

博士論文

Studies on the parasitic ciliate of marine teleosts *Cryptocaryon irritans*
for the development of new control methods against its infection

(海産真骨魚類に寄生する繊毛虫 *Cryptocaryon irritans* に対する
新しい防除法の開発のための研究)

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Contents

Abstract (in Japanese)

General introduction

Chapter 1 Influences of environmental factors on the development and the daily rhythms of *Cryptocaryon irritans*

1.1 Dormancy induced by a hypoxic environment in tomonts of *Cryptocaryon irritans*

1.2 Influences of photoperiod on the daily rhythms of *Cryptocaryon irritans*

1.3 Chapter Discussion

Chapter 2 Characterization of the proteases in the parasitic stage of *Cryptocaryon irritans* and its potential as vaccine antigens against its infection

2.1 Characterization of the proteases of the parasitic stage of *Cryptocaryon irritans*, and *in vitro* and *in vivo* effects of protease inhibitors on cryptocaryoniasis

2.2 Functional analysis of highly expressed cysteine proteases and serine carboxypeptidases in parasitic stage of *Cryptocaryon irritans*

2.3 Development of vaccines using recombinant proteases of *Cryptocaryon irritans* against its infection

2.4 Chapter Discussion

Chapter 3 General discussion

Acknowledgements

References

要旨

海産白点虫 *Cryptocaryon irritans* は、絶対寄生性の繊毛虫であり、ほとんどの海産真骨魚類の鰓や体表の上皮組織内に寄生し、その重篤な寄生は宿主の死亡を引き起こす。その生活史は、宿主組織内の寄生期虫体（トロホント）、成長して宿主を離脱した直後の虫体（プロトモント）、宿主離脱後にシスト壁を形成して水底にとどまるシスト期虫体（トモント）、シスト内で細胞分裂により形成され、水中に放出される感染仔虫（セロント）の4つのステージからなる。本種による疾病、海産白点病は、元来、水族館などの閉鎖的な環境で発生するものと考えられていた。しかし、現在では世界中の温帯・熱帯海域に分布し、海面網生簀養殖場など開放的な環境でも発生することが知られている。本病は国内外で頻発し、養殖魚の死亡による大きな産業被害を出し、地域経済に影響を与えることも多い。また、本疾病は持続的養殖生産確保法でも養殖漁場環境の指標疾病とされており、海産魚養殖の重要な疾病のひとつとなっている。

これまでに、海産白点病防除法の開発のために多くの研究が行われているが、食用魚に対して安全かつ効果的な薬剤やワクチンは開発されていない。現在の海面養殖では、網生簀を発生海域から移動させることが唯一の有効な対処法とされているが、生簀密度が高く、また、移動できる海域が限られていることから生簀の移動は実施困難なことも多く、本病が一旦発生すると大被害につながることが多い。このような背景から、新しい海産白点病の防除法の開発が望まれている。

近年、寄生虫被害を抑えるために総合的寄生虫（病害虫）管理（IPM: Integrated Parasite/Pest Management）が有効であると考えられている。IPM は、生物学的手法、物理学的手法、化学的手法など様々な方法を組み合わせることで、経済的に許容可能なレベルにまで寄生虫や害虫による被害を抑えるという考え方である。本病においても、単独では効果が十分でない防除策を新たな手法と組み合わせることで IPM の実施が可能であると考えられる。

そこで、本研究では海産白点病に対する IPM 手法の開発のための基盤として、海産白点虫の発生と環境要因の関係を検討するとともに、海産白点虫の感染におけるプロテアーゼの関与を明らかにし、プロテアーゼを抗原とした新しいワクチンの開発を試みた。

第1章 海産白点虫の発達と環境要因の影響

本虫のシスト期虫体を低溶存酸素条件下（低 DO）で維持すると、発達が抑制されることにより感染仔虫の放出が阻害されるが、好気条件（高 DO）に戻すと放出が再開することが知られている。また、虫体の宿主からの離脱や感染仔虫の放出には明確な日周リズムがあることも知られている。加えて、本病が主として水温低下期の秋に頻発することや、養殖場の環境に影響されること、それぞれの養殖場でも発生は年によって大きく異なることから、本病の発生には環境要因が強く関連していると考えられている。しかし、低 DO のシスト期虫体への影響や虫体の宿主離脱や感染仔虫放出における日周リズムの形成要因、光受容のメカニズムなど、環境要因が虫体の生理生態に与える影響については不明な点が多い。そこ

で第 1 章では、環境因子が虫体の発達や日周リズムに影響を及ぼすメカニズム解明の基盤的知見を得るために、低 DO がシスト期虫体の発達に及ぼす影響を詳細に検討するとともに、光周期が虫体の宿主離脱や感染仔虫放出の日周リズムに及ぼす影響を調べた。

シスト期虫体の低 DO 下での発達動態と生存を把握するため、シスト期虫体を期間をかけて低 DO や高 DO 条件に暴露し、酢酸カーミン染色法を用いシストの発達を経時的に観察した。その結果、いずれの発達段階のシスト期虫体でも低 DO 下では発達を停止し、それらを高 DO に戻すと発達を再開し、感染仔虫の放出に至った。さらに、シスト期虫体を低 DO で 1 ヶ月間培養し、発達を停止させても高 DO に移したところ、低 DO に収容していないシストと同等の感染仔虫放出率を示し、感染仔虫の感染力も同等であった。このことから、海底が低 DO 化する夏季には海底にシスト期虫体が発達を停止し、蓄積しており、水温躍層の崩壊や台風等による水底への酸素供給直後に発達を再開し、感染仔虫を放出することで本病が大発生することが示唆された。

次に、光周期が宿主離脱の日周リズムに与える影響を調べるため、感染仔虫に暴露して攻撃したブラックモーリー (*Poecilia* sp.) を二つの異なる光周期条件で飼育し、宿主から離脱した虫体を 3 時間毎に回収・計数した。その結果、宿主離脱虫体数のピーク時刻は、光周期を変えることで大きく変化し、光周期の変化により宿主離脱におけるリズムが変化することが示された。

また、光周期が感染仔虫放出における日周リズムの形成に与える影響を調べるため、細胞培養フラスコ内の海水中に宿主離脱直後の虫体を収容し、異なる光周期条件に暴露し、5 日後にシストから放出された感染仔虫を 3 時間毎に回収・計数した。その結果、仔虫放出時刻も光周期により変化することが示された。このことから、虫体の日周性が光周期により調節されていることが示唆された。

第 2 章 海産白点虫の寄生ステージで発現するプロテアーゼの特性とそれを標的としたワクチンの有効性

魚類が海産白点虫に対して免疫を獲得すること、ならびに獲得免疫には虫体表面に存在する不動態抗原が関与していることがよく知られている。しかし、繊毛虫の不動態抗原は株間での変異が高く、同種内であっても免疫効果を示さないことが知られている。そのため、ワクチン開発には異なる株間で共通に免疫効果のある抗原の探索が必要である。一方、寄生性原虫類において、虫体のプロテアーゼが宿主への侵入、摂餌、発達などの重要な寄生プロセスに関与していることが多数報告されている。海産白点虫においても、寄生期に複数のプロテアーゼ遺伝子が高発現することが報告されている。このことから、本虫の寄生にもプロテアーゼが重要な役割を持つことが予想されるが、その機能は明らかではない。そこで、第 2 章では、海産白点虫の感染および寄生期で発現するプロテアーゼをワクチン開発のための候補抗原として注目し、その特性・機能を調べ、これを抗原としたワクチンの有効性を検討した。

まず、海産白点虫の *in vitro* および *in vivo* アッセイ系を用いて、各種のプロテアーゼ阻害剤が虫体の生存と成長に与える影響を調べた。その結果、セリンおよびシステインプロテアーゼ阻害剤添加区において、虫体生存率の低下および感染虫体数の減少が示された。また、ザイモグラフィーにより、寄生期虫体は、セリンおよびシステインプロテアーゼを保有することが確認された。さらに、次世代シーケンサーによるトランスクリプトーム解析を行ったところ、感染仔虫および寄生期虫体でセリンおよびシステインプロテアーゼ遺伝子が高発現していることが示された。

そこで、感染仔虫と寄生期虫体で高発現していたセリンおよびシステインプロテアーゼの寄生への関与を確認するため、特に高発現していた遺伝子 4 種について、RNA 干渉法 (RNAi) により感染仔虫の遺伝子の発現を抑制し、これを用いてブラックモーリーを攻撃した。その結果、RNAi を施した虫体では、対照区と比較して、成長して宿主を離脱した虫体数が減少した。このことから、感染仔虫および寄生期虫体で高発現するプロテアーゼ遺伝子は宿主への感染に関与していることが示唆された。

この結果からプロテアーゼ分子で魚を免疫することにより海産白点虫の寄生を抑制できる可能性が考えられた。そこで、RNAi で標的とした 4 種のプロテアーゼについて組み換えタンパク質を大腸菌発現系によって作製し、これを注射ワクチンとしてトラフグ (*Takifugu rubripes*) を免疫したのち、感染仔虫による攻撃試験を行った。その結果、プロテアーゼを抗原として接種した 4 種の試験区はいずれも対照区と比較して宿主へ感染した虫体数が減少した。特に、そのうちの 1 種のシステインプロテアーゼを抗原として用いた場合は、感染虫体数が有意に減少した。さらに、異なる株の虫体破碎液を用いた酵素結合免疫吸着法 (ELISA) の結果から、ワクチン接種魚の抗体は異なる株にも反応することが確認された。このことから、海産白点虫のプロテアーゼによる免疫は、異なる株に対しても効果を示すことが示唆された。

総合考察

本研究では、低 DO がシスト期虫体の発達に及ぼす影響や、光周期が宿主離脱や感染仔虫の放出に及ぼす影響の詳細を把握することができた。これらは、海産白点病の発生には、溶存酸素条件、日照条件、潮汐などの環境因子が大きく関与している可能性を改めて示し、環境因子をより詳細にモニタリングすることで海産白点病発生予測技術を開発できる可能性が示された。また、従来、内在的であると考えられていた虫体の日周性が光周期によってコントロール可能であることが明らかになったことから、養殖環境や水槽の光周期をコントロールすることで海産白点病の発生を軽減できる可能性も示唆された。また、海産白点虫のプロテアーゼを抗原としたワクチンは、完全な防除は困難ではあるが、異なる株に対しても免疫効果があることが示され、有効なワクチン抗原になる可能性が示された。

本研究により海産白点病に対する IPM 手法の開発にむけて大きな手掛かりをあたえることができたと考えられる。

General Introduction

Cryptocaryon irritans

Cryptocaryon irritans Brown 1951 is an obligate parasitic ciliate of marine teleosts, which causes cryptocaryoniasis. This disease is also called as “marine white spot disease”, because visible pinhead-sized white spots are formed in the skin lesions in fish infected with the parasite (Cheung et al, 1979, Colomi and Burgess, 1997) (Fig. 1).

The parasite was first described by Sikama (1937) in the Aquarium of Tokyo Imperial University located in Shinmaiko, Aichi Prefecture in Japan, and the author proposed *Ichthyophthirius marinus* as the name of the parasite. However, Brown (1951) also found the parasite in the Aquarium of the Zoological Society of London, and, being unaware of the Sikama’s report, named the same parasite *C. irritans*. Although Brown’s ignorance of previous publication was questioned by Canella (1972), the name given by Brown has been accepted by the scientific community and *Cryptocaryon irritans* is used worldwide as the name of the parasite (See the review by Coloni and Burgess, 1997) at present.

The parasite invades the epithelial layer of the skin and gills of marine teleosts and disturbs the osmotic control and respiratory activity of its host, often causing mass mortalities in heavily infected fish (Colomi and Burgess, 1997). The occurrence of the parasite was originally thought to affect fishes only in closed environments, such as public or private marine aquaria (Nigrelli and Ruggieri 1966; Wilkie and Gordin 1969). However, cryptocaryoniasis now frequently occurs in open environments as well, such as marine floating net cages in tropical and subtropical waters in various marine waters worldwide (Colomi and Burgess, 1997). Cryptocaryoniasis causes mass mortalities of fishes and often results in large economic losses in aquaculture industry also in some countries other than Japan, such as China, Korea, and Taiwan (Chao et al., 1994; Yambot et al., 2003). For example, in South China, *C. irritans* has infected cultured fishes including greater amberjack (*Seriola dumerili*), orange spotted grouper (*Epinephelus coioides*), black seabream (*Sparus macrocephalus*), yellow croaker (*Larmichthys crocea*), pompano (*Trachinotus ovatus*) (Luo et al., 2008), and the direct economic loss due to the parasite amounted to around 16 million US dollars only in the Guangdong Province each year (Mai et al., 2015). Also in Japan, the disease repeatedly occurs and sometimes results in mass mortality in marine cultures fishes, such as red seabream (*Pagurus major*), greater amberjack (*Seriola dumerili*), tiger puffer (*Takifugu rubripes*) and Japanese flounder (*Paralichthys olivaceus*), and causes large economic losses in the

aquaculture industry and affects local economy (Yoshinaga and Nakazoe, 1997; Kochi Prefecture, 2005; Katata et al., 2006; Watanabe et al., 2011; Kadohara, 2013). In Wakayama prefecture in 2003 and Kochi prefecture in 2004, mass mortalities due to cryptocaryoniasis occurred and the economic loss amounted to 230 million yen and 1,430 million yen, respectively (Ando et al., 2008). Therefore, this parasite is a major threat to marine aquaculture in tropical and subtropical waters (Yoshinaga et al., 2011).

Life cycle of *C. irritans*

The life cycle of *C. irritans* consists of four developmental stages, namely, theront, trophont, protomont, and tomont (Wilkie and Gordin, 1969; Colorni, 1985, 1987) (Fig. 2). Free-swimming theronts invade the surface organs of fish, including the skin, fins, and gills, and transform to trophonts, the parasitic stage. Trophonts feed and grow, without cell division, in the epithelium of the surface organs. Fully developed trophonts leave the host as protomonts. Protomonts sink, settle on the substrate, and become encysted as tomonts. The tomont stage is the cell division phase; it repeats cell divisions without intervening growth to produce more than one hundred daughter cells, called tomites. This division involves asymmetric “budding,” resulting in an initial grouping of daughter cells at one pole followed by the division of the rest of the cell body (Brown, 1963; Nigrelli and Ruggieri, 1966; Dickerson, 2006). Fully developed tomites are then released into the seawater as theronts (Wilkie and Gordin, 1969; Colorni, 1985, 1987).

Control methods for cryptocaryoniasis

Integrated Pest/Parasite Control (IPM)

Eukaryotic pathogens, or parasites, have been becoming complex problems in the aquaculture industry, because effective control methods are less for parasite diseases in aquaculture than those for bacterial and viral diseases. Antibiotics conventionally used against bacterial pathogens are not in general effective to parasites, as cells in parasitic eukaryotes and those of host animals share basically similar metabolism, differently from the relation between prokaryotic bacteria and eukaryotic animal cells, and chemotherapies potentially lead to generation of drug-resistant pathogens. Vaccinations conventionally used against both bacterial and viral pathogens are not in general effective against parasites, as parasites are mostly provided with ability to avoid hosts' immune response. Similar problems are present in control of pests and

parasites in agriculture and livestock industries. However, the introduction of a strategy called “Integrated pest/parasite management” has succeeded in reducing the damage in various fields, including the agriculture and livestock industries.

Integrated pest/parasite management (IPM) is a strategy that uses combination of biological, cultural, and chemical tactics to reduce pest (parasites) amount to economically tolerable levels. This strategy was introduced in late 1950s for insect pest controls in agriculture and widely practiced during 1970s and 1980s (Stern, 1973). Food and Agriculture Organization of United Nations defines IPM as "the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment (<http://www.fao.org/agriculture/crops/core-themes/theme/pests/ipm>). IPM promotes the use of several measures such as insecticide applications, crop rotation, biological control, harvest management, and the use of pest-resistant varieties of crops to reduce pest populations below economic levels (Van Lenteren, 1995, 2000; Van Lenteren and Woets, 1988; Flint, 1987). This strategy is based on the postulate that a combination of several less effective methods can potentially reduce infection levels significantly, providing decent control of targeted diseases (Thamsborg et al., 1999).

Recently, IPM strategy has been proposed to apply in marine aquaculture (e.g. sea lice management in salmon culture and burrowing shrimp management in oyster culture) (Rae, 2002; Halwart, 2003; Dumbauld et al., 2006; Brooks, 2009). To prevent mass mortalities and economic losses caused by *C. irritans*, extensive researches have been conducted to develop control methods, including biological and physical controls, chemical controls and vaccinations as follows.

Biological and physical control

In closed environments, such as public or private marine aquaria, there are some effective biological and physical control methods for cryptocaryoniasis, such as immersion in hypersaline (40-45‰) and hyposaline (10‰) conditions; switching tanks (Colorni, 1987; Jiang et al., 2016); ultraviolet (UV) irradiation (Spotte, 1979); storing seawater for at least 24 hours before introducing into fish rearing tanks to inactivate infective theronts (Colorni and Burgess, 1997). However, these methods cannot be used for fish cultured in net cages in open sea.

In net cages in open sea, net cages containing fish are recommended to be transferred from original sites to sites far from the original sites, preferably to sites with stronger tidal current, in a bay. This is conducted to transfer fish far from places where tomonts from affected fish are supposed to accumulate on the seabeds, and to flow away protomonts that leave affected fish. This control method is conventionally used and effective to prevent heavy infections and resulting mass mortalities in net cages in Japan (Yoshinaga, 2001). However, delayed transfer often leads to mass mortalities of fish. If we can predict the occurrence of cryptocaryoniasis in net cages, it must be very helpful for reduction of the damage by the disease.

It is well known that cryptocaryoniasis occurs in autumn and after turbulence of water by storms and typhoons in fish cultured in net cages, suggesting that specific environmental factors are involved in the occurrences (Yoshinaga et al., 2001). Previous studies reported that tomonts of *C. irritans* stopped the development under a hypoxic conditions and resumed it after being transferred to a aerobic condition (Yoshinaga et al., 2001), and the swimming activity of theronts were not enough to reach the surface of water, where net cages are located, from the seabed, where tomonts stay (How et al., 2015). Dissolved oxygen concentration, which varies seasonally and is changed by vertical water turbulence appear to effect the occurrence of cryptocaryoniasis.

Furthermore, the presence of daily rhythms in protomonts leaving host fish and in release of theronts from tomonts were known (Burgess and Matthews, 1994). The daily rhythms also appear to affect the occurrence of cryptocaryoniasis; however, the mechanism of the rhythms is not clear yet.

Information on the details and mechanisms of the biological characteristics of *C. irritans*, especially the responses to different dissolved oxygen concentrations and those to daily light rhythms, are considered to lead to prediction of outbreaks of cryptocaryoniasis, and to development of biological and physical control methods of the disease. It is still difficult to predict the timing of the infections, and delayed detection of the parasite infection can lead to the death of cultured fish. Therefore, development of the effective control method is urgently required against cryptocaryoniasis in the open sea.

Chemical control

Ozone (Wilkie and Gordin, 1969); copper cation treatments (Colorni, 1987; Yin et al., 2019); formalin (Herwig, 1978; Moe, 1982; Rasheed, 1989); acriflavine, malachite green, methylene blue and quinine hydrochloride (Dujin, 1973; Herwig, 1978, 1979; Tookwinas, 1990; Nigrelli and

Ruggieri, 1966; Wilkie and Gordin, 1969) were reported to be effective to kill or inactivate tomonts and theronts in seawater. However, these chemicals can be used only for fish reared in aquaria, but not for food fish reared in net cages in open sea, where the disease has caused economic loss the most.

For culture fish in net cages in open sea, researches on oral medication have been conducted. Oral administration of lysozyme chloride appears to be an effective treatment of *C. irritans* and is approved for use on food fish in Japan ([http:// www.nval.go.jp/asp/asp_dbDR_idx.asp](http://www.nval.go.jp/asp/asp_dbDR_idx.asp)). In addition, antibiotic compounds, phytochemical compounds and medicinal plant extracts which have antiparasitic activity against *C. irritans* have been found; for example, antibiotic compounds including sodium salinomycin and Romet® 30 (a mixture of sulfadimethoxine with an ormetoprim) (Yoshinaga et al., 2011; Kawano et al., 2012) and phytochemical compounds including matrine and oxymatrine, both of which are alkaloids, epigallocatechin gallate (catechin), dihydroartemisinin (antimalarial drug), celangulin (botanical pesticide), L-DOPA, caprylic acid (medium-chain fatty acid) and honokiol (chemical compound obtained from the Japanese cucumber tree *Magnolia obovata* (Hirazawa et al., 2001a, 2001b; Goto et al., 2015b; Picón-Camacho et al., 2011; Zhong et al., 2018). Furthermore, oral administration of crude plant extracts from *Sophora flavescens*, *Areca catechu*, *Dendranthema indicum*, *Cyrtomium rhizoma*, *Scutellaria baicalensis*, and *Celastrus angulatus* have been proven to be effective against cryptocaryoniasis (Goto et al., 2015a; Liu et al., 2015). However, these treatments alone are not enough to control *C. irritans* infection. Moreover, the chemical treatments might result in drug-resistant parasites, and also cause environmental pollution and will be public concerns for food and environmental safety.

Vaccination

Fish immunity against *C. irritans* has recently attracted attention from researchers, because vaccines can reduce the environmental pollution and food safety risks caused by chemical treatments. Several studies demonstrated that fishes once infected with *C. irritans* acquired immunity against *C. irritans*, and that fish that were experimentally challenged with live theronts and recovered from the infection acquired almost complete protection against the parasite infection (Burgess and Matthews, 1995; Yoshinaga and Nakazoe, 1997). Some ciliates, such as *Tetrahymena thermophila*, *Paramecium aurelia* and *Ichthyophthirius multifiliis*, have immobilization antigens (i-antigens), which are present in cell surface membrane (Jones, 1965;

Bruns, 1971). The i-antigens elicit the production of antibodies that immobilize the cilia of ciliates *in vitro*, and are considered as vaccine candidates against ciliate infections. i-antigen in *C. irritans* was identified (Hatanaka et al., 2007), and vaccines, such as recombinant vaccine and DNA vaccines, against i-antigens of *C. irritans* were developed and found to be effective for protection of fish (Mo et al., 2019; Priya et al., 2011). However, there are many intraspecific variations in the i-antigens of ciliates. In previous studies on vaccination with *C. irritans* i-antigen, although i-antigen induced a strong immune response, the induced immunity was serotype-specific and recognized only the homologous serotype of i-antigen (Hatanaka et al. 2007; Misumi et al. 2011). Therefore, it is considered that vaccination using i-antigens is difficult to protect infection of various serotypes of *C. irritans*. However, fish immunized with *C. irritans* of different i-antigen serotypes elicited cross-protection against subsequent challenge by both homologous and heterologous serotypes (Misumi et al. 2011). This result suggested the presence of antigens commonly present in *C. irritans* of different serotypes of *C. irritans*, which may provide cross-serotype protection. Therefore, identification of such antigens may enable development of vaccines against various i-antigen serotypes of *C. irritans*.

Objectives of this study

As described above, the outbreak of cryptocaryoniasis is a serious problem for marine fish aquaculture. In this study, two aspects of those consisting of IPM were focused; one was biological and physiological control and the other was vaccination. In chapter 1, influences of dissolve oxygen concentration on the development of tomonts and those of light periods on the leaving of protomonts from fish and the release of theronts from tomonts were investigated to accumulate basic information on *C. irritans*, which is probably useful for further development of biological and physical control methods, especially for prediction of outbreaks of cryptocaryoniasis. In chapter 2, proteases highly expressed in *C. irritans* were investigated as antigens commonly present in *C. irritans* isolates having different serotypes of i-antigens and the efficacy of vaccinations with some recombinant proteases was examined.

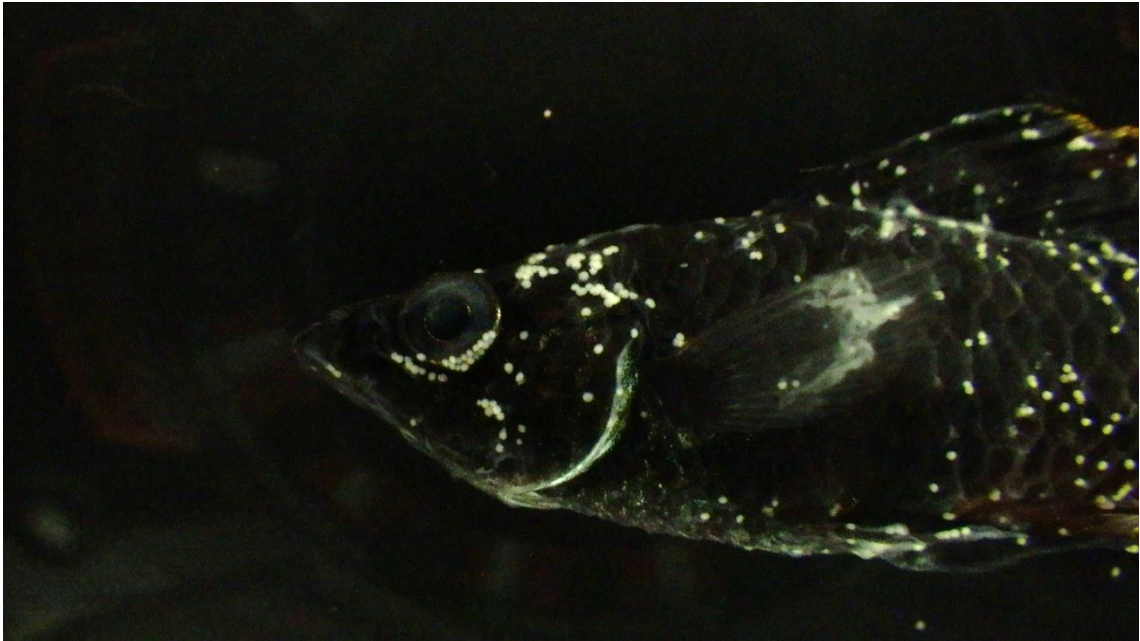


Fig. 1. A black molly (*Poecilia* sp.) infected with *Cryptocaryon irritans*.

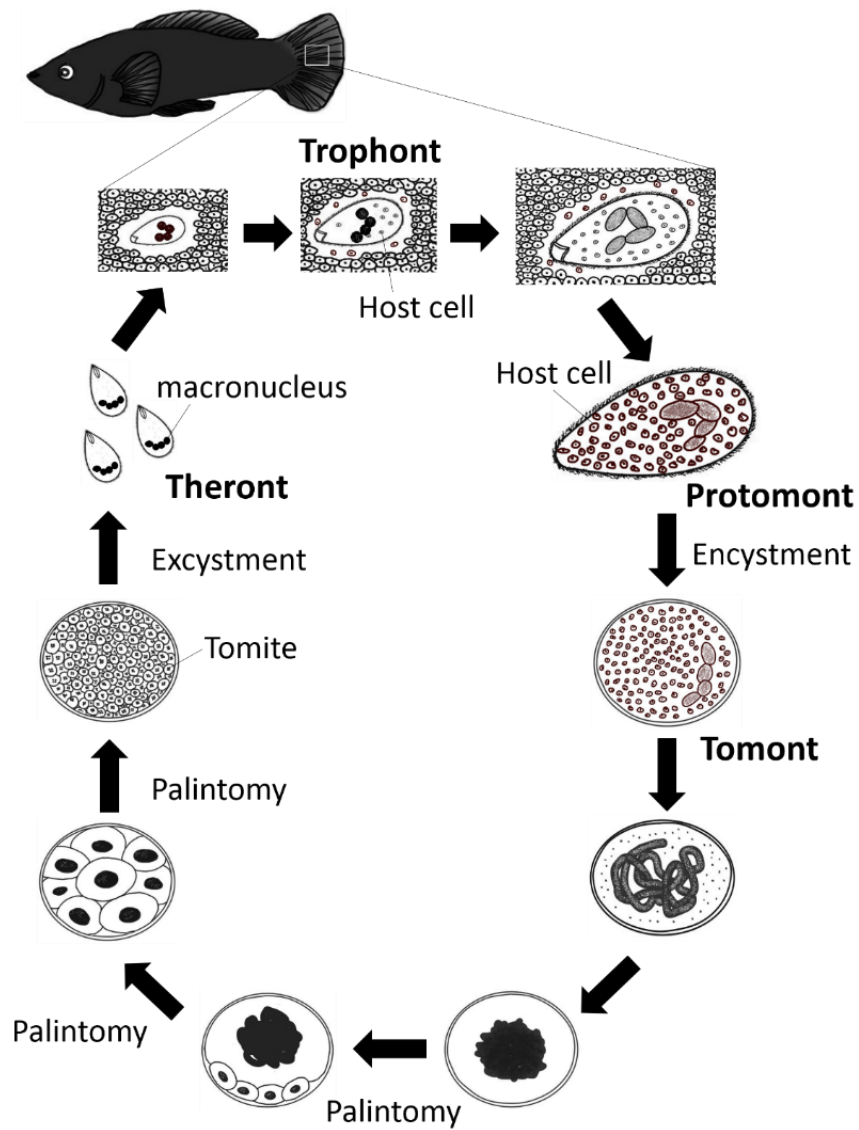


Fig. 2 The life cycle of *Cryptocaryon irritans*.

Chapter 1

Influences of environmental factors on the development and the daily rhythms of *Cryptocaryon irritans*

Section 1.1

Dormancy induced by a hypoxic environment in tomonts of *Cryptocaryon irritans*

Introduction

In Japan, outbreaks of cryptocaryoniasis frequently occur in floating net cages located in inner bays in autumn when water temperatures decrease (Yoshinaga, 2001; Kochi Prefecture, 2005; Katata et al., 2006). Yoshinaga (2001) reported that *C. irritans* tomont development was interrupted in a hypoxic environment, and resumed in an oxic environment. This suggests they become dormant in hypoxic environments. It may be presumed from this that tomonts remain in a dormant phase on the seabed around net-cage fish farms in summer, when the bottom layer of the water column becomes hypoxic because of the formation of a thermocline in inner bays. The frequent outbreaks of cryptocaryoniasis may then be related to the disappearance of the thermocline in autumn, when dissolved oxygen concentration become high on the seabed. Detailed information about the development and dormancy of tomonts in hypoxic environments, and on the dynamics of dissolved oxygen (DO) in seawater around marine net-cage farms, would be useful to predict outbreaks of cryptocaryoniasis. This would also be useful for determining appropriate timing to transfer fish net cages to sites with stronger tidal current; transferring net cages is a method conventionally used and effective to prevent heavy infections and resulting mass mortalities of fish in net-cage farms (Yoshinaga, 2001). However, little is known about the time required for theronts to be released after oxygen is supplied to the seabed, or how long tomonts can remain viable when kept in hypoxic environments. This information is necessary to know appropriate timing to transfer net cages. In addition, little is known about the internal structure of tomonts during the interruption, because observation of internal structure previously required serial sectioning and staining, both of which are labor- and time- consuming.

