

Picture 3. The procedures of TTXs extraction with acetic acid

3. RESULTS

3.1. Amounts of TTXs determined with TTX-ELISA kit in Lagocephalus *pufferfish*

Table 1 shows average and maximum amounts of TTXs determined by ELISA for totally 108 individuals of *Lagocephalus* pufferfish collected in Vietnam from 2017 to 2019. These include 83 individuals of *L. spadiceus* from northern, central and southern regions, 18 individuals of *L. cheesemanii* from central and southern regions, 6 individuals of *L. lunaris* from central and southern regions, and one individual of *L. inermis* from southern region in Vietnam. Hybrid individuals are often found in *Takifugu* pufferfish (Tasuno et al. 2019), whereas hybrid *Lagocephalus* was not found in this study by their appearance. As shown in Table 1, TTXs distributed to various tissues of *L. spadiceus*. Our TTX-ELISA kit designates not only TTX but also its analogs except for 4,9-anhydroTTX, although the reactivity is slightly different among different molecular species of TTXs (Sato et al. 2019). Thus, it should be noted that the concentration in Table 1 indicates an approximate estimation of total amount of TTXs except for 4.9-anhydroTTX.

Twenty-nine, thirty-five and nineteen individuals of *L. spadiceus* were collected from northern, central and southern regions, respectively (Table 1). The ELISA kit detected TTXs in intestine, liver, gonads and skin, but almost no TTXs in muscle except five individuals (>10 nmol/g) all collected from Vung Tau and Kien Giang in the southern region during non-spawning season (Supplementary Table S1).

Four individuals of *L. cheesemanii* were collected in the spawning season from Da Nang in the central region of Vietnam, and 14 individuals either in spawning or non-spawning season from Vung Tau in the southern region (Tables 1, Supplementary Table S1). Among these samples, one individual collected from Vung Tau in the spawning season showed high amounnt of TTXs in the intestine (63.9 nmol/g) and gonad (82.9 nmol/g) (Supplementary Table S1). One individual was also collected from Kien Giang in the southern region during spawning season, showing only marginal amount of TTXs in all tissues.

Four and two individuals of *L. lunaris* were collected from Da Nang in the central region and Kien Giang in the southern region in Vietnam, respectively (Table 1). All individuals from Da Nang showed appreciable amounts especially in the intestine (55.1 – 201.7 nmol/g), liver (18.5 – 289.5 nmol/g) and gonad (46.8 – 333.9 nmol/g) (Table 1 and Supplementary Table S1). The amount of TTXs in two individuals collected from the southern was quite low (<10 nmol/g). Such local difference in the distribution of TTXs was in contrast to that found in *L. cheesemanii* (Table 1).

Table 1. Average and maximum of total amount of TTX and its analogs determinded by ELISA in various tissues of *Lagocephalus* pufferfish collected in Vietnam

Species	Region	Location	Date of sampling	No. of individuals	Body weight (g)	Amount (nmol/g)									
						Intestine		Liver		Muscle		Gonads		Skin	
					Mean±SD	Mean±SD	Max.	Mean±SD	Max.	Mean±SD	Max.	Mean±SD	Max.	Mean±SD	Max.
L. spadiceus	North	Hai Phong	2017Sep~2018Dec	18	224±40	9.5 ± 27.9	120.8	20.6 ± 40.2	126.1	1.7 ± 1.5	7.0	34.6 ± 63.9	250.5	5.6 ± 12.9	56.9
		Thanh Hoa	2018Apr~Oct	11	225±63	1.2 ± 1.5	4.2	1.0 ± 1.8	6.7	0.9 ± 1.4	4.3	1.7 ± 1.7	5.0	0.8 ± 1.0	3.4
	Center	Nghe An	2017Sep~2018Oct	25	244±79	0.7 ± 1.3	5.4	1.0 ± 2.6	13.0	0.9 ± 1.9	9.5	4.7 ± 14.2	72.2	0.6 ± 0.8	3.2
		Da Nang	2018Jul~Dec	10	268±58	1.2 ± 1.3	3.5	9.0 ± 23.8	80.2	0.8 ± 0.6	1.9	9.5 ± 21.1	69.3	10.7 ± 28.3	95.5
	South	Vung Tau	2017Sep~2018Apr	9	148±61	36.3 ± 65.4	197.2	61.2 ± 103.5	270.8	13.3 ± 23.0	75.8	39.4 ± 47.5	135.1	19.9 ± 34.6	113.2
		Kien Giang	2018Jul~2019Oct	10	202±26	6.9 ± 6.4	20.9	6.8 ± 5.7	19.2	9.2 ± 11.8	42.1	24.5 ± 15.4	56.5	4.7 ± 2.8	10.0
L. cheesemanii	Center	Da Nang	2018Apr~Jul	4	403±82	0.4 ± 0.2	0.8	0.5 ± 0.2	0.8	0.3 ± 0.2	0.6	0.7 ± 0.3	1.2	0.3 ± 0.2	0.6
	South	Vung Tau	2018Apr~2019Jan	13	290±119	8.7 ± 16.4	63.9	4.3 ± 4.1	12.8	2.7 ± 2.7	11.1	9.7 ± 21.3	82.9	3.1 ± 1.8	7.4
		Kien Giang	2018Jul	1	294		0.7		0.3		4.5		1.7		1.1
L. lunaris	Center	Da Nang	2018Apr	4	657±119	115.4 ± 62.8	201.7	111.5 ± 105.9	289.5	26.0 ± 19.1	57.1	227.7 ± 109.8	333.9	44.6 ± 11.0	53.2
	South	Kien Giang	2018Jul	2	240±76	5.1 ± 3.4	8.5	5.6 ± 3.8	9.4	5.3 ± 4.0	9.3	6.1 ± 2.9	9.1	3.4 ± 1.4	4.7
L. inermis	South	Vung Tau	2018Nov	1	496		266.5		297.8		138.0				106.6

Only one individual of *L. inermis* was obtained from Vung Tau in the southern region of Vietnam (Table 1) and found to contain high amounts of TTXs in the intestine, liver and skin (>100 nmol/g) and even in the muscle (138.0 nmol/g).

