Doctoral Thesis

Ecological studies on parasitic copepods infecting fish fins, with special references to the life cycle and infection-site specificity

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CHAPTER 1

General Introduction

Parasitology is the science that deals with one of the several different kinds of symbiotic relationships including the parasitism (Bush et al. 2001). Organism involves in parasitism are called parasite and the host (Rohde, 1993). Bush et al. (2001) picked the definition of parasites according to the Oxford English Dictionary as the organism, which lives in or upon another organism and draw it nutrient directly from it. While, host is an organism that harbors a parasite, or a mutual or commensal symbiont, typically providing nourishment and shelter (Rohde, 1993). To define parasitism, one has to emphasize the damage parasites inflicted on hosts, the metabolic dependence of parasites on hosts, and ecological interactions between populations of these two species of living organisms (Bush et al. 2001).

Ecology has been defined as the study of interactions between organisms and their environments and among the organisms inhabiting these environments (Sobecka, 2012). This is a complex field of study, which is why it should be investigated on various levels. In the ecology of parasites, the niche which is the entirety of the parasite-host relationship and fragmentation, is of fundamental importance (Combes, 1995).

Parasite lives at the expense of the host, but it is also dependent on its host in many other aspects. Parasite may impose many impacts to the host. The nutritional, habitat, and dispersal exploitation of a parasite may lower the fitness of the host. They also have the ability to modify host behavior that may lead to their castration (Levri, 1998). Based on the way of invasion to a host, parasite can be classified into several categories. Endoparasite is the parasite confined within the body of the host while ectoparasite is typically restricted to the exterior part of the host's body (Sobecka, 2012). Mesoparasites are the in between group, with some part of the parasite buried into the host tissue, and some part is exposed to the outdoor world (Kearn, 2010). All parasitic nematodes and the majority of flatworms are endoparasites, while the majority of arthropods are ectoparasites.

Copepoda is a relatively a small group of arthropod with currently more than 11500 valid species worldwide (Boxshall & Hallsey, 2004). Half of known species of copepods developed symbiotic relationship with other organism (Huys & Boxshall, 1991) including as parasite. Boxshall (2005) recognized four major groups of copepods which involve in parasitism, namely Monstrilloida, Siphonostomatoida, Cyclopoida and Harpacticoida. Large amounts of the Siphonostomatoida involving 30 families are parasites of fishes. Copepod from Family Pennellidae Burmeister, 1835 and Caligidae are the common parasites on fishes (Boxshall, 2005).

Family Pennellidae contains 20 genera (Boxshall & Halsey 2004), most of which are known as mesoparasites (Kabata 1979). Pennellids have been described from non-commercial, deep-sea fishes (Shiino 1958; Izawa 1970, 1977; Boxshall 1986, 1989) coastal and shallow waters fishes (Nagasawa & Uyeno, 2010) and recently several species are reported to infect captive-kept fishes in cage-culture (Fukuda, 1999; Nagasawa et al. 2011; Maran et al. 2012; Ismail et al. 2013) and commercial aquarium (Okawachi et al. 2012). Only several species are well studied, such as *Lernaeocera branchialis* (Linnaeus, 1767), due to its severe impact to the aquaculture industry (Sproston, 1942, Kabata, 1962; Kabata, 1979; Pilcher et al. 1989; Brooker et al. 2007; Brooker et al. 2013; Khan et al. 1990).

In comparison, parasitic copepod from Family Caligidae is the most the most studied parasitic copepods due to its severe impact on economic losses to finfish aquaculture, particularly of salmonids (Boxshall & Defaye, 2006; Pike & Wadsworth,1999). Caligids copepod are very speciose family comprising 34 genera and more than 450 species (Ho & Lin, 2004; Boxshall & Halsey, 2004; Boxshall & Justine, 2005; Boxshall, 2008). In Japan, 6 caligid species were recognized as pests in finfish aquaculture (Ho & Lin, 2004, Ohtsuka et al. 2009; Maran et al. 2011).

In this present study, chapter 2 and 3 are detailing the research of *P. minuticaudae* particularly about the life cycle and ecology. However, instead in detailing the research for site-specificity in chapter 4 on *P. minuticaudae*, I choose *C. fugu* as the model animal for some reasons; (1) *C. fugu* and the hosts (*T. niphobles*) are easily obtainable from the water of Takehara Marine Station, Hiroshima University, (2) *C. fugu* imposed more economic significant in comparison to *P. minuticaudae* due to the heavy infection on *Takifugu rubripes* (3) the full genome information of the host, *Takifugu rubripes* is available in genomic database, thus will facilitate the screening of the candidate genes. Molecular approach and methodology

demanding high financial funding, thus the economic importance of *C. fugu* justifying the investment for the research.

Purpose of this study:

In this study, I aim to understand the fundamental of parasitism by exploring the life cycle, ecological aspect and site specificity mechanism of parasitic copepod. The study involving two parasitic copepods from the Order Siphonostomatoida, *Peniculus minuticaudae* (Pennellidae) and *Caligus fugu* (Caligidae). This two species share one similarity, where at least some part of its life stages are infecting the fins of the hosts (Ohtsuka et al. 2009; Maran et al. 2011; Nagasawa et al. 2011; Maran et al. 2012; Okawachi et al. 2012; Ismail et at. 2013). *P. minuticaudae* is less studied copepod in comparison to *C. fugu*. Accordingly, I take the opportunity to describe the complete life cycle and study the ecological aspect of this species.

C. fugu is a caligid copepod infecting high value species of puffers in Japan (Ohstuka et al. 2009). Due to its economic importance, research on *C. fugu* is more advances. Previous report on *C. fugu* includes many aspects such as the life cycle and development (Ohstuka et al. 2009), ecological (Maran et al. 2011), toxicity (Ikeda et al. 2006; Ito et al. 2006 Maran et al. 2007). Ohstuka et al. (2009) revealed that the developmental stages of this species show site-specificity to the fins of host, with a preference to the pectoral fins. However, host and site specificity of many parasites is not yet fully understood by the world scientist (Bron et al. 1993). Thus, in this study, I try to apply molecular techniques in combination to behavioral study to understand mechanism underlying the site-specificity of infective stage of *C. fugu* to the fins of puffer.

CHAPTER 2

Complete life cycle of a pennellid *Peniculus minuticaudae* Shiino, 1956 (Copepoda: Siphonostomatoida) infecting cultured threadsail filefish

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2.1 Introduction

The genus *Peniculus* von Nordmann, 1832 (Copepoda: Siphonostomatoida: Pennellidae) consists of 14 nominal species (Boxshall & Halsey, 2004; Maran et al. 2012). In Japan, three *Peniculus* species have so far been recorded: *P. minuticaudae* Shiino, 1956, *P. ostraciontis* Yamaguti, 1939 and *P. truncatus* Shiino, 1956 (Shiino, 1956; Yamaguti, 1939). *Peniculus minuticaudae* has so far been recorded from fishes of two different families: four fish hosts of the family Monacanthidae such as threadsail filefish *Stephanolepis cirrhifer* Temminck and Schlegel, 1850, unicorn leatherjacket filefish *Aluterus monoceros* Linnaeus, 1758, hairfinned leatherjacket *Paramonacanthus japonicus* Tilesius, 1809, black scraper *Thamnaconus modestus* Gunther, 1877 and one host of Chaetodontidae, brown-banded butterflyfish *Chaetodon modestus* Temminck and Schlegel, 1844 (Shiino, 1956; Nagasawa et al. 2011; Okawachi et al. 2012; Maran et al. 2012). *Peniculus ostraciontis* parasitized two boxfishes such as humpback turretfish *Tetrosomus gibbosus* Linnaeus, 1758 and triangular boxfish *T. concatenatus* Bloch, 1785 (Ostraciidae) (Yamaguti, 1939; Shiino, 1956), while *P. truncatus* was found to infect rockfish *Sebastes oblongus* Günther 1877 (Shiino, 1956) and Korean rockfish *S. schlegelii* Hilgendorf, 1880 (Sebastidae) (Maran et al. 2012).

Shiino (1956) first described the post-metamorphic female of *P. minuticaudae* recovered from wild *S. cirrhifer* collected from the waters of Shirahama, Wakayama

Prefecture, Japan. The post-metamorphic female of *P. minuticaudae* was recently redescribed from Japan (Okawachi et al. 2012) and Korean (Maran et al. 2012) waters. Recent reports indicated the extend of the infestation of *P. minuticaudae* on fishes kept in captivity such as in aquaculture facilities (Fukuda et al. 1999; Nagawasa et al. 2011; Maran et al. 2012) and in a commercial aquarium (Okawachi et al. 2012).

In general, the life cycle of pennellids is direct or indirect depending on species (Perkins, 1983). Some need two hosts, i.e., intermediate and definitive (Sproston, 1942; Ho, 1966; Perkins, 1983; Brooker et al. 2007), while some need only one host (Schram, 1979). Based on the discovery of different developmental stages (copepodid, late chalimus stages, pre-metamorphic adult female and adult male) on a host which was kept in an aquarium without any possible secondary host, it was suggested that *P. minuticaudae* could complete its life cycle on a single host (Okawachi et al. 2013). In the present study, we found all stages including copepodid, chalimi, adults and post-metamorphic females on the fins of cultured *S. cirrhifer*, indicating that *P. minuticaudae* could complete its life cycle on a single host. We also confirmed that the hatching stage of *P. minuticaudae* is copepodid, which is one of the two known types of hatching stage among copepods, naupliar or copepodid (Sproston, 1942; Ho, 1966; Schram, 1979; Perkins, 1983; Izawa, 1997; Brooker et al. 2012).

2.2 Materials and Methods

2.2.1 Observation of the first hatching stage

Ovigerous post-metamorphic adult females of *P. minuticaudae* (n = 10) were collected from the fins of *S. cirrhifer* captured from the sea cage aquaculture facilities of the Fisheries Research Center, Ehime Research Institute of Agriculture, Uwajima, Ehime Prefecture, Japan (33°16'92"N, 132°43'94''E) on 21 November 2011. Egg strings were carefully detached from the ovigerous females using fine forceps then transferred into vials containing filtrated sterilized seawater before being transported to Takehara Marine Science Station, Hiroshima, Japan (34°32'58"N, 132°92'33''E) for incubation. In the laboratory, the egg strings were transferred into Petri dish containing fresh filtrated sterilized seawater and incubated (NK system Biotron LH-200-RDSCT, Tokyo) at a temperature of ca. 22-25°C until hatching. Hatching of copepodids was confirmed by direct observation under a dissecting microscope Olympus SZX7. All hatching copepodids were immediately preserved in 70% ethanol for further study.

2.2.2 Description of developmental stages

Twenty individuals of *S. cirrhifer* (fork length range from 15-21 cm) were obtained from the Fisheries Research Center on 26 June 2011 and preserved in 10% neutralized formalin seawater individually in a plastic bag. They were screened to find the infection of copepods especially on the fins. Preserved solution was also filtered through a 300μm sieve to find any detached specimens. The collected specimens were preserved in 70% ethanol.

Observation was carried out for descriptions of all stages except adult male and post-metamorphic adult female since they are already well described (Shiino, 1956; Maran et al, 2012; Okawachi et al, 2012). Prior to observation, specimens were

cleared in a drop of lactophenol for 30 min, dissected and examined following the wooden slide procedure (Humes & Gooding, 1964). Drawing and measurements were made with the aid of a drawing tube attached to an Olympus BX50 differential interference contrast microscope. Specimens were measured intact using an ocular micrometer. Anatomical terminology follows Kabata (1979) and Huys & Boxshall (1991) and fish names conform to FishBase (2013).

2.2.3 Scanning electron microscope (SEM) analysis

About five to ten specimens of *P. minuticaudae* were used for the SEM microscopy analysis. The copepods were transferred to 70% ethanol and then dehydrated through a graded series of ethanol (90%, 99.5% and 100%) and finally by isoamyl alcohol. The samples were critical point-dried using CO_2 gas and ion-sputtered for observation with a scanning electron microscope JSM6510-LV (JEOL, Tokyo).

2.3 Results

2.3.1 Hatching of copepodid

Eight copepodids hatched out from a single egg string after 27 hours of incubation and seen directly its movement after hatching. Some other copepodids from the same egg string hatched with a layer of membrane which hindered their movement and sank to the bottom of the Petri dish. Some of them were not completely released from the egg string. The active copepodids were collected and preserved in 70% ethanol for description. After three days, the observation for all other egg strings was discontinued due to contamination.

2.3.2 Description

Copepodid (Figure 2.1A-J)

Body length (based on 6 individuals hatching from incubated egg string). Range: 2.60–3.26 mm; average±standard deviation: 2.87±0.23 mm; (based on 5 individuals collected from the host): 2.99–3.22 mm; 3.12±0.82 mm.

Body (Figure 2.1A) oval with dorsal surface highly pigmented from anterior part of cephalothorax to caudal rami (pigmentation omitted from illustration). Rostrum weakly developed, rounded. Cephalothorax incorporating first pedigerous somite, about twice as long as free post-cephalothoracic somites and caudal rami combined. Widest about mid-length. Naupliar eyes conspicuous. Second pedigerous somite wider than long; third pedigerous somite with anlagen of leg 3 (Figure 2.1K) represented by paired, laterally-located papillae, each bearing one short spine; third free somite shorter than preceding somite, unarmed; fourth free somite bearing caudal rami (Figure 2.1B) armed with single long spinulose seta (seta II) and five short naked setae. Inner surface of ramus having hairy row.

Antennule (Figure 2.1C) indistinctly 2-segmented, proximal segment bearing 3 setae; terminal segment armed with 13 setae and long aesthetasc. Antenna (Figure 2.1D, 2A) incompletely 3-segmented; proximal segment large; middle segment broad with 2 pointed processes posteriorly; 2 pairs of teeth-like protuberances along inner margin; terminal segment claw-like with spinules. Oral cone (Figure 2.1E) located on ventromedial line, labrum and labium not fused, arranged as equal halves. Mandible (Figure 2.1F) slender, proximal part cylindrical, distal part loosely inserted into mouth cone, flat with 10 teeth at tip. Maxillule (Figure 2.1G) indistinctly bilobed, carrying 1 and 2 distal setae, respectively. Maxilla (Figure 2.1H) 2-segmented; proximal segment large, rod-like; distal segment curved, ended with blunt tip with transverse striation on posterior part (see Figure 2.2B). Maxilliped absent. Legs 1 (Figure 2.1I)

and 2 (Figure 2.1J) with coxa, basis and unisegmented rami. Armature formula of legs shown in Table 2.1.

Remarks. The copepodid of *P. minuticaudae* collected from the host *P. japonicus* (Okawachi et al. 2012) is similar to the hatching copepodid of *P. minuticaudae* in our study except for the larger size. Through SEM examination, some features of antenna (Figure 2.2A) and maxilla (Figure 2.2B) are given in detail. The surface terminal segment of antenna is ornamented with small spinules and along the inner margin there are 2 pairs of tooth-like protuberances; the innermost element is bifurcated (Figure 2.2A). Among pennellids, only *P. minuticaudae* shows these features, but the antenna of *Cardiodectes* sp. (Ho, 1966) and *Lernaeenicus sprattae* Sowerby, 1806 (Schram, 1979) are similar to those of chalimi of *P. minuticaudae*. Recently, Brooker et al. (2012) redescribed the copepodid of *Lernaeocera branchialis* Linnaeus, 1767 and reported that, the distal border of the antenna is ornamented with blunt processes rather than a spine. Unlike *L. branchialis* where sexual dimorphism can be detected even at copepodid stage through the setal size (finer in female) in the caudal ramus (Brooker et al. 2012), no sexual dimorphism can be detected in *P. minuticaudae*.