We conducted this section to obtain detailed information on induction of dormancy and recovery from dormancy of *C. irritans* in a hypoxic environment and an oxic environment, respectively. We focused on the timing of nuclear development using the whole-body staining of the nuclei, which we developed in the present study. In addition, we also examined the release of theronts from tomonts recovered from one-month long dormancy and the infectivity of the released theronts to know the ability of theronts released from dormant tomonts.

Materials and Methods

Parasites

C. irritans was isolated from an ornamental blue surgeonfish (*Paracanthurus hepatus*), purchased in a local pet shop of Japan. We named this isolate as UT1 and used the isolate throughout all studies described in this thesis unless otherwise stated. The parasite was passaged and propagated on seawater-adapted black mollies (*Pocillia* sp.) (mean body length 2–3 cm), according to Yoshinaga and Dickerson (1994) with some modifications as previously described in Watanabe et al., (2016). Ten black mollies were exposed to 10,000 theronts (1,000 theronts/fish) in 1.5 L of filtered seawater in a 2 L plastic aquarium for 6 h in the dark at 25°C. Throughout the experiments described in this thesis, challenge and maintenance of fish were carried out in the dark at 25°C in an incubator unless otherwise stated. The challenged black mollies were transferred to another 2 L plastic aquarium containing 1.5 L of seawater and maintained with gentle aeration. After 3 days of challenge, infected black mollies were placed in a 1.5 L plastic aquarium containing 1 L of filtered seawater. Protomonts leaving the fish were allowed to settle and transform into encysted tomonts in the dark at 25°C. Encysted tomonts attached to the bottom of the aquarium were rinsed with filtered seawater and incubated in 50 mL of filter-sterilized seawater supplemented with antibiotics (500 IU/mL penicillin G potassium and 500 µg/mL streptomycin sulfate). The seawater in the aquarium was replaced with fresh filter-sterilized seawater supplemented with the antibiotics every day. Most tomonts released theronts 5–7 days after collection. Theronts in five replicates of 50 µL aliquots of suspension were placed in wells of 96-well plates, fixed with a drop of 20% formalin, and counted under an inverted microscope. After counting, theronts were used to challenge fish.

This process was repeated to propagate and maintain the *C. irritans* infection. Protomonts were collected soon after leaving their hosts, and before encystment. They were washed five times with filter-sterilized seawater supplemented by antibiotics, using Pasteur pipettes and glass bowls, before being used in the experiments.

Seawater for tomont incubation

Seawater (34–35 ppt salinity) taken from the surface of Kuroshio Current around the Izu Islands was purchased from Tokai Kisen Co. Ltd (Tokyo, Japan) and used for rearing fish and in

all experiments described in this thesis unless otherwise stated. The seawater was filtered with a 1 μm line filter for routine propagation and passaging of *C. irritans* on black mollies, or sterilized with a 0.22 μm membrane filter and supplemented with 500 IU/mL penicillin G potassium and 500 $\mu\text{g/mL}$ streptomycin sulfate for the incubation of tomonts. For incubation experiments, seawater was saturated with air by 30–60 min aeration. The dissolved oxygen (DO) concentration in the air-saturated seawater was 8.7–8.9 mg/L O_2 when determined by an optical oxygen monitor (Piccolo 2, BSA Co Ltd, Tokyo, Japan). The air-saturated seawater was used for the incubation of protomonts and tomonts in an oxic environment throughout this section (Section 1.1), unless otherwise stated.

Timing of tomont development in oxic and hypoxic environments

We compared the development of tomonts in an oxic environment (Fig. 1.1A) and an hypoxic environment (Fig. 1.1B). We prepared eight cell culture dishes (diameter 40 mm) each for oxic incubation and hypoxic incubation of tomonts, adding 2 mL of air-saturated seawater to each dish. Fifty protomonts were placed in each dish and transformed into tomonts. For oxic incubation, the eight dishes were placed in a humid chamber in an incubator (LH-30CCFL-8CT; Nippon Medical & Chemical Instruments Co., Ltd, Osaka, Japan). Seawater in the dishes was exchanged for fresh air-saturated seawater every day, and one dish was sampled every day for 8 days. The hypoxic environment was established using an anaerobic culture system for bacteria (AnaeroPack-kenki 5%; Mitsubishi Gas Chemical, Tokyo, Japan), which consisted of a jar and a bag containing both an oxygen absorber and a carbon dioxide generator. The DO concentration in seawater in the jar was 1.4–1.7 mg/L O_2 , determined by the Piccolo 2 optical oxygen monitor. The seawater was not changed, to prevent oxygen from entering the seawater in the dishes. The jar was opened every day to sample one of the eight dishes. When the jar was opened, the bag containing the oxygen absorber and carbon dioxide generator was exchanged for a new one.

Tomonts sampled from both oxic and hypoxic environments were stained with the acetocarmine stain to observe their nuclei. This staining method was developed in the present study, in which tomonts were covered with the acetocarmine solution (1% acetocarmine solution dissolved in 45% acetic acid solution supplemented with several drops of 4% iron alum solution) in the dishes for 10 min and decolorized with 1% hydrochloric acid in 70% ethanol. The stained tomonts were observed under a stereomicroscope (Olympus SZ61, Olympus, Tokyo, Japan) and

classified to their developmental stages (Fig. 1.2). These were: Stage 1, tomonts containing numerous acetocarmine-positive granules representing denatured nuclei of host cells; Stage 2, tomonts with a coiled macronucleus; Stage 3, tomonts with a massive nucleus; Stage 4, early stage of cell division; Stage 5, later stage of cell division; empty cyst, empty tomonts after theront release; dead cyst, dead tomonts. The same batch of protomonts collected from the same group of infected black mollies was used for oxic and hypoxic incubations. This experiment was repeated three times using different batches of tomonts collected on different occasions.

We also examined the development of tomonts in the oxic environment without exchanging the seawater to learn of any effects from the lack of exchange, as we also did not exchange seawater in the hypoxic incubation. We carried out the same experimental steps as in the oxic environment, but did not exchange seawater at all. This experiment was repeated three times using batches of tomonts collected on different occasions.

Effect of hypoxic environment on tomonts at different developmental stages

We incubated tomonts at various developmental stages in the oxic environment and then transferred them in the hypoxic environment (Fig. 1.1C). We incubated 20 dishes containing 50 tomonts in the oxic environment, exchanging the seawater daily. Five dishes were sampled each day from 1–4 days after incubation, to obtain tomonts at different developmental stages. Tomonts in one of the five dishes were immediately stained with acetocarmine. The remaining four dishes were transferred to the hypoxic environment where they were incubated without seawater exchange. Tomonts in one dish were stained with acetocarmine every day from 1–3 days and 7 days after transfer to the hypoxic environment. As a control, protomonts from the same batch were incubated in the oxic environment with daily exchange of seawater. Eight dishes containing 50 protomonts were prepared and tomonts developed from the protomonts in one of the dishes were stained with acetocarmine every day for 8 days. This experiment was repeated twice using protomonts collected on different occasions.

Recovery of tomonts from dormancy, induced by hypoxic incubation, by oxic incubation

We induced dormancy in tomonts by hypoxic incubation and then incubated them in the oxic environment to observe their recovery from dormancy (Fig. 1.1D, #7). Nine dishes containing 50 tomonts were incubated in the hypoxic environment without seawater exchange. After 7 days, all

of the dishes were transferred into the oxic environment for incubation. Seawater was then exchanged daily with air-saturated seawater. One dish was sampled daily to day 8, and tomonts in the dish were stained with acetocarmine and observed under a stereomicroscope. This experiment was repeated twice using tomonts collected on different days.

We also incubated tomonts in the oxic environment for 1–4 days to obtain specimens at various developmental stages. These were then incubated in the hypoxic environment for 1 week to induce dormancy at various developmental stages. Finally, we incubated the dormant tomonts in the oxic environment to observe their recovery from dormancy (Fig. 1.1D, #8–11). Forty dishes containing 50 tomonts were incubated in the oxic environment. Ten dishes were sampled every day after incubation for 1–4 days. Tomonts in one of the ten dishes were immediately stained with acetocarmine. The remaining nine dishes were transferred and incubated in the hypoxic environment for a further 7 days. Subsequently, one of the nine dishes was sampled and tomonts in the dish were stained with acetocarmine. The other eight dishes were transferred and incubated in the oxic environment with daily seawater exchange. One of the eight dishes incubated in the oxic environment was sampled every day for 8 days and the tomonts in the dish were stained with acetocarmine. This experiment was repeated twice using protomonts collected on different occasions.

Release and infectivity of theronts after tomont dormancy in the hypoxic environment

We examined the release of theronts from dormant tomonts kept in the hypoxic environment after a longer dormancy. We prepared four dishes containing 50 protomonts from the same batch. One dish was incubated in the oxic environment and the remaining three were incubated in the hypoxic environment. After 1 week, 2 weeks and 1 month (31 days), a dish incubated in the hypoxic environment was transferred and incubated in the oxic environment with daily seawater exchange (Fig. 1.1E). Tomonts releasing theronts or having completed theront release were counted under a stereomicroscope every day, when seawater was exchanged, for 8 days. We also counted theronts released from tomonts between 4 and 5 days after transfer to the oxic environment. Theronts released after 4 and 5 days from tomonts incubated in the oxic environment only were counted as a control. This experiment was repeated three times using protomonts collected on different days.

We also examined the effect of dormancy in the hypoxic environment on the infectivity of

theronts released from dormant tomonts, and on the development of tomonts obtained from fish challenged with theronts released from dormant tomonts. To examine infectivity, black mollies were infected with theronts released from tomonts incubated in the hypoxic environment for 1 month and subsequently incubated in the oxic environment. The experimental challenge was carried out according to the protocol of Yoshinaga et al. (2007) with some modification. Three black mollies were exposed to theronts (200 theronts/fish), within 3 h of their excystment from tomonts, in 300 ml of seawater without antibiotics in a 1 L plastic aquarium. After the challenge, the mollies were kept in 1.5 L of seawater in a 2 L plastic aquarium. Two days after the challenge, the fish were kept individually in 1 L of seawater in a 1.5 L plastic aquarium with gentle aeration; seawater was changed daily without antibiotics. Protomonts leaving the fish 3–5 days after the challenge were collected and counted. As a control, black mollies were challenged with theronts released from tomonts incubated in the oxic environment only, and protomonts leaving the fish were counted. Protomonts of the same batch were used for both the experiment and the control.

We collected protomonts from the black mollies challenged with theronts released from tomonts after 1-month dormancy and examined the release of theronts. Three dishes containing 50 protomonts from the challenged black mollies were incubated in the oxic environment. Theronts were counted on day 5, when their release peaked. As a control, three dishes containing 50 tomonts from the fish challenged with theronts from tomonts incubated in the oxic environment only (the previous control experiment) were incubated in the oxic environment. Theronts released 4 and 5 days after the beginning of incubation were counted. Protomonts of the same batch were used for both the experiment and the control.

Statistical analysis

Statistical analysis was performed on the long dormancy experiments. The percentages of tomonts kept in the hypoxic environment for different periods and released theronts were analyzed using one-factor repeated measure ANOVA after arcsine square root transformation. The numbers of theronts released and obtained from Petri dishes containing 50 tomonts were analyzed using one-factor repeated measure ANOVA.

The percentages of protomonts obtained from fish challenged with theronts released from tomonts after 1-month dormancy and control tomonts were compared using an F-test and Student's t-test after arcsine square root transformation. The numbers of theronts released from

tomonts obtained from the challenged fish were compared using an F-test and Student's t-test. Values $P < 0.05$ were regarded as statistically significant.

Results

Timing of tomont development in oxic and hypoxic environments

The timing of tomont development in the oxic environment with daily seawater exchange is shown in Figure 1.3 (upper chart). The mean percentage of each stage from three repetitions, carried out on different occasions, is shown in the figure. On the first day after encystment, the elongation of macronuclei (Stage 1) commenced. On day 2, many tomonts had a coiled macronucleus. Tomonts at Stage 3 showed a massive macronucleus, and Stage 4 (days 3 and 4) showed several cells in the early stage of cell division, but their percentages were relatively lower than other stages. Most tomonts entered Stage 5 on day 4, showing numerous tomites with a lobed macronucleus, representing the late stage of cell division, and empty cysts representing completed release of theronts began to appear. The percentage of empty cysts increased considerably on days 5 and 6, reaching almost 50%, but increased little thereafter. Even on day 8, 40% of tomonts were still at Stage 5, in which many tomites were left in the cysts and unreleased. When tomonts were kept in the oxic environment, but without seawater exchange, they showed a similar development to those incubated with seawater exchange (Fig. 1.3, lower chart). The percentage of empty cysts was higher in the oxic environment without seawater exchange. However, a precise comparison was not achieved, as the batches of protomonts used in this experiment differed from those used in the oxic environment with seawater exchange.

Tomonts incubated in the hypoxic environment showed little development (Fig. 1.4). Most stayed at Stage 1, displaying numerous acetocarmine granules, which represented the denatured nuclei of host cells.

Effect of the hypoxic environment on tomonts at different developmental stages

When tomonts at different stages generated by oxic incubation for 1–3 days were transferred and incubated in the hypoxic environment, development soon stopped. Development did proceed slightly, even under hypoxic incubation, but depended on the initial period of oxic incubation (Fig. 1.5). Tomonts incubated in the oxic environment for 1 day developed to some extent, and there was an increase in the percentage reaching Stage 2. However, the development thereafter changed

little even after hypoxic incubation for 7 days (Fig. 1.5A). The percentages in different developmental stages changed little in tomonts incubated in the oxic environment for 2 days (Fig. 1.5B). Although tomonts incubated in the oxic environment for 3 days proceeded to develop and some released theronts in the hypoxic environment, almost 70% of them did not release theronts (Fig. 1.5C). After hypoxic incubation for 4 days, tomonts began to release theronts after 1 day in the hypoxic environment. Around 70% released theronts (Fig. 1.5D); the final percentage of tomonts that released theronts did not differ much from tomonts incubated only in the oxic environment for 7 days (Fig. 1.5E).

Recovery of tomonts from dormancy induced by hypoxic incubation through oxic incubation

The vast majority of tomonts incubated in the hypoxic environment just after collection from fish (99%) were still at Stage 1 after incubation for 1 week, indicating that they developed little in the hypoxic environment. When the tomonts were transferred and incubated in the oxic environment, development resumed and as much as 80% released theronts in 8 days. Release of theronts peaked after 5 days of oxic incubation (Fig. 1.6A), similar to the tomonts incubated in the oxic environment (Fig. 1.3, upper and lower charts).

Tomonts at various developmental stages incubated in the oxic environment for 1–4 days and then incubated in the hypoxic environment for 1 week developed a little. Development was considerable after transfer to the oxic environment and theronts were released; as much as 80% in any group finally released theronts (Fig. 1.6 B–D). Tomonts incubated in the oxic environment for 0, 1 and 2 days showed peak theront release at 5, 4 and 3 days after transfer to the oxic environment, respectively. The total oxic incubation period (before and after hypoxic incubation) needed for tomonts to reach peak theront release was 5 days, except for tomonts incubated in the oxic environment for 3 and 4 days before the incubation in the hypoxic environment. Roughly 70% of those tomonts incubated in the oxic environment for 3 and 4 days had released theronts when hypoxic incubation ceased.

Long-term viability of tomonts in the hypoxic environment and infectivity of theronts released from dormant tomonts

All tomont groups with different hypoxic incubation periods released theronts, with most

released 5 days after tomonts were transferred into the oxic environment. The cumulative percentage of tomonts that had released theronts was greater than 70% over the 8-day oxic incubation period without significant differences among the groups (one-factor repeated measure ANOVA after arcsine transformation, $P>0.05$). The number of theronts released from 50 tomonts between 4 and 5 days after transfer into the oxic environment was: 6210 ± 550 (mean \pm standard deviation) at 0 days (control) incubated in the hypoxic environment before oxic incubation, 6506 ± 292 at 1 week, 7221 ± 312 at 2 weeks and 6016 ± 140 at 1 month. No significant differences were observed in the number of theronts among the groups (one-factor repeated measure ANOVA analysis, $P>0.05$).

The infectivity of theronts obtained from tomonts recovered after 1-month dormancy, represented by the percentage of protomonts recovered from challenged fish from the theronts used for challenge, was equivalent to that of the control. No significant difference was found based on the F-test and Student's t-test after arcsine transformation ($P>0.05$). Moreover, 6864 ± 630 theronts were obtained from tomonts recovered from the infected fish, and both the F-test and Student's t-test demonstrated no significant difference ($P>0.05$) compared with the control (7256 ± 401).

Section 1.2

Influences of photoperiod on the daily rhythms of *Cryptocaryon irritans*

Introduction

The parasite has clear daily rhythms in its life cycle. Detachment of trophonts from hosts and excystment of theronts from tomons occur from late at night to early in the morning (Yoshinaga and Dickerson, 1994; Burgess and Matthews, 1994; How et al., 2015). The time of day when protomonts detach from hosts and when theronts excyst from tomons appeared not to change, even when light and dark periods were temporarily reversed during the parasitic and cystic periods (Yoshinaga and Dickerson, 1994; Burgess and Matthews, 1994). Furthermore, theront excystment from tomons consistently occurred at the same time of day, even when protomonts collected on different days were cultured in the dark (Yoshinaga and Dickerson, 1994). Thus, the daily rhythms of this parasite appeared not to be the consequence of direct or immediate responses to light. This phenomenon implies that the parasite has an intrinsic daily rhythm (Yoshinaga and Dickerson, 1994). Burgess and Matthews (1994) suggested that the daily rhythms of protomont detachment and theront excystment would be influenced by photoperiods. However, how and when photoperiods influence these rhythms yet require clarification.

C. irritans is typically propagated by placing (or passaging) the parasite on live fish (Colorni, 1985; Burgess and Matthews, 1994; Yoshinaga and Dickerson, 1994; Dan et al., 2006), because *in vitro* culture methods for their propagation are not available. Yet, the trophonts of this parasite can be grown *in vitro*, to a certain extent (Yoshinaga et al., 2007). The serial passaging of the parasite on fish is laborious, especially because protomonts and theronts must be collected early in the morning. Furthermore, this process must be repeated at almost one-week intervals, without interruption, to propagate the parasite. This work hinders the progress of studies on *C. irritans*. By clarifying its photoperiods, it might be possible to manipulate its daily rhythms and, consequently, decrease the amount of labor needed for its propagation in the laboratory.

In this study, we investigated how photoperiods influence the daily rhythms of this parasite by exposing challenged fish and protomonts to different photoperiods.

Materials and Methods

Parasite propagation

Theronts, tomonts, and infected fish were obtained from routine propagation, following the methods of Section 1.1.

Effect of different photoperiods on the timing of detachment of trophonts from the host

Theronts were obtained from tomonts incubated for five days in plastic aquaria under a 12 h L/12 h D photoperiod (06:00–18:00 light, 18:00–06:00 dark), and were collected at 06:00 for use. Twenty black mollies were exposed to 2,000 theronts (100 theronts/fish) in filtered seawater in a 2 L plastic aquarium in the dark at 25 °C for 2 h (06:00–08:00). The challenged black mollies were separated into two experimental groups. Ten fish were transferred to each of two plastic aquaria containing 2 L filtered seawater and maintained with gentle aeration. The two aquaria were exposed to two different 12 h L/12 h D photoperiods (06:00–18:00 L and 18:00–06:00 D; and 15:00–03:00 L and 03:00–15:00 D), respectively, in incubators chambers (LH-30CCFL-8CT; Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) at 25 °C. The light intensity at the vertical level of the water surface in the incubator chamber and at the bottom of the incubator chamber was 6,450 lux and 2000 lux, respectively.

Every 3 h, from 58 h after the challenge (from 18:00 after 2 days), the 10 fish were transferred to a plastic net cage (mesh opening 6.4 × 6.4 mm) in another 2 L plastic aquarium containing 1.5 L fresh filtered seawater. The plastic net cage was used to separate the fish from the bottom of the aquarium, so they did not peck and eat the protomonts and tomonts. Protomonts that detached from the fish and sank to the bottom of the aquarium during each 3 h period were counted under a stereomicroscope, until 94 h after initiating the challenge (24:00 after three days). Fish were transferred between the two aquaria under light conditions (2000 lux) during the light period and under dark conditions (0.2–2 lux) during the dark period. This experiment was repeated three times using different batches of parasites at different times.

A similar experiment was carried out by challenging fish between 12:00–14:00. Theronts were obtained at 12:00 from tomonts incubated under a different photoperiod (15:00–3:00 L and 03:00–15:00 D), and were used for the challenge. This experiment was repeated three times using different batches of the parasite at different times.

Effect of different photoperiods on the timing of excystment of theronts

Ten infected black mollies were placed in a 2 L plastic aquarium containing 1.5 L fresh filtered seawater that was maintained with gentle aeration in an incubator at 25 °C under a photoperiod of 06:00–18:00 L and 18:00–06:00 D. In the morning, 3 days after the challenge, protomonts that had detached from the host at 03:00–06:00 were collected and washed five times with filter-sterilized seawater supplemented with antibiotics, using Pasteur pipettes and glass bowls. The protomonts were then incubated in 5 mL filter-sterilized seawater with antibiotics in 25 cm² cell culture flasks (Violamo, Osaka, Japan). Two flasks were prepared for the two photoperiod conditions (06:00–18:00 L and 18:00–06:00 D; 15:00–03:00 L and 03:00–15:00 D). One hundred protomonts were placed in each flask. From 18:00, 4 days after protomonts were collected (most theronts had not emerged before this time), the seawater in each flask was collected and replaced every 3 h for 30 h, and theronts in the seawater was counted. Seawater was replaced in the light during the light period and in the dark during the dark period. This experiment was repeated three times using different batches of the parasite at different times.

A similar experiment was carried out using protomonts collected at different times (12:00–15:00). Protomonts were obtained from infected black mollies that were maintained under a photoperiod of 15:00–03:00 L and 03:00–15:00 D. Protomonts mostly detached from fish from 12:00 to 15:00. After the protomonts were collected, the same process was followed as in the experiment when protomonts were collected from 03:00–06:00. This experiment was repeated three times using different batches of the parasite at different times.

Results

Effect of photoperiod on the detachment of protomonts from the host

Regardless of the time of day when fish were challenged or the photoperiod to which fish were exposed, protomonts mostly detached from fish in the last 6 h of the dark period, peaking in the last 3 h (Fig. 1.8). When fish were exposed to a photoperiod of 06:00–18:00 L and 18:00–06:00 D, protomonts mostly detached from 24:00 to 06:00, peaking at 03:00–06:00, regardless of the time of day of the challenge (Fig. 1.8 A-a and 1.8 B-a). When fish were exposed to a photoperiod of 15:00–03:00 L and 03:00–15:00 D, protomonts mostly detached from 09:00 to 15:00, peaking at 12:00–15:00, regardless of the time of day of the challenge (Fig. 1.8 A-b and 1.8 B-b).

Effect of photoperiod on theront excystment from tomonts

Regardless of the time of day when protomonts detached from fish and the photoperiod, theronts mostly excysted from tomonts during the last 9 h of the dark period, peaking between 6 h and 3 h before the end of the dark period (Fig. 1.9). When tomonts were incubated under a photoperiod of 06:00–18:00 L and 18:00–06:00 D, theronts mostly excysted from 21:00 to 06:00, peaking at 24:00–03:00. The two tomont groups detached from fish from 03:00 to 06:00 and 12:00 to 15:00 (Fig. 1.9 A-a and 1.9 B-a). When tomonts were incubated under a photoperiod of 15:00–03:00 L and 03:00–15:00 D, theronts mostly excysted at 6:00–15:00, peaking from 09:00 to 12:00 (Fig. 1.9 A-b and 1.9 B-b).

Section 1.3

Discussion

It is well known that tomont development is influenced by various factors, including water temperature (Wilkie and Gordin, 1969; Yoshinaga, 2001), DO (Yoshinaga, 2001) and salinity (Cheung et al., 1979; Coloni, 1985; Yoshinaga, 2001). To predict and prevent outbreaks of cryptocaryoniasis in marine net cages and land-based tanks, it is crucial to clarify how environmental factors affect the development of tomonts.

In the study by Yoshinaga (2001), tomont development was suppressed and no theront excystment was observed in a hypoxic environment (25% saturation DO, roughly 2 mg/L O₂), and development resumed when tomonts were transferred to an oxic environment. Similarly, Standing et al. (2017) also reported that no theront release was observed from tomonts in a hypoxic environment, and theront release was observed after the tomonts were transferred to an oxic environment. Although our results are consistent with these two other studies, our study is the first to demonstrate the ability of tomonts to become dormant in a hypoxic environment and to resume development in an oxic environment at any developmental stages. In addition, dormant tomonts incubated in the hypoxic environment for 1 month, as well as those incubated for 1 and 2 weeks, released theronts when transferred to the oxic environment, similar to those incubated in the oxic environment only. Theronts from tomonts that had experienced dormancy for 1 month infected fish. Therefore, it is clear that dormant tomonts can retain their viability and theront infectivity for at least 1 month.

Several dinoflagellates are also known to form dormant (resting) cysts. The cysts are formed when the surrounding environment becomes unfavorable, such as when nitrogen and phosphorus are depleted. The dormant cysts then germinate when the environment becomes favorable for the growth of vegetative cells (Pfiester and Anderson, 1987). Some factors that control germination of the dormant cysts are water temperature, light irradiation and DO. It is thus possible that the dormancy of tomonts of *C. irritans* may be a reaction to an unfavorable environment.

Hypoxic environments are often formed in bottom waters where vertical water exchange is poor. Fish in general avoid hypoxic environments and, therefore, tomonts that settle on hypoxic substrates must have limited opportunity to encounter and invade fish hosts. The nature of tomonts revealed in this study (their dormancy in hypoxic environments, and recovery from dormancy in

an oxic environment, and retained infectivity after dormancy) allow *C. irritans* to encounter and invade host, which must be advantageous for *C. irritans* in an evolutionary context. The DO concentrations may affect the survival of theronts as well as tomont development, but this subject requires further analysis.

Cryptocaryoniasis frequently occurs in red seabream and greater amberjack cultured in net cages in autumn and after typhoons (Yoshinaga, 2001; Kochi Prefecture, 2005; Katata et al., 2006). Yoshinaga (2001) suggested that the dormancy of tomont development in hypoxic environments and induction of theront release in oxic environments are key factors for disease occurrence. This is because oxic water flows into the sea bed in fish farms after the thermocline breakdown caused by cooling surface water (in autumn) or turbulence in the water column (by typhoons).

Some anecdotes and monitoring of cryptocaryoniasis in fish farms suggest a temporal relationship between the outbreaks and typhoons, although there are few documented reports. An outbreak started in red seabream cultured in Nomi Bay, Kochi Prefecture on 30 September 1997, 2 weeks after a typhoon hit the area on 16 September. Of the red seabream cultured in the bay, 30% died of the disease during the following month. Another outbreak occurred in greater amberjack in the same bay on 4 November 2004, 2 weeks after a typhoon on 20 October. In this incident, ~50% of cultured fish died of the disease during the following week (Kochi Prefecture, 2005). Monitoring of cryptocaryoniasis and gill fluke infection in red seabream in Nomi Bay in 2009 and 2011 showed that the prevalence and intensity of *C. irritans* increased for several days and up to 1 week after a typhoon passed close to the area (Watanabe et al., 2011; Kadohara, 2013). These reports and monitoring results suggest that cryptocaryoniasis often occurs within 2 weeks of a typhoon's passage. *C. irritans* needs almost 7–10 days to complete its life cycle at 25°C: 3 days as a trophont and 5–7 days as a tomont. The parasite cannot complete its life cycle twice in 2 weeks. Two-weeks also seems too short for the parasite to gradually propagate from unrecognizable low infection intensities to high intensities causing mass mortality of fish. It is reasonable to think that a certain number of tomonts had accumulated on the seabed before the outbreaks. Given that tomonts accumulate on the seabed during summer, their nature, that dormant tomonts at any developmental stage can resume their development when exposed to an oxic environment, may be a good explanation for sudden outbreaks of the disease in net-cage farms. However, it is still unclear whether tomonts can retain viability, even on the seabed under net cages, where bacteria and protozoa flourish on organic substances such as fish feces and

residual feed, generated by fish aquaculture. Further studies, such as an examination of tomont viability in microbe-rich environments and monitoring of their densities on the seabed, are still needed to learn more about the accumulation of tomonts on the seabed in cage-culture farms and the involvement of accumulated tomonts in outbreaks of cryptocaryoniasis.

Recently, How et al. (2015) demonstrated that theronts of *C. irritans* swam little and had a tendency to stay close to the bottom of the water column. The frequent occurrences of cryptocaryoniasis in fish in suspended net cages in autumn and after typhoons is likely associated with the low swimming ability of theronts as well as the resumption of tomont development in oxic environments. The breakdown of the thermocline in autumn and water turbulence during typhoons probably suspend theronts in the water column up to the height of the net cages in the surface water. Stress caused by upwelling of hypoxic water after the thermocline breakdown and turbulence during typhoons is also likely involved.

The study in section 1.2 demonstrated that protomonts detached from host fish and that theronts excysted from tomonts during the dark periods. In previous studies, the biological rhythm of the parasite remained unchanged, even when light and dark periods were temporarily reversed during the parasitic and cystic periods (Yoshinaga and Dickerson, 1994; Burgess and Matthews, 1994). Detachment and excystment were clearly not the consequence of direct and immediate responses of the parasite to light. Endogenous rhythms mediated by the photoperiod might be involved in the biological rhythm. However, it has not been clarified how and when light determined endogenous rhythms and the time required for rhythms to form.