3.2. Determination of toxin components by HPLC-FLD and LC-MS

The extracts from some individuals were further analyzed by HPLC-FLD. The extracts obtained from some individuals of *L. cheesemanii* and *L. inermis* and *L. lunaris* were found to contain appreciable amounts of TTX, 4-*epi*TTX and/or 4,9-anhydroTTX (Table 2). Considerable amount of TTX was detected in the intestine of *L. cheesimanii* and the gonads of *L. lunaris*. It was noted that two individuals of *L. spadiceus* also contained 4-*epi*TTX and/or 4,9-anhydroTTX in the liver, whereas another individual did marginal amount of TTX, 4-*epi*TTX and 4,9-anhydroTTX (< 0.1 nmol/g). TTX was also detected in the intestine of one individual of *L. cheesimanii* and in the muscle of another individual, though very little in the latter tissue. *L. lunaris* showed TTX at high frequency compared with *L. spadiceus* and *L. cheesimanii*, generally in the gonads.

Comparing with high amount of TTXs obtained by ELISA, the amount of TTXs in the muscle determined by HPLC-FLD was very marginal (Table 2). It has been reported that TDT, which is much less toxic compared with TTX, is contained in *Takifugu* pufferfish (Yotsu-Yamashita et al. 1995). Thus, it is necessary to determine the component(s) other than TTX, 6-*epi*TTX, 4-*epi*TTX and/or 4,9-anhydroTTX in the extract from the muscle, which was reactive with the TTX-ELISA kit, because the muscle part is commercially important as materials for processed food. While the muscle of *L. spadiceus* collected from the southern region contained TTXs, their amounts were too low to determine the components reactive with the TTX-ELISA kit by directly applying the extract to LC-MS/SIM). Thus, the muscle extract was prepared using some individuals of *L. spadiceus* (Table 1 and supplementary Table S1) and subjected to partial purification of the component(s) with the yield of 405 nmol as calculated by the TTX-ELISA kit. Partial purified fraction obtained by using charcoal treatment followed by Bio-Gel P-2 and Bio-Rex 70 column chromatography, together with monitoring employing the TTX-ELISA kit, was found to contain only TDT, but no TTX (Figure 2a). The extracts from other tissues including the intestine, liver and testis of sample No. 66 of *L. spadiceus* (Supplementary Table S1) also gave similar results (Figure 2b). It has been reported that TDT is much less toxic compared with TTX (Yotsu-Yamashita et al. 1995). In contrast, the ovary from sample No. 103, the testis and liver from samples No. 104, and the intestine from sample No. 105 of *L. lunaris*, which showed high amount of TTX by HPLC-FLD (Supplementary Table S2), clearly gave a large peak of TTX in the liver and gonads by LC-MS (Figure 3).

Table 2. Amounts of TTX, 6-epiTTX, 4-epiTTX and 4,9-anhydroTTX determined by HPLC-FLD, and total amount of TTX and its analogs by ELISA

Species	Region	Location	Year	Month	Sample No.	Tissue	HPLC-FLD				ELISA
							TTX	6-epi TTX	4-epi TTX	4,9-anhydro TTX	TTXs
L. spadiceus	North	Hai Phong	2018	Apr	7	L	<0.1	<0.1	<0.1	<0.1	126.2
					11	L	<0.1	<0.1	<0.1	<0.1	21.3
						G	<0.1	<0.1	<0.1	<0.1	14.7
						M	<0.1	<0.1	<0.1	<0.1	0.3
				Dec	13	M	<0.1	<0.1	<0.1	<0.1	1.4
	South	Vung Tau	2017	Sep	66	I*	0.2	<0.1	<0.1	<0.1	197.2
						L*	<0.1	<0.1	5.1	5.6	270.8
						M	<0.1	<0.1	<0.1	<0.1	75.8
						G*	<0.1	<0.1	<0.1	<0.1	57.7
						S	<0.1	<0.1	<0.1	<0.1	113.2
					67	L	<0.1	<0.1	<0.1	<0.1	205.8
						M	<0.1	<0.1	<0.1	<0.1	21.7
						S	<0.1	<0.1	<0.1	<0.1	16.1
			2018	Apr	68	I	<0.1	<0.1	<0.1	<0.1	4.3
						L	0.7	<0.1	2.1	<0.1	2.8
						G	<0.1	<0.1	<0.1	<0.1	135.1
					69	L	<0.1	<0.1	<0.1	<0.1	2.6
						G	<0.1	<0.1	<0.1	<0.1	6.8
						S	<0.1	<0.1	<0.1	<0.1	0.7
L. cheesemanii	South	Vung Tau	2018	Apr	90	I	9.7	<0.1	<0.1	<0.1	63.9
						L	<0.1	<0.1	<0.1	<0.1	11.3
						G	<0.1	<0.1	<0.1	<0.1	82.9
				Nov	91	I	<0.1	<0.1	<0.1	<0.1	5.6
						G	<0.1	<0.1	0.1	<0.1	7.9
			2019	Jan	97	M	<0.1	<0.1	<0.1	<0.1	11.1
					99	I	<0.1	<0.1	<0.1	0.3	1.1
						L	<0.1	<0.1	<0.1	0.3	0.3
						G	<0.1	<0.1	<0.1	0.1	8.0
						M	1.4	<0.1	<0.1	<0.1	3.2
L. lunaris	South	Da Nang	2018	Apr	102	I	<0.1	<0.1	<0.1	<0.1	55.1
						L	<0.1	<0.1	<0.1	<0.1	291.8
						G	2.1	0.2	<0.1	0.4	18.5
					103	I	1.7	<0.1	<0.1	0.7	148.9
						G (ovary)*	6.8	<0.1	<0.1	0.3	238.3
						L	0.6	0.7	<0.1	4.0	91.4
						S	<0.1	<0.1	<0.1	<0.1	50.1
					104	I	<0.1	<0.1	<0.1	<0.1	201.7
						L*	2.3	<0.1	<0.1	3.7	289.5
						G (testis)*	17.1	<0.1	<0.1	4.4	333.9
						M	0.1	<0.1	0.1	0.1	20.2
						S	0.1	<0.1	0.1	<0.1	49.3
					105	I	19.8	<0.1	<0.1	13.6	55.8
						L	0.1	<0.1	<0.1	<0.1	46.8
						G	0.3	<0.1	<0.1	0.1	46.8
						M	0.1	<0.1	<0.1	0.1	57.1
						S	0.5	<0.1	0.4	0.9	53.2
L. inermis	South	Vung Tau	2018	Nov	108	I	<0.1	<0.1	0.1	<0.1	266.5
						L	<0.1	<0.1	<0.1	<0.1	297.8
						M	<0.1	<0.1	<0.1	<0.1	138.0
						S	0.2	0.1	<0.1	<0.1	106.6