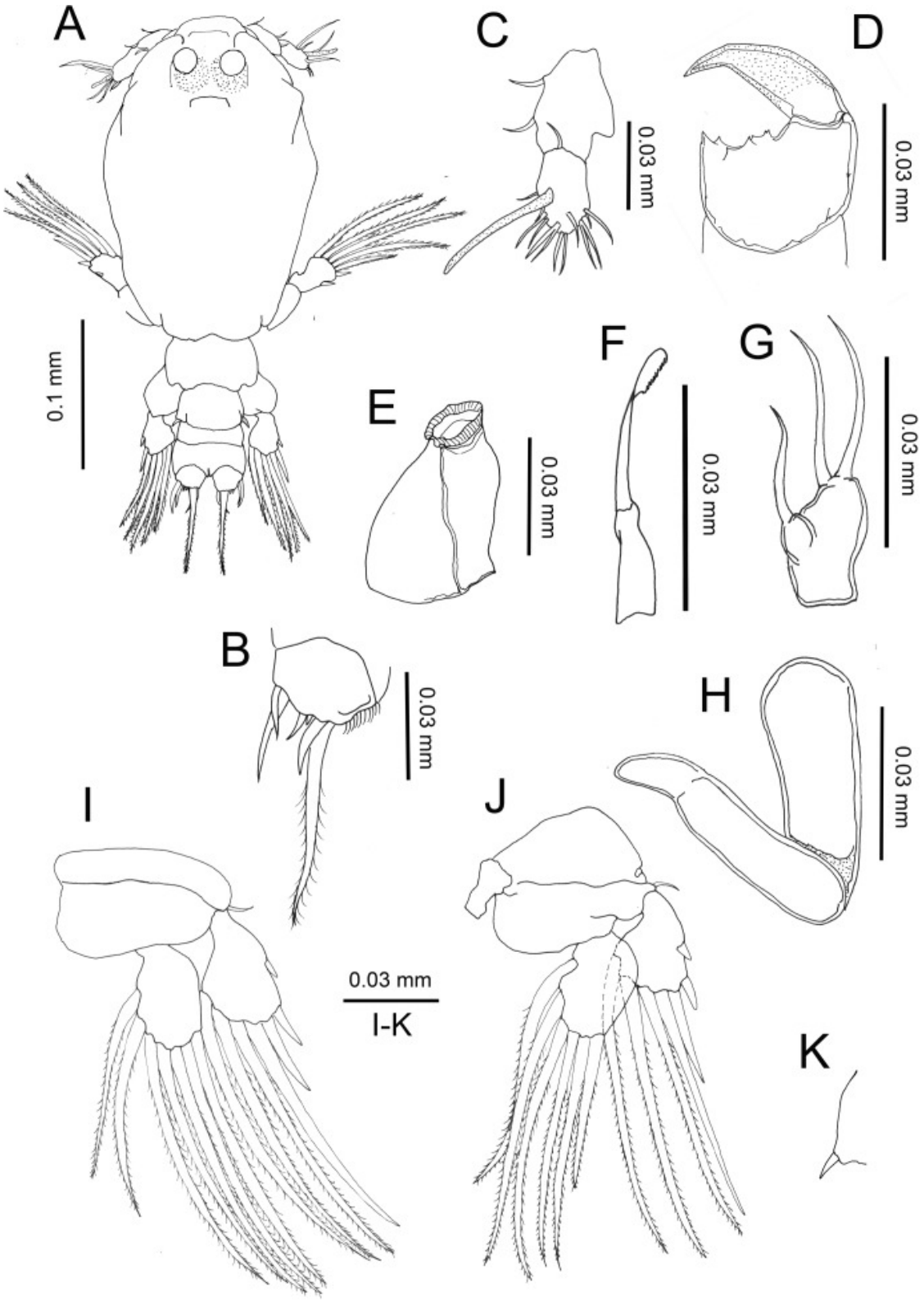

Figure 2.1 *Peniculus minuticaudae* Shiino, 1956. Copepodid stage: A, habitus, dorsal view; B, caudal ramus, dorsal view; C, antennule; D, antenna; E, mouth tube; F, mandible; G, maxillule; H, maxilla; I, leg 1, anterior surface; J, leg 2, anterior surface; K, leg 3, dorsal view. Scales in mm.

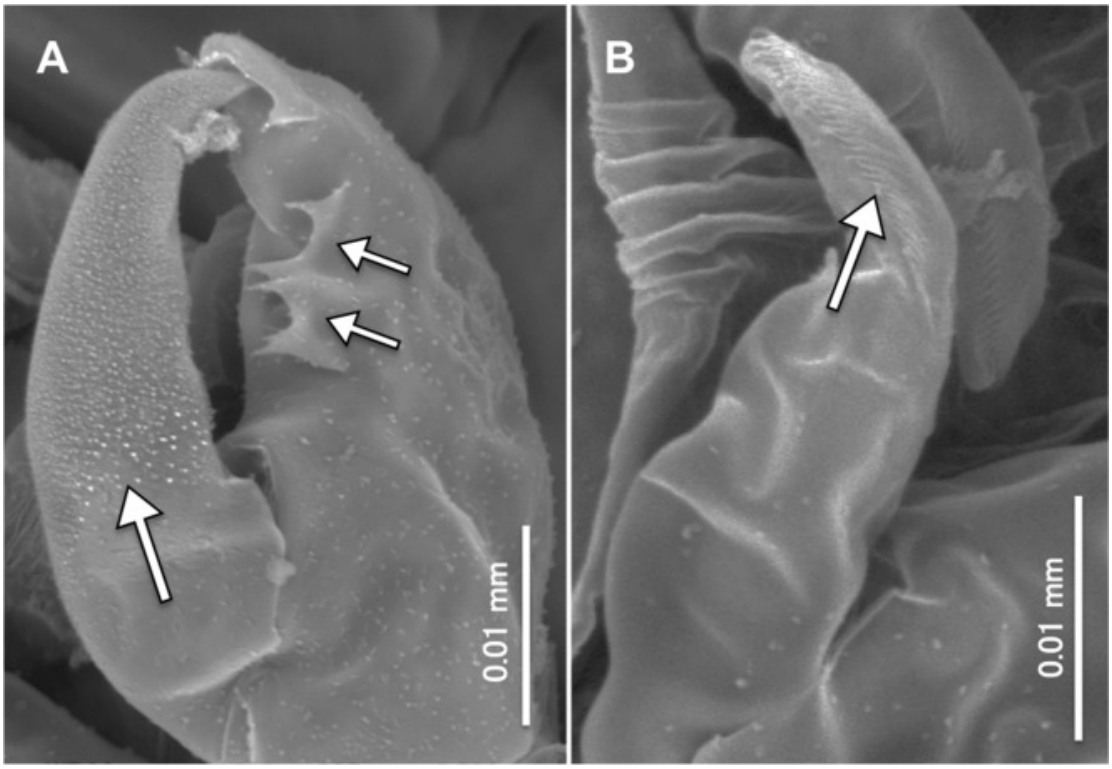

Figure 2.2 Scanning electron micrograph of *Peniculus minuticaudae* Shiino, 1956. A, antenna of copepodid stage. Arrows showing the spinules on terminal segment and the teeth-like protuberances on the second segment of the antenna; B, maxillae of copepodid stage. Arrow showing the transverse striation on posterior part of the maxillae. Scales in mm.

First chalimus, female (Figure 2.3A-J)

Body length (based on 5 individuals collected from *S. cirrhifer*): 3.13−3.35 mm; 3.22±1.10 mm.

Body (Figure 2.3A) slightly larger than that of copepodid. Cephalothorax about 1.48 times longer than free post-cephalothoracic somites combined. Frontal filament (Figure 2.3B) bearing single hood extending from cephalothorax, attached to fin rays using two short strands. Naupliar eyes present. Second pedigerous somite wider than long; third pedigerous somite unarmed; third and fourth free somites indistinctly segmented. Anal somite bearing short caudal rami (Figure 2.3C) armed with six naked setae of unequal length.

Antennule (Figure 2.3D) indistinctly 2-segmented, proximal segment bearing 3 marginal setae, distal segment having 13 fine setae and aesthetasc. Antenna (Figure 2.3E) indistinctly 3-segmented, chelate; proximal segment large; middle segment with 2 pointed processes inwardly; distal segment claw-like, with single minute seta basally. Mandible (Figure 2.3F), maxillule (Figure 2.3G) and maxilla (Figure 2.3H) same as in those of copepodid. Mouth cone not developed. Maxilliped absent. Legs 1 (Figure 2.3I) and 2 (Figure 2.3J) biramous, comprising protopod and both rami armed with six naked setae of unequal length. Armature formula of legs is shown in Table 2.1.

Remarks. The first chalimus differs from copepodid in the general appearance, the body shape, the presence of frontal filament, finer setae on the antennule, the structure of legs 1-3 and the absence of plumose setae on the caudal rami. The legs 1 and 2 of chalimus stages comprised of protopod, exopod and endopod. Setae on the rami become simple. No differentiation between spine and setae is found at this stage. In comparison to other pennellids, differences can be seen in the antenna and the maxilla. In *Cardiodectes* sp. the antennary terminal end and the terminal claw of the maxilla split into 3 processes (Ho, 1966).

First chalimus, male (Figure 2.3K-L)

Body length (based on 4 individuals collected from *S. cirrhifer*): 3.15−3.36 mm; 3.27±0.90 mm.

All features similar to those of female. Maxilliped (Figure 2.3L) projected as anlagen just behind maxilla. *Remarks.* The maxilliped anlagen of the first chalimus represent the first appearance of sexual dimorphism in *P. minuticaudae*. Such first

appearance of sexual dimorphism is also found in another pennellid of the first chalimus male of *Cardiodectes medusaeus* Wilson, 1908 [24].

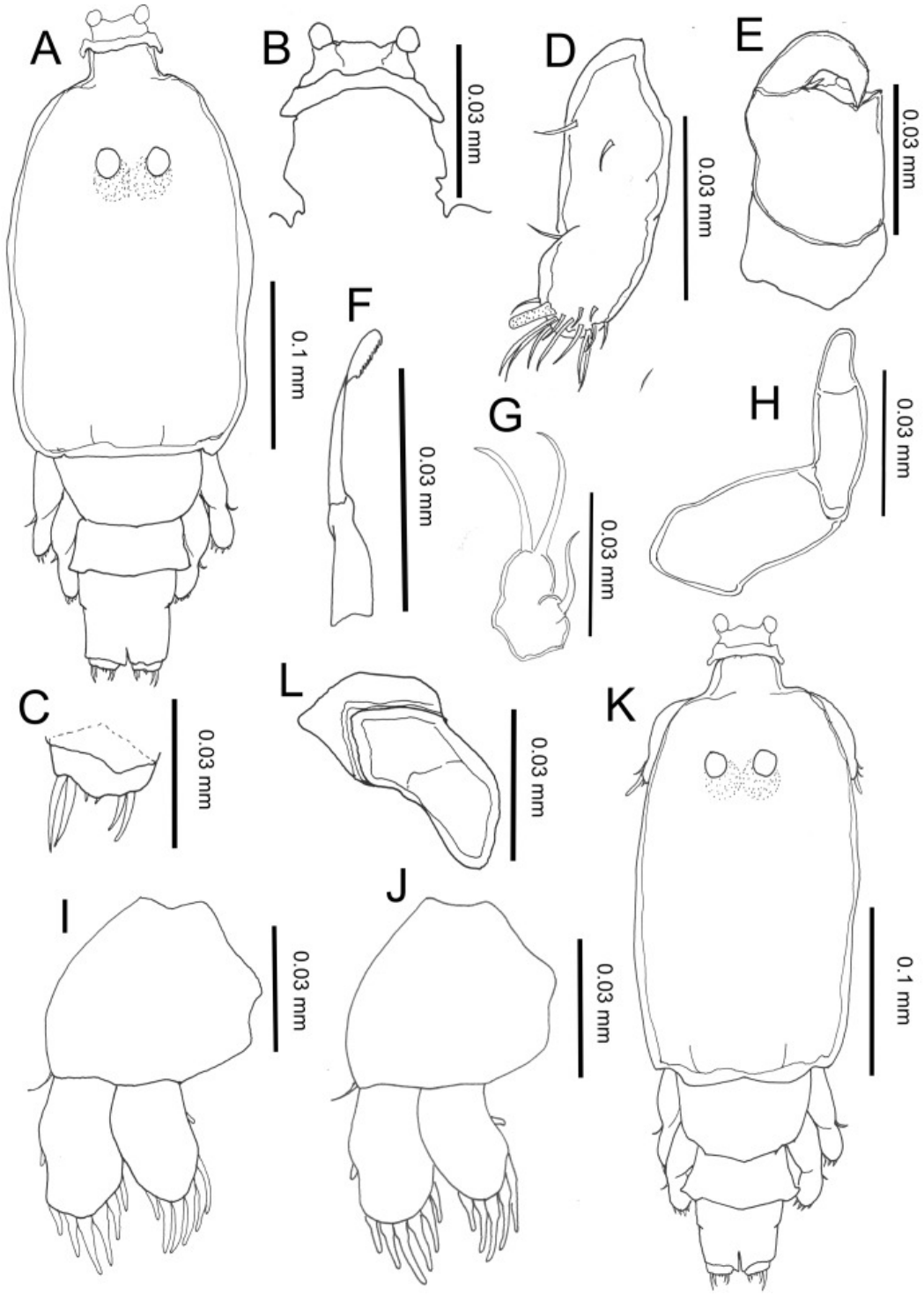

Figure 2.3 *Peniculus minuticaudae* Shiino, 1956. First chalimus stage, A, female, habitus, dorsal view; B, frontal filament; C, caudal ramus, dorsal view; D, antennule; E, antenna; F, mandible; G, maxillule; H, maxilla; I, leg 1, anterior surface; J, leg 2, anterior surface; K, male, habitus, dorsal view; L, anlagen of maxilliped. Scales in mm.

Second chalimus, female (Figure 2.4A-K)

Body length (based on 3 individuals collected from *S. cirrhifer*): 3.23−3.73 mm; 3.48±0.26 mm.

Body (Figure 2.4A) with elongated cephalothorax and 4 free somites. Cephalothorax about 1.41 times longer than free post-cephalothoracic somites combined. Frontal filament (Figure 2.4B) longer than in preceding stage; 2 remnants present at tip of frontal filament. Naupliar eyes present. Second pedigerous somites wider than long. Fourth pedigerous somite bearing anlagen of leg 4 ventrolaterally. Anal somite wider than long, bearing caudal rami (Figure 2.4C) with 6 naked setae of unequal length.