The present study demonstrated that the time of day that protomonts detach and theronts excyst changes when infected fish and tomonts are maintained under different photoperiod conditions. The rhythm of protomont detachment from hosts and excystment of theronts was formed and regulated by the photoperiod during the parasitic (trophont) and cystic (tomont) stages of the parasite, respectively. It was previously assumed that several generations may be needed for the parasite to form daily rhythms in response to photoperiods; however, in fact, 3 and 5 days were enough for the trophonts and tomonts, respectively, to respond to the photoperiods.

Some monogenean parasites of fish have daily rhythms that are mediated by photoperiods in their behavior, and that help them increase the chance of infection (Macdonald, 1975; Kearn, 1980). *C. irritans* is thought to have evolved in coral reefs, because most previous records of the parasite in wild fish populations were associated with coral reefs (Laird, 1956; Burgess, 1987;

Burgess and Matthews, 1994). Most fish species associated with coral reefs are diurnal, hiding at night in caves (Starck and Davis, 1966; Lowe-McConnell, 1987). Such host behavior would favor the parasite by providing a static target for host contact, in consideration of the findings by How et al. (2015) that theronts of *C. irritans* had very low swimming ability and tended to stay close to the bottom of the water column. It appears also important for the parasite to change its rhythms according to seasonally changing daily photoperiods to increase its chances of infecting fish and surviving.

It is known that outbreaks of cryptocaryoniasis do not occur every year, and it is expected that the occurrence of the outbreaks is affected by environmental factors other than dissolved oxygen concentration too. In the present study, the timings of protomont detachment from host fish and theronts release from tomonts changed depending on photoperiods. When the timing of rising tide overlaps the timings of the protomonts detachment and theront release, tomonts transformed from protomonts and theronts from tomonts appear to stay close to coastal areas where fish net cages are located, promoting the infection of fish with the parasite. Therefore, the photoperiod and tidal cycle also may be factors of the outbreaks other than seasonal disappearance of thermoclines.

Findings in previous and present studies being considered, water temperature, water depth, tidal cycle, thermocline and dissolved oxygen on seabeds are suggested as the major factors causing the outbreaks of cryptocaryoniasis. Intensive monitoring of the environmental factors will help to predict the occurrence of cryptocaryoniasis as well as to select appropriate sites for cage aquaculture. In addition, since the diurnal rhythms of the parasite, which had been considered to be intrinsic, were controlled by the photoperiod, the damage of cryptocaryoniasis may be reduced by controlling the photoperiods of aquaculture environments and aquaria, by artificial lighting of net cages and fish tanks

In addition, recently, antibiotic compounds, phytochemical compounds and medicinal plant extracts which have antiparasitic activity against *C. irritans* have been found; for example, antibiotic compounds including sodium salinomycin and Romet® 30 (Yoshinaga et al., 2011; Kawano et al., 2012); phytochemical compounds including matrine, oxymatrine, epigallocatechin gallate, dihydroartemisinin, celangulin, L-DOPA, and caprylic acid and honokiol (Hirazawa et al., 2001a, 2001b; Goto et al., 2015b; Picón-Camacho et al., 2011; Zhong et al., 2018); medical plant extracts of *Sophora flavescens*, *Areca catechu*, *Dendranthema indicum*, *Cyrtomium rhizoma*,

Scutellaria baicalensis, and *Celastrus angulatus* (Goto et al., 2015a; Liu et al., 2015). However, the importance of the administration timing was not mentioned. As it is expected that the effect of antiparasitic agents will gradually decrease in the seawater over time, optimizing the timing of administration will be helpful to increase the effect of antiparasitic agents. Results of this study also can be applied in the treatments of cryptocaryoniasis when preventive medication is approved in the future; for example, the medications might be more effective if applied to the seawater at the time of detachment from host or theront excystment.

The present study demonstrated that the daily rhythm of protomont detachment from host fish and theronts excystment from tomonts was changed by photoperiods. However, the mechanisms driving these processes require clarification. Ciliates including *C. irritans* do not have organelles for photoreception, to the best of our knowledge, but the presence of photoreceptive pigments was reported in some ciliates (Pill-Soon and Walker, 1981). Photoreceptive pigments might also contribute to the formation of the biological rhythms of *C. irritans*; however, such pigments have not yet been detected in the parasite. Another factor possibly affecting daily rhythms, especially for theront excystment, is the generation of peroxide in seawater by light. The generation of peroxide in seawater caused by light might cause the oxidation of fatty acids and the subsequent production of prostaglandins inside the embryo, which could induce the resting eggs of the rotifer, *Brachionus plicatilis*, to hatch (Hagiwara et al., 1995). Certain substances and processes might affect the physiological cascade *C. irritans* tomonts, potentially altering biological rhythms. Examples include hydroxy radicals generated in seawater as a result of irradiation with light and increases in water temperature due to with light. Host-mediated triggers could also influence the rhythm of protomont detachment, because light affects the physiology of fishes (Schwassmann, 1971). Thus, further researches are needed to clarify the mechanisms influencing how the photoperiod mediates the daily rhythms of *C. irritans*.

Furthermore, propagation of *C. irritans* has been conducted by passing the parasite on live fish (Coloni, 1985; Burgess and Matthews, 1994; Yoshinaga and Dickerson, 1994; Dan et al., 2006), because no *in vitro* culture method for their propagation is available, although trophonts of the parasite can be grown *in vitro* to an extent (Yoshinaga et al., 2007). Serial passaging on fish requires a great deal of labor and considerable time, and the process cannot be interrupted, even when parasites are not needed for experiments (Dan et al., 2009). Previously the propagation of *C. irritans* for most experiments using this parasite was carried out from late night to early in the

morning because important events (protomont detachment and theront excystment) occur during this period (Burgess and Matthews, 1994; Yoshinaga and Dickerson, 1994; How et al., 2015). Consequently, long and unconventional hours were required to work on this parasite. However, the results of the study showed that protomont detachment from the host and theront excystment could be controlled by altering the photoperiod to which infected fish and tomonts are exposed. Consequently, it is possible to obtain protomonts and theronts of *C. irritans* at any time of the day. Actually, we are currently obtaining protomonts and theronts at any time of day in our laboratory by controlling the photoperiod according the finding in this study. In addition, the viability of *C. irritans* is known to be gradually weakened as laboratory propagation cycles continue as staged. Burgess and Matthews (1994) and we have experienced similar phenomenon in our laboratory. This hinders the progress of studies on *C. irritans*. If the parasite could be preserved for a long period, it would considerably help studies on the parasite. According to Dan et al. (2009), tomonts were able to be preserved at low temperatures (12°C) for 4 months, but the percentage of successful theront release dropped after the preservation. In the present study, tomonts were preserved in a hypoxic environment using anaerobic chambers for at least 1 month without losing their viability or the infectivity of released theronts. Further study to determine how long dormant tomonts maintain viability in a hypoxic environment would be valuable from the view point of preservation of this parasite. Combination of preservations in hypoxic environments and in low temperature may considerably long-term preservation of tomonts possible, with maintaining their viability and the infectivity of theronts released from them. These knowledges will reduce labor time, facilitating progress in research on *C. irritans*.

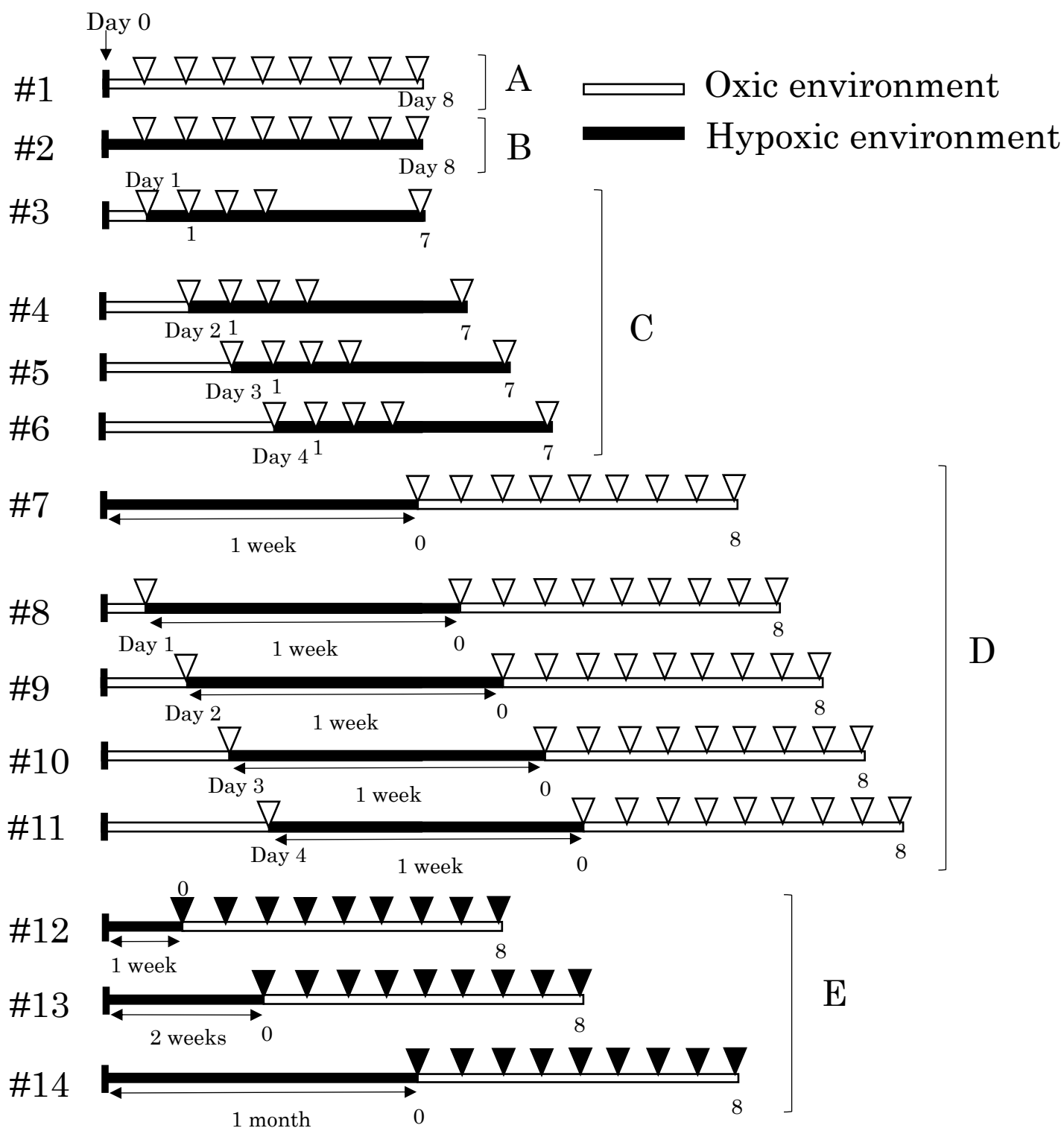


Fig.1.1 Timetable of tomont incubation and sampling. Open lines indicate incubation in the oxic environment, and solid black lines indicate incubation in the hypoxic environment. Open triangles indicate when sampling and staining with acetocarmine occurred. Solid black triangles indicate the observation days for theront release.

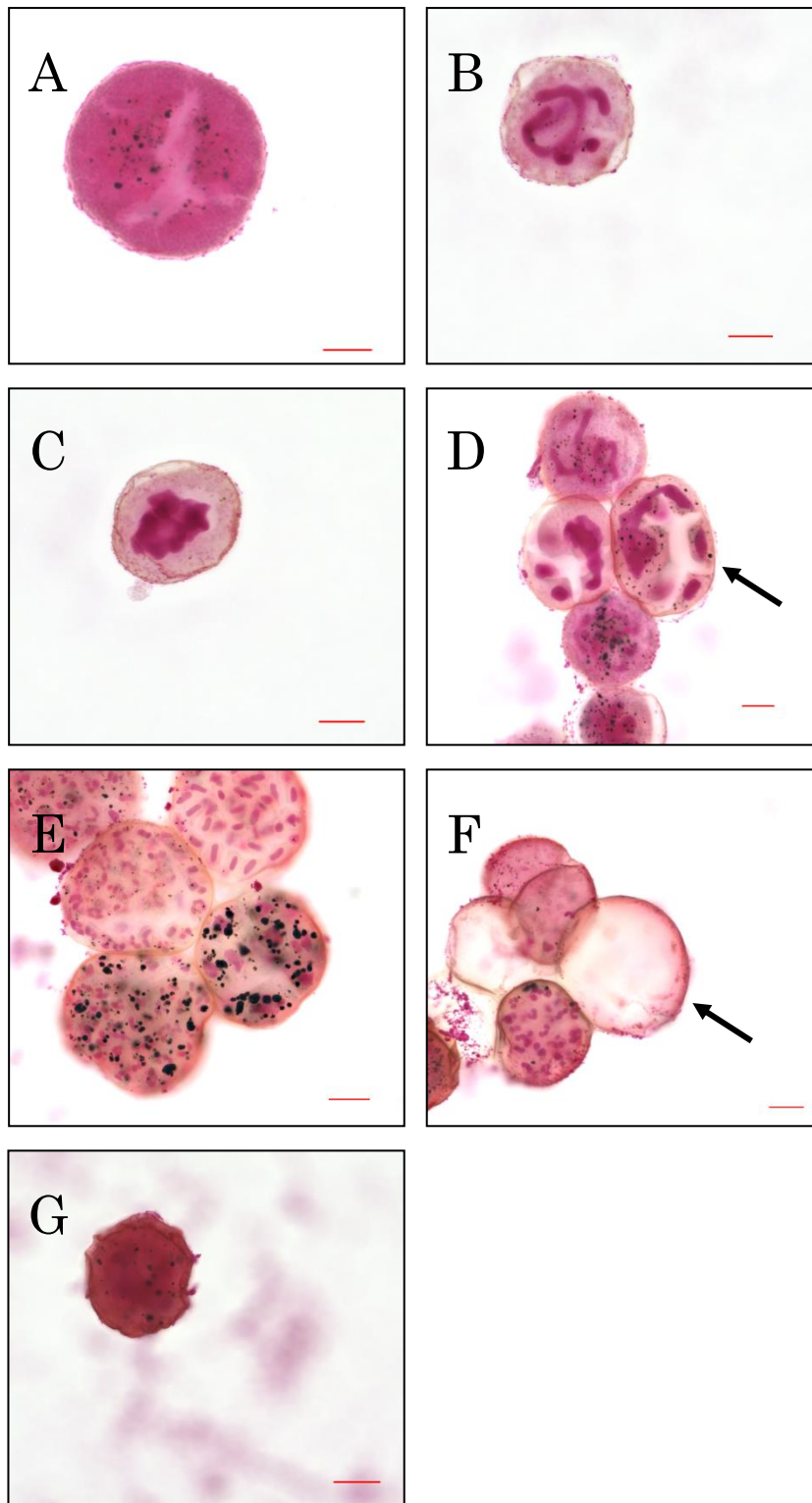


Fig.1.2 Tomonts stained with acetocarmine. A: tomont containing numerous acetocarmine-positive granules (Stage 1); B: tomont with a coiled macronucleus (Stage 2); C: tomont with a massive nucleus (Stage 3); D: early stage of cell division (arrow) (Stage 4); E: later stage of cell division (Stage 5) with tomites containing the lobed macronucleus; F: empty cyst after theront release (arrow) (Empty); G: dead cyst stained evenly and losing internal structures (Dead). Scale bars = 50 μ m.

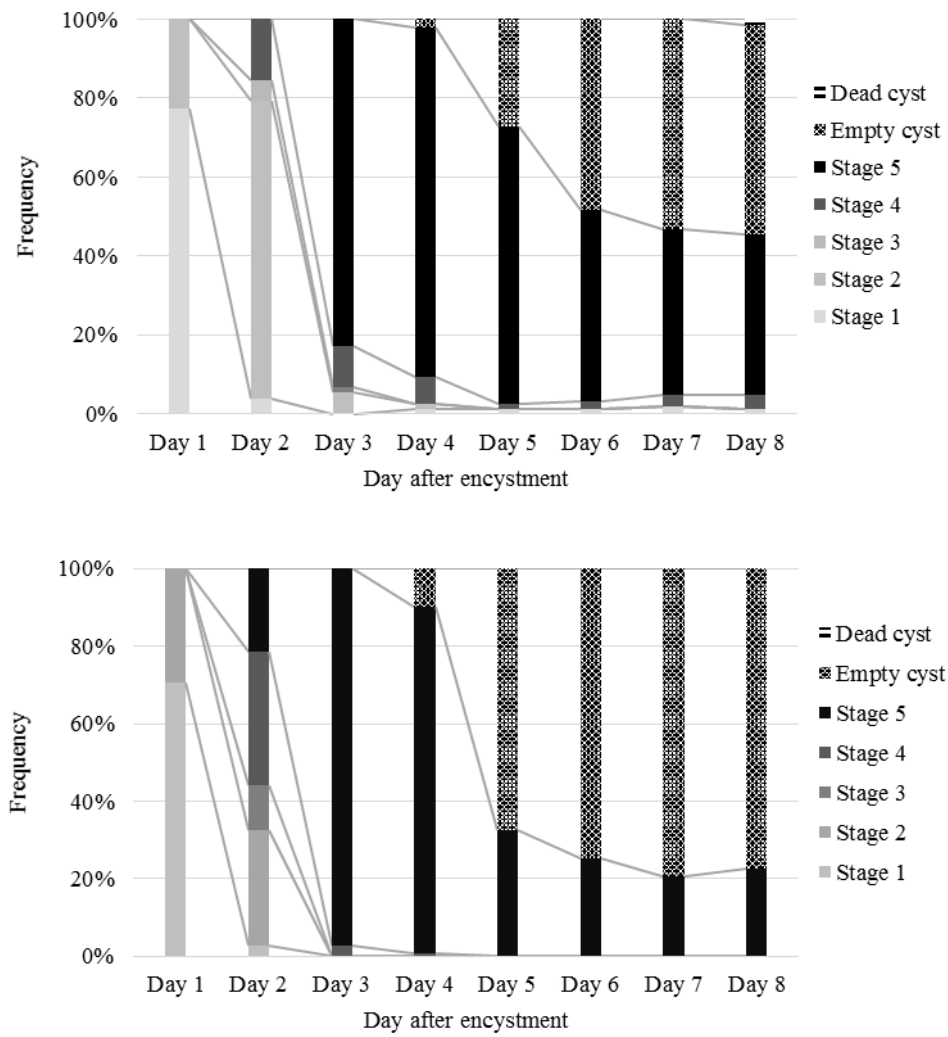


Fig. 1.3 Development of tomonts incubated in the oxic environment with water exchange (upper chart; Fig. 1A) and no water exchange (lower chart). See caption in Fig. 2 for stage definition. This experiment was repeated three times using different batches. Each column represents the mean of the repetitions.

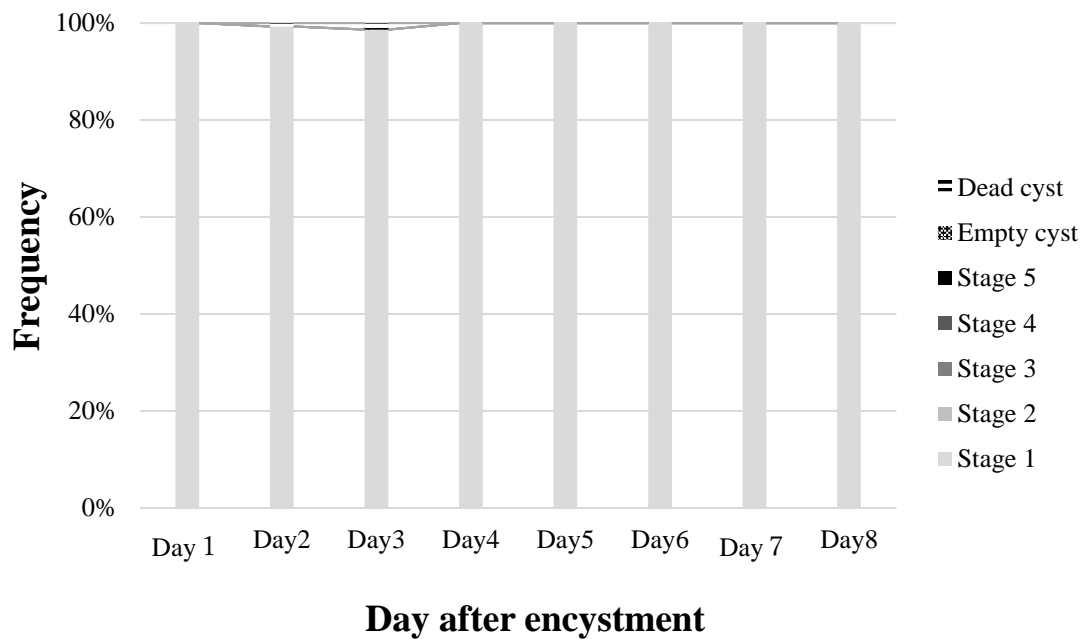


Fig. 1.4 Development of tomonts in the hypoxic environment (corresponding to Fig. 1B). This experiment was repeated three times using the tomonts from the experiment of tomonts incubated in the oxic environment with water exchange (Fig. 3, upper chart). Each column represents the mean of the repetitions.

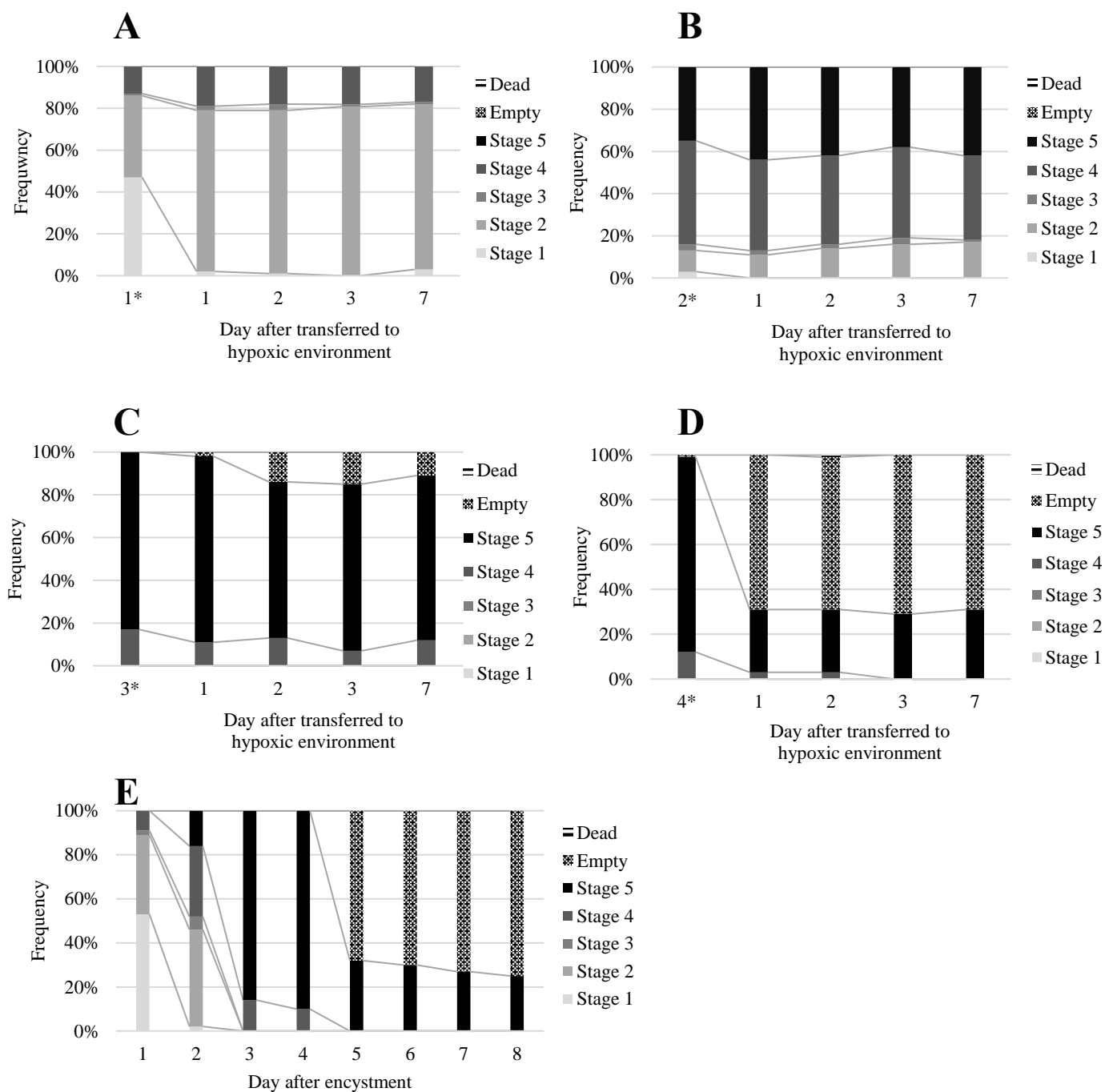


Fig. 1.5 Development of tomonts at different stages in the hypoxic environment. Asterisks indicate the days in the oxic environment (before transfer to the hypoxic environment). Tomonts transferred to the hypoxic environment after: A, 1-day oxic incubation (Fig. 1C, #3); B, 2-day oxic incubation (Fig. 1C, #4); C, 3-day oxic incubation (Fig. 1C, #5); D, 4-day oxic incubation (Fig. 1C, #6). E: tomonts incubated constantly in the oxic environment (control). This experiment was repeated twice using protomonts collected on different occasions. Each column represents the mean of the repetitions.

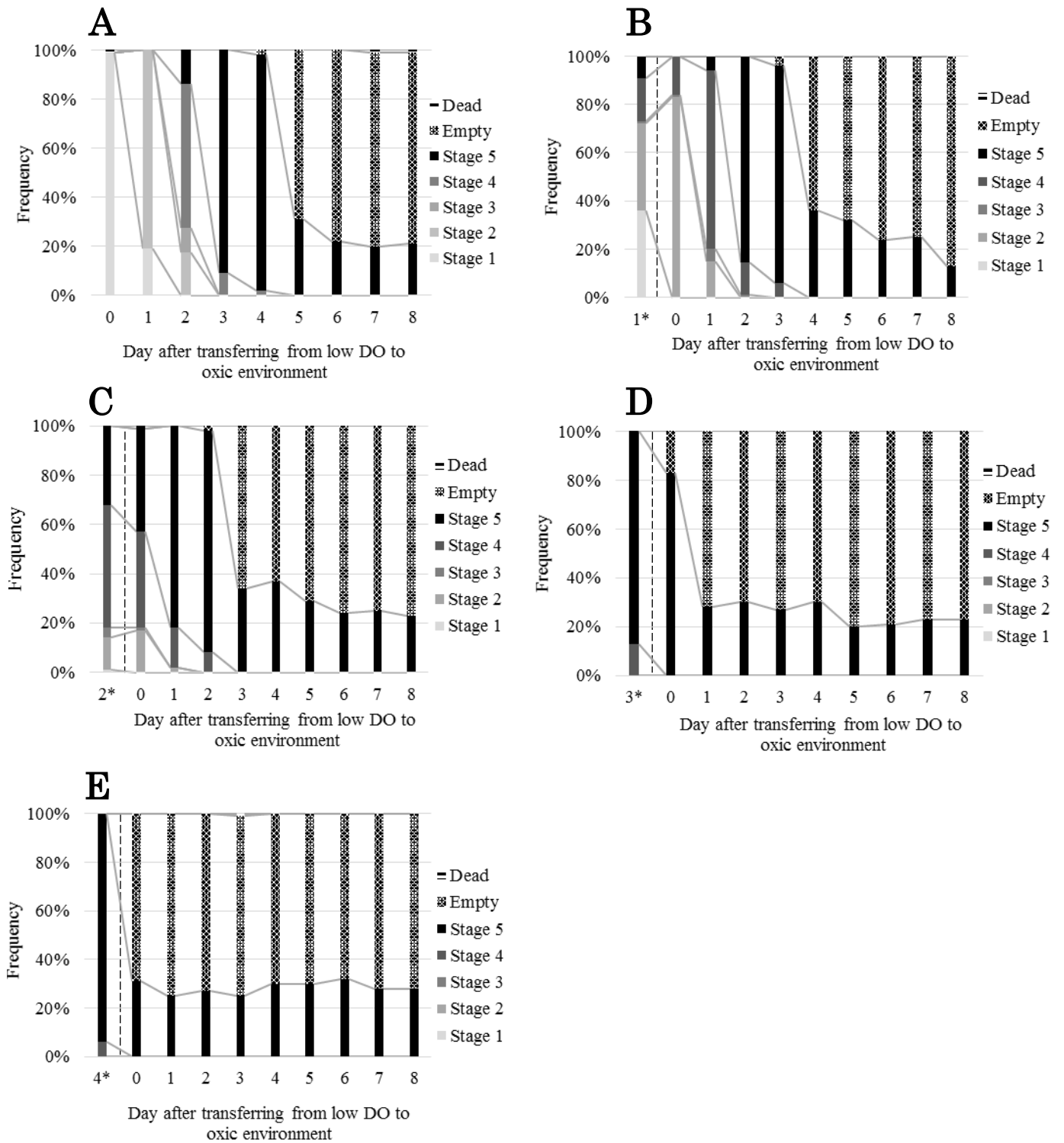


Fig. 1.6 Development of tomonts incubated in the hypoxic environment after various oxenic incubation periods and subsequently transferred to the oxenic environment. Asterisks indicate the days in the oxenic environment before incubation in hypoxic environment. A: tomonts incubated for 1 week in the hypoxic environment after leaving hosts and then incubated in oxenic environment (Fig. 1D, #7); B: tomonts incubated for 1 day in the oxenic environment, 1 week in the hypoxic environment and then transferred to the oxenic environment (Fig. 1D, #8); C: tomonts incubated for 2 days in the oxenic environment, then 1 week in the hypoxic environment and transferred to the oxenic environment (Fig. 1D, #9); D: tomonts incubated for 3 days in the oxenic environment, 1 week in the hypoxic environment and then transferred to the oxenic environment (Fig. 1D, #10); E: tomonts incubated for 4 days in the oxenic environment, 1 week in the hypoxic environment and then transferred to the oxenic environment (Fig. 1D, #11). This experiment was repeated twice using protomonts collected on different days. Each column represents the mean of the repetitions.