Samples numbers correspond to those listed in supplementary Table S1.*Sample Nos.66, 103 and 104 were further subjected to LC-MS analysis. Abbeviations used are: I, intestine; L, liver; M, muscle; G, gonads; S, skin.

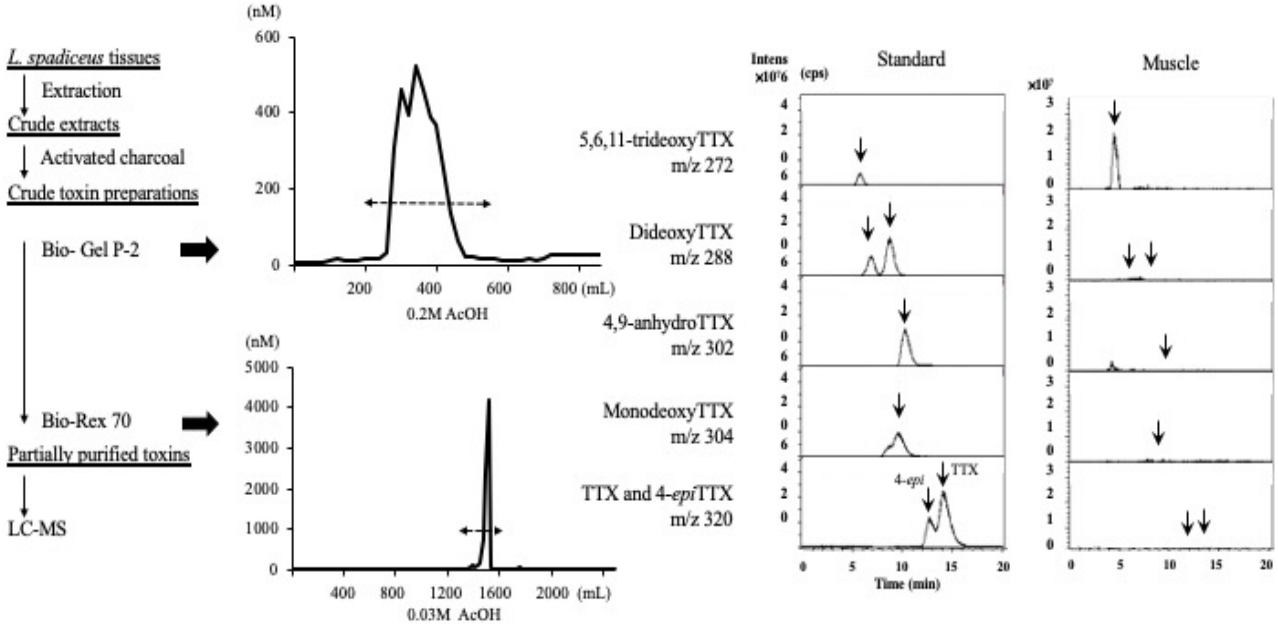


Figure 2a. Chromatograms of LC-MS for TTXs partially purified from the muscle extract of some specimens of *L. spadiceus* collected in Vietnam