Antennule (Figure 2.4D) indistinctly 2-segmented; proximal segment bearing 7 marginal setae; distal segment with 13 setae and aesthetasc. Antenna (Figure 2.4E) indistinctly 3-segmented, chelate; proximal segment broad; middle segment with 2 pointed processes inwardly; distal segment claw-like, with single minute seta at base. Mandible (Figure 2.4F), maxillule (Figure 2.4G) and maxilla (Figure 2.4H) similar to those of preceding stage. Maxilliped absent. Legs 1 (Figure 2.4I) and 2 (Figure 2.4J) biramous, comprising protopod with unisegmented rami. Leg 3 (Figure 2.4K) uniramous with 2 setae at tip. Armature of legs are given in Table 2.1.

Remarks. The second chalimus differs from the preceding stage in the frontal filament and the setation on legs. The frontal filament is quite prominent and more elongated in comparison to that of first chalimus female and two remnants are visible. In leg 1, one additional seta is seen on the posterior margin of protopod. In leg 2, one seta is added to both the exopod and the endopod. The characteristic features of leg segmentation and setation are similar to those of *L. branchialis* (Sproston, 1942) and *L. sprattae* (Schram, 1979). Leg 3 is represented by a single ramus equipped with 2

simple setae terminally and leg 4 by an anlagen on the fourth thoracic somite. In comparison, leg 3 of the second chalimus of *Cardiodectes* sp. bears 6 setae and the rudimentary protuberance of leg 4 is specific to the female only (Ho, 1966).

Second chalimus male (Figure 2.4L-M)

Body length (based on 4 individuals collected from *S. cirrhifer*): 3.28−3.8 mm; 3.45 ± 0.24 mm.

Body (Figure 2.4L) similar to that of female. Cephalothorax about 1.39 times longer than free post-cephalothoracic somites combined. Other features similar to those of female except for presence of maxilliped (Figure 2.4M). Maxilliped 2-segmented; proximal segment large and stout; distal segment tapering distally into blunt claw.

Remarks. Generally the body and appendages are similar to those of the female except on the presence of the maxilliped and the anal somite, which is slightly longer than female.

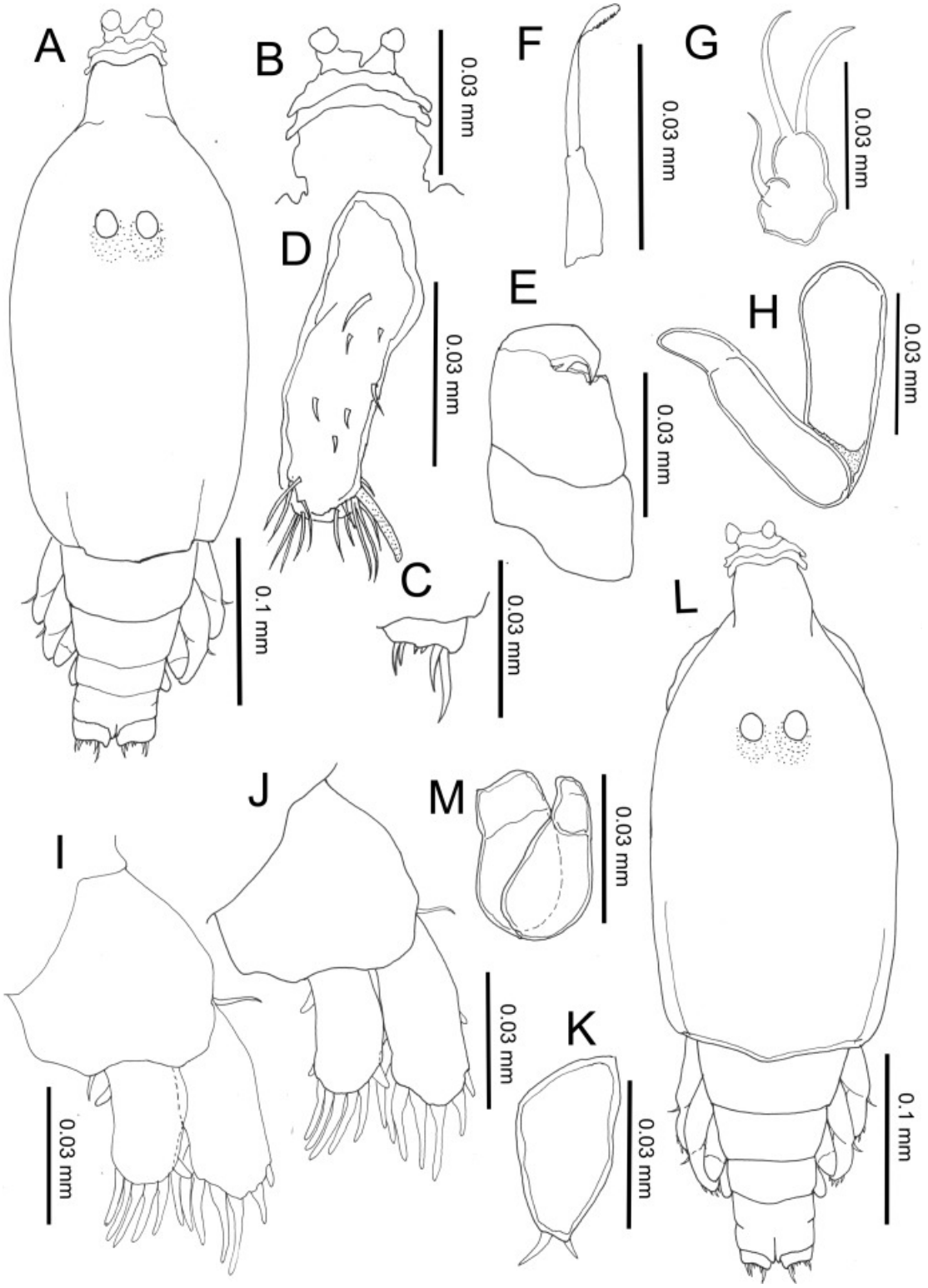

Figure 2.4 *Peniculus minuticaudae* Shiino, 1956 Second chalimus stage. A, female, habitus, dorsal view; B, frontal filament; C, caudal ramus, dorsal view; D, antennule; E, antenna; F, mandible; G, maxillule; H, maxilla; I, leg 1, anterior surface; J, leg 2, anterior surface; K, leg 3, anterior surface; L, male, habitus, dorsal view; M, maxilliped. Scales in mm.

Third chalimus, female (Figure 2.5A-L)

Body length (based on 5 individuals collected from *S. cirrhifer*): 4.41−4.47 mm; 4.44±0.28mm.

Body (Figure 2.5A) slender with cephalothorax about 1.5 times longer than free post-cephalothoracic somites combined, widest at mid-length. Frontal filament (Figure 2.5B) with 3 remnants. Fourth free somite representing genital somite. Anal somite bearing caudal rami (Figure 2.5C) with 6 setae of unequal length.

Antennule (Figure 2.5D) indistinctly 2-segmented; proximal segment bearing 15 setae on anterior margin; distal segment with 13 setae and aesthetasc. Antenna (Figure 2.5E), mandible (Figure 2.5F) and maxillule (Figure 2.5G), maxilla (Figure 2.5H) as in preceding stage. Legs 1 (Figure 2.5I) and 2 (Figure 2.5J) biramous, comprising protopod with unisegmented rami. Legs 3 (Figure 2.5K) and 4 (Figure 2.5L), uniramous, 2-segmented. All legs armed with naked setae. Armature of legs given in Table 1.

Remarks: Third chalimus stage has one additional free somite in comparison to second chalimus stage. Other differences are additional remnants on frontal filament, setation of antennule, development of third and fourth legs and also the setation of all legs.

Third chalimus, male (Figure 2.5M-N)

Body length (based on 4 individuals collected from *S. cirrhifer*): 3.89−3.94 mm; 3.92±0.32 mm.

Body (Figure 2.5M) stubbier with cephalothorax about 1.51 times longer than free post-cephalothoracic somites combined. Genital somite wider than long. All other features similar to those of female except for presence of maxilliped. Maxilliped

(Figure 2.5N) 2-segmented; proximal segment robust, unarmed; terminal segment tapering distally into blunt claw.

Remarks: Sexual dimorphism can be seen by general body appearance which is more stubbier than female; the presence of maxilliped and the size of genital somite which is shorter and wider in comparison to those of female.

Fourth chalimus, female (Figure 2.6A-L)

Body length (based on 4 individuals collected from *S. cirrhifer*): 4.14−4.51 mm; 4.35±0.16 mm.

Body (Figure 2.6A) with more distinct body segmentation. Cephalothorax about 1.5 times longer than free post-cephalothoracic somites combined. Frontal filament (Figure 2.6B) with 4 remnants. Nauplius eyes conspicuous. Caudal rami (Figure 2.6C) same as in preceding stage.

Antennule (Figure 2.6D) indistinctly 2-segmented, proximal segment bearing 18 setae, distal segment bearing 13 setae and aesthetasc. Antenna (Figure 2.6E) as in preceding stage. Mandible (Figure 2.6F), maxillule (Figure 2.6G) and maxilla (Figure 2.6H) as in preceding stage. Legs 1 (Figure 2.6I) and 2 (Figure 2.6J) biramous, each composed of protopod and 1-segmented rami. Legs 3 (Figure 2.6K) and 4 (Figure 2.6L) uniramous, 2-segmented. Armature of legs given in Table 1.

Remarks: This stage is easily distinguished from the preceding stage by: almost all appendages have characteristics close to the adult form; the four remnants on the frontal filament are clearly visible; the antennule is still indistinctly segmented but the segmentation is more visible compared to the previous stages; all legs have the equal number of elements in adults. The exopod and endopod are elongated with setae protruded from some indentation points, which in adults are separated into 2 segments.

Fourth chalimus female in the present study showed similarity to the late chalimus female described by Okawachi et al. (2012) except for the setation on legs 1 and 2 and the teeth count on mandible.

Fourth chalimus, male (Figure 2.6M-N)

Body length (based on 2 individuals collected from *S. cirrhifer*): 4.13–4.5 mm; 4.31±1.60 mm.

Body (Figure 2.6M) shorter than that of female. Cephalothorax longer than wide, about 1.5 times longer than free post-cephalothoracic somites combined. Appendages similar to those of female except for presence of maxilliped. Maxilliped (Figure 2.6N) 2-segmented; proximal segment robust, unarmed; terminal segment tapering distally into pointed claw having single element midway.

Remarks: General body length is shorter than female. The strong maxilliped of male represents the distinct sexual dimorphism. The body segmentation and maxilliped's form showed similarity to the maxilliped of late chalimus male described by Okawachi et al. (2012). However, the legs 1 and 2 setation between the fourth chalimus male of the present study is different from the late chalimus male described by Okawachi et al. (2012).

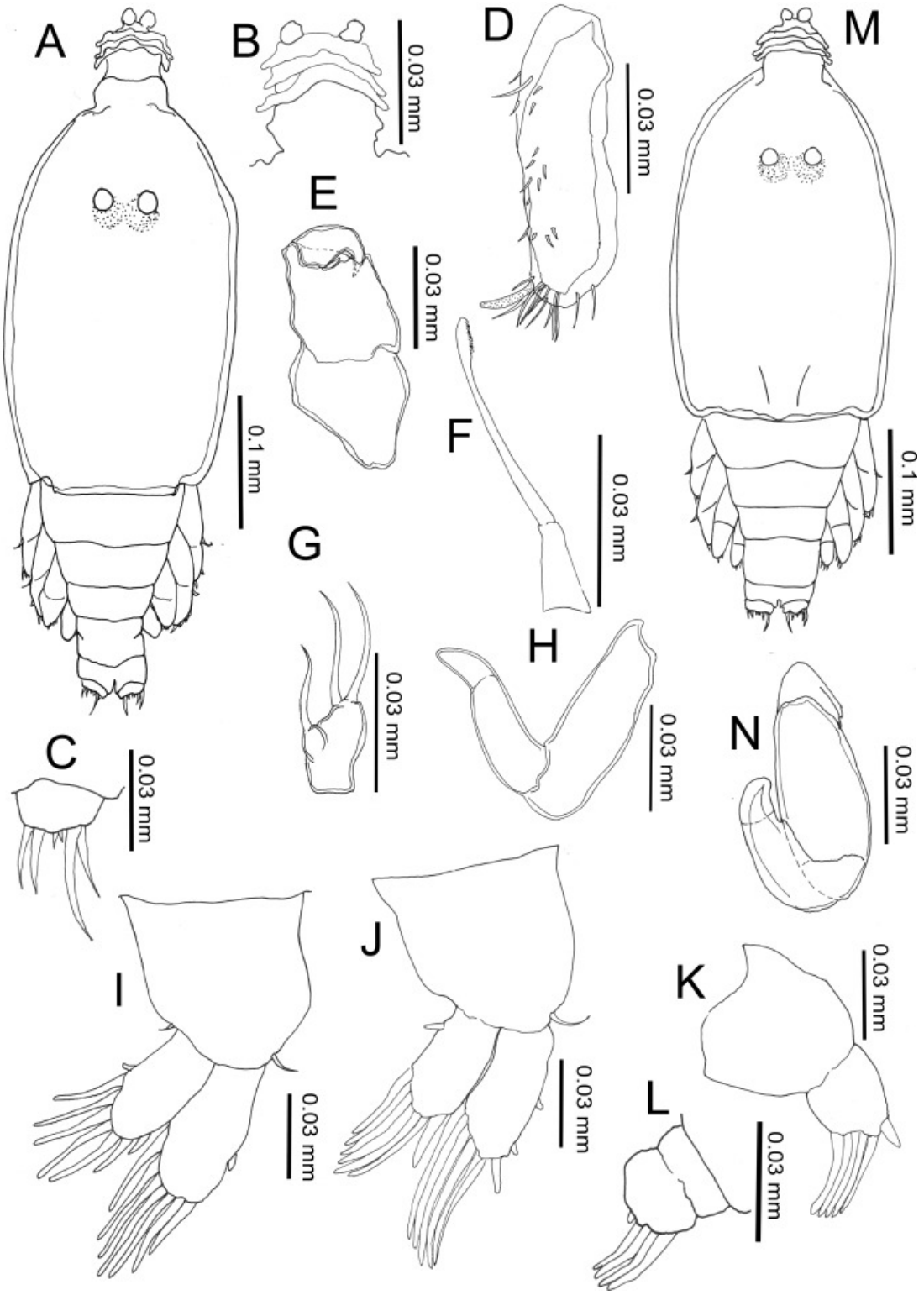

Figure 2.5 *Peniculus minuticaudae* Shiino, 1956. Third chalimus stage. A, female, habitus, dorsal view; B, frontal filament; C, caudal ramus, dorsal view; D, antennule; E, antenna; F, mandible; G, maxillule; H, maxilla; I, leg 1, anterior surface; J, leg 2, anterior surface; K, leg 3, anterior surface; L, leg 4, anterior surface; M, male, habitus, dorsal view; N, maxilliped. Scales in mm.