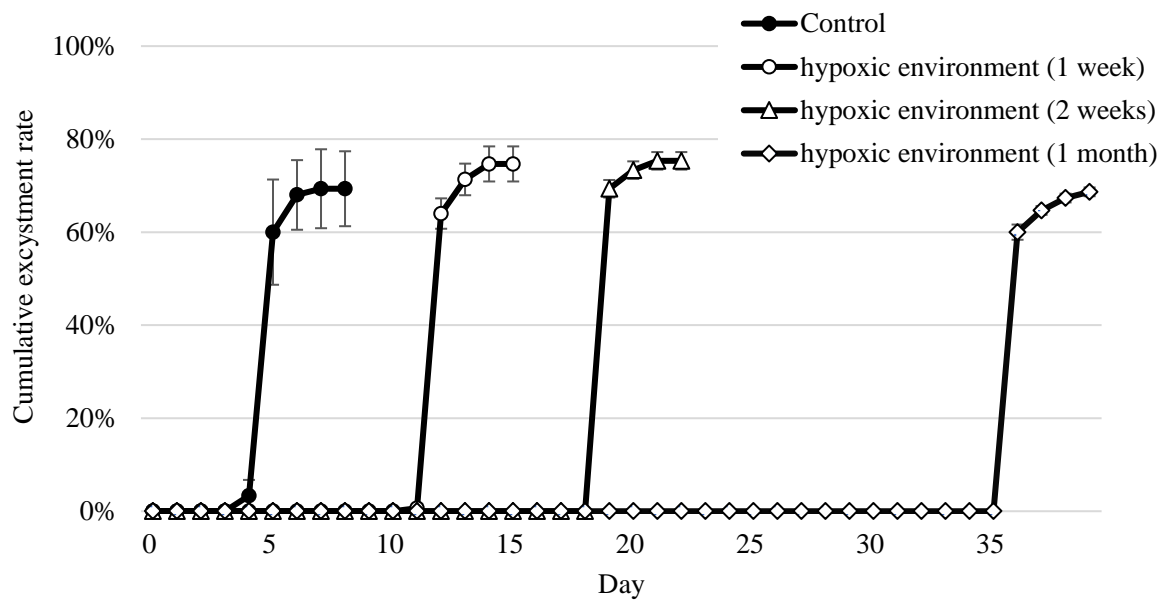
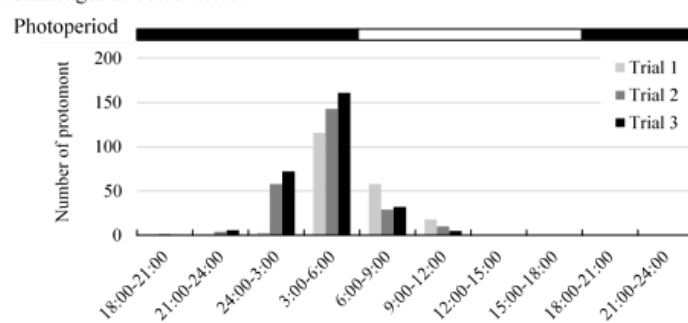
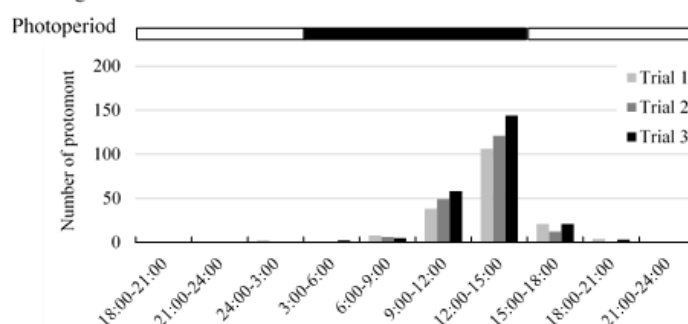


Fig. 1.7 Percentages of tomonts releasing theronts after incubation for 1 week, 2 weeks and 1 month in the hypoxic environment. Tomonts were transferred to the oxic environment after hypoxic incubation (Fig. 1E). Mean values of the cumulative percentages of tomonts that released theronts are plotted with the standard deviation (vertical bars).

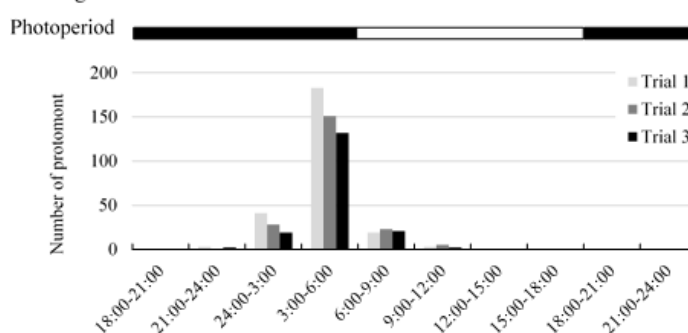
A-a: Challenged at 06:00–08:00.



A-b: Challenged at 06:00–08:00.



B-a: Challenged at 12:00–14:00.



B-b: Challenged at 12:00–14:00.

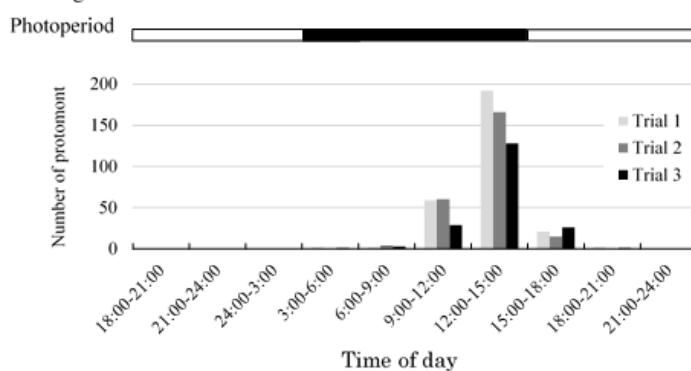
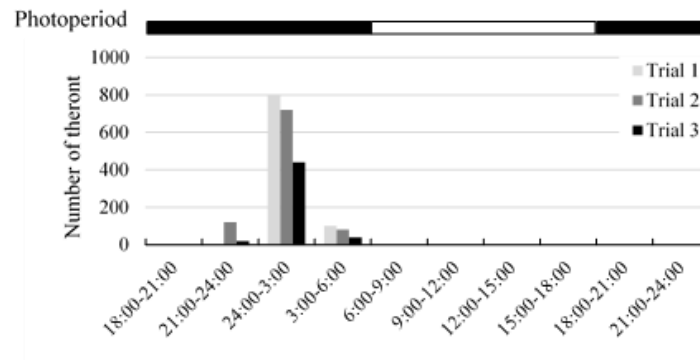
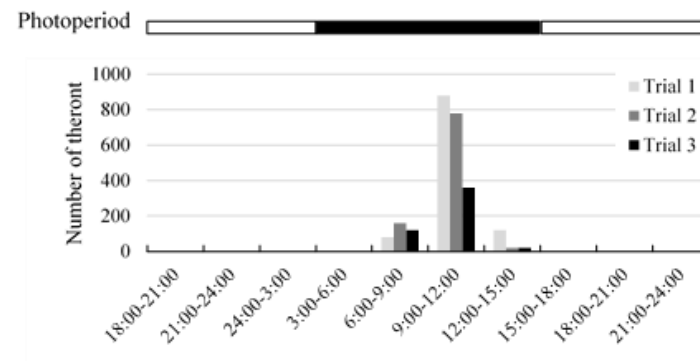


Fig. 1.8 Daily rhythm in the detachment of protomonts from challenged black mollies. Fish were challenged by exposure to theront suspension from 06:00 to 08:00 (A-a and A-b) and 12:00–14:00 (B-a and B-b), with photoperiod conditions of 06:00–18:00 light and 18:00–06:00 dark (A-a and B-a) and 15:00–03:00 light and 03:00–15:00 dark (A-b and B-b). The number of protomonts that detached from five fish is shown.

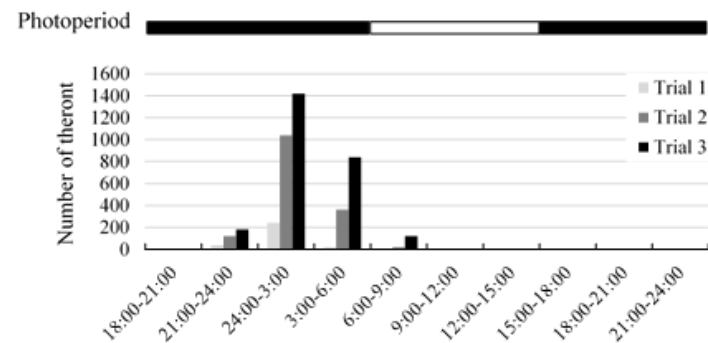
A-a: Protomonts that detached from fish at 03:00–06:00.



A-b: Protomonts that detached from fish at 03:00–06:00.



B-a: Protomonts that detached from fish at 12:00–15:00.



B-b: Protomonts that detached from fish at 12:00–15:00.

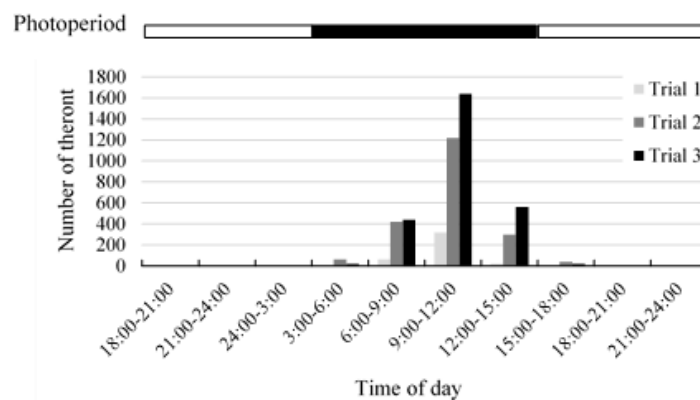


Fig. 1.9 Daily rhythm in the excystment of theronts from tomonts. Protomonts that detached from fish at 3:00–6:00 (A-a and A-b) and 12:00–15:00 (B-a and B-b) were incubated under photoperiods of 06:00–18:00 light and 18:00–06:00 dark (A-a and B-a), and 15:00–03:00 light and 03:00–15:00 dark (A-b and B-b). The number of theronts that excysted from 100 tomonts is shown.

Chapter 2

Characterization of the proteases in the parasitic stage of *Cryptocaryon irritans* and its potential as vaccine antigens against its infection

Section 2.1

Characterization of the proteases of the parasitic stage of *Cryptocaryon irritans*, and *in vitro* and *in vivo* effects of protease inhibitors on cryptocaryoniasis

Introduction

Many studies have focused on the development of control methods of cryptocaryoniasis, such as therapeutic drugs and vaccines, because the parasite is a major threat to marine aquaculture in tropical and subtropical waters. However, no treatments efficient enough to control infections with *C. irritans* have been developed, especially for cultured food fishes (Yoshinaga et al., 2011).

Recently, proteases are thought to play crucial roles in the infection and development of parasitic protozoans such as *Tritrichomonas foetus* (Metamonada), *Leishmania* spp. (Euglenozoa), *Tetrahymena* spp. and *Miamiensis avidus* (Ciliophora) (Leibowitz et al., 2009; Oliver et al., 2012; Tolbert et al., 2014; Narasaki et al., 2018) and have been recognized as potential targets of drugs and vaccines against protozoan parasites. In *C. irritans*, more than one hundred protease transcripts were identified by a transcriptome analysis, and some of them were highly expressed in the trophont stage (Mo et al., 2016). It is presumed from the previous studies that proteases play key roles in the infection and development of the trophont stage of *C. irritans* as well as other protozoan parasites. Although detailed information on the proteases highly expressed in the trophonts of the parasite would assist in the development of new chemotherapies or vaccines for cryptocaryoniasis, little is known about the characteristics of such proteases.

We conducted this study to determine the characteristics of *C. irritans* trophont proteases using zymography. In addition, we examined the effects of protease inhibitors on the infection, survival, and growth of the parasite both *in vitro* and *in vivo*.

Materials and Methods

Parasite

Cryptocaryon irritans used for present study was obtained from experimentally challenged tiger pufferfish (*Takifugu rubripes*) (body length 10–15 cm), which were purchased from a commercial hatchery (Marinotech Co. Ltd, Aichi, Japan) and raised in our fish-rearing facility. Five pufferfish were exposed to 20,000 theronts (4,000 theronts/fish) in seawater in a 60 L glass

aquarium for 6 h in the dark at 25°C. The theronts used for challenge were obtained by the propagation using black mollies (*Pocillia* sp.) as described in Chapter 1. The challenged pufferfish were transferred to another 60 L glass aquarium equipped with an overhead biological filter to prevent excessive infection, and maintained at 25°C. After 66 hours of the challenge, infected pufferfish were placed in a 20 L plastic aquarium containing 15 L of diluted seawater (9 ppt salinity). Trophonts detached from the fish were washed five times in phosphate-buffered saline (PBS) supplemented with antibiotics (500 IU/mL penicillin G potassium and 500 µg/mL streptomycin sulfate) using Pasteur pipettes and glass bowls under a stereomicroscope. The trophonts were incubated in Ø85 mm culture dishes containing 10 mL filter-sterilized (0.22 µm) seawater to allow them to transform into encysted tomites at 25°C. Most tomites released theronts 5–7 days after collection; the theronts were then collected and used to challenge fish. This process was repeated to propagate and maintain the *C. irritans* infection.

Trophont extract for gelatin zymography

Two thousand trophonts were collected and washed by PBS as described above. The trophonts in 200 µL of PBS were sonicated for 10 seconds on ice using an ultrasonic processor (UR-21P, TOMY SEIKO, Tokyo, Japan). The suspension was centrifuged at $10,000 \times g$ for 10 min and the supernatant was used for zymography. The concentration of protein in the solution was determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, California, USA) with bovine serum albumin as the standard.

Gelatin zymography

Effects of pH, temperature, and salinity on proteolytic activities

Gelatin zymography was carried out according to Hirazawa et al. (2006), with slight modifications. The trophonts extract (supernatant of sonicated trophonts) was mixed with 1/2 volume of 125 mM Tris–HCl buffer, pH 6.8, containing 4% sodium dodecyl sulfate (SDS), 20% glycerol, and 0.01% bromophenol blue. The extract, of which the amount of protein was approximately 25 µg, was loaded into 8 wells of 10% polyacrylamide gels containing 0.1% gelatin (w/v) from cold fish skin (Sigma-Aldrich) and separated by electrophoresis (200 V, 45 min). After electrophoresis, the gel was immersed in 100 mM Tris–acetate, pH 7.0 containing 2.5% Triton

X-100 (renaturing buffer) with gentle shaking for 30 min at around 20°C to renature the proteins denatured by SDS. After renaturation, the gel was divided into 8 strips each containing one lane.

To examine the effects of pH, temperature, and salinity on proteolytic activities of proteases contained in the trophont extract, the strips were separately incubated under different conditions. For the effects of pH, the strips were incubated separately at 37°C for 6 h in 100 mM of each of eight following incubation buffers: glycine–HCl at pH 4.0; Tris-acetate at pH 5.0, 6.0, and 7.0; Tris–HCl at pH 8.0 and 9.0; borate at pH 10.0 and 11.0. For the effects of temperature, the strips were incubated in Tris-acetate at pH 7.0 for 6 h at eight different temperatures (5, 10, 15, 20, 25, 30, 35, and 40°C). For the effects of salinity, the gel strips were incubated in Tris-acetate at pH 7.0 for 6 h at three different salinities (0, 0.9, and 3.5% NaCl). All buffers used for incubation were supplemented with 5 mM CaCl₂ and 200 mM ZnCl₂.

The incubated gel strips were stained with 1.0% Coomassie Brilliant Blue (CBB) R-250 in 40% methanol, 10% acetic acid, and 50% distilled water, and then de-stained using 40% methanol, 10% acetic acid, and 50% distilled water. Proteolytic activity was visualized as transparent bands not stained by CBB R-250.

Effect of protease inhibitors on gelatinolytic activities

Four protease inhibitors, namely, E-64 (Peptide Institute Inc., Osaka, Japan) inhibiting cysteine protease, Leupeptin (Peptide Institute Inc.) inhibiting serine protease and cysteine protease, Pepstatin A (Peptide Institute Inc.) inhibiting aspartic protease, and EDTA (Ethylenediaminetetraacetic acid) inhibiting metalloprotease were used in this experiment. The 100x stock solutions of E-64, Leupeptin and EDTA were prepared in distilled water. The 100x stock solution of Pepstatin A was prepared in ethanol. The stock solutions of inhibitors were diluted 100 times with Tris-acetate at pH 7, which was used as the conventional incubation buffer throughout this study, unless otherwise stated. The final concentrations of E-64 and Leupeptin were 100 µM, and those of EDTA and Pepstatin A were 1 mM and 20 µM, respectively. The trophont extract was loaded into each well of the gel and separated by electrophoresis and then the gel was immersed into the renaturing buffer as described above. Gel strips obtained were immersed in incubation buffers containing different inhibitors. For negative controls, gel strips were immersed in incubation buffer supplemented with each solvent. After incubation, proteolytic activity was visualized in the same method as described above.

***In vitro* assay of effect of protease inhibitors on the development of the parasite**

The double-layered medium consisting of a cell layer and agarose layer, previously developed for the *in vitro* culture of *C. irritans* by Yoshinaga et al. (2007), was used for the *in vitro* assay to determine the effect of protease inhibitors (E-64, Leupeptin, or Pepstatin A) for the development of *C. irritans*, as following method.

FHM cells, a cell line derived from fathead minnow *Pimephales promelas*, were seeded at 2.5×10^5 cells/cm² in 6-well cell culture plates, and were stabilized and formed a cell layer on the bottom of the wells. Each protease inhibitors (E-64, Leupeptin, and Pepstatin A) were dissolved in the appropriate solvent at 100 times as their final concentration. Then, the stock solutions were diluted 100 times with 0.5% low gelling agarose solution containing Leibovitz's L-15 medium, which was supplemented with antibiotics (500 IU/mL penicillin G potassium and 500 µg/mL streptomycin sulfate dissolved in 0.85% NaCl solution), and 10% fetal bovine serum (FBS) for use. The final concentrations of E-64 and Leupeptin were 10, 100, and 200 µM, and those of Pepstatin A were 1, 10, and 20 µM. We loaded 5 ml of the medicated agarose solutions onto a layer of FHM cells that were seeded on the bottom of 6-well cell culture plates and gelatinized the solution in the refrigerator (4°C). A theront suspension containing 100 theronts in 15 µL seawater were inoculated at the center of the cell layer beneath the gel with a 200 µL pipette tip and incubated at 25°C. Dead parasites were discriminated by rupture and disappearance of body, and live parasites in the media were counted with an inverted microscope at 6 h post-inoculation and every 24 h after until day 7. The number of the live parasites at 6 h post-inoculation was defined as 100% of "Percentage of parasite survival". We also measured the length of each individual of trophonts on images using cellSens (Olympus Corporation, Tokyo, Japan) at day 1, 3, and 5; ten trophonts were measured from each of the three wells for each concentration and condition, and mean and frequency distribution of parasite length were obtained by the measurements. The control medium was supplemented with the solvent used to dilute each protease inhibitor and inoculated with theront suspension. The assays were carried out using different batches of theronts at different times for different protease inhibitors. The assays were carried out in triplicate in three wells. In order to examine the cytotoxic effects of each protease inhibitor on the parasite and FHM cell as well, we inoculated about 100 theronts to the liquid L-15 medium supplemented with each inhibitor at the highest final concentration of the *in vitro*

assay (E-64 and Leupeptin: 200 μ M, Pepstatin A: 20 μ M) without FHM cells. We also cultured FHM cells in liquid L-15 medium supplemented with each of the inhibitors at the highest final concentration, without inoculating the theronts.

***In vivo* effects of protease inhibitors for parasite invasion to host fish**

Black mollies were experimentally infected with theronts in the presence of protease inhibitors. Fifteen back mollies (3–4 cm) were randomly divided into three groups. Within 3 h after being released from tomonts, each fish was individually exposed to 300 theronts in 100 ml of seawater supplemented with E-64 (final concentration was 100 μ M), Leupeptin (100 μ M), or distilled water (control) in a beaker for 1 h. The stock solutions of each protease inhibitor were diluted 100 times with seawater. Subsequently, each fish was individually transferred to 500 ml of seawater without protease inhibitors and reared at 25°C for 4 days; seawater was replaced daily. Parasites that left the fish as protomonts 3 to 4 day after the exposure, were collected from the bottom of the beakers and counted.

Statistical analysis

In the *in vitro* assay, the percentages of parasite survival between control and protease-inhibitors supplemented groups were analyzed using the Kruskal-Wallis test followed by the Shirley-Williams test. The mean length of the parasite was analyzed using the Dunnett T3 test. In the *in vivo* assay, parasite recovery was compared using the Steel test after an arcsine transformation. Values at a probability level of $P < 0.025$ were considered significant in the one-sided Shirley-Williams test. In other tests, $P < 0.05$ was regarded as significant.

Results

Gelatin zymography

Zymography of trophont extract revealed proteases at approximately 30, 40, and 70 kDa (Fig. 2. 1). The gelatinolytic activities of 30 and 40 kDa proteases were greater at a low pH (pH 4.0–5.0) and increased at high temperatures (35–40°C). However, their activities decreased at high salinity conditions (3.5%). In contrast, the gelatinolytic activity of 70 kDa proteases were greater at neutral to weakly alkaline conditions (pH 7.0–8.0) and increased at 15–35°C. Their activity was maintained at high saline conditions (3.5%) (Fig. 2.1 A–C).

The activity of 30 and 40 kDa proteases was suppressed by Leupeptin, which is a serine and cysteine protease inhibitor, and E-64, a cysteine protease inhibitor. The activity of 70 kDa protease was suppressed only by Leupeptin. However, none of these proteases were affected by EDTA (metalloprotease inhibitor) and Pepstatin A (aspartic protease inhibitor) (Fig. 2.1 D, E). The gelatinolytic activities in trophont homogenate are summarized in Fig. 2.1 F.

The position of gelatinolytic bands of muscle homogenate and mucus of tiger pufferfish were different from the trophonts homogenate (Fig. 2.1 G).

***In vitro* assay of effect of protease inhibitors for the parasite**

In the double layer medium, theronts were transformed to trophonts. The percentage of surviving trophonts frequently exceeded 100% in inoculated theronts in both the control groups and media supplemented with inhibitors at low concentrations during the initial 1–2 days after inoculation (Fig. 2.2 A, C, E). It is likely that the number of theronts soon after inoculation (day 0) into the double layer medium was underestimated because theronts actively moved for several hours after inoculation.

In the E-64 supplemented group (100 μ M, 200 μ M), the percentages of surviving trophonts were significantly lower than the control group on day 1, 2, and 3. When E-64 were supplemented at 200 μ M, the percentages significantly decreased on day 3 and day 4 as well (Fig. 2.2 A). In the Leupeptin-supplemented group (200 μ M), the percentages of surviving trophonts were significantly lower than the control group on day 1 and 2 (Fig. 2.2 C). In the Pepstatin A supplemented group (20 μ M), the percentages of surviving trophonts were significantly lower than the control group on day 1, 3, and 5 (Fig. 2.2 E). The sizes of the trophonts treated with protease inhibitors were slightly smaller than those of the control group on day 5, although these values were not significantly different from the control groups (Fig. 2.2 B, D, F).

The cytotoxic effect of each protease inhibitor on the parasite and FHM cells was not found in this study (data not shown).

***In vivo* assay of effect of protease inhibitors for the parasite invasion**

The percentages of recovered parasites from the control group, the E-64 and Leupeptin groups were $43.3 \pm 0.05\%$ (mean \pm SD), $30.2 \pm 0.05\%$, and $39.2 \pm 0.06\%$, respectively. The percentage of recovered parasites in the groups treated with E-64 and Leupeptin were lower than that of the

control group. More specifically, the percentage of recovered parasites in the E-64 group was significantly lower than that of the control group ($P < 0.05$).

Section 2.2

Functional analysis of cysteine and serine type proteases of *Cryptocaryon irritans* with RNA interference

Introduction

Recently, Mo et al. (2016) reported that more than one hundred protease transcripts were identified in *C. irritans* by transcriptome analysis using a next generation sequencer, and some serine and cysteine proteases were highly expressed in the theront and trophont stages. Therefore, serine and cysteine type proteases might play important roles in the infection and development of *C. irritans*. In section 2.1, the presence of serine and cysteine proteases in trophonts of *C. irritans* was confirmed and the inhibitors against these proteases decreased the viability and infectivity of the parasite. These results suggest that serine and cysteine proteases are important for the infection process of *C. irritans* and can be target molecules for the development of chemotherapy and vaccinations against cryptocaryoniasis. However, the molecules of the proteases involved in the infection process remain unidentified.

RNA interference (RNAi) is a cellular mechanism induced by double-stranded RNA (dsRNA) for post-transcriptional gene silencing in cells (Ullu et al., 2004). RNAi is an evolutionary conserved phenomenon, based on a common mechanism throughout in the eukaryotic cells. This leads to the sequence-specific destruction of endogenous RNAs that match the dsRNA (Dykxhoorn et al., 2003; Tijsterman et al., 2002; Cottrell et al., 2003). In ciliates, the inhibition of gene expression by RNAi has been reported from *Paramecium tetraurelia*, *Euplotes aediculatus*, *Oxytricha nova* and *Stylonychia lemnae* (Galvani and Sperling, 2002; Paschka et al., 2003). RNAi was expected to be a powerful tool to analyze the biological function of molecules of *C. irritans* as well.

In this study, we conducted transcriptome analyses of *C. irritans* using next generation sequencing to find protease genes highly expressed in the theront and trophont stages, and examined the roles of four highly-expressed proteases in the invasion and infection of *C. irritans* using RNAi technique.

Materials and Methods

Parasites

Trophonts, tomonts and theronts of the same isolate of *C. irritans* used in Section 2.1 was obtained as described in Section 2.1.

For RNA extraction, detached trophonts were collected after washing with PBS, and tomonts were collected at day 3 after encysted. Theronts were collected according to the protocol of Mo et al., (2016) with slightly modified. Briefly, theronts were cooled in ice-bath for 10 min to reduce swimming ability, and collected after centrifugation at 2,000×g for 5 min. Each stages of *C. irritans* were cryopreserved in liquid nitrogen until RNA extraction.

Transcriptome analysis

RNA extraction

RNA was extracted from theronts (5.0×10^6 cells), trophonts (2,000 cells) and tomonts (2,000 cells) using a NucleoSpin® RNA Plus (Macherey-Nagel, Dürren, Germany) according to the manufacturer's instructions. The RNA samples were sent to Filgen, Inc. (Nagoya, Aichi, Japan) for RNA sequencing. Procedures for transcriptome analysis were also conducted by Filgen, Inc. as follows.

RNA quantification and qualification

RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Library preparation for Transcriptome sequencing

A total amount of 1.5 µg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under

elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H Minus). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150–200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 µl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index primers. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

Clustering and sequencing

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq platform and paired-end reads were generated.

Quality control, assembly

Raw data (raw reads) were firstly processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. At the same time, Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on clean data with high quality. The resulting high-quality sequences were de novo assembled into contigs and transcripts with Trinity software (<http://trinityrnaseq.sf.net>). Gene function was annotated based on the following databases: Nr (NCBI non-redundant protein sequences); Nt (NCBI non-redundant nucleotide sequences); Pfam (Protein family); KOG/COG (Clusters of Orthologous Groups of proteins); Swiss-Prot (A manually annotated and reviewed protein sequence database); KO (KEGG Ortholog database); GO (Gene Ontology).

Estimation of gene expression levels

Gene expression levels were estimated by RSEM (Li et al, 2011) for each sample. Clean data were mapped back onto the assembled transcriptome. Readcount for each gene was obtained from the mapping results.

Differential expression analysis

Prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by edgeR program package through one scaling normalized factor. Differential expression analysis of two samples was performed using the DEGseq (2010) R package. P-value was adjusted using q-value (Storey et al, 2003). $q\text{-value} < 0.005$ & $|\log_2(\text{foldchange})| > 1$ was set as the threshold for significantly differential expression.

cDNA cloning of highly expressed protease or peptidase genes at theront and trophont stages

Highly expressed protease and peptidase genes (two genes of serine proteases and two genes of cysteine proteases) selected from the results of RNA-seq and used as targets for the following RNAi experiments (Table 2.1). These serine protease and cysteine protease genes were named respectively as *sp1* and *sp2*, *cp1*, and *cp2*. The complete cDNA sequences of the four genes were determined using the SMART RACE cDNA Amplification kit (Clontech, Palo Alto, CA, USA) following the manufacturer's instructions. The primers used for RACE PCR were described in Table 2.1. The obtained RACE PCR products were purified by FastGene Gel/PCR Extraction Kit (NIPPON genetics, Tokyo, Japan) and incubated with Ex Taq polymerase (Takara Bio, Shiga, Japan) to add 3' A overhang and then cloned into pGEM-T easy vector (Promega, Madison, WI, USA). These constructed plasmids were used to transform into competent quick DH5 α *Escherichia coli* (TOYOBO, Osaka, Japan) transformed according to the manufacturer's instructions and sequenced by Eurofins Genomics (Tokyo, Japan). The target genes were subjected to analyses with Blastx and Wolf PSORT (Horton et al, 2006) for their identity and cellular localization.