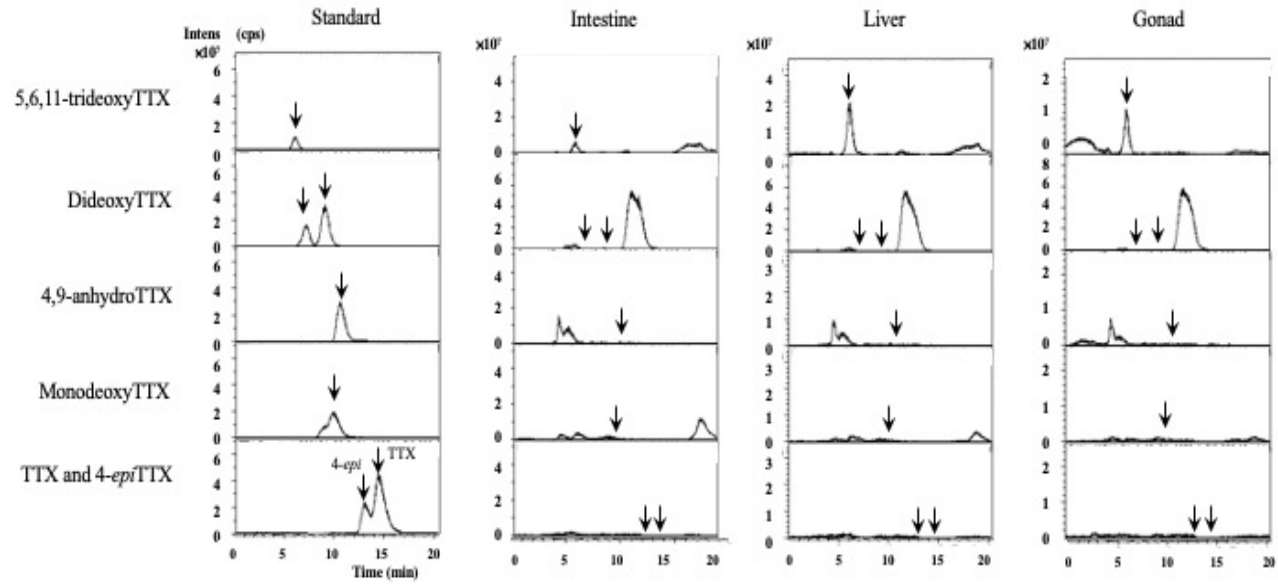


Figure 2b. Chromatograms of LC-MS for TTXs of the extracts from the intestine, liver and gonads of *L. spadiceus* sample

Figure 2 showed chromatograms of LC-MS for TTXs partially purified from the muscle

extract of some specimens of *Lagocephalus spadiceus* collected in Vietnam (a), and the extracts from the intestine, liver and gonads (b) of *L. spadiceus* sample No. 66 (Table S2 and supplementary Table S1). Authentic TTX and its analogs were prepared from *Takifugu* pufferfish and used as the standard. Arrows indicate elution peak positions of authentic TTX and its analogs.

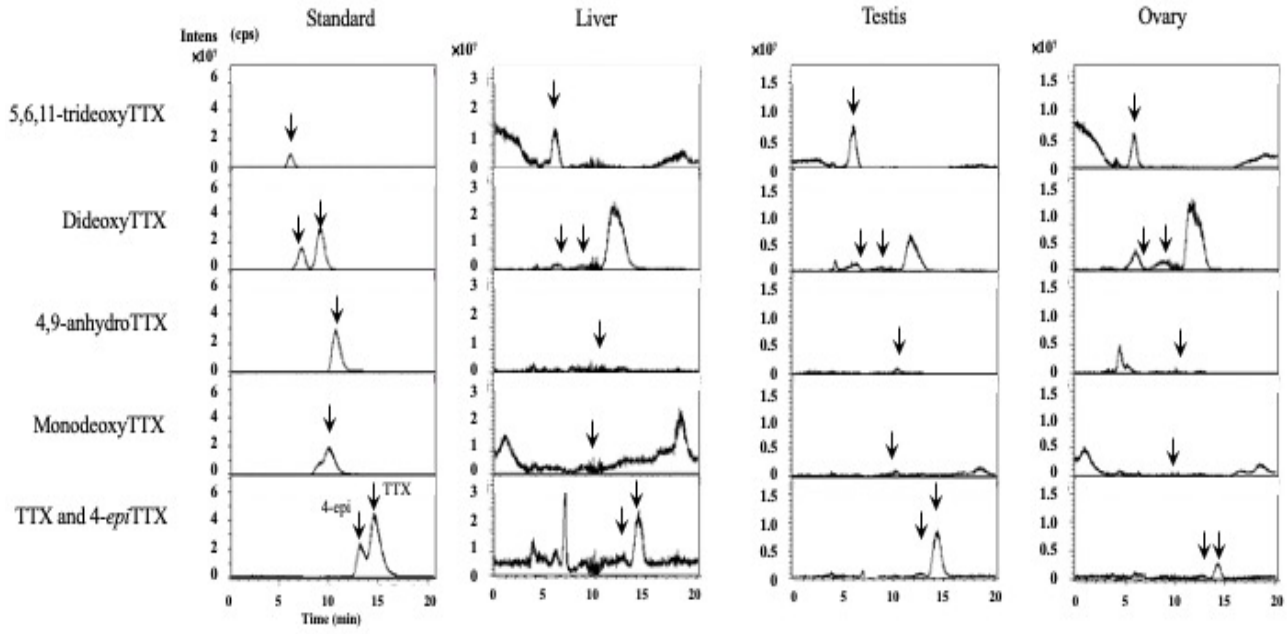


Figure 3. Chromatograms of LC-MS for TTXs partially purified from the extracts of various tissues of *Lagocephalus lunaris*

Figure 3 showed the liver, testis and ovary from sample of *L. lunaris* clearly gave a large peak of only TTX by LC-MS.

4. DISCUSSION

Lagocephalus pufferfish are commonly captured not only in Japan but also in Vietnam and neighboring countries. Among various *Lagocephalus* pufferfish, *L. spadiceus* and *L. cheesemanii* have been believed to be non-toxic and their muscles are frequently consumed after processing to slightly salted and dried food in Japan due to their reasonable prices for market distribution. However, several reports claimed that not only *L. lunaris* but also *L. spadiceus* and *L. cheesemanii* caught in the region of the South China Sea are possibly toxic even for muscle (Harada 1979; Tabeta and Kumagai 1980; Hwang et al.1992). Thus, the present study was undertaken to thoroughly examine the presence of TTXs in 108 individuals of *Lagocephalus* pufferfish collected in Vietnam covering the entire coastal region from near Hai Phong in the north to near Kien Giang in the south, using ELISA, HPLC-FLD and LC-MS.