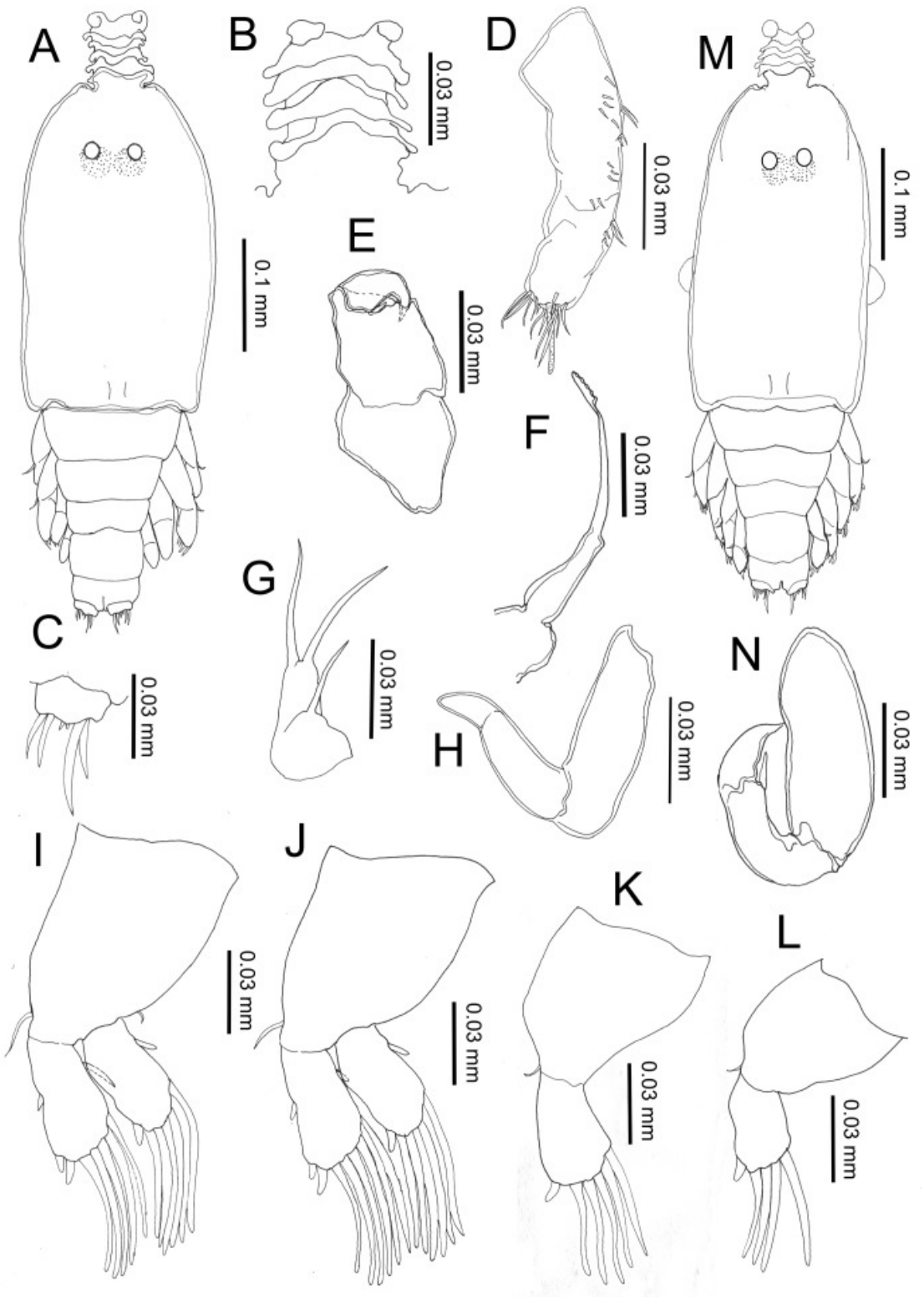

Figure 2.6 *Peniculus minuticaudae* Shiino, 1956. Fourth chalimus stage. A, female, habitus, dorsal view; B, frontal filament; C, caudal ramus, dorsal view; D, antennule; E, antenna; F, mandible; G, maxillule; H, maxilla; I, leg 1, anterior surface; J, leg 2, anterior surface; K, leg 3, anterior; L, leg 4, anterior surface; M, male, habitus, dorsal view; N, maxilliped. Scales in mm.

Pre-metamorphic adult female (Figure 2.7A-L)

Body length (based on 6 individuals collected from *S. cirrhifer*): 5.60–6.60 mm; 5.90±0.40 mm.

Body (Figure 2.7A) slender with distinct 5 post-cephalothoracic somites. Cephalothorax with large, conical rostrum, longer than wide, about 1.5 times longer than free post-cephalothoracic somites combined. Temporary frontal filament with five remnants (Figure 2.7B). Nauplius eyes conspicuous. Genital complex long, with transverse striation. Abdomen short, wider than long; carrying caudal rami with 6 setae of unequal length (Figure 2.7C).

Antennule (Figure 2.7D) 4-segmented, with armature formula of 9, 6, 8, 13+ae. Antenna (Figure 2.7E) 2-segmented, chelate; proximal segment robust, bearing 2 pointed processes on inner margin; terminal segment claw-like with minute seta at base. Oral cone well developed, located at midventral surface of cephalothorax. Mandible (Figure 2.7F) rod-like with 10 teeth and pointed tip. Maxillule (Figure 2.7G) bilobed with 1 and 2 setae at tip, respectively. Maxilla (Figure 2.7H) 2-segmented; proximal segment with single process anteriorly; distal segment with transverse striation and 2 rows of fine setulose ornamentations. Legs 1 (Figure 2.7I) and 2 (Figure 2.7J) with coxa, basis, and 2-segmented rami. Legs 3 (Figure 2.7K) and 4 (Figure 2.7L) with coxa, basis and 2-segmented exopod only. All rami armed with plumose setae. Armature of legs given in Table 2.1.

Remarks: Adult male of *P. minuticaudae* was first described by Okawachi et al. (2012). Sexual dimorphism between adult male and pre-metamorphic adult female can be distinctly seen on the body segmentation, the antenna and the genital structures. The body segmentation of the male is composed of 7 post-cephalothoracic somites including 2 abdominal somites. The pre-metamorphic adult female has only 5 post-

cephalothoracic somites including 1 abdominal somite. The antenna of the male is similar to those of chalimus stages while, in the female, it shows swelling on the proximal part. Adult female that has undergone metamorphosis shows huge morphological differences in comparison to the pre-metamorphic adult female. The body segmentation of post-metamorphic adult female has been reduced due to the incorporation of fourth pedigerous somite with the expansion of genital complex to form the trunk region (Shiino, 1956; Okawachi et al. 2012; Maran et al. 2012) . The abdominal segment has also become indistinctly segmented from the trunk region (Shiino, 1956; Okawachi et al. 2012; Maran et al. 2012). The caudal rami that located at posterior end of pre-metamorphic female have been pushed towards the posteroventral part of the post-metamorphic female. The post-metamorphic females also lack antennules and the rami on the legs (Shiino, 1956; Okawachi et al. 2012; Maran et al. 2012) which are retained in pre-metamorphic female for swimming purposes

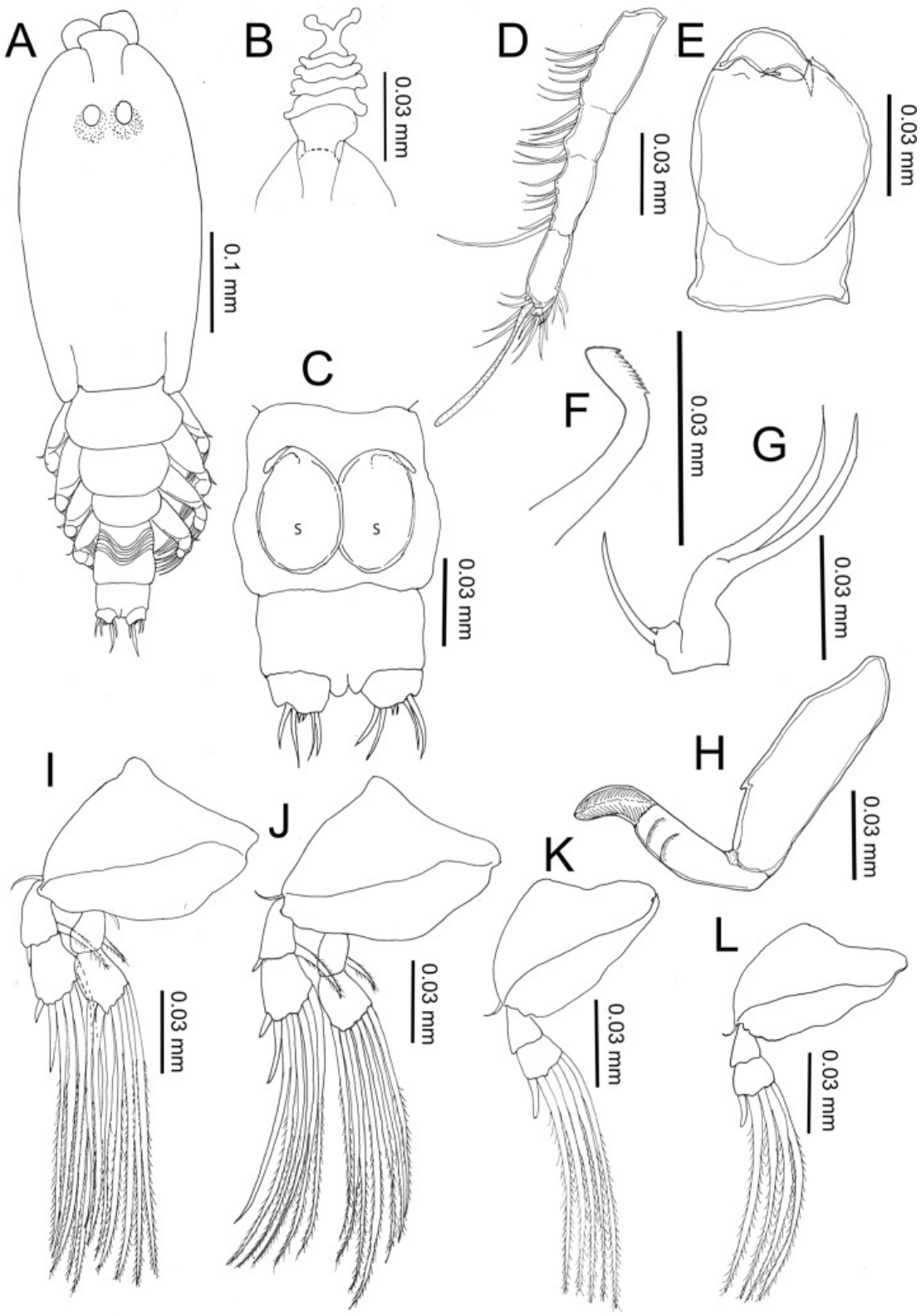

Figure 2.7 *Peniculus minuticaudae*, adult female. A, habitus, dorsal view; B, frontal filament; C, posterior region, ventral view, s=spermatophore. D, antennule; E, antenna; F, mandible; G, maxillule; H, maxilla; I, leg 1, anterior; J, leg 2, anterior; K, leg 3, anterior; L, leg 4, anterior. Scales in mm.

Table 2.1 Armature formula of legs of six different stages in the life cycle of *Peniculus minuticaudae* Shiino, 1956 (Roman and Arabic numerals indicating spines and setae, respectively)

Stage/Leg	**Segmentation**			
Copepodid	**Coxa**	**Basis**	**Exopod**	**Endopod**
Leg 1	0-0	1-0	II, II, 3	7
Leg 2	0-0	1-0	II, II, 3	6
Chalimus I	**Protopod**		**Exopod**	**Endopod**
Leg 1	1-0		7	7
Leg 2	1-0		7	6
Chalimus II				
Leg 1	1-1		7	7
Leg 2	1-0		8	8
Leg 3	0		2	-
Chalimus III				
Leg 1	1-1		8	8
Leg 2	1-0		9	8
Leg 3	0-0		6	-
Leg 4	0-0		5	-
Chalimus IV				
Leg 1	1-1		9	8
Leg 2	1-0		10	8
Leg 3	1-0		6	-
Leg 4	1-0		5	-
Pre-metamorphic adult female	**Coxa**	**Basis**	**Exopod**	**Endopod**
Leg 1	0-0	1-1	I-1; I, I, 5	0-1; 7
Leg 2	0-0	1-0	I-1; I, I, 6	0-1; 7
Leg 3	0-0	1-0	0-0; I, 5	-
Leg 4	0-0	1-0	0-0; I, 4	-

2.4 Discussion

2.4.1 Complete life cycle of *P. minuticaudae*

In this study, the presumed complete life cycle of *P. minuticaudae* based on the discovery of all stages from a single host *S. cirrhifer* is given (Figure 2.8). This is the first layout of the complete life cycle proposed for the genus *Peniculus*. Overall, the life cycle of *P. minuticaudae* consists of six developmental stages separated by moult, which is composed of one infective copepodid (Figure 2.8A), four chalimi (Figure 2.8B-E) and adult (Figure 2.8F and 2.8G). Through the observation of hatching process of the egg strings incubated under laboratory conditions, we could confirm that the hatching stage of *P. minuticaudae* is copepodid. Figure 2.9A shows the copepodid in the egg sac just before hatching. The hatched infective copepodid has the ability to actively swim and search for a host (Boxshall, 2005). After the infection of copepodid on the hosts, particularly the fins, they moult into chalimus stages. *Peniculus minuticaudae* have four chalimus stage prior to final moulting to adult stage. The presence of complete and well developed swimming legs of pre-metamorphic adult female and adult male suggested that they have abilities to detach from a host for copulation, or to search for another suitable host or site of final settlement.

The copulatory process of *P. minuticaudae* (Figure 2.8I) is likely to be similar to that of other pennellids as described by Ho (1966) and Schram (1979). Soon after copulation, the fertilized pre-metamorphic female detaches and swims to find a new settlement site (Figure 2.8J). After the final settlement (Figure 2.8K), pre-metamorphic adult female undergoes massive differential growth and finally becomes post-metamorphic adult female (Figure 2.8L) and produces eggs. Pre-copulation (2.8H) was also observed between adult male and various stages of female. Pre-copulation amplexus between adult male and first chalimus stage female is shown in

Figure 2.9B. The male grasped the female at the base of its frontal filament. Such precopulatory behaviour was also reported in *L. branchialis* (1989).