Protease or peptidase genes knock down by RNAi

dsRNA synthesis

dsRNAs for the targeted 4 genes were synthesized using *in vitro* Transcription T7 Kit (Takara Bio, Shiga, Japan), following the manufacturer's instructions. Briefly, a partial open reading frame (ORF) region (101 bp) of the each target gene was PCR amplified from pGEM-T easy vectors using primers in Table 1 (T7_*sp1F*-R, T7_*sp2F*-R, T7_*cp1F*-R, and T7_*cp2F*-R). A partial sequence of *β-lactamase* of pGEM-T easy vector was also PCR amplified (T7_*β-lacF* and T7_*β-lacR*, Table 1). These PCR products were purified using FastGene Gel/PCR Extraction Kit. For each gene, sense and antisense single-stranded RNA (ssRNA) were synthesized using a same reaction tube to anneal and produce dsRNA. The template DNA and any single-stranded RNA remaining in the reaction were digested by RNase A and DNase I treatments. Finally, dsRNA was purified by ethanol precipitation. The quality of dsRNA was checked by agarose gel electrophoresis and quantified using a SimpliNano (GE Healthcare, NJ, USA).

dsRNA inducing method by using electroporator

Theronts (1.0×10^5 parasites) were collected and washed 5 times with L-15 medium. Then the parasites (5.0×10^4 parasites) were placed in 1.5 mL microtubes containing sterile L-15 medium along with $0.1 \mu\text{g}/\mu\text{L}$ of either target-dsRNA or *β-lactamase*-dsRNA. Each suspension was transferred to an electroporation cuvette (2-mm gap) (Nepa Gene, Chiba, Japan), and electroporation were performed with the Bio-Rad Micro Pulser under conditions of 1500 V/cm and 10 μF , with a time constant of 0.6 ms. Electroporated theronts were transferred into fresh 1 mL of L-15 medium and incubated for 30 min at 25°C for recovery. For the negative controls, we also established two groups (electroporated parasites without dsRNA and parasites without electroporation) to know the effect of electroporation and dsRNA induction for the parasite.

Quantification of RNAi efficacy

After the electroporation and recovery, 1,000 theronts were counted and used for the following challenge experiment ("*Challenge experiment with dsRNA transfected theronts*").

To quantify gene silencing, the remaining theronts (approximately 4.9×10^4 parasites) were kept in L-15 medium for 24 h and the total RNA was extracted using a NucleoSpin® RNA Plus, treated by DNase I (Takara Bio, Shiga, Japan) to remove genomic DNA and purified using a NucleoSpin® RNA Clean-up (Macherey-Nagel, Dueren, Germany). Purified RNA was transcribed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka,

Japan), and then expression levels of target gene of either target-dsRNA or β -lactamase-dsRNA treated samples were quantified with Quantitative-PCR (qPCR) using newly designed primers (qF_sp1, qR_sp1, qF_sp2, qR_sp2, qF_cp1, qR_cp1, qF_cp2, and qR_cp2), which were expected to amplify a 150 bp fragment (Table 2.1). Expression level of *C. irritans EF-1 β* was used as internal control for normalization, using specific primers qF_EF-1 β and qR_EF-1 β (Table 2.1) newly designed based the transcriptome data obtained in this study. qPCRs were performed in 10 μ L using the THUNDERBIRD SYBR qPCR Mix in accordance with the manufacturer's protocol (TOYOBO, Osaka, Japan) on a CFX Connect real-time PCR detection system (Bio-Rad, Hercules, CA, USA). PCR amplification was performed using as follows: an initial denaturation at 95°C for 1 min, 40 cycles of 95°C for 10 sec and 60°C for 30 sec. Melting curve analysis was performed immediately after the amplification protocol with the defaulted program of the CFX Connect real-time PCR detection system to confirm specificity of primer annealing. qPCR products were sent to Eurofins Genomics and sequenced to confirm that the target DNA fragments were correctly amplified. qPCR data were analyzed by Bio-Rad CFX manager software. Control reactions were performed using the same procedures without the addition of template cDNA, as the negative control of PCR. Genomic DNA contamination was also checked using samples without reverse transcription. All the amplifications were performed in duplicate. Standard curves (10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 molecules of pGEM-T easy vector containing the target) were performed for both *EF-1 β* , and target genes. Based on these curves, the total number of molecules were calculated for *EF-1 β* and target genes in target-dsRNA and β -lactamase-dsRNA treated samples (control). Relative expression was determined as number of target gene molecules/number of *EF-1 β* molecules. This procedure was carried out against the four target genes.

Challenge experiment with dsRNA transfected theronts

dsRNA transfected-theronts prepared as above from each group were also used the challenge experiment. One thousand theronts were added to 200 mL beakers and five beakers were prepared for each group. In each beaker, one black molly (*Poecilia* sp.) with an average size of 3-4 cm was exposed to electroporated or control theronts so that five fish were used for each experimental group. Subsequently, the black mollies were individually kept at 25°C in 300 mL of seawater with gentle aeration. Three days after the experimental challenge, fish was anesthetized and the

trophonts remaining on the skin of fish and the detached protomonts were counted under a stereoscopic microscope.

To confirm the result of the challenge experiment, this procedure was repeated again only with electroporated theronts with target dsRNA of target genes and with *β -lactamase*-dsRNA without the two negative control groups, because little effect of the electroporation and dsRNA transfection on infectivity of the parasite had been confirmed in the first challenge experiment .

Efficiency of dsRNA transfection

To know the efficiency of dsRNA transfection, electroporation was performed using fluorescein-labeled dsRNA of *β -lactamase*. The procedure of dsRNA transfection was the same as described above. The labeled dsRNA was prepared using Label IT Nucleic Acid Labeling Kit (Mirus Bio, Madison, WI, USA) according to the manufacturer's instructions. Theronts subjected to the electroporation were kept in L-15 for 6 h and observed with a fluorescence microscope (BZ-X800; Keyence, Tokyo, Japan).

Statistical analysis

In the first trial of RNAi experiment, differences of parasites numbers between the treatment groups were compared using Kruskal–Wallis followed by Steel–Dwass test. In the second trial, Mann–Whitney test was performed to compare the parasites numbers of target-dsRNA treated and *β -lactamase*-dsRNA treated groups. Treatments were considered to be significantly different if $p < 0.05$.

Results

Transcriptome analysis

Totals of 91.27, 84.72, and 84.38 million raw reads were obtained from theronts, trophonts and tomonts, respectively, by Illumina Hiseq 4000 sequencing (Table 2. 2). After the removal of adaptors, low-quality reads (reads with more than 50% low quality base, Quality value $sQ \leq 20$), reads with more than 10% N (N represent the base with uncertainty), and reads < 20 bp, 87.31, 79.34, and 79.26 million clean reads were obtained from the theront, trophont, and tomont libraries with the clean-read ratio of 93.93%, 95.66% and 93.65%, respectively. A total of 85,033 unigenes were then obtained after de novo assembly, with a total length of 72,097,052 bp, an

average length of 848 bp, and N50 of 1,080 bp. 34.07% of the unigenes were annotated in Nr database, and 67.19% of unigenes were annotated in at least one database. Ten unigenes annotated as host fish protein were removed from this analysis. The ratio of annotated genes was summarized in Table 2.3.

More than one thousand of protease/peptidase transcripts were identified in the transcriptome. Some serine and cysteine proteases/peptidases (Theront: 29 genes, Trophont: 18 genes) were highly expressed (FPKM>100) in theronts and trophonts. From these proteases/peptidases, several serine and cysteine proteases/peptidase genes (10 genes) were highly expressed in theront and trophont stages (FPKM>200), but less expressed in tomonts (fold change < -2.0). Therefore, these genes were considered to be important genes at the invasion and parasitic stages of the parasite, and were targeted for subsequent RNAi experiments.

Isolation of target protease gene cDNA

Complete cDNA sequence of target genes were determined from trophonts by RACE PCR (Fig. 2.3 A–D). Blastx search showed that the target genes had the highest identity with proteins of some ciliates; the most homologous protein of *sp1*, *sp2*, *cp1* and *cp2* are respectively serine carboxypeptidase family protein of *Tetrahymena thermophila* (GenBank XP_001018531.2); serine carboxypeptidase family protein of *Oxytricha trifallax* (EJY70217.1); papain family cysteine protease of *Tetrahymena thermophila* (XP_001026313.1); and hypothetical protein of *Paramecium tetraurelia* (XP_001436818.1), which has a peptidase_C2 domain (Calpain family cysteine protease). Analysis of protein localization by WoLF PSORT suggested that *sp1*, *sp2* and *cp1* are an extracellular protein, and *cp2* is an extracellular or plasma membrane protein.

Except for the 4 mainly focused genes, several highly expressed serine and cysteine protease (peptidase) genes were found in the results of the transcriptome analysis, but complete cDNA sequences of these genes were not determined in this study; thus, the 4 genes (*sp1*, *sp2*, *cp1* and *cp2*) were used as targets for RNAi experiment.

RNA interference

In the first trial, fewer trophonts and protomonts were collected in all of the groups challenged with RNAi-treated theronts than controls (Fig. 2.4 A–D). Especially, the number of parasite were significantly lower in the RNAi-treated against *cp2* group than in any control groups ($p < 0.05$)

(Fig. 2.4 D). In addition, target genes transcripts in RNAi-treated theronts were 17–47% less than in those with *β -lactamase* -dsRNA (Fig. 2.4 A–D).

In the second trial, fewer trophonts and protomonts were collected in all of the groups challenged with RNAi-treated theronts than the control (*β -lactamase* group) (Fig. 2.4 E–H). Especially, the number of parasites in the RNAi-treated groups (against *sp2* and *cp2*) were significantly lower than control groups ($p < 0.05$) (Fig. 2.4 F, H). The amount of target genes transcript was 13–46% less in RNAi-treated groups than in the negative control group (*β -lactamase* group) (Fig. 2.4 E–H).

Most of theronts transfected with fluorescein-labeled *β -lactamase*-dsRNA with the electroporation showed fluorescent, indicating the high efficiency of transfection with the electroporation (Fig. 2.5).

Section 2.3

Development of vaccines using recombinant proteases of *Cryptocaryon irritans* against its infection

Introduction

Vaccination experiments have already been conducted against cryptocaryoniasis. Previous studies showed that immobilization-antigen (i-antigen), which is a cell surface membrane protein of *C. irritans*, induced strong immune response in host fish, but the immune response was serotype-specific and recognized only the homologous serotype of i-antigen (Hatanaka et al. 2007; Misumi et al. 2011). However, fish immunized with live theronts of different *C. irritans* i-antigen serotypes induced cross-protection against subsequent challenges by homologous and heterologous serotypes (Misumi et al. 2011). This result suggests the existence of antigens that are common to all *C. irritans* serotypes and are able to provide cross-serotype protection (Misumi et al. 2011). Therefore, identification of antigens that are common to all serotypes is required for the development of *C. irritans* vaccines.

In section 2.3, RNA interference against four protease and peptidase genes (highly expressed in theronts and trophonts) reduced infectivity and/or growth of the parasite on the host, suggesting that these protease genes highly expressed in the theronts and trophonts play important roles in the infection and development of *C. irritans*, possibly via invasion, food uptakes, and digestion. Therefore, these proteases can be target molecules of new vaccines development against cryptocaryoniasis.

In this section, recombinant proteins of the four protease and peptidase genes were produced and evaluated as target molecules for vaccination antigens.

Materials and Methods

Parasites

Two isolates of *C. irritans* were used in this study. One of them (UT1) was isolated from an ornamental blue surgeonfish (*Paracanthurus hepatus*) purchased in a local pet shop and passaged in our laboratory for more than 3 years. The isolate UT1 was used for challenge experiments. The other isolate K1 was obtained from red sea bream (*Pagrus major*) in the Aquaculture Research

Institute of Kindai University (Shirahama, Wakayama, Japan), and this isolate was collected from bottom deposits of a fish tank rearing red sea bream in the institute in November, 2019. The isolate K1 was used for confirmation of the cross-reactivity of the antibodies of immunized fish.

Both of the isolates were passaged and propagated separately on seawater-adapted black mollies (*Pocillia* sp.) (mean body length 3–4 cm) as described in Chapter 1.

Comparison of i-antigen of two isolates of *C. irritans*

To compare the amino-acid sequences of i-antigen between UT1 and K1, cDNA were synthesized from total RNA extracted from theronts of both isolates as described in section 2.2 and the cDNA of their i-antigens were obtained amplified by PCR amplification using primers 5'-YWGCWGATTGGAMHGGWAC -3' and 5'-AGTACTWAATTCATGAATAT -3', which were designed based on an i-antigen sequence obtained from transcriptome data in this study and three i-antigen sequences available in Genbank; G32 and G37 (Hatanaka et al., 2007, 2008) and Chiayi (Yambot et al., 2003). PCR amplification was performed using KOD one PCR Master Mix (TOYOBO, Osaka, Japan) as follows: 40 cycles of 98°C for 10 sec, 45°C for 5 sec and 68°C for 5 sec. Each PCR product was purified by FastGene Gel/PCR Extraction Kit and cloned into pGEM-T easy vector using competent quick DH5α *E. coli* and sequenced by Eurofins Genomics as described in Section 2.2. Amino acid sequences of the i-antigens were deduced from their sequences.

Production of recombinant proteins of target genes

Preparation of codon changed sequences of target genes

Codon usage is different between ciliates and bacteria. UAA and UAG specify glutamine in ciliates instead of stop signals in bacteria. UAA and UAG in the original sequences of two serine carboxypeptidase genes (*sp1* and *sp2*) and two cysteine protease genes (*cp1* and *cp2*) were replaced with CAA and CAG codons for amino acids, and modified for expression for *Escherichia coli* by Eurofins Genomics (Tokyo, Japan). We obtained modified-DNA of target genes that were inserted into pEX-A2J2 vector from Eurofins Genomics (Fig. 2.6).

Recombinant proteins expression

For expression of four proteases in *E. coli*, the partial sequences (*sp1*_partial (p), *sp2*_p, *cp1*_p, and *cp2*_p) of the optimized protease genes were amplified from the DNA vectors by PCR using specific primers (Table 2.4). These primers contain restriction sites to insert the amplified fragments into the corresponding cloning sites in the pET-26b or pET-28b expression vector (Novagen). PCR amplification was performed as follows using KOD one PCR enzyme (TOYOBO, Shiga, Japan): 35 cycles of 98°C for 10 sec, 60°C for 5 sec, and 68°C for 1 sec. Each amplified fragment was extracted from agarose using Fastgene Gel/PCR extraction kit (NIPPON genetics, Tokyo, Japan), digested with restriction enzymes (BamHI and EcoRI, BamHI and XhoI, or EcoRI and XhoI) and ligated into pET-26b or pET-28b, respectively. These final vectors (pET-26b-*sp1*_p, pET-26b-*sp2*_p, pET-26b-*cp1*_p, and pET-28b-*cp2*_p) were sequenced by Eurofin to confirm that the target sequences were correctly inserted.

These constructs for four genes were transformed into *E. coli* BL21 (DE3) cells, and colonies that grew on LB plates containing kanamycin (30 µg/mL) and contained the final vectors were isolated. Isolated clones were cultured in 200 mL of LB broth containing Kanamycin (30 µg/mL) at 37°C with agitation (180 rpm) until an optical density at 600 nm (OD₆₀₀) of 0.5–1.0 were obtained. Then, isopropyl β-D-1-thio-galactopyranoside (IPTG) was added to a final concentration of 1 mM to induce protein expression, and the cultures were further incubated for 5 h in the same condition.

The cultured cells were collected by centrifugation, washed 3 times by phosphate buffered saline (PBS), and resuspended in 10 mL of 8M Urea/PBS. The cell suspensions were lysed by sonication (twice for 30 s on ice), and incubated overnight at 4°C. Then, the suspensions were centrifuged at 15, 000 g for 30 min at 4°C and the each supernatant containing the target recombinant proteins were collected.

Recombinant protein purification

The affinity chromatography was performed under denaturing conditions employing TALON Metal Affinity Resin (Clontech, Palo Alto, CA, USA) according the manufacturer's protocol. Briefly, the collected supernatant from each culture was added to the TALON resin, and mixture was gently agitated with a rocking shaker (BCMe700S) (Bio Craft, Tokyo) for 60 min. Then, the resin was washed three times with 10 bed-volume of 8M urea/PBS pH 7.4, 5 mM imidazole. Recombinant protein was eluted with 5 bed-volume of 8M urea/PBS pH 7.4, 150 mM imidazole.

The mixture was gently agitated for 30 min, centrifuged at 700 g for 2 min, and obtained the supernatant that contained target recombinant protein. The recombinant protein purified by affinity chromatography were dialyzed in a number of steps, ending with PBS, pH 7.4. The purified polypeptide was concentrated using Amicon ultra centrifugal filter units (Millipore), according to the manufacturer's protocol.

Protein gel electrophoresis and Western blot analysis

The purified recombinant proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and Western blotting.

Each 6.5 μ L of the purified protein solution, containing 3 μ g of the recombinant protein, was mixed with 2.5 μ L of 4 \times NuPAGE™ LDS sample buffer (Thermo Fisher Scientific Inc, MA, USA) and 1.0 μ L of NuPAGE™ Sample Reducing Agent (10X) (Thermo Fisher Scientific Inc), and heated at 70°C for 10 minutes. The samples (3 μ g of recombinant protein) were loaded into wells of a 12% acrylamide gels for SDS-PAGE at 200 V for 45 minutes (SDS BioRad Mini-PROTEAN Tetra System). The SDS gels were stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes in a semi-dry transfer chamber (ATTO WSE-4020) at 153 mA for 30 minutes. The membrane was blocked with 5% skim milk for 2 h at 25°C. After washing with PBS plus 0.1% Tween 20 (PBS-T) three times, the membrane was incubated overnight in 5% skim milk containing anti-6 \times His monoclonal antibody (Wako chemicals, Osaka, Japan) as first antibody at 1:10,000 dilution at 4°C. Next day, the membrane was washed with the PBS-T, incubated with a 1:5,000 dilution of anti-mouse IgG monoclonal antibody HRP conjugate (Proteintech, Tokyo, Japan) as secondary antibody with gentle shaking for 1h at 25°C, and washed again. Chromogenic detection was carried out using Ez West Blue (ATTO, Tokyo, Japan) as an HRP detection substrate.

Fish immunization and challenge experiments

Immunization

Naïve fingerlings of the Tiger puffer (*Takifugu rubripes*) were purchased from Marinetech Co. Ltd (Aichi, Japan). These fish were kept in a 500 L recirculation tank equipped with a biological filter at 25°C and 30 ppt salinity and fed daily with commercial feed until satiation.

The concentrations of recombinant proteins dissolved in PBS was quantified using BCA Protein Assay Kit (Takara) following manufacturer protocols. Each protein solution was dissolved in PBS to give desired concentrations and conjugated with the same volume of Freund's complete adjuvant (Wako chemicals) by repeated passage through a double-hubbed emulsifying needle until a stable emulsion was formed. The concentration of protein in each conjugate was adjusted so that the dosage of 1 μg protein/g mean fish weight when 100 μL of conjugate was injected as vaccine to each fish.

Efficiency of immunization with injection of the recombinant proteins of target genes were examined twice. In first trial, naïve tiger puffer (mean weight 6.0 g, $n = 20/\text{group}$) were anesthetized in 10 L seawater containing 5 mL of 2-phenoxyethanol. Each fish was injected interperitoneally with 100 μL vaccines containing 6.0 μg of sp1_p, sp2_p, cp1_p, or cp2_p depending on groups. Fish were injected with PBS, or a conjugate of PBS and Freund's complete adjuvant in control groups. Immunized fish were kept in the 500 L recirculation tank and fed every day.

After two weeks from first vaccination, five fish from each group were anesthetized, and the blood were collected from the hepatic vein using a 1 mL syringe and 25 G needle. Blood from each fish was stored at 4°C overnight, and centrifuged at 12,000 \times g for 5 min at 4°C. Then, sera were collected and stored at -80°C for ELISA assays to investigate the antibody titer against the antigens.

Remaining fifteen fish were weighed for boost immunization. The amount of protein in 100 μL vaccines was changed to 11.5 μg , because the mean body weight of fish increased to 11.5 g. The fifteen fish were injected with the same vaccine used for the first immunization as the booster according to the same protocol for the first immunization. Immunized fish were fed and kept under the same conditions used after the first immunization.

Two weeks after the boost immunization, fish blood was collected from five fish from each group, from which sera were obtained and preserved with the same protocols used after the first immunization for ELISA assays. For challenge experiment, eight to ten fish from each group were gathered together and transferred into an aquarium (90 \times 45 \times 45 cm) equipped with an overhead biological filter containing 150 L seawater (salinity 35‰) at 25°C for challenge. After acclimating the fish for three days, 57 fish in total were challenged by 28,500 theronts (500 theronts/fish) in the aquarium in the dark at 25°C with the water flow stopped for 6 h. The challenged fish were

maintained in the aquarium at 25°C. After 66 h, each challenged fish was individually placed in a 3 L plastic aquarium containing 2 L seawater supplemented with antibiotics (100 IU/mL penicillin G potassium and 100 µg/mL streptomycin sulfate). Parasites that left the fish as protomonts from 3 to 5 days after exposure were counted from the bottom of the aquaria.

In the second trial, which was carried out to confirm the reproducibility of the result obtained in the first trial, the procedures of the immunization and challenge experiments were the same as those in first trial, except that the amount of protein in the vaccine conjugates were changed according to the weight of fish. In first immunization, fish were immunized with interperitoneally injection of vaccines containing 18.6 µg of proteins in 100 µL, because mean weight of fish was 18.6 g. In boost immunization, fish were immunized with the vaccines containing 22.5 µg of proteins, because mean weight of fish was 22.5 g. For challenge experiment, 57 fish in total were challenged by 28,500 theronts (500 theronts/fish) as described above.

Enzyme-linked immunosorbent assay (ELISA)

To investigate antibody titers in the sera of fish immunized with the recombinant proteins, ELISA was carried out according to Lokanathan (2016), in which antibody against recombinant proteins of *C. irritans* in *Lates calcarifer* was assayed, with slightly modification for present study.

Recombinant proteins of the four target proteases were diluted at 10 µg/ml in carbonate buffer (0.05 M Na₂CO₃ and 0.05 M NaHCO₃, pH 9.6) and 100 µL of the diluted protein solution was added to each well of 96-well microtiter plates. The plates were incubated overnight at 4°C to coat the wells with the recombinant proteins. On the next day, after the well content being discarded, 100 µL of 5% skim milk in PBS was added and incubated for an hour at 37°C to block non-specific binding sites. The plates were washed three times with PBS plus 0.1% Tween 20 (PBS-T). Then, 50 µL of fish sera, which were diluted 100 times with 1% skim milk in PBS and incubated in advance for an hour at 37°C to reduce nonspecific reactions of antibodies, was added to each well and incubated for 2 h at 37°C. For the negative control, 50 µL of 1% skim milk in PBS was added to well instead of the diluted fish sera. The plates were washed five times with PBS-T, and 50 µL of anti-tiger puffer IgM monoclonal antibody (kindly provided from Dr. Hiroaki Suetake, Fukui Prefectural University, Fukui, Japan) diluted 1,000 times with 1% skim milk in PBS was added to each well and incubated for an hour at 37°C. The plates were washed five times with PBS-T, and 50 µL of goat anti-mouse IgG HRP conjugate (Proteintech, Tokyo,

Japan) diluted 10,000 times with 1% skim milk in PBS was added to each well and incubated for an hour at 37°C. After the plates being washed 10 times with PBS-T, 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) solution (Wako chemicals) was added to each well and incubated at 25°C for 30 min in the dark for color development. After the incubation, the color development was stopped by addition of 100 µL of 1M H₂SO₄. Absorbance of each well at 450 nm were measured with a plate reader (iMark, Bio-Rad).

In addition, to investigate the cross-reactivity of the antibody in the sera of fish immunized with the recombinant proteins (2 weeks after the boost immunization in the second trial) against different strains, ELISA was carried out using lysates of two different isolates of *C. irritans*. Sonicated *C. irritans* theront homogenates of each isolate were diluted in carbonate coating buffer at 10 µg protein/mL, and 100 µl of the homogenate was added into each well of a 96-well ELISA plate and incubated overnight at 4°C to coat the well with proteins of *C. irritans* theronts. After this step, ELISA was carried out with the same measures used for detection antibody against recombinant proteins. As a control, the sera of fish inoculated with PBS mixed with adjuvant were used.

Statistical analysis

Numbers of parasites recovered from fish in the challenge experiment and absorbance in ELISA were analyzed using Kruskal–Wallis followed by Steel–Dwass test. 0.05 was used as the significant level in the analyses.

Results

Amino acid sequences of i-antigen of UT1 and K1

Partial amino acid sequences of the i-antigen of UT1 (295 residues) and K1 (285 residues) were aligned and compared using Clustal Omega program (<https://www.uniprot.org/align/>) (Fig. 2.7). Sequence identity of these amino acid sequences was 62.8%. Blastx search showed that the both i-antigen of UT1 and K1 sequences had the highest identity to i-antigen of isolate of Japan (BAG16623), with values of 80% and 77%, respectively.

The expression of *C. irritans* recombinant proteins

All of the target proteins were expressed in insoluble fractions (pellet after sonication). After solubilization in urea, purification by TALON resin, and dialyzation, these recombinant proteins became soluble proteins. By the SDS-PAGE and Western blots, it was confirmed that target recombinant proteins were correctly expressed and purified (Fig. 2.8). The molecular weights of recombinant sp1_p, sp2_p, cp1_p, and cp2_p were estimated as 25.4 kDa, 26.5 kDa, 19.9 kDa, and 22.8 kDa respectively, close to the molecular weights expected from amino acid sequences (27.9 kDa, 29.6 kDa, 16.9 kDa, and 23.8 kDa, respectively). The yields of these proteins purified from 200 mL bacterial culture were 1.0-3.0 mg.

Challenge experiments

In the first trial, the numbers of parasites recovered from fish immunized with PBS, PBS with adjuvant, sp1_p, sp2_p, cp1_p, and cp2_p groups were 134.0 ± 27.8 (mean \pm SD), 132.2 ± 27.4 , 104.7 ± 26.9 , 121.0 ± 17.2 , 110.8 ± 31.7 , and 85.9 ± 25.8 , respectively (Fig. 2.9 A). The mean numbers of those parasites recovered from the immunized groups were lower than those of the control groups (PBS and PBS with adjuvant). Especially, the number of parasites recovered from fish immunized with cp2_p was significantly lower than those from the control groups ($p < 0.05$).

In the second trial, the numbers of parasites from fish immunized with PBS, PBS with adjuvant, sp1_p, sp2_p, cp1_p, and cp2_p were 74.7 ± 23.6 , 71.0 ± 13.4 , 57.3 ± 13.8 , 61.6 ± 15.8 , 55.4 ± 17.0 , and 46.1 ± 12.2 , respectively (Fig. 2.9 B). Similarly to the first trial, the mean numbers of parasites recovered from fish immunized with the *C. irritans* recombinant proteins were lower than those from the control fish. Especially, the numbers of parasites recovered from fish immunized with cp2_p was significantly lower than those from the control fish ($p < 0.05$).

Antibody titer

In each immunized group, the sera obtained from fish immunized with each recombinant protein showed high degree of antibody response against the protein at 2 weeks post the first immunization and 2 weeks post the boost immunization (Fig. 2.10). The antibody titer was significantly higher in the serum of the immunized fish than in that in naïve fish (control) ($p < 0.05$). The sera of fish immunized with each recombinant protein showed reaction to sonicated theronts of both UT1 and K1 as well, with significant difference from the sera from fish inoculated with a conjugate of PBS and adjuvant (Fig. 2.11).

Section 2. 4

Discussion

In section 2.1, gelatinolytic activity was found in three bands (approximately 30, 40, and 70 kDa) in the zymography of trophonts (Fig. 2.1). The inhibition of 70 kDa band activity by Leupeptin (inhibitor of serine and cysteine proteases), but not E-64 (inhibitor of cysteine proteases), EDTA (inhibitors of metalloproteinase), or Pepstatin A (inhibitor aspartic proteases), indicated that the band represented a serine protease. The inhibition of 30 and 40 kDa activity by E-64 and Leupeptin indicated that the bands represented cysteine proteases. These results indicate that trophonts actually possess serine and cysteine proteases, supporting the results of Mo et al. (2016), who showed that serine and cysteine protease genes were expressed at the trophont stage of *C. irritans*.

Given that seawater is alkaline and saline, the high activity of the serine protease of 70 kDa in neutral to weakly alkaline conditions (pH 7.0–8.0), and at high salinities (3.5%), suggests that the serine protease might be most effective in seawater. Theronts swim in seawater and invade host tissue; therefore, this protease would be involved in the invasion. However, the proteolytic activity of theronts was not examined. In contrast, trophonts of *C. irritans* harbor host tissue where osmolality is comparable to that in the 0.9% NaCl solution, and phagocytes (food vacuoles) become acidic during phagocytosis. The high activity of 30 and 40 kDa cysteine proteases under acidic conditions (pH 4.0–5.0) and low salinities (0 and 0.9 % NaCl) suggest that cysteine proteases are most effective in the phagocytes of trophonts in host tissue, where osmolality is comparable to that of the 0.9% NaCl solution. However, further research is necessary to elucidate the underlying mechanisms. In addition, because we focused on particularly active proteases in this study, we characterized only three proteases. According to Mo et al. (2016), trophonts expressed more than one hundred protease genes. When the incubation time of zymography was extended, numerous bands showing protease activity appeared in the gels (data not shown). Thus, it might be necessary to focus on more proteases to understand their function in this parasite.

In *in vitro* assay using protease inhibitors, it is suggested that proteases have important functions in survival and growth of the parasite by the significant decreasing in survival and the dose-dependent delay of growth in trophonts in media supplemented with the protease inhibitors

(E-64, leupeptin, and Pepstatin A). The significant differences in survival were mostly evident at the beginning of the *in vitro* assay. The percentage of survival between the inhibitor group and the control group were almost identical at the end of the experiment (Fig. 2.2 A, C, E). As all the inhibitors (E-64, leupeptin, and Pepstatin A) were peptides, they might have gradually denatured and lost activity over the course of the experimental period.