As a result, the muscle of *L. spadiceus* and *L. cheesemanii* from northern and central regions in Vietnam was found to be practically not toxic as revealed by ELISA, HPLC-FLD and LC-MS. Interestingly, the muscle of *L. spadiceus* from the southern region in Vietnam contained relatively high amount of TTXs as revealed by ELISA. Because the TTX antibody has been demonstrated to be reactive not only TTX but also its analogs including TDT, the toxicity of which is known to be about 90 folds lower than that of TTX (Yotsu-Yamashita et al. 1995). The maximum amount of TTXs by ELISA in muscle was 75.8 nmol/g for *L. spadiceus* from the southern region (sample No. 66; Table 1, Supplementary Table S1). However, it contained no TTX as revealed by HPLC-FLD (Table 2). Moreover, the muscle extracts collectively prepared from some individuals of *L. spadiceus* were found to be almost exclusively composed of TDT, but no TTX by LC-MS (Figure 2). The amount of 75.8 nmol/g is roughly equivalent to 1 MU/g referring to the toxicity of TDT as 1/90 of TTX. Thus, the muscle of *L. spadiceus* and *L. cheesemanii* are considered to be safe in terms of human consumption as far as specimens collected in the present study, because the safety standard in Japan allows the limit of 10 MU/g (Life Health Bureau, Ministry of Health and Welfare of Japan, 1991), where lethal potency of 1 MU is determined by the official method for TTX using ddY strain male mice weighing 19 - 21g within 30 min by intraperitoneal injection. Apart from food hygiene, it was interesting to know whether or not the extract of the liver with the highest amount of 270.8 nmol/g TTXs among various tissues from *L. spadiceus* and *L. cheesemanii* captured in Vietnam (sample No. 66 in supplementary Table S1) have toxin compositions similar to that observed for the muscle extract as mentioned above. Again, no TTX and its analogs

except TDT were found by either HPLC-FLD or LC-MS (Table 2 and Figure 2b). *L. spadiceus* and *L. cheesemanii* captured in Japanese waters are supposed to be weakly, or almost non-toxic. On the other hand, *Takifugu* species are often contain TTX and its analogs in high concentrations, with the same level of TDT. In addition, Jang et al.(2010) reported that freshwater puffers *Pao* (former *Tetraodon*) sp. also possess high amount of TTX with TDT. TTX was not detected in *L. spadiceus* collected in Vietnamese waters, whereas TDT seemes to be dominant. The results of present study may partially explain the differences of toxicity between pufferfish species.

L. lunaris showed high reactivity in the TTX-ELISA kit especially with the extracts from intestine, liver and gonads, though much lower with that of muscle (Table 1). Because *L. lunaris* contained TTX and TDT as the major components (Table 2 and Figure 3), this fish is really toxic.

Only one individual of *L. inermis* was available in the present study. *L. inermis* are also often found in waters around southern Japan. This fish is regarded as an edible pufferfish species in Japan, because muscle, skin and testis are non-toxic in contrast to the liver, which is known to be highly toxic (Life Health Bureau, Ministry of Health and Welfare of Japan, 1991). However, Harada (1979) and Hwang et al. (1992) claimed that *L. inermis* collected near Taiwan were toxic even for muscle. Furthermore, Nagashima et al. (2012) reported the incidence of food poisoning due to ingestion of toxic *L. inermis* captured in Japan. In contrast, *L. inermis* captured in Vietnam in the present study was found to contain no TTX in any tissues by HPLC-FLD, although high amounts of TTXs were detected in various tissues including the muscle by ELISA. Thus, it is urgent to investigate TTX distribution to *L. inermis* in Vietnam further.

So far, pufferfish toxin, mainly composed of TTX, has been quantified by mouse bioassay (Sato and Kodama 2015). However, mouse bioassay has negative impacts in terms of animal welfare and as well its sensitivity is not high as like instrumental analyses. Recently, LC-MS as well as HPLC-FLD have been developed to detect TTXs (Yotsu et al. 1989; Yotsu-Yamashita et al. 2013). Currently, however, only a limited number of authorized references of TTX analogs are available for quantification by such highly efficient instrumental analyses. On the other hand, immunochemical methods have high sensitivity and specificity, and a number of samples can be treated at a time. The present study demonstrated that our newly developed TTX-ELISA kit was very useful to determine the distribution of TTXs in various tissues of *Lagocephalus* pufferfish collected from different areas. Furthermore, the TTX-ELISA kit was also applicable to monitor isolation or purification of TTXs. However, highly efficient instrumental analyses such as fluorometric HPLC-FLD or LC-MS are still necessary, if identification of components

reactive with the kit is required. Thus, application of immunochemical and instrumental analyses in combination is a powerful tool not only for food safety management but also for research related to TTX.

We proposed the estimated conversion pathway of TTXs in toxic organisms (Sato et al. 2019), by referring to Yotsu-Yamashita et al. (2005). In this pathway, TDT is the origin of TTX and we detected a large amount of TDT, but almost no TTX, in not only muscle but also other various tissues including intestine, liver and gonads of *L. spadiceus* (Figure 2). On the other hand, *L. lunaris* contained both TDT and TTX at high concentrations in liver and (Table 2 and Figure 3). Thus, it is interesting to clarify the mechanisms involved in the conversion of TDT to TTX in *L. lunaris* by endogenous specific enzyme(s) or exogenous factors such as microbes. We are currently trying related investigations.