In the present study, all stages of *P. minuticaudae* from copepodid to post-metamorphic adult female were found infecting the fins of *S. cirrhifer*. Five copepodids were found clinging on the hosts by grasping the fin ray tissue using its powerful antenna. Unlike *L. branchialis* (Brooker et al. 2012) and *L. sprattae* (Schram, 1979), no copepodid of *P. minuticaudae* was found having a frontal filament, which suggests that the intrusion of frontal filament might occur very shortly before the first moult. Chalimus stages attached to the host by means of their frontal filament and can be distinguished by counting the remnants on the frontal filament, which increase by stage corresponding to each moult (Sproston, 1942; Ho, 1966; Schram, 1979). Adult male, pre-metamorphic, metamorphic and post-metamorphic adult females were found attached to the fin rays using their antenna. In this study, two adult females were found attached to the fin ray of a host by means of frontal filament. Ho (1966) also observed adults of *Cardiodectes* sp. attached to the host using frontal filament. It is believed that frontal filament is used for a temporary attachment (Boxshall, 2005; Ohstuka et al. 2009; Maran et al. 2013) and soon they will detach and swim for new settlement site. Metamorphing adult females can be distinguished from the pre-metamorphic females by their slightly enlarged antennae and elongated genital segment. The pre-metamorphic females found on the fin of a host normally carrying spermatophores. In addition to the strong grasping of the antennae, the whole cephalothorax region of the post-metamorphic female usually covered by the scarred tissues of the fins. The feeding activity of the parasite might induce the proliferation of the fin tissues and finally scar that developed is covering the whole cephalothorax region of the post-metamorphic female. As the ratio of head to whole body of post-

metamorphic female are 1: 5.84 (Okawachi et al. 2012), the scar tissue creating a secure attachment for the parasite. In the case of fish burdening high density of parasites, some pennellid specimens were found infecting the skin near the fins.

Okawachi et al. (2012) summarized that the life cycle of pennellids can be divided into four phases, i.e., first free-living, first sessile or chalimus phase, second free-living and second sessile phase. Two swimming stages i.e., the infective copepodid and the fertilized pre-metamorphic female determine the settlement site for the first and second sessile phase, respectively (Sproston, 1942; Schram, 1979; Perkins, 1983). These two stages of *P. minuticaudae* were found to infect a single host, on the same site particularly the fins, together with all other stages. From the findings we could confirm the suggestion of Okawachi et al. (2012) that *P. minuticaudae* could complete its life cycle on a single host.

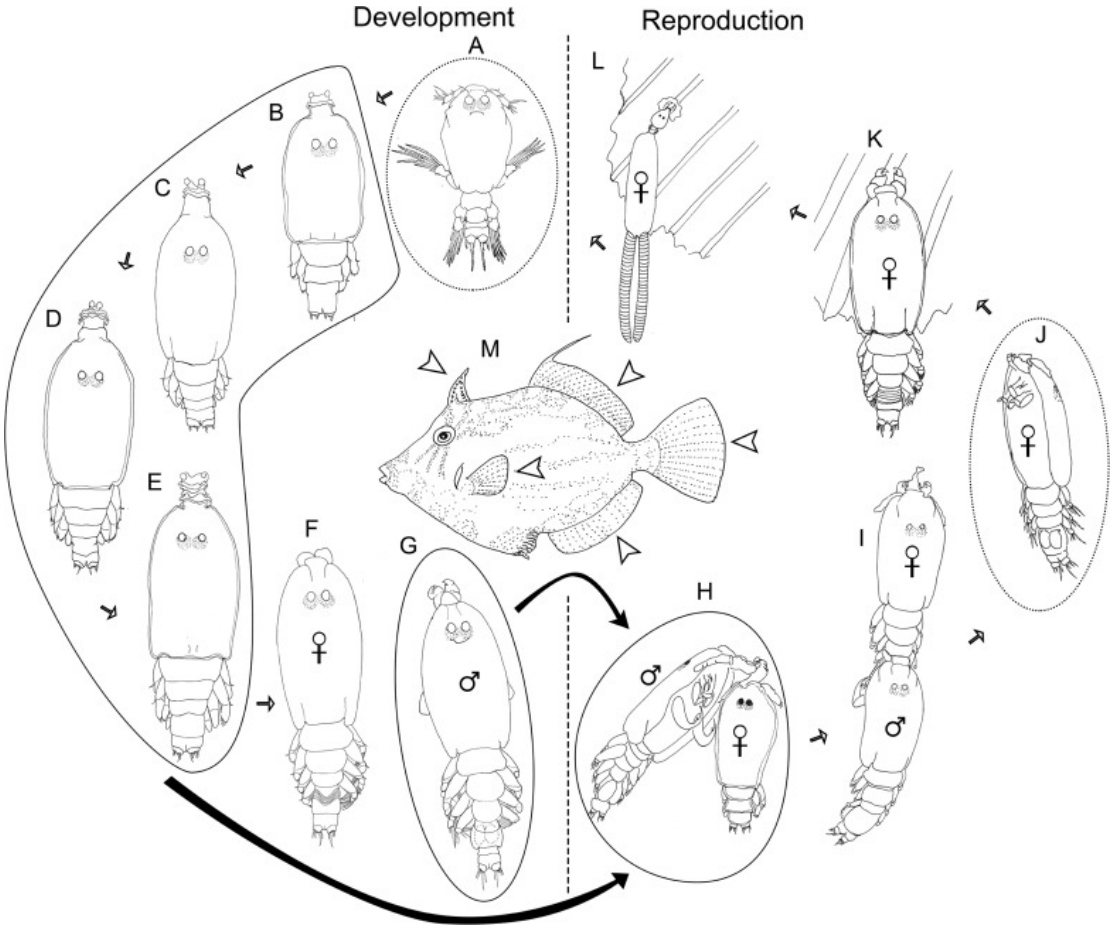

Figure 2.8 Schematic life cycle of *Peniculus minuticaudae*. A, infective copepodid; B, chalimus I; C, chalimus II; D, chalimus III; E, chalimus IV; F, pre-metamorphic adult female; G, adult male; H, pre-copulation guarding of adult male to chalimus I female; I, copulation of adult male to pre-metamorphic adult female; J, fertilized pre-metamorphic adult female with spermatophores detach from the temporary frontal filament and swimming for new settlement site; K, fertilized pre-metamorphic adult female clinging to the new settlement site on the fin ray of host; L, ovigerous post-metamorphic female on the fin of fish host; M, fish host, threadsail filefish (*Stephanolepis cirrhifer*). Arrows showing infection sites of *P. minuticaudae* on the host. Line circles (___) indicating stages involves in precopulation. Dotted circles (…) indicating free-swimming stages.

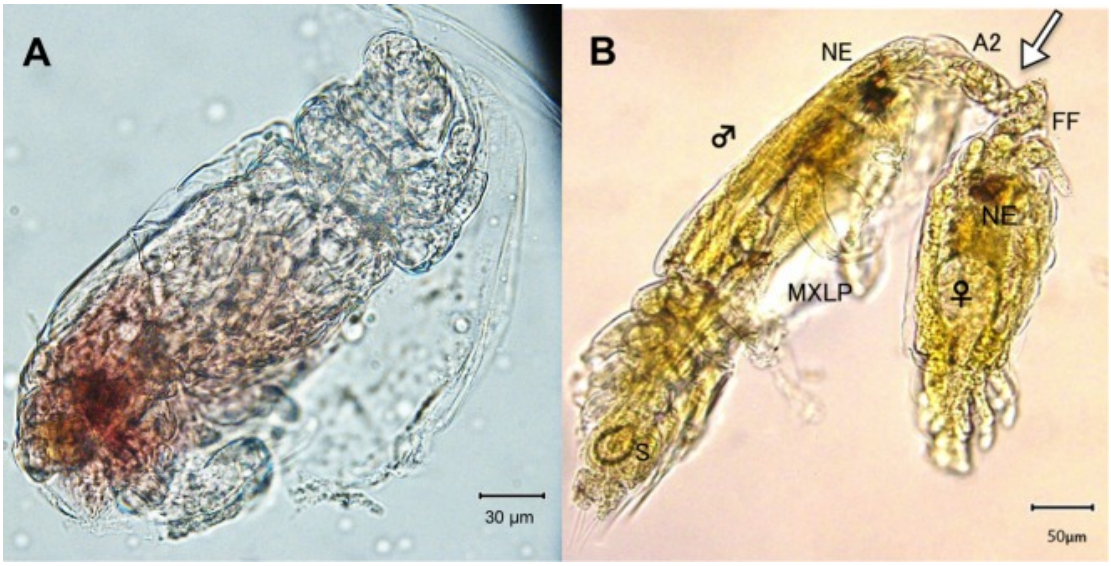

Figure 2.9 *Peniculus minuticaudae* Shiino, 1956. A, copepodid in the egg sac just before hatching; B, precopulation guarding of adult male with chalimus I female. Arrow showing the male's antennae which grasp to the female's frontal filament. NE=nauplius eyes, MXLP=maxilliped, S=spermatophore, A2=antennae, FF=frontal filament.

2.4.2 Comparison of life cycle among pennellids

The complete life cycle of pennellids has so far been described only for three genera and species: *L. branchialis* (Sproston, 1942; Brooker et al. 2012), *C. medusaeus* (Ho, 1966; Perkins, 1983) and *L. sprattae* (Schram, 1979) and now the fourth genera *P. minuticaudae*. The present study on *P. minuticaudae* sheds new insights into the life cycle of pennellids. The characteristics including the life cycle of all four genera (Pennellidae) are compared in this study (Table 2.2).

The basic life cycle of copepods comprises two phases with six naupliar stages and five post-naupliar stages prior to adult stage (Boxshall, 2005). However, naupliar phase abbreviation is a common phenomenon for siphonostomatoid copepod and the brief summary of the naupliar stages abbreviation among siphonostomatoid copepods was reported by Izawa (2012). Some siphonostomatoids of the families Lernaeopodidae H. Milne-Edwards, 1840 (Kabata, 1973), Nicothoidae Dana, 1852 (Ohstuka et al. 2005; Ohstuka et al. 2007) and Pennellidae (Ho, 1966; Perkins, 1983;

Table 2.2 Comparison on the life cycle and characteristics of four pennellids (*Lernaeocera branchialis, Cardiodectes medusaeus, Lernaeenicus sprattae* and *Peniculus minuticaudae)*.

Pennellids / Characteristics	*Lernaeocera branchialis* Linnaeus, 1767	*Cardiodectes medusaeus* Wilson, 1917	*Lernaeenicus sprattae* Sowerby, 1806	*Peniculus minuticaudae* Shiino, 1956
Developmental stages	8 (2 naupliar, 1 copepodid, 4 chalimi, adult)	5 (1 copepodid, 3 chalimi, adult)	8 (2 naupliar, 1 copepodid, 4 chalimi, adult)	6 (1 copepodid , 4 chalimi, adult)
Host(s) needed to complete life cycle	Double	Double	Single	Single
Intermediate host	Mainly fishes from the family Pleuronectidae	Pelagic gastropods mainly from the families Cavolinidae and Janthinidae	-	-
Definitive host	Fish of Gadidae	Fish of Myctophidae	Fish of Clupeidae	Fishes of Monocanthidae and Chaetodontidae
Infection site of copepodid stage	Gill lamellae	Gill lamellae/ mantle tissues	Body surface and fins	Fins
Infection site of post-metamorphic female	Burrowing through the gill arch to reach the heart of the fish host	Burrowing from various parts of the ventral surface of the fish host to reach the heart	Eyes	Fins
Possible food source of larval stages and post-metamorphic female	Blood	Blood	Blood, coelomic and tissues fluid	Presumably epithelium tissue and mucous
Range size of post-metamorphic female	20 to 50 mm	8.5 to 15 mm	12 to 18 mm	5 to 6 mm
References	Sproston [28], Brooker *et al.* [6, 7], Kearn [18]	Ho [11], Perkins [24]	Schram [25], Kearn [18]	Shiino [26], Nagasawa *et al.* [19], Venmathi Maran *et al.* [29], Okawachi *et al.* [23], present study

Izawa, 1997) showed the most abbreviated naupliar stage by skipping the whole stages within the eggs and hatches directly as infective copepodid (Boxshall, 2005; Izawa, 2012). While the abbreviation of naupliar stage is common, siphonostomatoid copepods are suggested to retain the basic five post-naupliar stages prior to adult (Boxshall, 2005; Ohtsuka et al. 2009; Maran et al. 2013). However, due to the transition from free-living to parasitic mode of life, after the settlement of infective copepodid to the host, most siphonostomatoid copepodids parasitizing fishes undergoes copepodid form modification by attaching to the host by means of frontal filament and these forms are called as chalimus (Ho & Lin, 2004; Boxshall, 2005; Ohstuka et al. 2009, Maran et al. 2013).

Among pennellids, the complete life cycle is known for four genera and species. Among those four genera, *L. branchialis* and *L. sprattae* retain the naupliar phase and in total having seven developmental stages prior to adult (two naupliar, one copepodid, four chalimus). While, *C. medusaeus* and *P. minuticaudae* show naupliar stage abbreviation and hatch directly as infective copepodid stage. *Peniculus minuticaudae* shares the similarity in the pattern of post-naupliar stages with other two genera *L. branchialis* and *L. sprattae* by having one copepodid and four chalimus stages prior to adult. However, *C. medusaeus* was reported with lacking of one chalimus stage in compared to other pennellids (cf. Table 2.2) (Ho, 1966; Perkins, 1983). Since abbreviation of post-naupliar stages is not common among siphonostomatoid copepods, revision on the life cycle of *C. medusaeus* might be helpful to explain the peculiarity.

In general, pennellids need two hosts (intermediate and definitive) to complete its life cycle (Boxshall & Halsey, 2004). Infective copepodid settles on an intermediate host, and after copulation fertilized pre-metamorphic female finds the

definitive host for the final settlement. The information on the intermediate and definitive hosts for *P. minuticaudae*, *L. branchialis*, *L. sprattae* and *C. medusaeus* are briefly provided (cf. Table 2). Among pennellids, *L. branchialis* and *C. medusaeus* need two hosts (Sproston, 1942; Ho, 1966; Perkins, 1983), while *L. sprattae* (Schram, 1979; Anstensrud & Schram, 1988) and *P. minuticaudae* (present study) could complete their life cycle on a single host. For *P. minuticaudae*, our observation showed that all developmental stages infected at the same site, particularly on the fins. In the case of *L. sprattae*, infection site of the adult female after copulation differs from that of infective copepodid and chalimi stages. Adult female particularly infects the eyes of the fish host, while other developmental stages infect the fins and the body surface of the host (Schram, 1979; Anstensrud & Schram, 1988).

The size of post-metamorphic adult female of *P. minuticaudae* is the smallest in compared to three other pennellids (cf. Table 2.2). Among pennellids, *Peniculus*, *Peniculisa*, *Exopenna* Boxshall, 1986, and *Parinia* Kazachenko & Avdeev, 1977 are categorized as ectoparasite, while the rest are known as mesoparasites (Kabata, 1979; Boxshall, 1986). In *L. branchialis*, *L. sprattae* and *C. medusaeus*, the whole thoracic region are burrowed into the body of a host (Schram, 1979; Perkins, 1983; Brooker et al. 2007). Judging from the way of attachment, it is suggested that post-metamorphic adult female of *P. minuticaudae* might ingest the epithelium and mucous from the fin, in contrast to other pennellids, which are known as blood-feeding parasites (Perkins, 1983; Kearn, 2010; Brooker et al. 2007). The feeding type might influence the difference of sizes among pennellids.