In *in vivo* assay, it is suggested that cysteine proteases contribute to the infection of the parasite, because the lower recovery of the parasites from fish challenged with theronts in seawater supplemented with E-64 (cysteine proteases inhibitor). As the theronts were only exposed to the protease inhibitors during the invasion period, the cysteine type proteases might have been involved in the invasion of the host. However, the results of zymography suggested that the 30 and 40 kDa cysteine proteases were more active in the phagocytes of the parasite than the seawater. The lack of consistency might suggest that the cysteine proteases detected in the zymography differed from that inhibited by E-64 in the challenge experiment. These results indicate that there are cysteine proteases that were not detected by the zymography but are related to theront invasion.

Leupeptin also acts as an inhibitor of cysteine proteases, but it did not significantly affect the number of parasites that were recovered. Narasaki et al. (2018) reported a similar phenomenon, in which the *in vitro* proliferation of *M. avidus* was suppressed in a medium supplemented with E-64, but not one supplemented with leupeptin. E-64 inhibits cysteine proteases by binding directly to their active site, whereas leupeptin is a competitive transition state inhibitor; thus, the lack of consistency between the results for E-64 and leupeptin might be attributed to a difference in their inhibition mechanisms.

In present study, it is clear that trophonts actually possess serine and cysteine proteases, and inhibitors of serine and cysteine proteases suppressed the survival and growth of *C. irritans* *in vitro*, as well as the infection, and probably invasion, of the parasite *in vivo*. These results strongly support the view that proteases play important roles in the infection process, including invasion and food digestion of *C. irritans*. Similarly, proteases are important for the successful invasion by merozoite of the erythrocytes of host *Plasmodium knowlesi* and *P. chabaudi* (Breton et al., 1992; Hadley et al., 1983; Greenbaum et al., 2002). Serine and cysteine proteases are necessary for the lysis of host cells and the nutrient uptake in the fish ciliate parasite *M. avidus* (Narasaki et al., 2018). The protozoan, *Perkinsus marinus*, infects oysters and produces a serine protease that is capable of digesting the connective tissue of oysters by degrading extracellular matrix proteins

(La Peyre, 1996). In addition, according to Mehdi (1991) and Hayashi et al. (1991), E-64 and leupeptin have low membrane permeability; thus, these inhibitors do not penetrate the plasma membrane or the vacuole membrane to access the plasma. Therefore, these inhibitors may mainly inhibit extracellular proteases or those in the food vacuole of the parasite. Considering these reports and results of present study, it is suggested that the serine and cysteine proteases of *C. irritans* are important for the infection process and could be target molecules for the development of chemotherapy and vaccinations against cryptocaryoniasis.

However, due to the difficulty in recovering enough parasites, the proteases detected by zymography could not be purified. Therefore, the transcriptome analysis and RNAi were carried out to select target protease genes in section 2.2, because it is considered that proteases highly expressed in theronts and trophonts are important factors for the parasite infection.

In section 2.2, RNA interference was achieved for the first time for *C. irritans* by the delivery of dsRNA using the electroporation technique. It is considered that the RNAi method can be used for functional analysis of various genes of *C. irritans*, such as identification of pathogenic factors. The range of suppression of gene expression was 13–47% in present study. It is known that the length, concentration, and sequence of exogenous dsRNA are important for effective RNAi in insects (Bolognesi et al., 2012; Miller et al., 2012; Li et al., 2011). To make the RNAi method better, it is necessary to modify the methods considering these factors.

Suppression of activities of proteases and peptidases by the RNA interference method reduced the number of infecting *C. irritans*. This result indicated that the target-proteases play important roles in the infection processes, including invasion and development of *C. irritans*, suggesting that inhibition of the activity of proteases and peptidases with vaccination and chemotherapies targeting them may prevent the infection. In *Plasmodium falciparum*, which causes malaria in humans, a cysteine protease is known to be involved in the invasion and development of the parasite, and inhibition of the cysteine protease prevents the parasitic invasion and development. Therefore, the cysteine protease has been suggested as a potential target for antimalarial drugs (Schrevel et al., 1988; Mayer et al., 1991). Similarly, the proteases and peptidases of *C. irritans* could be target molecules for the development of chemotherapy and vaccination against cryptocaryoniasis. In addition, we focused on only four genes in present study, but some other proteases and peptidases were also highly expressed in theronts and trophonts. These other genes are considered also important in the invasion and parasitic stages, and might be important

candidates for drug and vaccine development. Research focusing on those genes are necessary as well to develop new control methods.

In section 2.3, fish were successfully immunized with interperitoneally injection of recombinant protein of cp2 of *C. irritans*. Dalton et al (1996) reported that vaccines against secreted proteases of the liver fluke of the cattle and sheep, *Fasciola hepatica*, reduced the parasite counts, fecal egg counts, and egg viability, speculating the proteases were involved in tissue penetration, nutrition, and protection from the host's immune system. Sajid and Mckerrow, (2002) suggested vaccination with proteases may be effective in general against parasitic organisms in their review on parasites proteases. The protease cp2 can be a good candidate as an antigen for further development vaccination against *C. irritans* similarly to proteases against other parasites, although the efficacy as vaccination with the protease was limited; the reduction of parasite recovery by vaccination with cp2 was 34.1% and 37.4% in the present challenge experiments. Vaccination with other three proteases showed reduction of parasite recovery although the numbers of recovered parasite was not significantly different from those in the controls, and induced the significant production of antibodies against them, as well as vaccination with cp2. Other three proteases still remain as antigen candidates for vaccination, as use of other vaccination methods such as DNA and RNA vaccinations may improve the efficacies.

The proteases of *C. irritans* were highly expressed in trophonts and theronts and predicted as an extracellular protease. The protease can be considered to be secreted outside of the parasite cells and to play important roles in invasion, feeding and/or nutrient uptake in the vacuoles of *C. irritans*. The parasite takes up surrounding body fluids, tissue debris, and whole cells from the host fish after transferring to trophonts (Coloni, 1985). In *I. multifiliis*, the freshwater counterpart of *C. irritans*, host fish IgM was found inside organelles of trophonts. This indicates that trophonts of *I. multifiliis* are able to ingest antibodies of host fish (Cross 1993; Cross and Matthews 1992, 1993; Jørgensen et al., 2011). *C. irritans* also may take up host antibodies induced by the vaccinations. Therefore, if the proteases are expressed in the food vacuoles of the parasite, the interruption of the function of the proteases in food vacuoles via the action of neutralizing antibodies is not surprising. The antibodies induced by the vaccination with the proteases may have disturbed functions of target proteases inside food vacuoles of the parasite, which can be one explanation of reduction of parasite recovery in immunized fish. However other mechanisms including cellular immunity may be involved the acquired protection. To understand the function

of the proteases and mechanism of the acquired immunity with the proteases will assist in future vaccine development against *C. irritans*, which can be a target for further researches on the parasite and vaccine developments.

Previous researches for vaccine development against *C. irritans* have focused on i-antigen of the parasite. However, the i-antigen is known to be highly diverse, and the epitopes much vary among isolates or strains of the parasite, similarly in many other ciliates. Therefore, i-antigens currently considered to be an appropriate target for the development of vaccines (Misumi et al., 2011). In this study, the sera of fish immunized with the protease cp2 of UT1 isolate also reacted to the theronts of another K1 isolate, suggesting that cp2 is a common antigen for different isolates. Further studies are needed to clarify the uniformity or diversity of cp2 and other proteases of *C. irritans* as vaccine antigens.

In fish vaccinated with the protease antigens, the titer of IgM against the antigens increased. IgM in the peripheral blood of the vaccinated fish was considered to be transported or diffused from the blood to the epithelial tissues, bind to the target proteases of *C. irritans*, and inhibit its function. However, the cutaneous IgT produced in lymphoid tissue of the skin epithelium bound to trophonts of *I. multifiliis* in rainbow trout (*Oncorhynchus mykiss*) immunized against the parasite and the binding of IgT was not observed in naïve fish (Jørgensen et al., 2011; Buchmann, 2019), suggesting the potential role of IgT in the immunological reaction to the parasite. In the study, IgT and other immunological agents such as cellular defense as well IgM may have been involved in the reduction of parasite recovery and further studies on their mechanisms of acquired protection in immunized fish are required.

The effects of vaccination with the proteases were limited to with the levels of protection ranging from 8.3–34.1% (first trial) and 13.2–37.4% (second trial) compared with a control group (a conjugate of PBS and adjuvant). A significant difference was observed only in fish vaccinated with cp2. One of the reasons for the low efficacy may be the presence of plural proteases having a similar function. One third at least of eukaryotic genomes are considered to comprise of genes having similar functions (Rubin et al. 2000; Li et al. 2001). Loss one gene can be tolerated because others having similar function can buffer the loss (Conant and Wagner, 2003). According to the present result of the RNA-seq, many types of cysteine protease and serine carboxypeptidase are expressed in theronts and trophonts. Some of the protease genes were annotated with the same genes that were targeted by the vaccine in this study. Therefore, there is a possibility that these

similar genes complemented the functions of the vaccine targets, and lowered the effect of vaccinations in this study.

Degradation of antibodies of the host by parasite cysteine proteases has been documented in *Trichomonas vaginalis*, *T. foetus*, *Entamoeba histolytica*, *Giardia muris*, *G. lamblia*, *Trypanosoma cruzi*, *Gymnorhynchus gigas*, *Haemonchus contortus*, *Fasciola hepatica*, *F. gigantica*, *Taenia solium*, *T. crassiceps* and *Spirometra* (Sajid and Mckerrow, 2002). The immunoglobulins of immunized fish might be degraded by *C. irritans* proteases, and the parasite evaded the immune response of fish. This might be one of other for limited effects of the vaccines in the present study.

Table 2. 1

Primers used to amplify target genes.

Primers	Sequence 5'-3'	bp
5'_RACE_sp1	ATCCAGTTCCAATTGGTTAGTCAACATA	26
3'_RACE_sp1	GCCTTGCACTGTGCCTGGATTATGC	25
5'_RACE_sp2	TTAAAATTGTCTTTATCTGTAAATCCA	28
3'_RACE_sp2	TGCAACTGGAGAGGAACCGAGGAATG	26
5'_RACE_cp1	ATGCCAGATCTTCTATCTATGCATTT	26
3'_RACE_cp1	ATTCGAAACAAGGGATGTGAAGGAGGA	28
5'_RACE_cp2	TGTCTTGAAAATTCTTTATCGTCTCCAT	28
3'_RACE_cp2	AGTTGGGCAGCAAATGGTTGGTGT	26
T7_sp1F	TAATACGACTCACTATAGGGCTATAACTTTAGTTTAGTTGAT	42
T7_sp1R	TAATACGACTCACTATAGGGGCACATCCATATTGCAACTGCTC	43
T7_sp2F	TAATACGACTCACTATAGGGAAAAATAGAAAAATTTTATAATAGG	46
T7_sp2R	TAATACGACTCACTATAGGGAAATTTAATCCTTCAATGTTTCGTG	45
T7_cp1F	TAATACGACTCACTATAGGGAAAGAATGTAAAAATCTTAAATG	43
T7_cp1R	TAATACGACTCACTATAGGGAAACCACATTACTATTAGTCTT	41
T7_cp2F	TAATACGACTCACTATAGGGTATAGGAAAATAATATAATAGAG	43
T7_cp2R	TAATACGACTCACTATAGGGCAGTAATAAAATTTCCAAAAATC	43
T7_β-lacF	TAATACGACTCACTATAGGGTGAGGCACCTATCTCAGCGATCTGTC	46
T7_β-lacR	TAATACGACTCACTATAGGGGGCCAGATGGTAAGCCCTCCCGTATC	46
qF_sp1	CTTGCACTGTGCCTGGATTAT	21
qR_sp1	CAATCTCCCATTAACGCAGAA	21
qF_sp2	AATGGCTTGAAAAATTCCTCT	22
qR_sp2	CATTTCTATAGCAGCCCCCTT	22
qF_cp1	TGAAGAAGCCTGAAGGTCCA	20
qR_cp1	GTAGAGGAAAATGCCCAGCA	20
qF_cp2	TTTTCAAGACACAACCCAAACA	22
qR_cp2	GTGCTCCTGTCTTGCATCAT	21
qF_EF-1β	CGATGGAAGGTTTAGTTTGAA	22
qR_EF-1β	ACTTCATCTTCCCATGCCAAT	21
sp1_pF	GGGGGATCCGCAGTTTGAGTTGAAGGAATGTCAG	34
sp1_pR	GGGGAATTCAGGATTTTGTACTTCATCTTTGACAC	35
sp2_pF	GGGGGATCCGTACGATATTCAGTTCAACCAGTGC	34
sp2_pR	GGGGAATTCTTATAGCTCTGAATAGGTCGGTTG	33
cp1_pF	GGGGATCCCAAGTACATTGGGGAAGAGGAGAAG	34
cp1_pR	GGGCTCGAGAGCTACGGGATAGGATGCCCCATC	33
cp2_pF	GGGGAATTCAGAAGAAGCGAGTAGCATCTCAG	32
cp2_pR	GGGCTCGAGTATGAGCCACCTGGTGGTATAG	31

Table 2. 2

Summary of *C. irritans* transcriptome produced by Illumina Hiseq 4000 sequencing

	Theront	Trophont	Tomont
Sequencing			
Raw reads (Pair-end)	91275682	84720088	84387764
Clean reads (Pair-end)	87315480	79341214	79268634
Clean reads ratio	93.93%	95.66%	93.65%
Assembly			
Number of unigenes		85033	
Total length (bp)		72092934	
N50 length (bp)		1080	
Mean length (bp)		848	
Largest transcripts (bp)		14959	

Table 2. 3

The ratio of successfully annotated genes

	Number of unigenes	Percentage (%)
Annotated in NR	28977	34.07
Annotated in NT	34209	40.23
Annotated in KO	10131	11.91
Annotated in SwissProt	19603	23.05
Annotated in PFAM	14221	16.72
Annotated in GO	16289	19.15
Annotated in KOG	13508	15.88
Annotated in all Databases	1837	2.16
Annotated in at least one Database	57135	67.19
Total Unigenes	85033	100

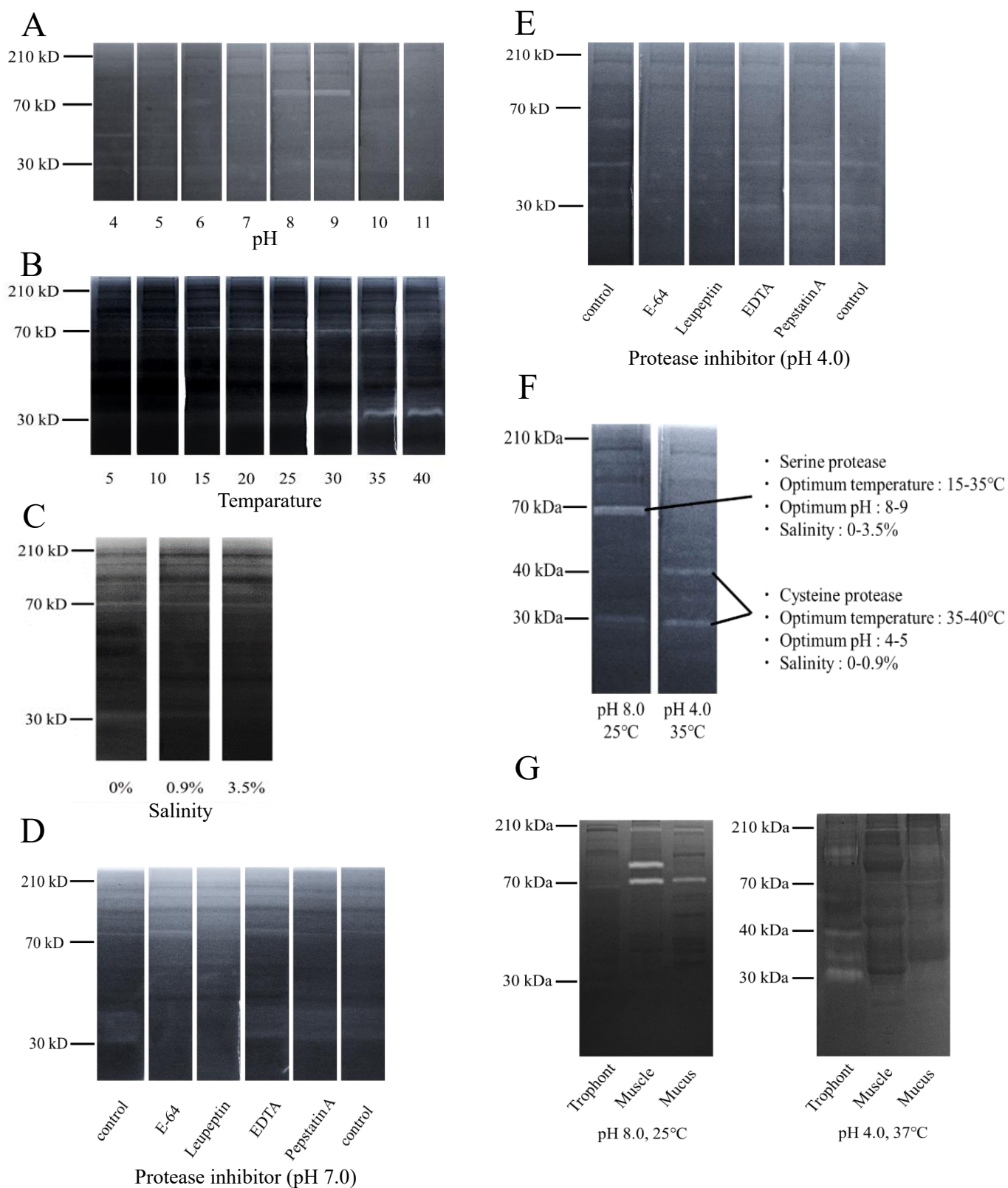


Fig. 2. 1. Gelatinolytic activity in trophont homogenate and the effects of pH, temperature, and protease inhibitors. (A) Effects of pH on gelatinolytic activity in trophonts. (B) Effects of temperature on gelatinolytic activity in trophonts. (C) Effects of salinity on gelatinolytic activities in trophonts. (D) Effects of protease inhibitors on gelatinolytic activity in trophonts at pH 7.0. (E) Effects of protease inhibitors on gelatinolytic activity in trophonts at pH 4.0. (F) Summary of gelatinolytic activity in trophont homogenate. (G) Gelatinolytic activity in trophont homogenate and muscle homogenate of tiger pufferfish and mucus.

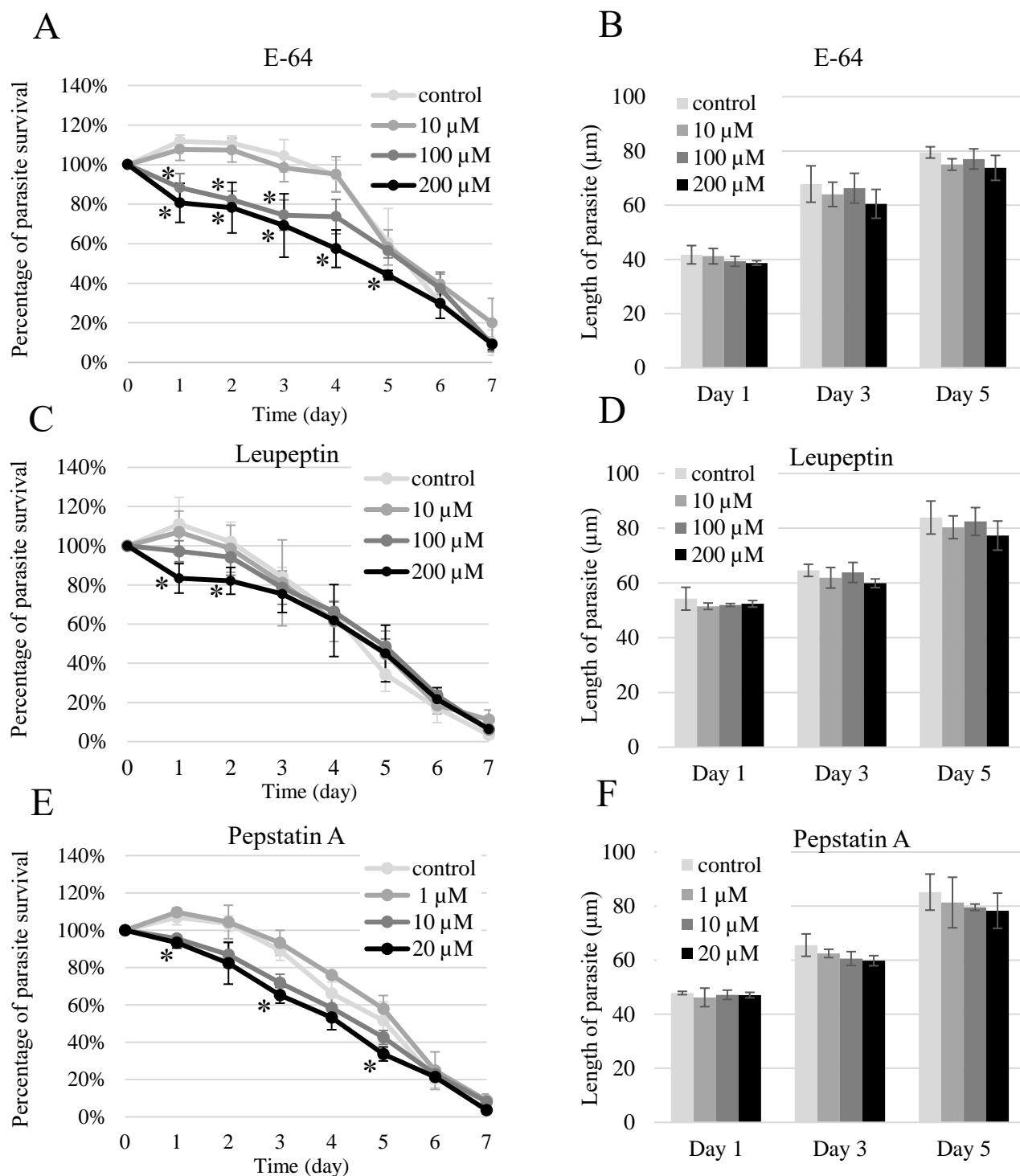


Fig. 2. 2. Effects of protease inhibitors against survival (A, C, E) and growth (B, D, F) of *Cryptocaryon irritans* in vitro. (A, B) E-64 supplemented group. (C, D) Leupeptin supplemented group. (E, F) Pepstatin A supplemented group. Mean values of the percentage of parasite survival and parasite length are plotted with the standard deviation (vertical bars). Asterisk indicates a significant difference ($p < 0.025$) in one-sided Shirley-Williams test.

A : *spl*

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1 ATG AAA GTT TTT ATC TGC ACT TTA TTA GCT TTA ACT TCT GTT CTA GCC AAT CCC GAC ATA TTT TTG AAT GAA GAT CAA CAT CCT GGA TTT
1 M K V F I C T L L A L T S V L A N P D I F L N E D Q H P G F

91 GTC AAA TTA AAA GAT AAG GAT GAT TTG TTT TTT TGG TTG TTT AAA TCT AGA AGT GAT AAA AAC TCT GAT CCT TTG GTA ATT TGG CTA ACT
31 V K L K D K D D L F F W L F K S R S D K N S D P L V I W L T

181 GGG GGA CCA GGT TGC TCT TCA GAA TTG GCT ATA TTT TAT GAA AAT GGT OCT TTT TCA ATC AAC TAA AAT CTA ACC CTA AAA AAA AAT AAA
61 G G P G C S S E L A I F Y E N G P F S I N Q N L T L K K N K

271 TAC TCT TGG AAT GAA AGA ACC AAC TTA TTA TAT GTT GAC TAA CCA AIT GGA ACT GGA TTT TCT CAT GGG GAA AAA GAT TTT GTA CAT
91 Y S W N E R T N L L Y V D Q P I G T G F S H G E K K D F V H

361 AAT GAA ACC ATG GTA TCA AAA GAT TTT TAT TAG TTC ATG GAA GGA TTT TTA GAA TAA TTC OCT GAG TAT AAA GGT AGA GAT CTT TAT AIT
121 N E T M V S K D F Y Q F M E G F L E Q F P E Y K G R D L Y I

451 ACT GGA GAA TCT TAT GCA GGA CAT TAT GTT CCG GCT AIT TCT GCT TAT CTT CAT TAG AAA AAA AAT CCT GAT AIT AAT CTC AAA GGC TCA
151 T G E S Y A G H Y V P A I S A Y L H Q K K N P D I N L K G S

541 GCA ATA GGA AAT GGA CTT GTT AAT OCT TAT TTA CAA TAT CCA GAA TAT GCT ACT TTT GCA TAT GAA AAT AAA TTG GTT TCT AAA ATG AAA
181 A I G N G L V N P Y L Q Y P E Y A T F A Y E N K L V S K M K

631 TAC AAA ATT TTG CAA TTT GAA TTA AAA GAA TGC CAA AAA AAA ATA AAA AGT GGA GAT TGG GAT AGA GCA TTA ATA ATA TGT AAT CTT CCT
211 Y K I L Q F E L K E C Q K K I K S G D W D R A L I I C N L P

721 GTG GAA TAA AIT TTA GGA AAT OCT CAA AAA TTT AAT ACC TAT GAT ATA AGA AAG OCT TGC ACT GTG OCT GGA TTA TGC TAT AAC TTT AGT
241 V E Q I L G N P Q K F N T Y D I R K P C T V P G L C Y N F S

811 TTA GTT GAT AAA TTT TTG GCA AGA GAA GAT GTT ATA GAA GCT TTA AAT GTT AAA GGA AGA AAA TGG AGC AGT TGC AAT ATG GAT GTG CAT
271 L V D K F L A R E D V I E A L N V K G R K W S S C N M D V H

901 TCT GGG TTA ATG GGA GAT TGG ATG AGG AAT TTA TCA TAA AAA GTC AIT TAT TTG TTG AAA AAT GAT AIT AAA GTT CTT ATA TAT GCT GGA
301 S A L M G D W M R N L S Q K V I Y L L K N D I K V L I Y A G

991 GAC AAA GAT TTC ATA TGC AAC TGG AGA GGA AAT GAA AAA TGG GTA AAT GAA TTA ACT TGG TTC TAT AAA GAA TAA TTT AAA AGT CAA TAA
331 D K D F I C N W R G N E K W V N E L T W F Y K E Q F K S Q Q

1081 TAC TAA GAG GTA TTT AIT GAC AGC AAA AGC TAT GGA AAA AIT AAG TTT TAT AAG AAT TTC GCA TTC TTC AGG GTT TAC GAA GCT GGG CAT
361 Y Q E V F I D S K S Y G K I K F Y K N F A F F R V Y E A G H

1171 ATG GTT CCT ATG GAT TAA CCA ATG GCA GCT TTG AAG ATG CTT GAC CAT TTT AIT TAC AAG TGG TAG OCT AAT TCA ATA GAT TAT AAA TAA
391 M V P M D Q P M A A L K M L D H F I Y K W Q P N S I D Y K Q

1261 TCG AAA AAA AIT GTT ACT GAA TGA
421 S K K I V T E *
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B : *sp2*

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1 ATG AAT TAC AAT GCA TTT AAG GTT ATG CTT TTA GTT GCT TTT TTA AIT AAT AIT TTG CAA ACA ATA TCT CAT TCT OCT TTT TAA AGT AGT
1 M N Y N A F K V M L L V A F L I N I L Q T I S H S P F Q S S

91 TCT CAT GGT TTT AIT CCT TTA TCA GAT ACA TCA GAT ATA TTT TAT TGG CTG TTT GAT TCC TAA TCA TAA CCA AAT ACA GAT CCC CTT GTA
31 S H G F I P L S D T S D I F Y W L F D S Q S Q P N T D P L V

181 ATA TGG CTG TCT GGT GGT CCC GGT TGT GCT TCA ACT TTT GCT TTA TTT TAT GAA AAT GGA CCT TAC AGT TTA AAT TAA AAT TTG TCT TTA
61 I W L S G P G C A S T F A L F Y E N G P Y S L N L S L

271 TCT GTA AAT CCA TAT TCA TGG AAT TAA TAA GCA AAT ATA ATG TAT GTT GAT CAA CCA AIT GGT ACA GGA TTC TCT AAA TCA AGT GAA TAA
91 S V N P Y S W N Q Q A N I M Y V D Q F I G T G F S K S E Q

361 GAA TTA GCC TAA AAT TAA GAA TAA AIT GCC TAA AAA TTC GCT TAA TTT ATG ACT AAA TGG CTT GAA AAA TTC OCT CTA TTT AAA AAT AGA
121 E L A Q N Q E Q I A Q K F A Q F M T K W L E K F F L F K N R

451 AAA ATT TTT ATA ATA GGA GAA AGT TAT GCA GGA AAA TTT ATA TCT AGC AIT TGC AAT CAT TTG CTT TCA CGA AAC ATT GAA GGA TTA AAT
151 K I F I I G E S Y A G K F I S S I C N H L L S R N I E G L N

541 TTA AAA GGG GCT GCT ATA GGA AAT GGG TGG GTA GAT CCT TTA AGT CTA TTT ACA AAA TAA ACC TAA TAT GCC TTA GAA AAT AAT TTA ATA
181 L K G A A I G N G W V D P L S L F T K Q T Q Y A L E N N L I

631 AAT GCT ATA GAT AAG ACA GAA TAT GAT AIT TAA TTT AAT TAA TGC TTT AAT TTT CTT AAA TAA GGT GAT TAT AAT TAA AGT TTT TAA TCG
211 N A I D K T E Y D I Q F N Q C F N F L K Q G D Y N W T M C S

721 TGC TAT TAA CCT TTT AAT AAA ATA AIT AAT ATA ACA AAT ATA AAT CCA TAT GAT ATA AGA TTA TAA TGC TAA AIT AAA CCA TTT TGC TAT
241 C Y Q P F N K I I N I T N I N P Y D I R L Q C Q I K P F C Y

811 GAT TTT TCT CCG TTA GAA GAA TAT TTC TAA TAA TAA CAT ATA AIT TAA AAA CTA TAA GTA TAA AAT AGG AAT TGG ACA ATG TGC AGT GAT
271 D F S P L E E Y F Q Q H I I Q K L Q V Q N R N W T M C S D

901 GAT ATA TTT GCG AAA TTA ATG GAT GAT TTT TTA AGA GAT ACT AAT CAG GAT TTT GTT AAT CTT TTA AAT TAA GAT TTC AAA AIT TTG GTT
301 D I F A K L M D D F L R D T N Q D F V N L L N Q D F K I L V