As described above, the present study was conducted to thoroughly survey the presence of TTXs in the extracts from various tissues of *Lagocephalus* pufferfish including *L. spadiceus*, *L. cheesemanii, L. lunaris* and *L. inermis* collected from various coastal and ocean areas off Vietnam by using the TTX-ELISA kit. The tissue extracts of selected individuals were also subjected to HPLC-FLD and LC-MS analyses to investigate toxin components, revealing that the muscle from *L. spadiceus* and *L. cheesemanii* is non-toxic as far as samples collected in the present study are concerned. Further survey is necessary to confirm our results in terms of food safety and food hygiene by collecting more samples to be analyzed.

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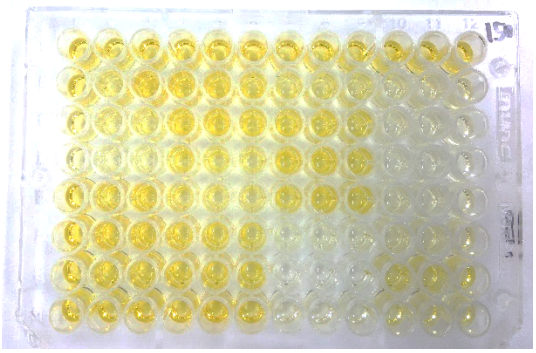
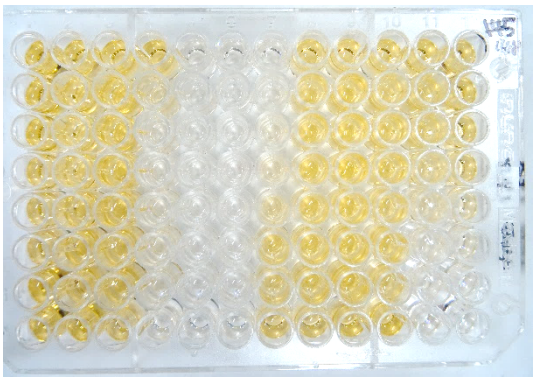
APPENDIXES



Picture S1a. Plate 150



Picture S1a. Plate 145

Supplementary Picture S1. The analyzed ELISA plates

Supplementary Table S1. Total amount of TTX and its analogs determined by ELISA in various tissues from each individual of *Lagocephalus* pufferfish collected in Vietnam (Abbreviation used are: I, intestine; L, liver; M, muscle; G, gonads; S, skin; SS, spawning season)

Species	Region	Location	Year	Month	Sample No.	Body weight (g)	Remark					
							I	L	M	G	S	
L. spadiceus	North	Hai Phong	2017	Sep	1	202	3.0	0.9	0.4	4.6	0.7	
					2	179	0.4	0.4	0.8	3.1	0.5	
					3	239	12.7	123.8	1.8	250.5	56.9	
					4	264	1.1	0.8	0.4	1.1	0.7	
					5	239	1.4	0.5	1.7	3.3	1.0	
					6	304	2.1	0.7	1.2	7.1	1.7	
			2018	Apr	7[a]	179	4.3	126.2	7.0	50.5	6.3	SS
					8	181	1.4	0.8	0.6	1.8	2.3	SS
					9[b]	210	5.9	7.2	1.6	14.6	4.5	SS
					10[b]	201	2.8	0.5	0.8	3.0	2.0	SS
					11[a,b]	211	1.3	21.3	0.3	14.7	2.5	SS
					12[b]	200	3.7	7.3	3.4	69.9	3.6	SS

			Dec	13[b]	202	2.4	1.6	1.4	20.8	1.4	
				14[b]	179	120.8	70.7	2.3	153.8	5.0	
				15[b]	239	1.0	1.8	1.5	1.4	1.7	
				16[b]	264	2.8	1.8	1.8	1.9	0.0	
				17[b]	239	2.2	3.0	2.3	4.1	2.1	
				18[b]	304	1.9	2.5	1.3	5.5	2.3	
	Thanh Hoa	2018	Jul	19	204	0.1	0.1	0.3	0.3	0.2	SS
				20	243	0.2	0.1	0.2	1.5	0.1	SS
				21	242	0.2	0.5	0.3	0.8	0.3	SS
				22	274	0.8	1.0	4.3	0.7	2.2	SS
				23	326	4.2	1.7	3.2	1.8	1.4	SS
				24	272	3.3	0.3	0.4	3.3	0.2	SS
			Oct	25	111	0.2	0.2	0.2	0.2	0.1	
				26	158	0.1	0.3	0.1	5.0	0.2	
				27	174	3.6	6.7	0.1	4.6	3.4	
				28	197	0.1	0.3	0.2	0.2	0.2	
				29	276	0.3	0.1	0.2	0.7	0.3	
Center	Nghe An	2017	Sep	30	157	0.8	1.2	0.1	4.1	0.3	
				31	178	0.2	0.2	0.4	0.3	0.4	
				32	100	0.5	0.1	0.1	4.7	0.2	
				33	176	0.1	0.2	0.5	1.7	0.5	
				34	101	0.0	0.3	0.0	0.1	0.1	
				35	185	0.1	0.1	1.6	0.2	1.9	
		2018	Apr	36	358	0.2	0.2	0.1	3.6	0.2	SS
				37	249	0.2	0.1	0.1	3.9	0.2	SS
				38	227	0.1	0.2	0.1	0.1	0.1	SS
				39	239	0.1	0.2	0.1	2.1	0.1	SS
				40	241	0.2	0.2	0.2	0.3	0.1	SS
				41	301	0.2	0.4	0.1	2.0	0.2	SS
				42	346	0.2	0.1	0.2	0.2	0.1	SS
			Jul	43	214	0.2	0.1	0.2	0.2	0.1	SS
				44[b]	304	1.1	1.0	1.3	8.3	1.1	SS
				45	282	2.1	1.5	1.7	1.4	1.3	SS
				46	276	3.7	1.3	2.3	2.1	0.2	SS
				47	323	0.0	0.1	9.5	0.1	0.1	SS