To date, the pathogenicity of *P. minuticaudae* has not yet been studied in detail. However, the findings of high prevalence and intensity on cultured fishes (Fukuda, 1999; Nagasawa et al. 2011, Maran et al. 2012) and the mortality of

aquarium-kept fishes (Okawachi et al. 2012) showed that *P. minuticaudae* could act as a potential pest to harm the fishes kept in captivity (Nagasawa et al. 2011; Okawachi et al. 2012; Maran et al. 2012). In the present study, we revealed that *P. minuticaudae* has abbreviated life cycle and hatch directly as infective copepodid, and could infect the same host and infection site. Now that we have confirmed a complete life cycle of *P. minuticaudae* with six stages (1 copepodid, 4 chalimi and adult) and expect that it could make a significant implication in the aquaculture industry especially on the control strategies.

CHAPTER 3

Ecology of *Peniculus minuticaudae*

3.1 Introduction

Recently, several authors have reported the heavy infection of *Peniculus minuticaudae* Shino, 1956 on fish hosts kept in captivity. Nagasawa et al. (2011) recorded the infection of *P. minuticaudae* on two hosts *Stephanolepis cirrhifer* (Temminck & Schlegel, 1850) and *Thamnaconus modestus* Gunther, 1877 collected from different cage-culture facilities in Mie and Oita Prefectures, western Japan with the data on the prevalence and intensity and the total number of parasites harbored and its distribution on fish hosts. Okawachi et al. (2012) reported several stages of *P. minuticaudae* in aquarium-held fishes from Kagoshima Prefecture, southern Japan. Venmathi Maran et al. (2012) redescribed adults of *P. minuticaudae* from marine ranched *T. modestus* in Korea. Further, recently Venmathi Maran et al. (2014) reported the seasonal occurrence of the parasite from the same locality.

With the increasing and spreading incidence of *P. minuticaudae* on economically important fishes in Japan and Korea, it is essential to survey the ecology of this parasite on cage-cultured fish. In this chapter, I examined the population dynamics of *P. minuticaudae* infecting *S. cirrhifer* cultured in cage-culture facility in Uwajima Bay, Ehime Prefecture, western Japan.

3.2 Materials and methods

3.2.1 Sampling site

The sampling site (Figure 3.1) Ehime Prefectural Fisheries Experimental Station (EPFES), Uwajima, Ehime Prefecture is located in Uwajima Bay, Shikoku Island, western Japan (132° 31’ 00’’ E; 33°12’72’’ N) (Fig. 3.1).

Figure 3.1 Location of the sampling site Ehime Prefectural Fisheries Experimental Station.

3.2.2 Hosts sampling and parasites examination

Monthly samplings of the hosts were carried out from September 2011 to August 2012. Each host fish was collected by a scooping net, and then preserved in 10% formalin in an individual plastic bag immediately after capture. All bags were monthly kept in a plastic or polystyrene container and transported from EPFES to Takehara Marine Science Station, Hiroshima University (TMSS), western Japan. In-situ surface water temperature and salinity upon sampling were simultaneously recorded.

Ten randomly selected fish hosts were examined every month which bring in total 120 fishes were examined throughout the sampling period. In the laboratory, fish

hosts were measured for standard length (SL) and total length (TL), weighted using precision balance (Metler Toledo PB602-S, Switzerland) and examined individually for parasite infection. The whole body of fish hosts was examined for parasites. Host fins which the parasites exclusively infected were cut and examined in details under a dissecting microscope (Olympus SZX7, Tokyo, Japan). However many individuals were detached from the host fins during the transportation from EPFES to TMSS. Therefore preservative formalin solution in each plastic bag was sieved using a 100 μm modified plankton sampler to retain all detached specimens. All specimens were identified to each developmental stages and sex, counted and recorded, following Ismail et al. (2013). Copepods were preserved in vials containing 70% ethanol and labeled according to fish host number and month.

Length of egg strings and total eggs count in the egg strings were counted and recorded for (n=15) sample of ovigerous females collected in June 2012.

3.2.3 Data analysis

Chi-square test was used to analyze the differences of proportion of infected to uninfected host (prevalence) (Rosza et al. 2000). One sample t-test was used to analyze the differences of total count of copepodid, post-metamorphic and pre-copulation couples throughout the sampling period (Zar, 1984). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc was used to compare the abundance of parasite infections. For ANOVA, the data were square-root (x + 0.5)-transformed prior to analysis to normalize distribution and equalize variance (Itoh & Nishida, 2008). For all statistical analysis, the software SPSS version 20 was employed at the significant level of 0.05.

3. 3 Results

3.4.1 Environmental parameters

Figure 3.2 shows the fluctuation of water temperature and salinity in Uwajiwa Bay where the fish sampling was carried out. Salinity ranges from 32.13 to 32.88 psu with not much fluctuation throughout the sampling period. Water temperature remarkably showed a seasonal fluctuation ranging from 14.2 to 26.2 °C with the lowest temperature recorder in February and highest in August.

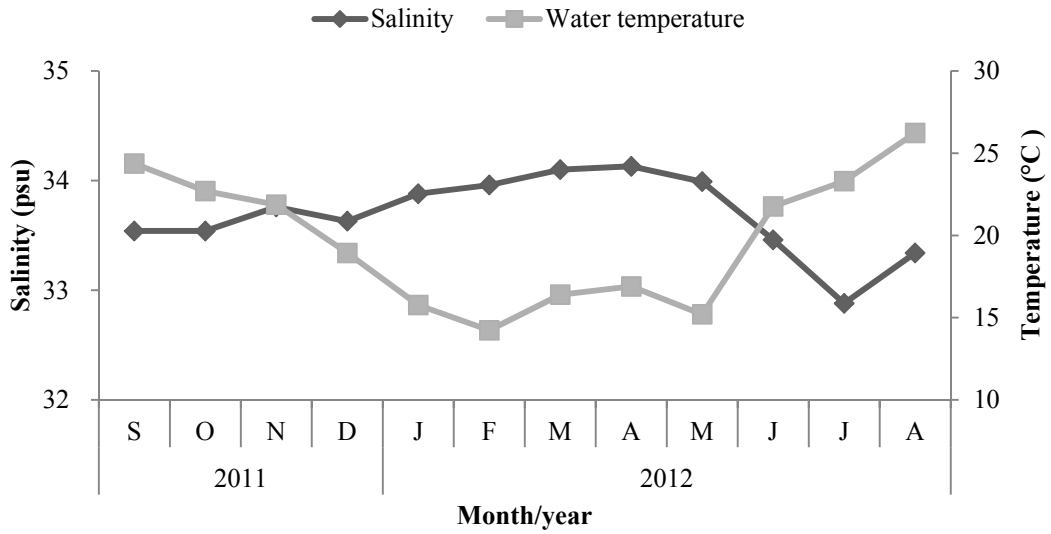

Figure 3.2 Salinity and water temperature in Uwajima Bay from September 2011 to August 2012.

3.4.2 Fish hosts

Figure 3.3 shows the correlation of standard length and weight of host fishes examined throughout the study period. The standard length of fish ranged from 16.92 to 22.04 cm. The weight of fish ranges from 176.5 to 274.5 g. The standard length is significantly correlated with the weight of fishes ($r2 = 0.96$, $p<0.05$).

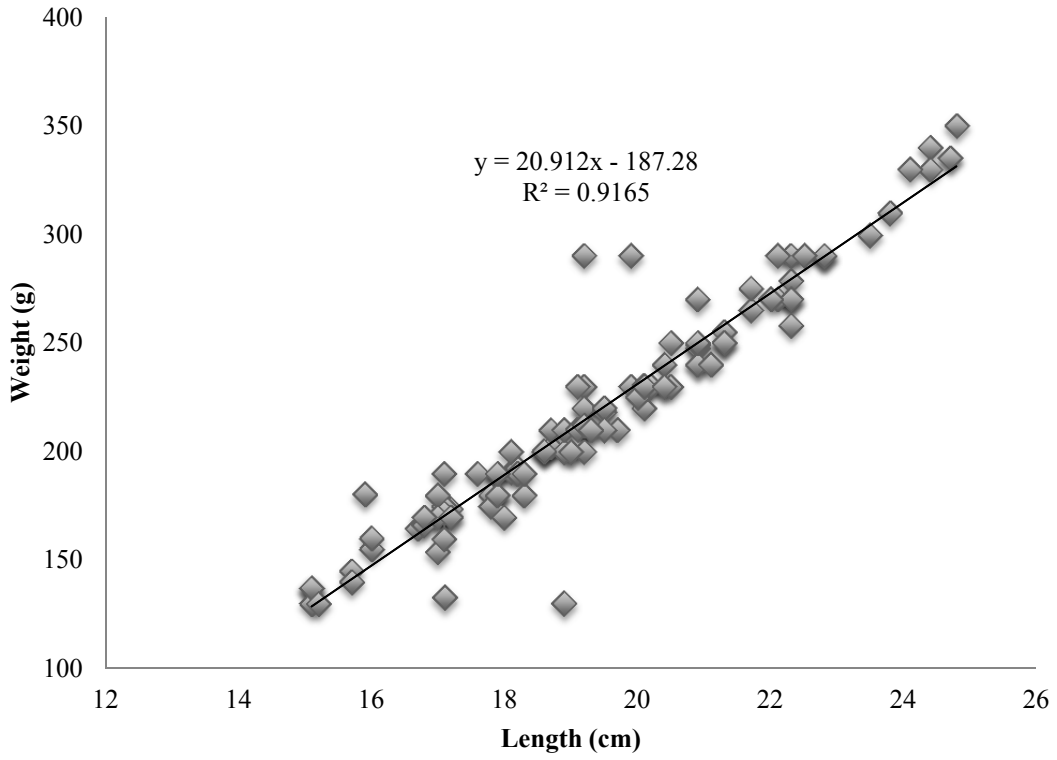

Figure 3.3 Standard length and weight of *Stephanolephis cirhiferr* examined from September 2011 to August 2012

3.4.3 Seasonal occurrence

Figure 3.4 shows the prevalence *Peniculus minuticaudae* infecting *Stephanolepis cirrhifer* from September 2011 to August 2012. The prevalence of all developmental stages of *Peniculus minuticaudae* ranged from 30% to 100% throughout the study period. The prevalence remained high from September 2011 to June 2012, but decreased starting July to August 2012. The prevalence throughout the sampling period was significantly different (Chi-square test: $X_{2=}171.1$, $p<0.05$).

A total of 1,645 parasites were collected throughout the study period. The mean intensity (total parasites/infected hosts) ranged from 0.4 to 45.2 parasites per host (Figure 3.5). The lowest mean intensity was recorded in July and August 2012 (0.4 respectively) while the highest mean intensity (45.2) was recorded on June 2012. The minimum intensity of parasites on a single host was 0, while the maximum intensity of parasites burdening a single host were 123 parasites. The intensity of parasites collected throughout the sampling period is significantly different (Kruskal-Wallis, $H_{11, x}=59.58$, $p<0.05$). The detailed result is shown in Appendix A.

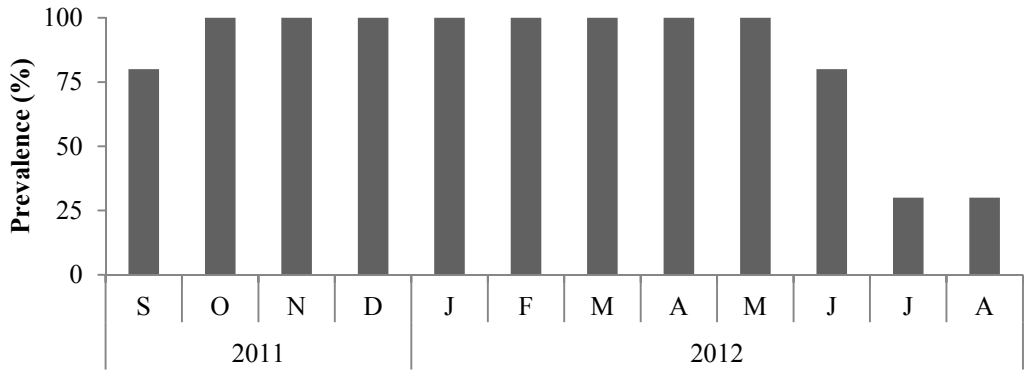

Figure 3.4 Prevalence of *Peniculus minuticaudae* infecting *Stephanolephis chirhiferr* from September 2011 to August 2012

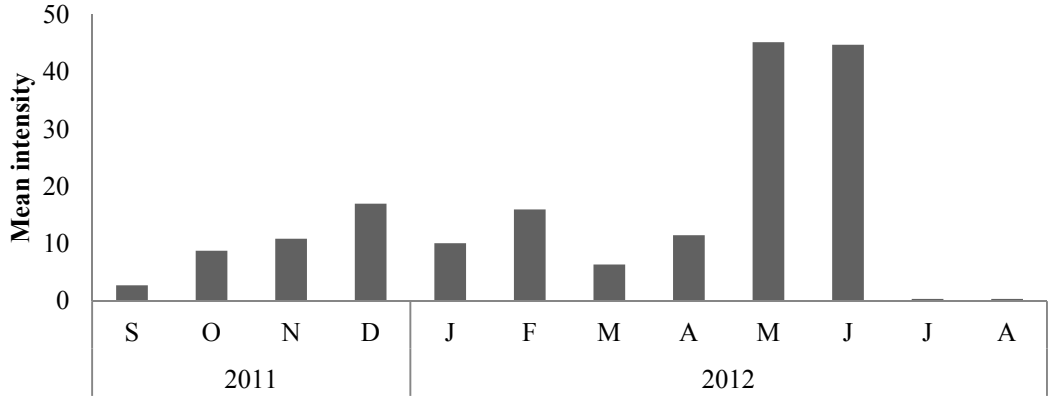

Figure 3.5 Mean intensity (total parasites/infected hosts) of *Peniculus minuticaudae* infecting *Stephanolephis chirhiferr* and from September 2011 to August 2012

3.4.4 Composition of developmental stages

Figure 3.6 shows the composition and abundance of parasites of all developmental stage throughout the study period. Adult females were categorized into four; pre-metamorphic, metamorphing, post-metamorphic and ovigerous post-metamoprhic. The ovigerous females are the post-metamorphic adult females carrying egg sacs. They occurred all year round except in July 2012 with the abundance ranged from 0 to 109 individuals, contributing to 0 to 49% of the monthly parasites population. From May to June 2012, ovigerous females steadily composed 24 to 25% of the parasites population. Post-metamoprhic female ore the fully matured females

but not carrying any egg sacs were found throughout the sampling period. The abundance of this stage ranged from 1 to 129 individuals contributing to 13 to 67% of the overall parasites count every month. Metamorphing females are the adult females with the transition morphology characteristics between the pre-metamorphic and post-metamorphic adult females. The abundance of metamorphing females ranged from 0 to 19 individuals, composing up to 33% of the monthly parasite counts. Pre-metamorphic females were identified by having 4 functional swimming legs, some are carrying spermatophores. The lowest total count of this stage were recorded as 0 individuals in September, February 2011 and August 2012. While the highest abundance was 38 individuals in May 2012.Except for July 2012 (33%), the count of pre-metamorphic females only contributed to less than 10% of the monthly parasites counts every month.