991 TTC AGT GGT GAC AAA GAT TAT GTA TGC AAC TGG AGA GGA ACC GAG GAG TGG TTA GAA AAT TTA AAT TGG AAT TAA AAA GAG GAT TTT AAT
331 F S G D K D Y V C N W R G T E E W L E N L N W N Q K E D F N

1081 AAT CAA TAA TAC ACT GAT GTG ACA CTA GAA AAT CAA ACT GTA GGA AAA ATA AAA ACA CAA GGA CCT TTA AGT TTA TAT ATA ATA TAT TAA
361 N Q Q Y T D V T L E N Q T V G K I K T Q G P L S L Y I I Y Q

1171 GCT GGA CAT ATG GTA CCT ATG GAT AAG CCA GAT TTA AGC CTA AAA ATG CTT AAT GAT TTT ATG AAT AAT AGA CCA ATA TCA TCA TAT TGA
391 A G H M V P M D K P D L S L K M L N D F M N N R P I Q S Y *
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C: *cp1*

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1 ATG TAA AAA TTA TTC CTT GCT CTT TTA GCC ACA GCT GCT ATA TTC AGC ACA GCT GTC TAT ATA ATC CAT AAA CAA AAA ACT CAC CAT ACC
1 M Q K L F L A L L A T A A I F S T A V Y I I H K Q K T H H T

91 TAA CCT TAA TTT AGT CTT CCA ACT CAC CTC TAT GAC AAC TAT ATT CAT TGG AAA AAC AGA TTC CAA AAA TCA TAC TCT TCT CTT GAA GAA
31 Q P Q F S L P T H L Y D T Y I H W K N R F Q K S Y S S L E E

181 ATG CAT AGA TAG AAG ATC TGG CAT GAA AAT TTC ATG AAA ATC AAA ATT CAC AAT GAA AAG AAT GAT TAG ACC TTT AGT TTA GGT CTT AAC
61 M H R Q K I W H E N F M K I K I H N E K N D Q T F S L G L N

271 CAA TTT ATG GAT TTA ACT TAA GAT GAG TTC AAA TAA ACT TAT TTG AAT ATG AAG AAG CCT GAA GGT CCA TAA AAG AAT GTT AAA ATC TTA
91 Q F M D L T C Q D E F K Q I Y L N H K K F E G F Q K N V K I L

361 AAT GAA TCT TAC TTA CCT CAC AGT GTT GAT TGG AGA GCA AAA GGA GCT GTT ACT GCT GTC AAA GAC TAA TAG TAA TGT GGT TTA TGC TGG
121 N E S Y L P H S V D W R A K G A V T A V K D Q Q Q C G S C W

451 GCA TTT TCC TCT ACT GGT TCC TTA GAA GGT GCT TAT TTC CTT AAA ACA GGA AAA TTA TTA AGC TTT TCA GAG CAA TAG CTA GTT GAT TGT
151 A F S S T G S L E G A Y F L K T G K L L S F S E Q Q L V D C

541 TCA GCT AAA TTC GGA AAC AAG GGA TGT GAA GGA GGA CTT ATG GAC TAT GCT TTT GCT TAT GTT GAA AAA AAT GGA ATC ACC ACT GAA GAT
181 S V K F G N K G C E G G L M D Y A F A Y V E K N G I T T E D

631 AAG TAC AAA TAT ATT GGT GAA GAA GAG AAA TGC AAA CAT AAT TAA GGA ATG AAA TTT GTT TAA AAA CAC TTT GAT GTT AAA GCA GGA AGT
211 K Y K Y I G E E E K C K H I Q G M K F V Q K H F D V K A G S

721 ACT AAT TAA CTT AAA GCT GCT TTG AAA CAA TAG CTT GTT AGT GTA GCT GTG GAT GCT TTA GAA TGG TAA TTC TAT TCT GGA GGA GTC TTT
241 T N Q L K A A L K Q Q P V S V A V D A L E W Q F Y S G G V F

811 AAT AAT TGT GGA AAA CAA TTA GAT CAT GGA GTT TTG GCT GTA GGA TAT CAA GGA GAT GAA TTC TGG AAT GTT AAA AAC TCA TGG GGA AAA
271 N N C G K L A V G Y Q G D E F W I V K N S W G K

901 TCA TGG GGA GAA GAA GGT TAT ATC AGA GTA AAA ATG GGA GAT ACT TGC GGT ATT GAA GAT GGA GCT AGT TAT CCA GTT GCC TGA
301 S W G E E G Y I R V K M G D T C G I E D G A S Y P V A *

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D: *cp2*

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1 ATG CTA AAA AAT TAA TTT TTA TGC TTT TTA TAC TTT ACC TTA CTA ATC ACT CTA ATT TTT GAG GTA AAT GCC TAA ACT GAA GAA GCT TCT
1 M L K L N Q F L C F L Y F T L L I T L I F E V N A Q T E E A S

91 TCG ATT TCC GAA TGC AAC CGT CAT GGC TTT ATT TAT GAA AAA ACT TGT TTT TGT GCA AIT TAG TAT TCA GGA ACC TAA TGT CAA AAG GAA
31 S I S E C N R H G F I Y E K T C F C A I Q Y S G T Q C Q N E

181 GAT GAT CGT TAA TGG ATA AAT ACA CAT TCA ATA TGT GAT GAA CTT TTC TCA AGA TTA GAT CAT TTA ATG AAC ATA TCT CCT GAT CAT TAT
61 D D R Q W I N T H S I C D E L F S R L D H L M N I S P D H Y

271 GAT GGA GAC GAT AAA GAA TTT TCA AGA CAC AAC CCA AAC ACT ATA GGA AAA TAA TAT AAT AGA GAT CCA GAC TAC AAA TTT CCA TGG ATA
91 D G D F D K H N F N T I G K Q Y N R D F D Y K F F W I

361 TAA ATG CAT AAA TTG TCC AGT GAT TTT AAG ATT TTT GGA AAA TTT ATT ACT GCA AAT GAT GCA AGG CAA GGA ACA CTT AGA GAT GGT TAT
121 Q M H K L S S D F K I F G K F I T A N D A R Q G T L R D G Y

451 CTA TTA GCA GCA TTT GCT TCT TTA GCA AAT ATA AGA GAT GGT TAA ATT ATA AAA GAT GTA TTC ATG ACA AAG GGA GAA AAT AAA AAA CAT
151 L L A D A F A S L A N I R D G Q I I K D V F M T K G E N K A H

541 ATC TAT ACA ACA AGA TGG TTA ATA AAT GGA AAA CCA AGA TAT GTC TCA GTG GAT GAT TGG ACT GTT GGA AGA GAA TCG TAT TCA AAA ATT
181 I Y T T R W L I N G K P R Y V S V D D W T V G R E S Y S K I

631 TTT TCT TAA AAT AAA GAA GAT GAA GAT TTC TGG TCA CCT ATT CTT TAA AAA TCA TGG GCT AAA ATA TAT GGA AGT TAT TCA AAT ACT TTA
211 F S Q N K E D E F I L Q K S W A K I Y G S Y S N T L

721 TAT GGT ACA TAC GAA GAA GTG ATT ACT GCA TTA ACT CAA GCT CCT ATT ACT AGT ATA TAA CAC TAA AAC ACT TAA ATA TAA ATC CTT ATT
241 Y G T Y E E V I T A L T Q A P I T S I Q H Q N T Q I Q I L I

811 GAA ATA ATA AAG GAT TGT ATA AAA GAA TAC CCA TTA GTA GCT TCT ACT AAT GAA AAT AAA TTA GGA ATA AAC ATT TTT CCA AAT TCC
271 E I I K D C I K K E Y P L V A S T N E N K L G I N I F Q N S

901 TTT TTC GCT ATC CTA TAA TTG TAT GAA ATA AAA TTA GAT GAT AAT CAG AAA CAA ACA CTA ATT TTA ATT TAT AAT CCA AGG ACT TAT ATT
301 F F A I L Q L Y E I K L D D N Q K Q T L I L I Y N P R T Y I

991 AAA GAT TAC TCT CAT AAT CTT TGG AAT GAA AAT AGC AAT AAA TGG ACT GAA AAT GTT AAA TCT TAA GTT AAA GAA TAT GAA GAA AAA
331 K D Y S H N P W N E N S N K W T E N V K S Q V K E Y E E K K

1081 AAT GGA TAT ATT TTC GTT TCA ATT GAA GAT TTT CAT AGA TAT TTT ACA AAA ACA ATT TGG GCA AAA GTT GAA AAA GAT TAT TTT GTA TTT
361 N G Y I F V S I E D F H Y F T K T I W A K V E K D Y F V F

1171 TAC AAG GAA ATT CGA ATG GCG GCT AAT ACT AAT ACT TAT ATT ACT TAA TTT ACT TAT AAT GGA CTT GAA GAA AAG ATT TAT GTA AAA ATT
391 Y K E I R M A A N T N T Y I T Q F T Y N G L E E K I Y V K I

1261 CCT AAG GCT GAT TCT AGA GTC ACA TTA AAA AAT TGC TTA TAA TAA TAT TAT GTT AAA AGT ATG ACA ATA AAT GAT CCT TTA GGT AAA ATT
421 P K A D S R V T L K N C L Q Q Y V K S M T I K D P L G K I

1351 TAT GAA GGA AAC TTC TAA GAA ATA ATA AAA CCT TAA AAA GGA ATT TAT GAA ATA TCT GCT AAA GTG ACT TTA GGT GAA TGG TCT AAT AGA
451 Y E G N F Q E I I K P Q K G I Y E I S A K V T L G E W S N R

1441 TAT TTT GTT TTT AAT ATA TAT GCT CPT AAA AAT TCT GTT AAA TTT GAA GAA TTT CTA GAG ATT AAA AGT GAA ATT ATA GAA ATA CPT GAA
481 Y F V F F N I Y A F K N S V K F E E F L E I K S E I I E I C E E

1531 AAA GAA AAA AAT GTA GAT AAG ACA GAC AAA CCC GAG GAA AAA GAG GTA ATC AAA AAA CCA GAA GAA CAA AAA ATT ACT GAT AAA ATA AAT
511 K E K N V D K T D K P E E K E V I K K P E E Q K I T D K I N

1621 ATA AAT AAA GAA TAT GAT AAA ATT TCT GTT TTA ACT AAT CCA AAT GAT AAT TAA ATC GAG ACA AAT ATT AAA TGC CTT AAT GAT TGC AGT
541 I N K E Q D K I S V L T N F N D N Q I E T N I K C L N D C S

1711 CAT AAT GGT ATT TGC GAT AGA TAA TCA GGA ATT TGT TAT TGC TTT GAT TAG TAT GAT GGA TTA GAT TGT TCT TAG AAA ATC AAA TTA AAT
571 H N G I C D R Q S G I C Y C F D Q Y D G L D C S Q K I K L N

1801 ACT TAA TGC AGA AAT TAT GAT AGA TAA TGC TAT AGT TGG GCA GCA AAT GGT TGG TGT TTA GAT GAA AAT GAA AAT ATA ATG AAA TAA AAG
601 T Q C R N L D R Q C Y S W A A N G W C L D E N E N I M K Q K

1891 TGT CCT TTA TCA TGT TAT GAA AAA GGA ATT GAA TAG TAC GAT TAT TGT AAG CAT AGC TCT AAA CTA ATT ATA GAG ACT AAT TAG AAA ATT
631 C P L S C Y E K G I E Q Y D Y C K H S S K L I I E T N Q K I

1981 AAA AAA TTA ATC AGT TAT TCA AAA AAT CAA TAA TAT TAT GAT AAT TCA TAA TAA AAA TTG AAG ACT AAT TAT TTC TTA AAT TTT ATA ATG
661 K K L I S Y S K N Q Q Q F D N S Q Q K L K T N Y F L N F I M

2071 ATA TCA ATA TCA TTT TTC ATT ATT CTT AGA CAA AAC AAT TGA
691 I S I S F F I I L R Q N N *

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Fig. 2. 3. Nucleotide sequences and amino acid sequences deduced from nucleotide sequences of *sp1* (A), *sp2* (B), *cp1* (C), and *cp2* (D). Black bars indicate the position of primers for qPCR. Red boxes indicate the template sequence for dsRNA synthesis.

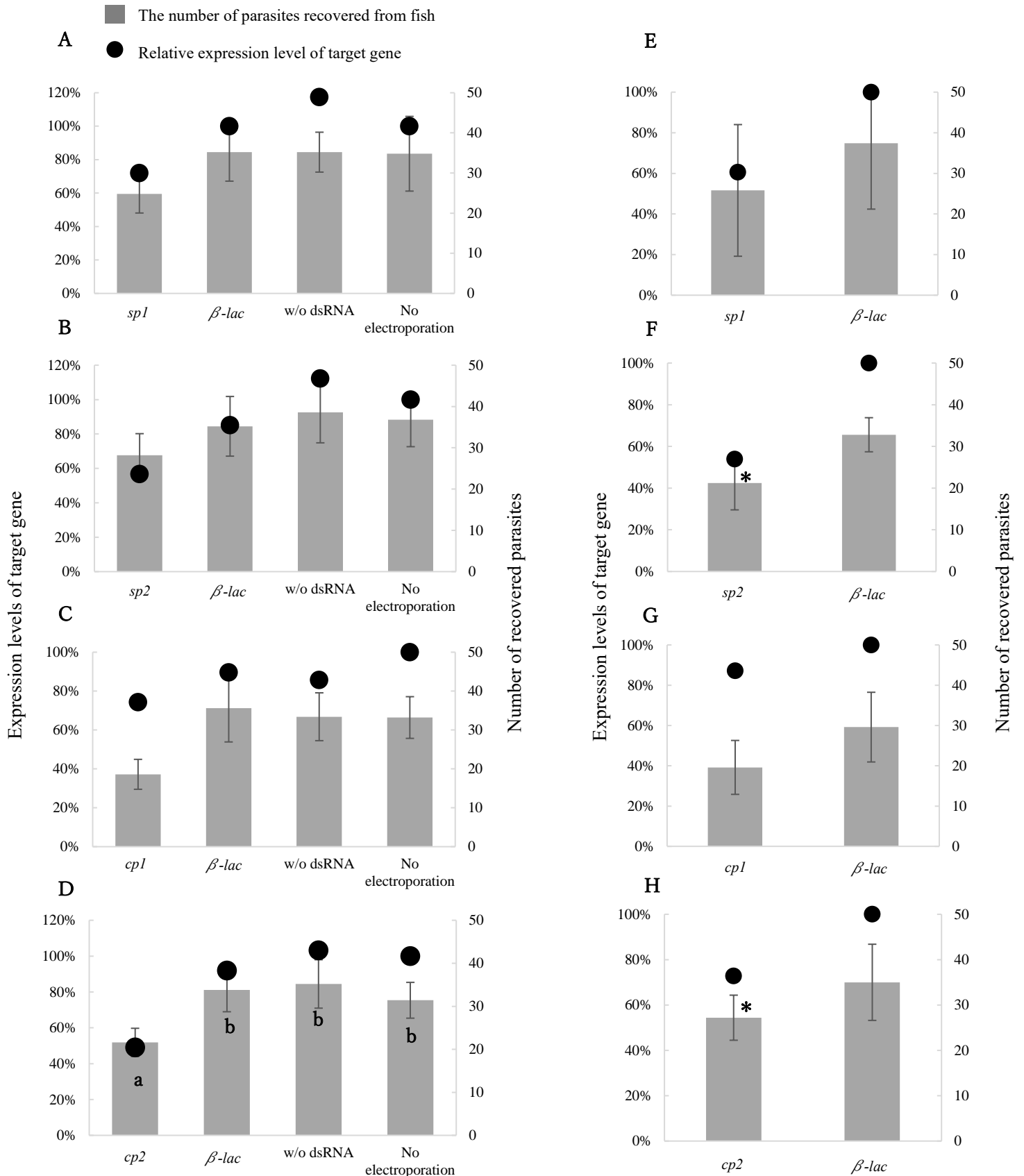


Fig. 2. 4. RNA interference experiment. A–D: trial 1. E–H represent the results of trial 2. Closed circles represent the percentage ratios of the expression level of target genes to that of negative controls (No electroporation in A–D interfered with β -lactamase-dsRNA in F–H). Columns and vertical bars represent the mean numbers and SD of protomonts leaving each of 5 fish. The labels with different letters on columns indicate significant difference in numbers of trophonts ($P < 0.05$, Steel–Dwass test). In A–D Asterisk (*) indicates the significant difference in numbers of trophonts between control treated group is significant ($p < 0.05$).

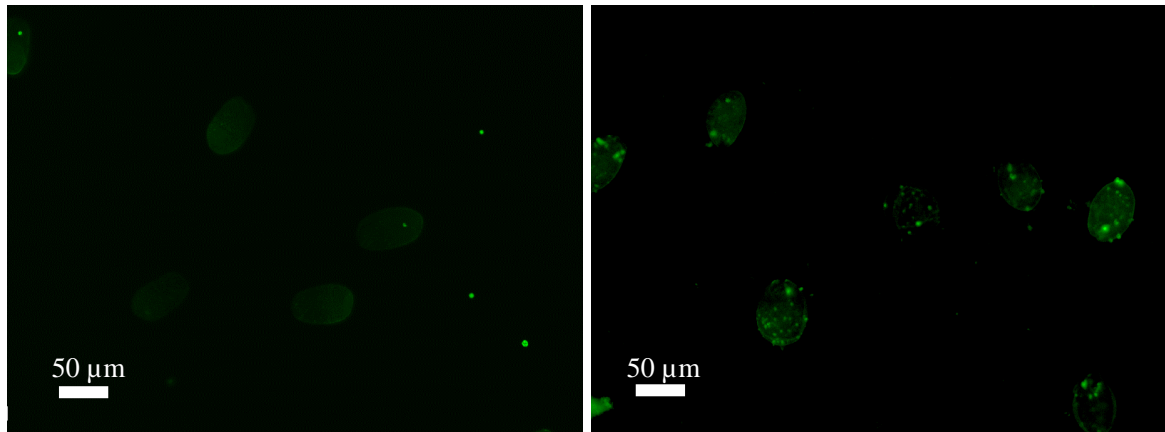


Fig. 2. 5. Theronts transfected with dsRNA of *β -lactamase*. A: native theronts. B: theronts transfected with the fluorescein-labeled dsRNA.

A : *sp1*

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1 ATG AAA GTG TTC ATC TGC ACT CTG CTG GCC TTA ACC TCG GTA CTG GCT AAC CCT GAC ATT TTC CTC AAC GAA GAC CAG CAT CCC GGT TTC
1 M K V F I C T L L A L T S V L A N P D I F L N E D Q H P G F

91 GTC AAA CTC AAG GAC AAA GAT GAC CTG TTT TTC TGG CTG TTT AAA TCC AGG AGC GAT AAG AAC TCC GAT CCA CTG GTT ATC TGG CTT ACA
31 V K L K D K D L F F W L F K S R S D K N S D P L V I W L T

181 GGA GGA CCC GGA TGT TCG TCA GAG CTG GCG ATT TTC TAC GAG AAT GGT CCC TTT AGC ATC AAC CAG AAT CTG ACG CTG AAG AAA AAC AAG
61 G G P G C S S E L A I F Y E N G P F S I N Q N L T L K K N K

271 TAC TCA TGG AAC GAG AGA ACC AAC CTC TTG TAC GTC GAC CAA CCG ATA GGA ACT GGC TTT TCT CAT GGC GAG AAG AAG GAC TTC GTC CAC
91 Y S W N E R I N L L Y V D Q P I G T G F S H G E K K D F V H

361 AAC GAA ACC ATG GTG TCC AAA GAC TTC TAC CAG TTT ATG GAA GGC TTT CTC GAG CAG TTT CCT GAG TAC AAA GGC AGG GAT CTG TAC ATT
121 N E T M V S K D F Y Q F M E G F L E Q F P E Y K G R D L Y I

451 ACA GGG GAG AGT TAC GCC GGA CAC TAC GTC CCA GCC ATT TCC GCC TAT CTC CAC CAA AAA AAG AAC CCC GAT ATT AAC CTC AAG GGA TCT
151 T G E S Y A G H Y V P A I S A Y L H Q K K N P D I N L K G S

541 GCC ATT GGA AAT GGG CTT GTC AAC CCC TAT CTG CAG TAT CCG GAG TAT GCA ACC TTT GCA TAC GAG AAC AAG CTC GTG TCA AAG ATG AAG
181 A I G N G L V N P Y L Q Y P E Y A T F A Y E N K L V S K M K

631 TAC AAA ATC CTG CAG TTT GAG TTG AAG GAA TGT CAG AAA AAG ATC AAG AGC GGT GAT TGG GAC AGA GCC CTG ATC ATC TGC AAT CTC CCT
211 Y K I L Q F E L K E C Q K K I K S G D W D R A L I I C N L P

721 GTG GAG CAG ATC CTC GGT AAT CCT CAA AAG TTC AAT ACC TAC GAC ATA CGG AAA CCG TGT ACA GTG CCT GGG CTT TGC TAC AAC TTC AGC
241 V E Q I L G N P Q K F N T Y D I R K P C T V P G L C Y N F S

811 TTA GTG GAC AAG TTC CTG GCT AGG GAA GAC GTG ATA GAA GCC CTG AAC GTT AAA GGC CCG AAA TGG AGT TCC TGC AAC ATG GAT GTA CAC
271 L V D K F I C A N W R E D V K G R K W S S C N M D V H

901 TCT GCA CTA ATG GGC GAT TGG ATG CGC AAT TTG AGC CAG AAA GTC ATA TAT CTG CTT AAG AAC GAC ATC AAG GTG CTG ATC TAT GCT GGC
301 S A L M G D W M R N L S Q K V I Y L L K N D I K V L I Y A G

991 GAC AAG GAC TTT ATC TGC AAT TGG CGA GGC AAC GAG AAA TGG GTG AAC GAG TTG ACG TGG TTC TAC AAG GAG CAG TTC AAG TCT CAG CAG
331 D K N E K N W R E G A L N V N E L T W F Y K E Q C N M D V H

1081 TAT CAG GAG GTG TTC ATT GAC TCC AAA AGT TAC GGG AAG ATC AAA TTC TAC AAG AAT TTC GCG TTT TTC CGT GTT TAC GAA GCT GGG CAC
361 Y Q E V F I D S K S Y G K I K F Y K N F A F F R V Y E A G H

1171 ATG GTT CCC ATG GAT CAG CCA ATG GCT GCG CTG AAA ATG CTA GAC CAC TTC ATC TAC AAA TGG CAA CCA AAC AGC ATC GAC TAT AAG CAG
391 M V P M D Q P A A L K M L D H F I Y K W Q P N S I D Y K Q

1261 AGC AAA AAG ATC GTC ACT GAG TGA
421 S K K I V T E *

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B : *sp2*

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1 ATG AAC TAC AAC GCG TTC AAG GTG ATG CTT CTG GTG GCT TTT CTC ATC AAC ATA CTG CAG ACT ATC AGC CAT TCC CCT TTC CAG AGT TCC
1 M N Y N A F K V M L L V A F L I N I L Q T I S H S P F Q S S

91 TCT CAC GGC TTC ATA CCT CTA TCT GAC ACC TCC GAC ATC TTT TAC TGG CTG TTT GAC AGC CAG AGT CAG CCC AAT ACC GAT CCT CTT GTC
31 S H G F I P L S D T I F Y W L F D S Q S P N T D S Q P L V

181 ATC TGG CTC AGT GGA GGG CCA GGT TGT GCC TCC ACA TTT GCC CTG TTC TAC GAG AAT GGA CCG TAC TCA CTG AAT CAA AAC TTG TCA CTC
61 I W L S G G P G C A S T F A L F Y E N G P Y S L N Q N L S L

271 TCC GTT AAC CCC TAC TCT TGG AAC CAG CAA GCG AAC ATT ATG TAC GTC GAT CAG CCC ATA GGC ACT GGG TTT AGC AAG TCG TCA GAG CAG
91 S V N P Y S W N Q Q A N I M Y V D Q P I G T G F S K S S E Q

361 GAG CTG GCA CAG AAT CAG GAA CAG ATA GCT CAG AAG TTT GCC CAG TTC ATG ACC AAA TGG TTG GAG AAA TTC CCG CTC TTC AAA AAT CGC
121 E L A Q N Q E Q I A Q K F A Q F M T K W L E K F P L F K N R

451 AAG ATC TTC ATC ATT GGC GAG TCA TAT GCA GGC AAG TTC ATC AGC TCT ATC TGC AAC CAC CTG CTT TCT AGG AAC ATC GAA GGG CTA AAC
151 K I F I I G E S Y A G K F I S S I C N H L L S R N I E G L N

541 CTG AAA GGT GCT GCT ATA GGG AAT GGA TGG GTA GAT CCC TTA AGC CTC TTT ACC AAA CAG ACC CAA TAT GCC CTG GAG AAT AAC CTG ATC
181 L K G A A I G N G W V D P L S L F T K Q T Q Y A L E N N L I

631 AAT GCC AIT GAC AAG ACA GAG TAC GAT ATT CAG TTC AAC CAG TGC TTT AAC TTT CTC AAG CAA GGA GAC TAT AAC CAG TCG TTT CAG TCC
211 N A I D K T E Y D I Q F N Q C F N F L K Q G D Y N Q S F Q S

721 TGT TAT CAG CCT TTC AAC AAG ATC ATT AAC ATC ACC AAC ATC AAT CCC TAT GAC ATT CGT CTG CAA TGC CAG ATT AAG CCC TTT TGC TAC
241 C Y Q P F N K I I N I T N I N P Y D I R L Q C Q I K P F C Y

811 GAT TTC AGT CCA CTG GAG GAG TAC TTT CAG CAG CAG CAC ATC ATC CAG AAG CTG CAA GTG CAG AAC CGG AAC TGG ACG ATG TGT TCC GAC
271 D F S P L E E Y F C Q Q K L Q V Q N R N W T M D S D

901 GAC ATC TTT GCC AAG CTG ATG GAC GAT TTC CTG AGG GAC ACT AAC CAA GAC TTT GTC AAT TTG CTC AAC CAG GAC TTC AAG ATC CTG GTC
301 D I F A K L M D D F L R D T N Q D F V N L L N Q D F K I L V

991 TTC TCG GGA GAC AAA GAC TAC GTG TGC AAT TGG AGA GGT ACA GAA GAG TGG CTT GAA AAC CTC AAC TGG AAC CAG AAA GAA GAC TTC AAT
331 F S G D K D Y V C N W R G I I E E W L E N L N W N Q K E D F N

1081 AAC CAG CAG TAC ACG GAT GTG ACC CTC GAG AAT CAG ACA GTT GGC AAA ATC AAA ACG CAG GGA CCA TTA AGC TTG TAC ATT ATC TAC CAA
361 N Q Q Y T D V T L E N Q T V G K I K T Q G P L S L Y I I Y Q

1171 GCA GGC CAC ATG GTG CCA ATG GAT AAA CCG GAT CTG AGC TTG AAG ATG CTG AAC GAC TTC ATG AAC AAC CGA CCT AIT CAG AGC TAT TGA
391 A G H M V P M D K P D L S L K M L N D F M N N R P I Q S Y *

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C: *cp1*

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1 ATG CAG AAG CTC TTT CTG GCT CTG TTA GCT ACA GCA GCC ATC TTT AGC ACC GCA GTC TAC ATT ATC CAC AAA CAG AAG ACA CAC CAT ACT
1 M Q K L F L A L L A T A A I F S T A V Y I I H K Q K T H H T

91 CAG CCA CAG TTC TCT CTT CCG ACT CAC CTG TAT GAC ACC TAC ATC CAC TGG AAG AAT CGG TTT CAG AAA TCC TAC TCC TCT CTG GAG GAG
31 Q P Q F S L F T H L Y D T Y I H W K N R F Q K S Y S S L E E

181 ATG CAT AGG CAG AAG ATC TGG CAC GAG AAC TTC ATG AAA ATC AAG ATC CAC AAC GAG AAG AAT GAC CAG ACG TTC TCT TCT GGA CTG AAC
61 M H R Q K I W H E N F M K I K I H N E K N D Q T F S T L G G A C T G N

271 CAG TTC ATG GAC CTA ACC CAG GAC GAG TTC AAG CAG ATC TAC CTG AAC ATG AAG AAG CCT GAA GGT CCA CAG AAG AAC GTG AAA ATC CTG
91 Q F M D L T Q D E F K Q I Y L N M K K P E G P Q K N V K I L

361 AAC GAG TCG TAC CTC CCC CAT AGC GTT GAT TGG AGA GCC AAA GGT GCA GTA ACC GCT GTG AAA GAC CAA CAA CAG TGT GGC TCA TGT TGG
121 N E S Y L P H S V D W R A K G A V T A V K D C A A C A G C G G C T A T G T T G

451 GCC TTT AGC AGT ACC GGA AGC CTG GAA GGA GCG TAC TTC CTG AAA ACC GGC AAA TTG CTC TCC TTT TCG GAG CAA CAG CTT GTC GAC TGC
151 A F S S T G S L E G A Y F L K T G K L L S F S E Q Q L V D C

541 AGC GTC AAA TTC GGG AAT AAG GGG TGT GAG GGA GCG TTG ATG GAC TAT GCT TAT GTG GAG AAG AAC GGC ATA ACT AGC GAA GAC
181 S V K F G N K G C E G G G G T T M D Y A F A Y V E K N G I T T E D

631 AAG TAC AAG TAC ATT GGG GAA GAG GAG AAG TGC AAG CAC ATT CAA GGG ATG AAA TTC GTG CAG AAA CAC TTC GAC GTT AAA GCC GGA TCA
211 K Y K Y I G E E E K C K H I Q G M K F V Q K H F D V K A G S

721 ACG AAC CAG CTG AAA GCG GCC TTA AAA CAG CAG CCT GTG TCA GTG GCT GTC GAT GCC CTC GAA TGG CAG TTC TAC TCT GGA GGC GTG TTT
241 T N Q L K A A L K Q Q P V S V A V D A L E W Q F Y S G G V F