				48	382	5.4	13.0	2.2	72.2	3.2	SS
			Oct	49	146	0.5	2.9	0.2	2.7	2.4	
				50	168	0.3	0.4	0.2	0.4	0.2	
				51	277	0.2	0.3	0.1	1.0	0.4	
				52	254	0.2	0.3	0.3	0.5	0.3	
				53	258	0.5	0.0	0.0	0.5	0.0	
				54	347	0.0	0.1	0.4	0.2	0.6	
	Da Nang	2018	Jul	55	209	0.3	0.5	0.4	0.6	0.6	SS
				56	208	0.3	0.2	0.3	0.3	0.3	SS
				57	198	0.1	0.1	0.2	0.2	0.2	SS
				58	225	0.3	0.1	0.1	1.3	0.1	SS
		2018	Dec	59	279	1.1	1.2	0.4		1.2	
				60	280	0.5	0.7	1.1	2.1	0.7	
				61	324	3.2	0.6	0.6	3.4	1.5	
				62	380	0.5	1.4	1.3	3.3	3.4	
				63	285	3.5	4.7	1.9	69.3	3.7	
				64	290	2.5	80.2	1.5	5.2	95.5	
South	Vung	2017	Sep	65	97	11.6	0.0	8.0	104.7	32.7	
	Tau			66[a,b]	88	197.2	270.8	75.8	57.7	113.2	
				67[a,b]	101	105.5	205.8	21.7	40.1	16.1	
		2018	Apr	68[a,b]	137	4.3	2.8	3.6	135.1	3.7	SS
				69[a,b]	108	1.8	2.6	0.5	6.8	0.7	SS
				70[a,b]	118	0.8	2.7	0.5	3.3	1.0	SS
				71[a,b]	231	0.7	0.3	1.7	1.2	0.7	SS
				72[a,b]	212	1.2	1.2	0.2	4.2	0.2	SS
				73[a,b]	236	1.0	3.8	7.3	1.6	7.0	SS
	Kien	2018	Jul	74	201	1.3	0.2	0.6	3.5	1.2	SS
	Giang			75	265	7.0	1.5	1.4	42.1	1.6	SS
				76[b]	227	2.9	19.2	2.7	56.5	3.3	SS
				77	194	1.8	3.3	3.3	26.3	3.5	SS
		2019	Oct	78	188	3.3	3.3	1.6	20.1	2.2	
				79	177	16.4	9.9	11.8	30.5	9.1	
				80	180	20.9	12.3	13.9	27.9	6.0	
				81	201	8.6	10.9	7.4	21.2	5.3	
				82	188	2.2	2.8	42.1	11.1	4.8	

					83	195	4.4	4.4	7.2	5.9	10.0	
L. cheesemanii	Center	Da Nang	2018	Apr	84	290	0.8	0.3	0.2	1.2	0.2	SS
					85	482	0.2	0.2	0.2	0.8	0.1	SS
				Jul	86	436	0.3	0.8	0.1	0.3	0.4	SS
					87	406	0.2	0.7	0.6	0.7	0.6	SS
	South	Vung Tau	2018	Apr	88	250	2.6	1.8	0.5	3.3	2.3	SS
					89	182	2.1	1.3	1.2	3.1	1.1	SS
					90	187	63.9	11.3	1.1	82.9	2.9	SS
					91	258	7.9	5.6	1.5	6.7	3.1	SS
				Nov	92	471	1.7	0.4	0.7	0.8	1.1	
					93	441	0.6	0.3	0.5	4.2	2.5	
					94	537	5.6	2.4	3.7	7.0	4.5	
			2019	Jan	95	192	1.6	2.3	4.7	3.0	3.4	
					96	237	2.8	5.2	2.2	3.5	7.4	
					97	289	5.8	9.3	11.1	1.9	0.4	
					98	257	14.7	12.8	3.7	0.5	2.3	
					99	298	1.1	0.3	3.2	8.0	5.4	
					100	171	2.9	3.0	1.4	0.9	4.3	
		Kien Giang	2018	Jul	101	294	0.7	0.3	4.5	1.7	1.1	SS
L. lunaris	Center	Da Nang	2018	Apr	102	788	55.1	18.5	5.2	291.8	25.7	SS
					103[b]	666	148.9	91.4	21.7	238.3	50.1	SS
					104[b]	675	201.7	289.5	20.2	333.9	49.3	SS
					105	499	55.8	46.8	57.1	46.8	53.2	SS
	South	Kien Giang	2018	Jul	106	294	8.5	9.4	9.3	9.1	4.7	SS
					107	187	1.7	1.8	1.3	3.2	2.0	SS
L. inermis	South	Vung Tau	2018	Nov	108	496	266.5	297.8	138.0		106.6	

CHAPTER 4. BIRTH AND DEVELOPMENT OF PUFFERFISH FOOD CULTURE IN VIETNAM

1. Vietnam's expectations for pufferfish

Thanks to the cooperation among Vietnamese and Japanese researchers and specialists, we have done studies on sensory acceptability of pufferfish and on toxic testing of Vietnamese pufferfish (Chapter 2 and 3). The results show that pufferfish will be well accepted as part of Vietnam's food culture. The toxic analysis suggested that the muscles of Vietnam's *L. spadiceus* Shirosabafugu and *L. cheesemanii* Kurosabafugu are safe as food. Through these studies, I have confirmed the potential success of pufferfish food culture in Vietnam. However, to achieve it, we need to make further efforts.