The total count of adult males ranged from 0 to 91 individuals, composing 0 to 25% of the overall parasites count every month. The chalimus counts reported in this study were the combination of four different chalimus stages (Chalimus I, II, III and IV). Chalimus stages present throughout the sampling period but absent in July and August 2012. The abundance ranged from 0 to 85 individuals and they composed 0 to 25% of the total counts of parasites every month. The copepodid is the hatching stage of *P. minuticaudae*, they also can be found in the parasite population all year round except for July and August 2012. The total count every month ranged from 0 to 22 copepodids and they composed up to 18% of the parasites abundance.

The abundance of parasites collected every month was significantly varied seasonally (One-way ANOVA, $F_{11, 108} = 21.3$, $p<0.05$). The abundance in May and June were significantly higher in comparison to all months of the sampling period (post-hoc test, $p<0.05$) while the total parasite counts in July and August 2012 were significantly lower than the abundance of parasites collected in December 2011 until June 2012 (post-hoc, $p<0.05$). The detailed count of the parasites according to different developmental stages throughout the study is exhibited in Appendix A.

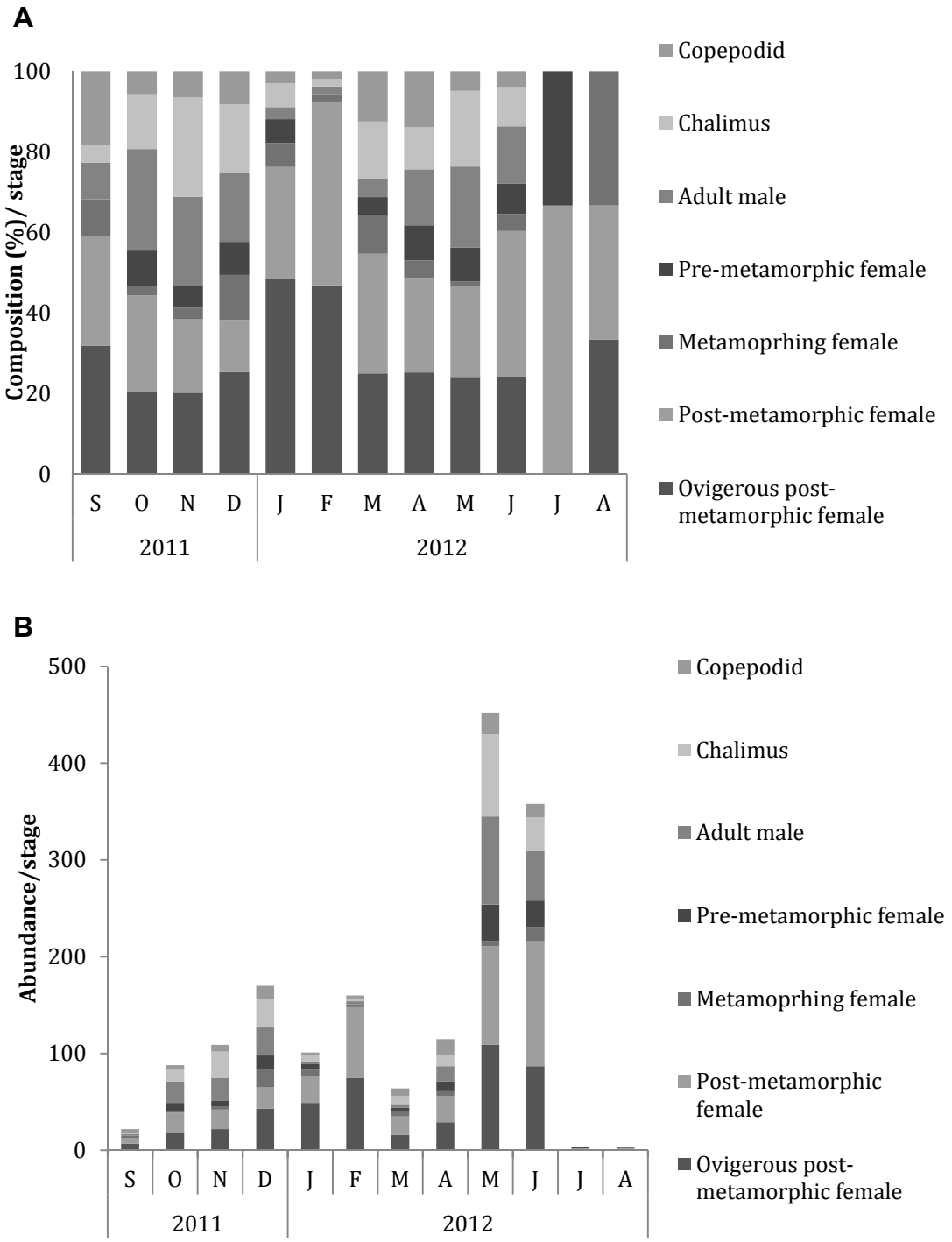

Figure 3.6 (A) Composition and (B) abundance of *Peniculus minuticaudae* per stage, infecting *Stephanolephis chirhiferr* from September 2011 to August 2012

3.4.5 Distribution of *Peniculus minuticaudae* on the host

From the overall collection of parasites found in this study, 66% of *Peniculus minuticaudae* were found attached to the fins and the remaining (specimens detached

from hosts) were collected from the preservative liquid (10% formalin) from the plastic bags containing the fish hosts (Figure 3.8). Only adult females (pre-metamophic, metamorphing post-metamoprhic and ovigerous post-metamorphic) were found firmly attached on the fins. All developmental stages (copepodids and chalimi) together with swimming stages adult male and some adult females (pre-metamophic, metamorphing and post-metamorphic) were found easily detached from the hosts. The detailed counts of individuals of *P. minuticaudae* attached on and detached from the fish hosts were tabulated in Appendix B.

Hence, only site-preference of adult females (pre-metamophic, metamorphing and post-metamorphic) attached on the fins (N=1086) were charted to different fins of infection site (Figure 3.7). Thirthy-eight percent of the adult females were found attached to the second dorsal fins of the hosts. The second most preferred site was both pectoral fins (23%). Eleven and 7% of the parasites were collected from anal and caudal fins, respectively. One percent of the parasites also can be found attached to the first dorsal of the hosts.

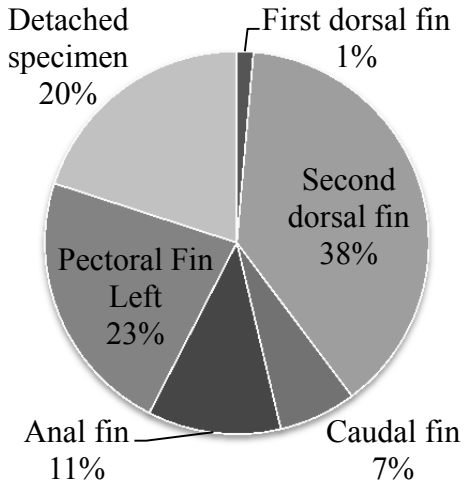

Figure 3.7 Distribution of adult females (pre-metamophic, metamorphing, post-metamorphic and ovigerous post-metamorphic) of *Peniculus minuticaudae* on the fins of *Stephanolephis chirhiffer* (N=1086).

3.4.6 Correlation of length of egg string and number of eggs

The number of eggs per string was significantly correlated with the length of egg string (r^2= 0.986, p<0.01) (Figure 3.x). Egg strings length ranged from 1.48 to 2.92 mm (mean±standard deviation; 2.1 ± 0.41 mm). The total eggs count per string

ranged from 31 to 51 eggs (mean ± standard deviation; 42 ± 7). In average, an ovigerous female bearing a pair of egg strings may carry a total of 84 eggs.

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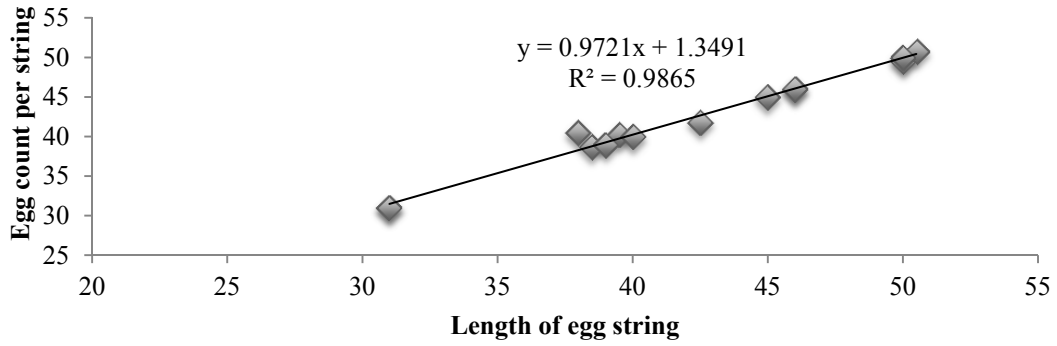


Figure 3.8 Relationships between length of egg string and number of eggs per string of post-metamorphic adult females bearing eggs collected in June 2012

3.4.7 Occurrence of precopulation couples

In total, 20 pre-copulation couples of adult male with various stages of *Peniculus minuticaudae* were found. Adult males were found clasping the antennae to the base of frontal filament of chalimi or the rostrum of copepodids. The composition of *P. minuticaudae* guarded by adult males were as follows (Figure 3.9); copepodid (5%), chalimus I female (20%), chalimus II female (5%), chalimus III female (10%), chalimus IV female (50%) and chalimus IV male (10%). The occurrence of pre-copulation couples throughout the sampling period shows significant seasonal changes (One-sample t-test, $p<0.001$). Figure 3.10 shows the total count of pre-copulation couples found throughout the sampling period. Nine out of 20 pre-copulation couples were found in May 2012. Two couples were found in October and November 2011 respectively. One couples was found from December 2011 until February 2012.

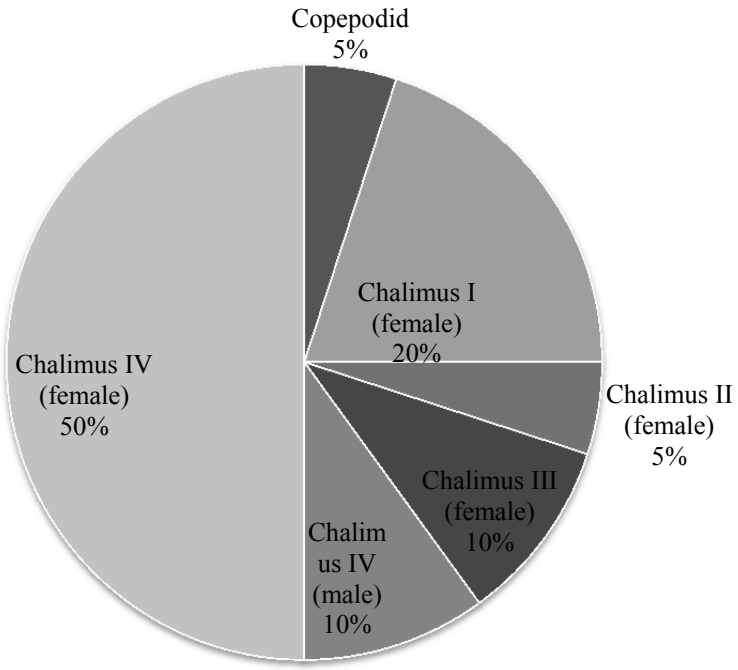


Figure 3.11 Composition of developmental stages of *Peniculus minuticaudae* involved in precopulation guarding with adult male (N=20).

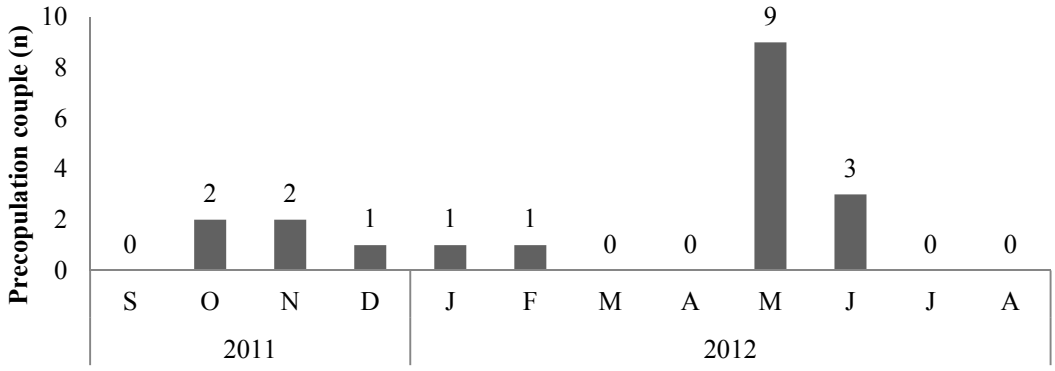


Figure 3.12 Total count of precopulation couples found from September 2011 to August 2012

3.4 Discussion

In this study, the observation of *Peniculus minuticaudae* infection on threadsail filefish cultured in cage-culture facility were carried out for 12 months, involving four different season in Japan. January to March are categorized as winter, April to June are spring, July to September are summer and October to December are autumn. Several parameters were calculated in order to observe the seasonal variation of the parasites population. The prevalence's of *P. minuticaudae* were high in all season except for summer, due to sharp decrease recorded in July and August 2012. In those two months, which are the most final sampling periods for this study, the abundances were also very low. The results were contradictory with a study on *P. minuticaudae* infection on black scraper *T. modestus* and Korean rockfish *Sebastes schlegelii* in cage culture facility in southern coast of Korea reported by Maran et al. (2014). In the previous study, high infections were recorded in late summer (September and November). However, both studies were involving small sample size of hosts, thus to accurately determined the seasonal prevalence and intensity of *P. minuticaudae*, larger sample size and longer observation period are required.

The post-metamorphic adult females of *P. minuticaudae* have the ability to produce offspring. The adult female which attached to the host by embedding its antennae deep inside the fins rays and usually found with cephalothorax region encapsulated in hyperplasial inflammation tissues of the host's fin (Ismail et al. 2013) present all year round even when the abundance were very low in July and August 2012. The fitness of adult female particularly the post-metamorphic stages might be higher in comparison to other life stages due to its strong and secured attachment and possibly the stage might have better nutrient absorption due to its well-developed mouth cone. The composition of *P. minuticaudae* suggests that for this species, breeding could occur all year round whenever the post-metamorphic female encounters optimal condition for reproduction. However, the conditions that influence the reproduction capability need further investigations. The increasing water temperature after winter might be one of the factors, since the abundance of parasites particularly the ovigerous, post-metamoprhic females and copepodids show increment pattern starting from May 2012. Additionally almost 50% of precopulation couples were collected in the same month (May 2012) suggesting that spawning activity were

intensified during this period. Since the intensity of parasites were getting higher during spring, more parasite management and control should be intensified during the period to avoid heavy parasite infestation to the cultured hosts.

Our data on the attachment site of adult females (pre-metamorphic, metamorphing and post-metamorphic) is well agreed with Nagasawa et al. (2011) and Maran et al. (2014). Almost 40% of the parasite specimens were found firmly attached to the second dorsal fins. Located on the dorsal area of the host with large surface area, second dorsal fins might be preferred by the parasites as it facilitates easy attachment during first (infective copepodid) and second (pre-metamorphic) infection.