811 AAC AAT TGC GGC AAG CAG TTG GAC CAT GGT GTT CTC GCG GTC GGA TAC CAA GGT GAT GAG TTC TGG ATC GTG AAA AAC TCC TGG GGC AAG
271 N N C G K Q L D H G V L A V G Y Q G D E F W I V K N S W G K

901 AGT TGG GGA GAA GAG GGT TAT ATT CGC GTG AAG ATG GGC GAT ACA TGC GGC ATA GAG GAT GGG GCA TCC TAT CCC GTA GCC TGA
301 S W G E E G Y I R V K M G D T C G I E D G A S Y P V A *
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D: *cp2*

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1 ATG CTG AAG AAT CAG TTC TTG TGC TTT CTC TAC TTC ACC CTG CTG ATC ACC CTG ATC TTC GAG GTG AAT GCA CAG TCA GAA GAA GCG AGT
1 M L K N Q F L C F L Y F T L L I T L I F E V N A Q T A E E A S

91 AGC ATC TCA GAG TGC AAT CGC CAT GGG TTC ATT TAC GAG AAG ACG TGC TTC TGT GCG ATC CAA TAT AGC GGT ACA CAA TGT CAG AAC GAG
31 S I S E C N R H G F I Y E K T C F C A I Q Y S G T Q C Q N E

181 GAC GAC AGG CAG TGG ATT AAC ACC CAT AGC ATC TGC GAC GAA CTC TTT TCC AGG TTG GAC CAC CTC ATG AAT ATC TCA CCT GAT CAC TAT
61 D D R Q W I N T H S I C D E L F S R L D H L M N I S P D H Y

271 GAC GGA GAT GAC AAA GAG TTT AGC CGA CAT AAT CCC AAT ACC ATC GGT AAG CAG TAT AAC CGA GAT CCA GAC TAC AAA TTT CCA TGG ATC
91 D G D D K E F S R H N P N T I G K Q Y N R D P D Y K F P W I

361 CAG ATG CAC AAG CTG AGT TCT GAC TTC AAG ATT TTC GGA AAG TTC ATC ACT GCC AAT GAC GCA AGG CAA GGA ACC CTG AGA GAT GGC TAC
121 Q M H K L S D F K I F G K F I T A N D A R Q G T L R D G Y

451 TTA CTG GCC GCT TTT GCT AGC TTA GCT AAC ATC CGT GAT GGT CAG ATC ATT AAG GAT GTG TTC ATG ACC AAA GGT GAG AAT AAG AAG CAC
151 L L A A A F A S L A N I R D G Q I I K D V F M T K G E N K K H

541 ATC TAT ACC ACC AGG TGG CTC ATA AAC GGA AAG CCT CGS TAC GTT AGC GTG GAT GAT TGG ACT GTC GGT CGC GAG TCG TAT TCA AAG ATC
181 I Y T T R W L I N G K P R Y V S V D D W T V G R E S T Y S K I

631 TTC TCA CAG AAC AAG GAG GAT GAG GAC TTT TGG TCG CGC ATC CTG CAG AAA TCG TGG GCG AAG ATT TAC GGC TCT TAC TCC AAC ACT CTG
211 F S Q N K E D E D F W S P I L Q K S W A K I Y G S Y S N T L

721 TAC GGC ACT TAT GAG GAG GTC ATA ACC GCC CTT ACT GAC GCT CCC ATT ACC AGT ATA CAG CAC CAA AAC ACG CAA TGT CAG ATC CTC ATC
241 Y G T Y E E V I T A L T Q A P I T S I Q H Q N T Q I Q I L I

811 GAG ATA ATC AAG GAC TGC ATC AAA AAA GAG TAC CCT CTG GTC GCA TCT ACC AAT GAG AAC AAG CTA GGA ATC AAC ATT TTC CAG AAC TCC
271 E I I K E I K I F L V A S T N E N K L G I N I F Q N S

901 TTC TTT GCC ATC TTG CAG TTG TAT GAA ATA AAG CTC GAC GAT AAC CAG AAA CAA ACA CTG ATT CTA ATT TAC AAT CCC AGA ACG TAC ATC
301 F F A I L Q L Y E I K L D D N Q K Q T L I L I Y N P R T Y I

991 AAA GAC TAC AGT CAC AAT CCG TGG AAC GAG AAC AGC AAC AAG TGG ACT GAA AAC GTG AAG AGC CAG GTC AAG GAA TAC GAG GAG AAG AAG
331 K D Y S H N P W N E N S N K W T E N V K S Q V K S Y E E G K K

1081 AAC GGG TAC ATT TTC GTG TCA ATA GAG GAC TTT CAC CGC TAC TTT ACA AAG ACC ATC TGG GCC AAA GTT GAG AAG GAT TAC TTT GTC TTC
361 N G Y I F V S I E D F H R Y F T K T I W A K V E K D Y F V F

1171 TAC AAA GAA ATA CGC ATG GCT GCT AAT ACC AAC ACC TAC ATA CCG CAG TTC ACA TAC AAC GGA CTG GAG GAA AAG ATC TAT GTG AAG ATT
391 Y K E I M A A N T N A T Y I T Q F T Y N G L E E K I Y V K I

1261 CCA AAG GCC GAC TCT AGG GTT ACT CTC AAG AAT TGT CTG CAG CAG TAC TAC GTG AAA TCT ATG ACA ATT AAA GAC CCT CTG GGC AAA ATT
421 P K A D S R V T L C K N C L Q Q Y V V K S M T I K D P L G K I

1351 TAT GAG GGG AAC TTT CAG GAA ATA ATC AAA CCC CAG AAA GGC ATT TAC GAA ATC AGC GCC AAA GTT ACG CTT GGC GAG TGT TAT AAC CGG
451 Y E G N N F Q E I I K P Q A K I Y E I S A K V T L G E E W S N R

1441 TAC TTT GTG TTT AAC ATT TAT GCC CCG AAG AAC TCC GTG AAG TTC GAA GAG TTC CTG GAA ATC AAA TCC GAG ATT ATC GAG ATC CCC GAA
481 Y F V F N I Y A P K N S V K F E E F L E I K S E I I E I F E

1531 AAA GAG AAA AAC GTT GAC AAG ACA GAC AAA CCC GAA GAA GAG GTA ATT AAG AAG CCT GAG GAG AAG ATC ACA GAG AAG ATC AAC
511 K E K N V D K T D K P E E K E V I K K P E E G Q K I T D K I N

1621 ATC AAT AAG GAG CAG GAC AAG ATC TCC GTA CTG ACC AAC CCG AAC GAC AAC CAG ATT GAG ACG AAC ATC AAA TGC CTC AAC GAC TGC TCC
541 I N K E Q D K I S V L T N P N D N Q I E T N I K C L N D C S

1711 CAT AAC GGC ATC TGT GAC CGT CAA TCC GGG ATC TGC TAC GTC TTT GAC CAG TAT GAT GGC TTA GAC TGC AAG CAG AAA ATC TCA AAG ATT
571 H N G I C D R Q S G I C Y C F D Q Y D G L D C S Q K I K L N

1801 ACT CAG TGT CGS AAT CTG GAT AGA CAG TGC TAC TCT TGG GCA GCC AAT GGA TGG TGT CTT GAC GAG AAC GAG AAT ATC ATG AAA CAG AAG
601 T Q C R N L D R Q C Y S W A A N G W C L D E N E N I M K Q K

1891 TGT CCA TTG AGC TGT TAT GAG AAA GGG ATA GAG CAG TAT GAT TAC TGC AAA CAG TCC TCC AAA CTG ATC ATT GAG ACA AAC CAG AAG ATT
631 C P L S C Y E K G I E Q Y D Y C K H S S K L I I E T N Q K I

1981 AAA AAG CTG ATC TCG TAC TCC AAG AAC CAA CAG CAG TFC GAC AAC TCA CAA CAG AAA CTC AAA ACG AAC TAC TTT CTG AAC TTC ATC ATG
661 K K L I S Y S K N Q Q C Y S Q Q K L K T N Y F L N F I M

2071 ATC AGT ATC TCT TTC TTC ATC ATA CTT AGA CAG AAC AAC TGA
691 I S I S F F I I L R Q N N *
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Fig. 2. 6. Modified nucleotide sequences of *sp1* (A), *sp2* (B), *cp1* (C), and *cp2* (D). Red boxes indicate the regions of recombinant proteins for immunization.

UT1	1	YVVVKASCSASCSYKLGSIKITPKIGSETTKTTWAGTAHSTDSTDVTLGSGNCKYVTVV	60
K1	1	FIVVKASCADSCSYKLGSIKITETAGSTSTKTTWAGTASSTDSTDVTVASGKCSYVTSV	60
		:*****: *****:***** .:*** :***** *****:.*:*.*** *	
UT1	61	TSSTAGTAVTVLSDADECTVLSGMCTTQGGKQTAAKINFKRDMDLATKPFQTTYKQWSV	120
K1	61	TSGTADAADVILNDADECTVATGICTNQGQKQTTKGKINFKRDMDLATKPFQTTFKQFKV	120
		.*.:** :*.***** :*:**.***** .*****:***:*. *	
UT1	121	IKPKTSTTNTAVSDQSANCVTEADMIDTAVDAKDIVGTLKLTEACGTCSDWTSKELKIS	180
K1	121	IKPKTSTTTAAASDQTADCVTEAEMIDTTLDAKDIVGTLKLSEAACGTCSDWISKELKIS	180
		*****.:*.***:*.*****:*****:*****:*****:***** *****	
UT1	181	QHDSSKKYMTIAGTIKGKTAASDCTNKLTAKECYAAKKDANNW---AVFGCTTLQSPT	237
K1	181	QHDSTKKYMTLAGTIKGKSAASDCTNKLTDTEKCYAAKKMQTIGLYLVLLCNHLQEVF	240
		****:*****:*****:***** ***** . .*: *. **.	
UT1	238	---GGIPIVKATASGKTTILTMQWISGSDNCKVVG EVTSTSTNAQKLISVLAPFVLLQLS	294
K1	241	LLQKLLPVVKQ-----HILWHGLVDLI-IVKLLESLHLE-----LQALQRFSQVLA--	285
		:** : * . * : * : * : :..* * :*	
UT1	295	F	295
K1	286	-	285

Fig. 2. 7. Partial amino acid sequences of i-antigen of UT1 and K1. Asterisks (*) indicate positions which have a single, fully conserved residue. Colons (:) indicate conservation between groups of strongly similar properties. Periods (.) indicate conservation between groups of weakly similar properties.

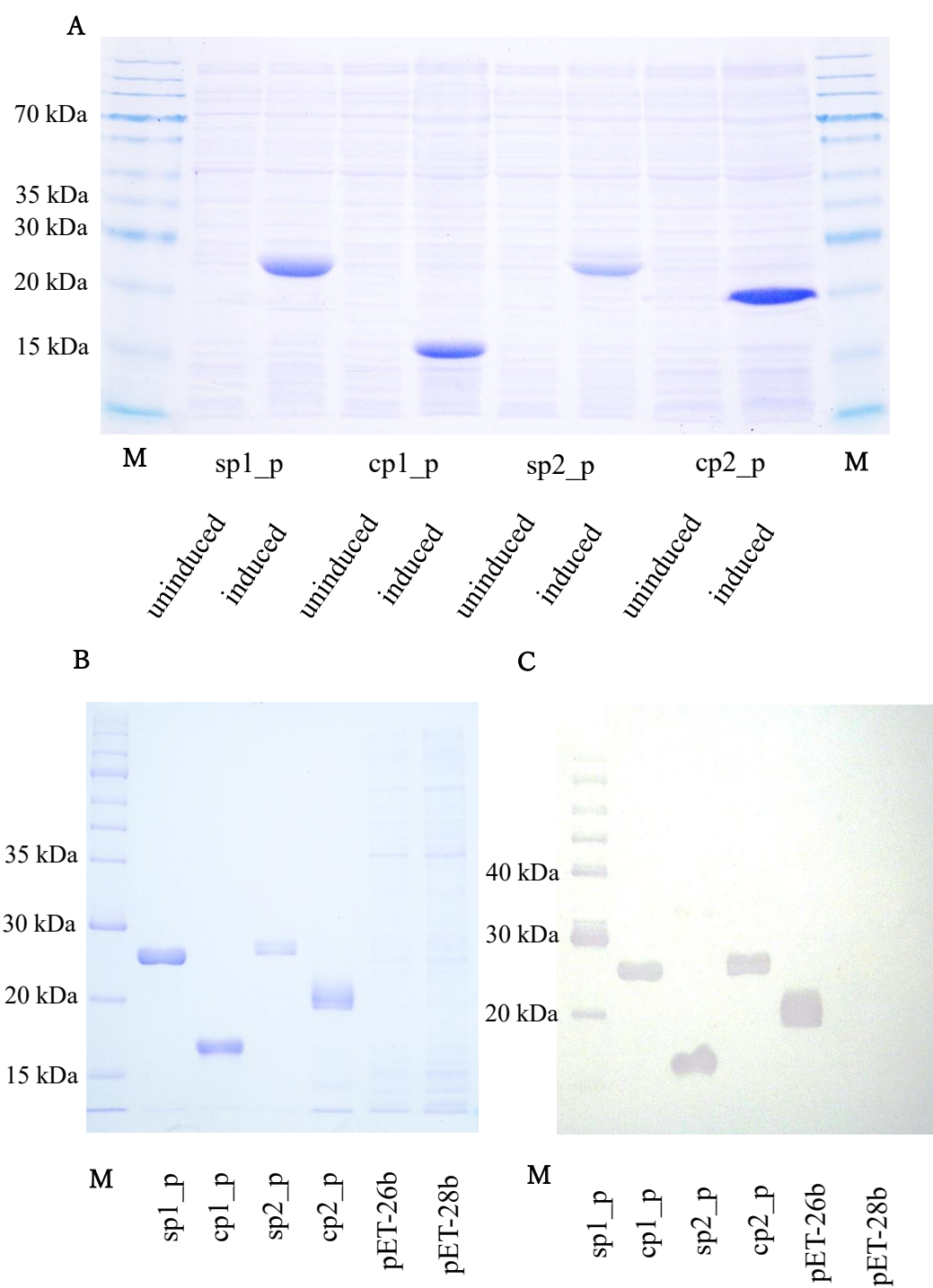


Fig. 2. 8. Protein expression by transformed *E. coli*. A: SDS-PAGE profiles of *E. coli* lysates before and after expression induction with IPTG. B: SDS-PAGE profiles of purified proteins. C: Western blots of purified proteins. Lysates of empty pET-26b and pET-28b vector-transfected cells were used for negative controls for western blot. M: molecular makers.

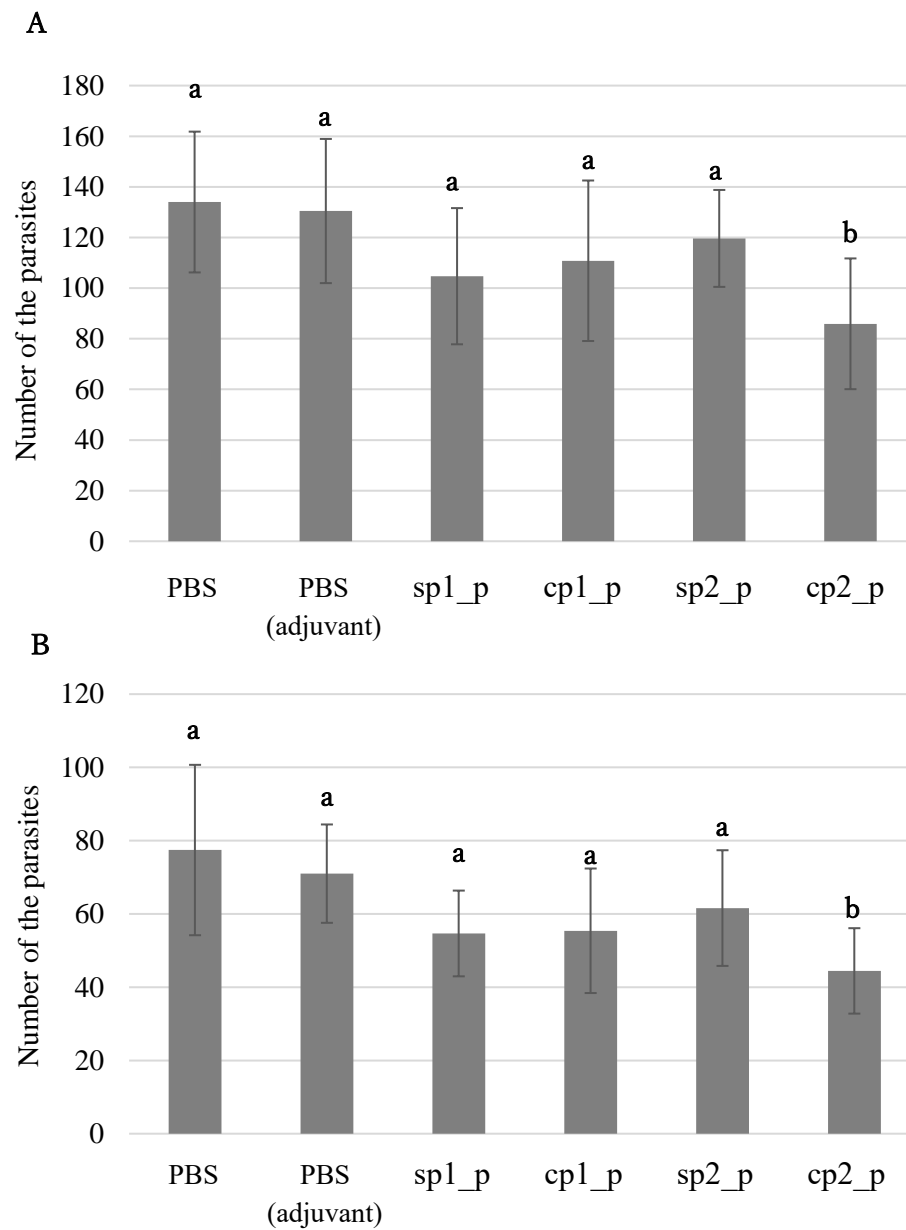


Fig. 2. 9. Number of parasite of *C. irritans* recovered from or detected in fish immunized with recombinant proteins in challenge experiments. A: first trial. B: second trial. Number of parasites are shown as mean \pm SD. Different letters denote significant difference in number of recovered parasites ($P < 0.05$).

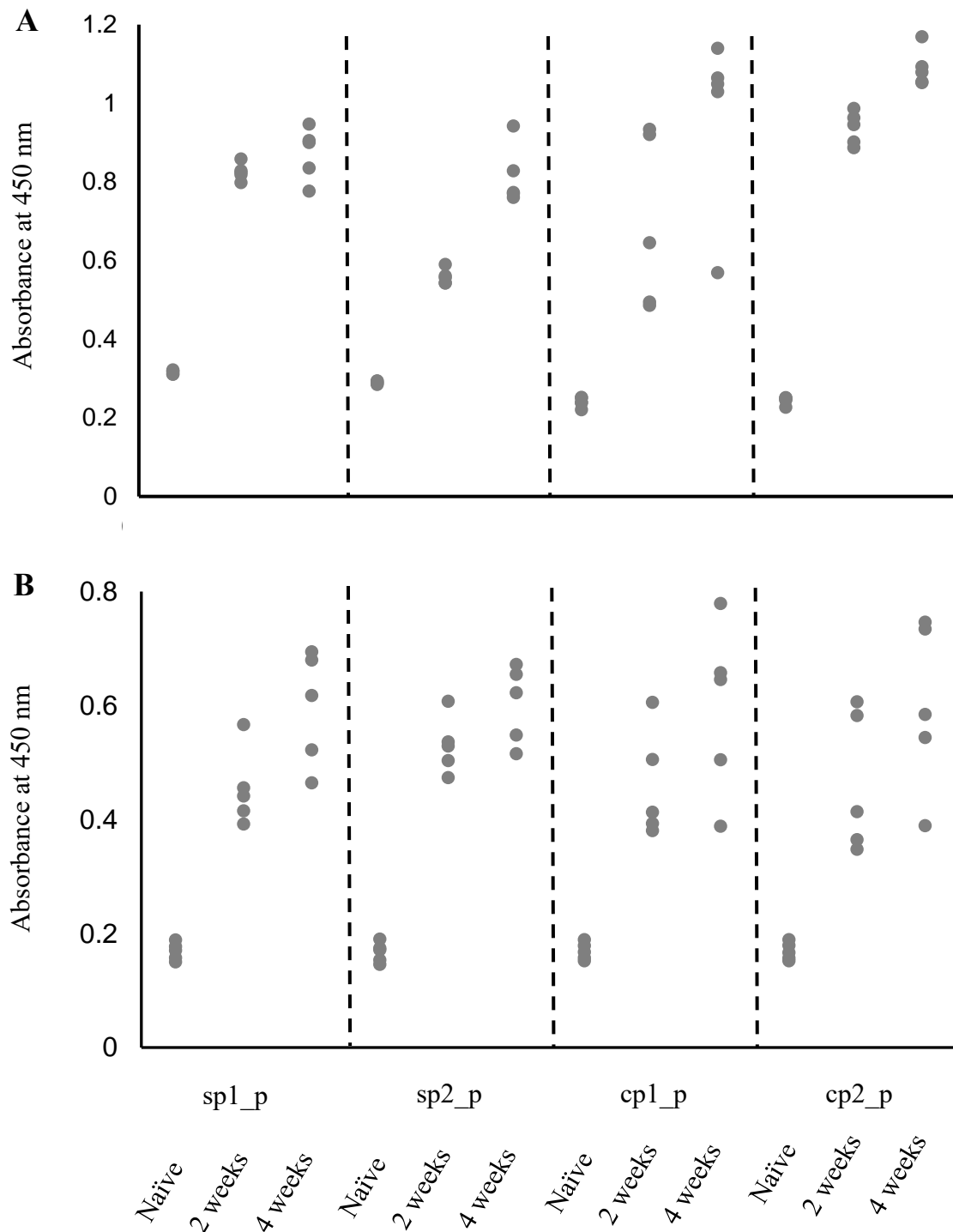


Fig. 2. 10. ELISA assay of antibodies against recombinant proteins of *C. irritans* in sera of fish immunized with the proteins for challenge experiments. A: fish immunized for first trial. B: fish immunized for trial. Absorbance values in ELISA for fish unimmunized (naïve), 2 weeks post the first immunization (2 weeks) and 2 weeks post the boost immunization (4 weeks) are individually plotted.

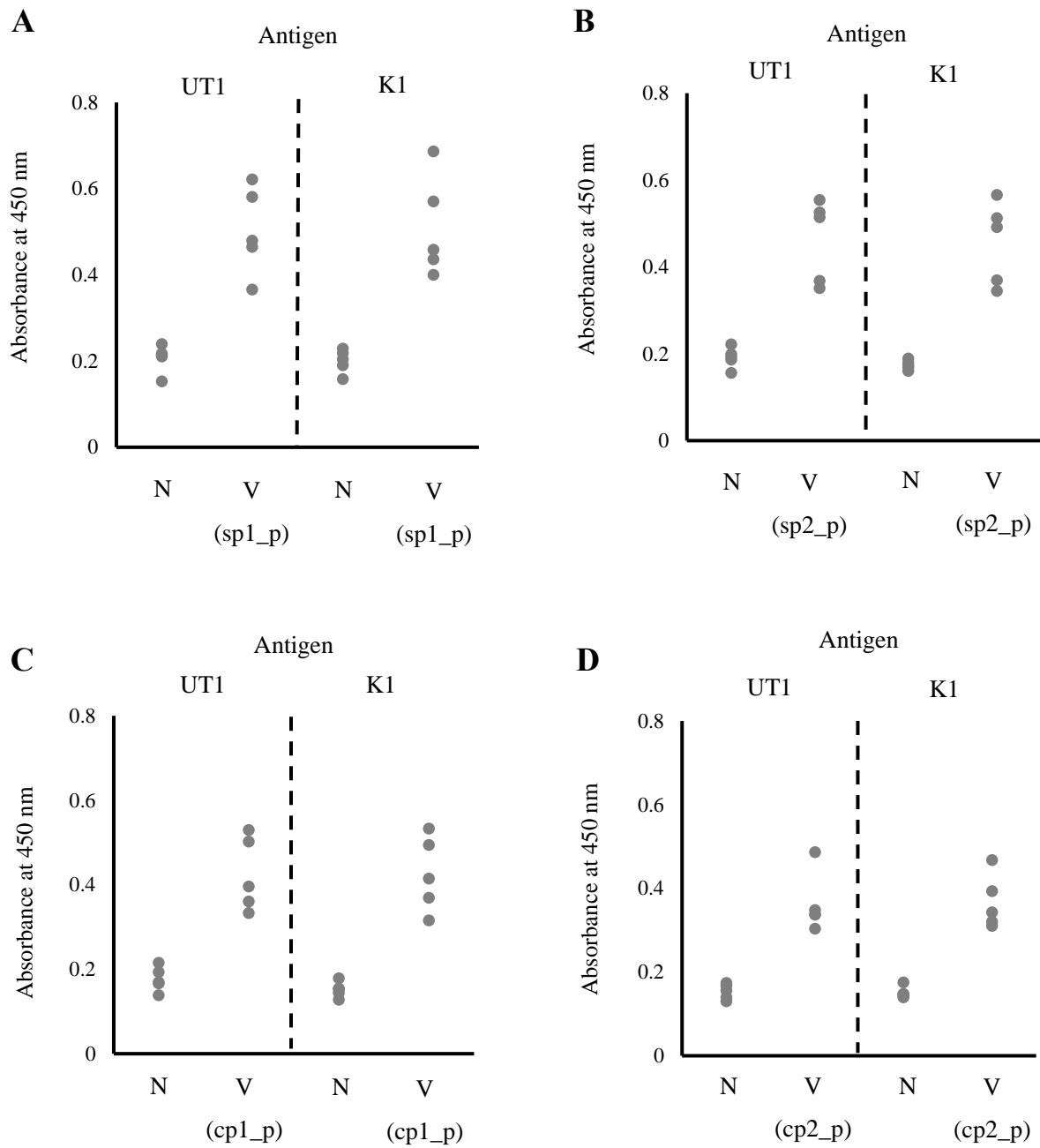


Fig. 2. 11. ELISA assay of antibodies against lysates of two different strains (UT1 and K1) in sera of fish immunized with the proteins for second trial of challenge experiments. A-D: fish immunized with sp1_p, sp2_p, cp1_p and cp2_p, respectively. Absorbance values in ELISA for fish unimmunized (N) and fish at 2 weeks post the boost immunization are individually plotted.

Chapter 3

General Discussion

As aquaculture has developed, a lot of fish diseases have been reported and have caused serious problems. So far, medicines have been used to reduce the threat of diseases. However, drug-resistant bacteria and parasites due to repeated use of medicines have been reported in aquaculture (Alderman and Hastings, 1998; Aaen et al., 2015). Also, it is concerned that spillover from medicinal treatments to reduce the diseases is potentially harmful to other organisms such as lobsters and shrimps (Fairchild et al., 2010). Therefore, it has been required to reduce the amount of medicines and develop control methods, such as vaccines, instead of chemical medication.

In Chapter 1, we demonstrated the ability of tomonts to become dormant in a hypoxic environment and to resume development in an oxic environment at any developmental stage; it has also demonstrated the potential long-term viability of dormant tomonts in hypoxic environments. This accumulation of tomonts may be a key factor for the autumn outbreaks of cryptocaryoniasis in floating net cages in temperate waters. Also, we demonstrated that the biological rhythm of the parasite is mediated by the photoperiod during the parasitic and cystic periods. These results indicate that tomonts can accumulate on the seabed during the summer when it becomes hypoxic; water temperature, water depth, tidal cycle, thermocline and dissolved oxygen on seabeds are suggested as the major factors causing the outbreaks of cryptocaryoniasis.

In Chapter 2, we found that recombinant proteases of *C. irritans* induced partial protection against the parasite infection and that the antibodies in sera of fish vaccinated with a recombinant protease of the parasite reacted with lysates of a different isoalte of the parasite. Previous experimental vaccines using i-antigen of *C. irritans* have been considered difficult to be put into practical use against cryptocaryoniasis because the vaccines using i-antigens cannot induce cross-serotype protection. The proteases may be good candidates for vaccine antigens common to many strains of *C. irritans*.

Both knowledge obtained in Chapter 1 and 2 can be applied to develop an IPM system against cryptocaryoniasis. For example, early warning system by monitoring environmental factors such as water temperature, depth, tidal cycle, disappearance of thermocline and dissolve oxygen

concentration on seabeds, and immunization with vaccines against proteases can be applied in floating net cages. When infection of *C. irritans* is confirmed, oral administration of antiparasitic compounds such as sodium salinomycin and Romet® 30 that were proven to have therapeutic effect against *C. irritans* infection (Yoshinaga et al., 2011; Kawano et al., 2012) and transferring net cages containing fish to open sea also can reduce the infection. IPM system against cryptocaryoniasis has not been tried so far, but combinations of several less effective methods can potentially reduce infection levels significantly. This may not only reduce the severity of cryptocaryoniasis but also reduce the dependence on one chemotherapeutic compound for treatment. Furthermore, it will be a help to reduce the risk of the generation of drug-resistant parasites and environmental pollutions. However, further studies, such as those about the relationship between environmental factors and occurrence of cryptocaryoniasis, are still needed to develop the warning system for cryptocaryoniasis, because our results were obtained from experiments in our laboratory. Also, since research on vaccine candidates except i-antigen has just started, it is important to identify other antigens of the parasite that can induce protective antibodies for future vaccine development.

Thus, the results obtained in the present study are thought to have provided a great help in the development of future control methods against cryptocaryoniasis. However, there are some unclear points about cryptocaryoniasis that remain and therefore many issues to be studied. In several bacterial and viral fish diseases, effective control methods, such as drugs and vaccines, have been developed and succeeded in controlling. On the other hand, few cases have succeeded in controlling fish parasitic diseases. It is considered that the control of cryptocaryoniasis is difficult, similarly to other parasitic diseases. Therefore, in order to prevent cryptocaryoniasis, research from various aspects other than the present study also will be required. As such, the road to controlling cryptocaryoniasis, which was reported about 80 years ago in Japan, still seems to be long.

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