Luckily for our activities, the Vietnamese government intends to increase added value in the agricultural and fishery sectors, make them internationally competitive, and become major industries. The 2014 New Year statement by the Prime Minister and the Prime Minister's decision No.1684/QĐ-TTg showed the importance of the development of agriculture and fisheries with the following statement, "the restructuring toward sustainable development will be tackled by linking the increased value addition of the agriculture and fishery industries" [1].

In 2019, the resolutions of the 12th National Congress of the Communist Party of Vietnam, "IV. Suggestions and strategies for socio-economic development" called for the "promotion of the development of agriculture, forestry and fisheries by applying the progress of science and technology to increase the economic yield per unit area. The government document "Vietnam's Fisheries Development Strategy by 2020 (approved)" also states "4. Improvement of value addition and quality; the fishing industry should develop in the direction of improving quality and sustainability based on the enhancement of the product value and quality, food safety, environmental protection, and resource protection" [2].

Marine resources along the coast are declining, and the development of aquaculture technology has become a policy issue. Joining the Free Trade Agreement (FTA) is expected to intensify competition with China, India, Thailand and Indonesia. The aquaculture industry, currently led by shrimp, is thought to need new value-added processed products. Furthermore, the "National Nutrition Strategy aiming for achievement by 2030", recommends daily intake of fish, milk, and vegetables in order to improve the nutritional balance of the people's diet [3]. By utilizing Japanese pufferfish food culture and technology, we can expect to increase the economic value of this unused marine resource in Vietnam.

2. Toxic analysis laboratory

In order to lift the law banning use of pufferfish [4] and establish the food culture of pufferfish in the future, it is necessary to investigate the toxicity of pufferfish inhabiting the waters of Vietnam. The animal test is recognized as the only official method for testing pufferfish toxin in Japan [5]. It seems desirable to adopt this method in Vietnam, following the Japanese method as well (under preparation). First, it is necessary to build a mouse analysis laboratory for the pufferfish toxicity test at Vietnam National Institute of Nutrition (NIN). NIN will be registered to become the responsible institute for toxin analysis and food safety. Relevant parties (Jumonji University) in Japan will transfer research techniques to Vietnamese researchers. Next, each sample taken from tissue of potentially toxic pufferfish will be divided into two parts. Half one will be analyzed at the analysis center in Japan, and the other will be analyzed at NIN's analysis lab. Analytical techniques will be verified when the results at both facilities match.

After the analytical technology in Vietnam was confirmed to be reliable, large-scale safety tests will be able to be conducted at any time in Vietnam. NIN will provide the Vietnamese government with sufficient scientific evidence to amend the current law related to pufferfish.

3. Pufferfish processing licence system

There are hundreds of types of pufferfish; in Japan, there are 21 types of pufferfish currently recognized legally as edible [6–8]. Depending on the species of pufferfish, the edible parts, and even in the same species the toxic amount of each part can vary greatly. Therefore, only those with adequate knowledge are allowed to process pufferfish. To obtain the Japanese pufferfish processing license, both two years cooking experience and a regular cooking license are required (I took the test on 25th January, 2021).

In the test there are two parts, a written test and a skill test [6–8]. The written test is on general knowledge about pufferfish, the law, nutrition, hygiene, the history and culture of food, and the handling of poisonous parts; the skill test is on the type of pufferfish and the separation of edible parts from inedible parts. Since the introduction of the license system for the processing of pufferfish in Japan, the number of poisoning cases has plummeted to almost zero, and the rare cases are mainly caused by laymen who do not have the knowledge or skill to handle pufferfish [6–8]. In order to provide pufferfish as a safe food in Vietnam, it will be necessary to introduce a licensing system similar to the one in Japan. Japanese pufferfish is expensive, dishes are also made very elaborately, for example, sashimi is arranged on a large plate in the shape of chrysanthemums, cranes,

Mount Fuji, Osaka Castle, etc. to enhance the asthetic appeal and to encourage use in celebrations.

4. Proposal for puffer fish industry development

If the ban on pufferfish is lifted in Vietnam, it will be possible to enjoy pufferfish as a food with a cheaper price than in Japan. In Japan, pufferfish dishes are regarded as delicious but are considered a luxury item; many Japanese are able to eat pufferfish only a few times in their lives, or even never. If we can develop pufferfish food tourism in Vietnam, it could be an attractive tourist option. Pufferfish tourism could highlight Vietnamese attention to food and willingness to try new dishes. Pufferfish can also be expected to be a substantial economic impact on tourism and export businesses.

In addition, to ensure the stability of the pufferfish supply, as well as to increase the production of high-value pufferfish species such as Yoritofugu, the project of pufferfish cultivation and processing is essential. This will be a series of cooperative projects between Vietnam and Japan, aiming to transfer and establish a system from pufferfish farming to processing in Vietnam. In these projects, cooperation of researchers from Japanese universities such as Jumonji University, seafood companies such as Mitsui Suisan Co., Ltd., and support from Official Development Assistance (ODA) of the Japanese government are important. When that model is successful, Vietnam pufferfish resources will be able to meet domestic demand and exports abroad.

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