Kabata (1979) suggested that adult male of Pennellid copepods will die shortly after copulation. However, in this study, a total of 244 adult males were found detached from the host, suggesting they were loosely attached to the host prior to the fish sampling. Previously Izawa (2008) reported the aggregation of 108 adult males of unknown species of Pennellid on the branchial lamellae of *Brotula multibarbata* Temminck & Schlegel, 1846. Thus, the life span of adult male pennellids might need further investigation.

Precopulatory guarding is common among various copepod orders (Boxshall, 1990). For *P. minuticaudae*, the adult males were found to grasp the base of frontal filament chalimi or the rostrum of copepodids. In this study 20 adult males were found in precopulatory guarding with various stages of female chalimi, copepodid and even with two chalimus IV males. Adult males were also found in precopulatory amplexus with pre-metamorphic adult females and adult males with a frontal filament, suggesting that the guarded copepod might just molt into adult and copulation might occur anytime soon during the preservation of the hosts. According to Kelly & Snell (1998), copepod males have the ability to detect diffusible sex pheromone released from females with antennulary chemosensors, and then identify the candidate mate by recognizing specific surface glycoprotein on the urosome and caudal rami of females. The findings that this species was actively involved in precopulatory guarding await further investigation. This study implies that even early female chalimus stages may release sex pheromone.

To date, *Peniculus minuticaudae* have been reported to infect four fish host of Monacanthidae (Nagasawa et al. 2011; Maran et al. 2012; Ismail et al. 2013) a single species from family Chaetodontidae (Okawachi et al. 2012). Maran et al. (2014)

reported that, in the cage area where seven species of hosts are cultured together, *P. minuticaudae* exclusively infecting *T. modestus* (Monacanthidae). The infection on the *Chaetodon modestus* Temminck and Schlegel, 1844 occurred in a commercial aquarium, with limited area and choice of host. From the observation, it is suggested that *P. minuticaudae* is a host-specific to Monacanthidae fishes, with the infection on *C. modestus* considered as accidental infection due to limited choice of host.

Appendix A Data of composition of *Peniculus minuticaudae* in different life stages from September 2011 to August 2012

Parasite stages/ sampling time	2011					2012							TOTAL
	S	O	N	D	J	F	M	A	M	J	J	A	
Post-metamorphic female	6	21	20	22	28	73	19	27	102	129	2	1	**450**
Ovigerous post-metamorphic female	7	18	22	43	49	75	16	29	109	87	0	1	**456**
Metamoprhing female	2	2	3	19	6	3	6	5	5	15	0	1	**67**
Pre-metamorphic female	0	8	6	14	6	0	3	10	38	27	1	0	**113**
Adult male	2	22	24	29	3	3	3	16	91	51	0	0	**244**
CHIV female	0	5	7	8	3	2	0	3	19	15	0	0	**62**
CHIV male	0	2	5	8	1	0	4	2	20	8	0	0	**50**
CHIII female	0	2	2	2	1	0	0	0	5	0	0	0	**12**
CHIII male	0	0	3	1	0	0	2	0	4	3	0	0	**13**
CHII female	0	0	1	6	0	1	1	1	7	3	0	0	**20**
CHII male	0	1	4	2	1	0	0	1	10	1	0	0	**20**
CHI female	0	2	3	2	0	0	2	2	16	4	0	0	**31**
CHI male	1	0	2	0	0	0	0	3	4	1	0	0	**11**
Copepodid	4	5	7	14	3	3	8	16	22	14	0	0	**96**
TOTAL	**22**	**88**	**109**	**170**	**101**	**160**	**64**	**115**	**452**	**358**	**3**	**3**	**1645**

Appendix B The distribution of *Peniculus minuticaudae* on the fish host

***P.minuticaudae* stages/ Fins**	**First dorsal fin**	**Second dorsal fin**	**Caudal fin**	**Anal fin**	**Pectoral Fin Left**	**Pectoral Fin Right**	**Detached specimen**	**TOTAL**
Post-metamorphic female	5	166	47	58	44	67	63	**450**
Ovigerous post-metamorphic female	9	203	17	56	77	50	44	**456**
Metamoprhing female	1	43	5	8	2	3	5	**67**
Pre-metamorphic female	0	5	2	0	0	1	105	**113**
Adult male	0	0	0	0	0	0	244	**244**
CHIV female	0	0	0	0	0	0	62	**62**
CHIV male	0	0	0	0	0	0	50	**50**
CHIII female	0	0	0	0	0	0	12	**12**
CHIII male	0	0	0	0	0	0	13	**13**
CHII female	0	0	0	0	0	0	20	**20**
CHII male	0	0	0	0	0	0	20	**20**
CHI female	0	0	0	0	0	0	31	**31**
CHI male	0	0	0	0	0	0	11	**11**
Copepodid	0	0	0	0	0	0	96	**96**
TOTAL	**15**	**417**	**71**	**122**	**123**	**121**	**776**	**1645**

CHAPTER 4

Site-specificity of infective copepodid stage of *Caligus fugu* to the fins of puffer fish: molecular evidence

4.1 Introduction

Caligus fugu (Yamaguti, 1936) is a parasitic copepod from the family Caligidae (Copepoda: Siphonostomatoida) which is highly host-specific to puffer fishes such as *Takifugu* spp. The parasitic copepod from family Caligidae also known as "sea louse", which is known to cause serious economic loss to aquaculture industry worldwide (Ho and Lin 2004). In Japan *C. fugu* was recorded to infect several species of pufferfish including the tiger puffer *Takifugu rubripes* (Temminck & Schlegel, 1850), grass puffer *Takifugu niphobles* and the panther puffer *Takifugu pardalis* (Temminck & Schlegel, 1850) (Yamaguti, 1936; Ikeda et al., 2006; Ohtsuka et al. 2009), fine-patterned puffer, *Takifugu poecilonotus* (Temminck & Schlegel, 1850), *Takifugu alboplumbeus* (Richardson, 1845) and brown-backed toadfish, *Lagocephalus wheeleri* Abe, Tabeta & Kitahama, 1984 (Nagasawa, 2011). Pufferfish industry in Japan has been nowadays facing an economic problem from the heavy infection of parasites on high value fish, the tiger puffer *T. rubripes* (Ohtsuka et al., 2009; Maran et al., 2011). Towards constructing an effective parasite management, studies on various aspect of the pufferfish and its parasites including *C. fugu* has been carried out intensively these recent years. On *C. fugu*, previous studies are covering the fundamental biology (Ohstuka et al. 2009; Maran et al. 2011), life cycle and development (Ohstuka et al. 2009), seasonal occurrence (Maran et al. 2011), symbiont relationship with other organism (Okawachi et al. 2012) and toxicology with particular emphasize on tetrodotoxin (Ikeda et al. 2006, Maran et al. 2007, Ito et al. 2006).

The development of *C. fugu* involves eight life stages; two naupliar, one copepodid, four chalimus and adult (Ohtsuka et al. 2009). The infective stage the copepodid; which have the ability to locate and establish itself on a host (Ohtsuka et al. 2009). The distal area of *C. fugu* copepodid antennules' is equipped with two aesthetascs and 11 setae, five of which forked terminally; which suggest it is a

powerful tool in detecting mechanical or chemical stimulation during host and site searching activity. The copepodid are also armed with powerful antenna and maxilliped that can strongly grasp into the host tissues once settlement is achieved. The copepodid of *C. fugu* were exclusively attached to the fins of pufferfish, with the highest preference on the pectoral fin (Ohtsuka et al. 2009). The site-specificity raises a question; how do infective copepodid differentiate between the fins from other body part of the host?

To date, no detail explanation is available regarding the recognition of host and site-specificity of *C. fugu* to the puffers. However, previous studies suggest that like most of other aquatic species, copepod generally do communicate via chemical interaction, intraspecies, interspecies and with the surrounding environment (Mordue & Birkett, 2009). In natural condition, copepods have the ability to response or release semiochemical; the chemical cues that induce specific behavior in a target organism. Semiochemical can be divided into two categories; allelochemicals and pheromones. Of these, allelochemical are the substance that mediates interspecific interaction and the substances that give an adaptive advantage to the receiving organism are called kairomones (Mordue & Birkett, 2009). According to Burke & Lodge (2002) among aquatic species, fish tend to emit kairomones which can be detected by zooplankton. To date, most of the studies regarding the semiochemical interaction in parasitic copepod and their host are focusing on the most economically harmful sea lice, *Lepeophtheirus salmonis* (Krøyer, 1837), a salmonid fish-specific parasite. Studies reported that host searching behaviour of copepodid and adult stages of sea lice *L. salmonis* involves behavioural responses towards host kairomones that enables them to recognize their salmonid host from other non-host fish (Devine et al. 2000; Ingvarsdóttir et al. 2002b; Bailey et al. 2006).

According to Mordue & Birkett (2009) host location of sea lice is related to two aspects; orientation towards the natural environment of the host and host recognition. Infective copepodid are known to response to environmental parameters such as light (Bron et al. 1993; Pike & Wadsworth 2000; Genna et al. 2005) and salinity (Bron et al. 1993; Heuch et al. 1995; Bricknell et al. 2006) to bring them to the location where potential host may be found. Once potential host is detected either by the perception to light changes (Flamarique et al. 2000; Browman et al. 2004; Genna et al. 2005) and mechanical vibration from the swimming activity of the host (Heuch & Karlsen 1997; Heuch et al. 2007), copepodid also may be able to detect the chemical cues

released by the fish host (Devine et al. 2000; Ingvarsdóttir et al. 2002b; Bailey et al. 2006). All these cues will activate host encounter search pattern where the copepodid will increase the duration and frequency of turning behaviour, involving circles and helices occurs, within the normal sinking and swimming behaviour (Genna et al. 2002). Upon initial contact to the host, copepodid will grip the host tissue using its maxillipeds and probing tissue surface with the anterior end of the cephalothorax. If the host and site is suitable, the copepodid will start hooking the antennae deeply and released frontal filament, however if the site is not suitable, the copepodid may leave the host or move to a preferred infection site (Bron et al. 1991).

Semiochemical studies in copepods need an integrated methodology (Mordue & Birkett, 2009) in order to identify the chemical substance involved, and to observe the response and behavior of copepods towards in different chemical and stimulation. To date, the methodology that has been used in previous study includes behavioral observation in y-tube arena, chemical techniques to extract and identify specific semiochemical (Devine et al. 2000; Ingvarsdóttir et al. 2002; Genna et al. 2005; Bailey et al. 2006; Pino-Marambio et al. 2007) and neurophysiological testing (Ingvarsdóttir et al. 2002b; Fields et al. 2007).

The objectives of this chapter are:

1. To determine if the stimulation of puffer conditioned water could stimulate the active swimming behavior of copepodid *Caligus fugu*
2. To determine the possibility that the chemical attractant exist in the puffer conditioned water is in a form of protein
3. To determine whether recombinant protein library of *T. rubripes* pectoral fins stimulate the active swimming behavior of copepodid *C. fugu*
4. To screen the genes highly expressed in the fins compared to the skin of *T. rubripes*, which are supposed to be related with tissue specificity

Investigation to answer the objectives number 1, 2 and 3 were carried out by observing the copepodid swimming behavior in a y-tube arena. For objective number 1 and 2, swimming behavior of copepodid were observed under the stimulation of puffer-conditioned water and heated puffer-conditioned water. For objective number 3, copepodid behaviors were observed when copepodid were stimulated with a series of

diluted culture medium of insect cells transfected with full-length cDNA library of the pectoral fins of *T. rubripes*.

To achieve objective 4, Suppression Subtractive Hybridization (SSH) technique was used to obtain a list of secreted protein encoding genes expressed exclusively on the pectoral fins of *T. rubripes*. So far chemical separation methodology was used to identify semiochemicals (Devine et al. 2000; Ingvarsdóttir et al. 2002b; Bailey et al. 2006), but in the present study I applied SSH methodology, which is proven to be very efficient in amplifying cDNA fragments of genes expressed differentially in different tissues (Diatchenko et al. 1999). Since the copepodid of *C. fugu* shows high preference to the fin, in particular, pectoral fins of the host in comparison to adult which roam on the body surface of the host (Ohtsuka et al. 2009). If fin secrets or leaks semiochemicals, genes encoding semiochemicals themselves (if they are secreted proteins) or enzymes synthesizing and/or modifying them should exist in pectoral fin more abundantly compared to in other tissues. SSH is an useful technique to screen such kind of genes. Because the epidermis of fins and skin share many similarities histologically, genes expressed in these two tissues are supposed to be highly overlapping with only very small differences. Thus, I chose to use pectoral fins and skin of *T. rubripes* for SSH methodology. Another advantage of using this approach is that vast genetic information of *T. rubripes* is available. *T. rubripes* is one of the model animals with almost the whole genome has been sequenced and the results of the assembly were reported, and the fifth assembly is available online (http://www.fugu-sg.org/) at the moment.

4.2 Material and methods

4.2.1 Bioassay

4.2.1.1 Copepod culture

Grass puffer, *T. niphobles* were fished from neighboring waters near Takehara Marine Science Station, Hiroshima University, Japan (34.33° N, 132.9167° E) and kept in a tank as source for parasites in this study from May to August 2012. To obtain the copepodid, ovigerous females of *C. fugu* were collected from the hosts, carefully picked using fine forceps and transferred to Petri dishes containing filtered-autoclaved seawater (ca. 32 psu). The Petri dishes were covered with aluminium foils

pricked with ball pen to make holes; to avoid over-evaporation of the medium culture. A maximum of 5 ovigerous females were placed in one Petri-dish to avoid overcrowding. The ovigerous females were incubated in total darkness, with controlled temperature (20 to 22 °C) in an incubator (NK System Biotron, Nippon Medical & Chemical Instruments Co. Ltd) until first batch of nauplii hatched. When the first batch of nauplii was seen, all the ovigerous females were transferred to a new Petri dish. After all or nearly all eggs hatched, the females were transferred back to the tank containing fish hosts. The naupllii were incubated in the same incubator and allowed to grow to copepodid stage before being used in the bioassay testing. The copepodids used in this study were in average day 3-4 post-hatching based on a preliminary testing. The 3-4 day copepodid seemed to be most active for infection.

4.2.1.2 Y-tube bioassay system

Y-tube bioassay system was constructed from a modified model of Bailey et al. (2006). Figure 4.1 shows the schematic illustration of the bioassay system. The y-tube was constructed using a Y-shaped glass tube with a 4 mm diameter bore. The length of the arms and the main leg were 4.5 cm respectively. The arms of the y-tube were connected to a silicon tube (7 mm diameter bore) (42 cm length) which is connected to tube connectors to allow the end to be sealed with pipette bulb. Sealing the end of the tubes using pipette bulbs create a vacuum condition to maintain the same level of water in both arms prior each experiment. The end of the main legs of the y-tube was sealed with plankton net (100 μm mesh size) to avoid copepodid passing to the outflow. The outflow of the Y-tube was made by connecting the end of main leg silicon tube to a tube connector and smaller silicon tube (diameter bore 3 mm). A clipper and a flow controller were used to control the water flow of the system.

Figure 4.2 shows the basic procedures used for all bioassay experiments. The technique of using the pipette bulbs in this experiment was very efficient to avoid the mixing of different water from stimulus and control arm. After both arms of the y-tube were filled with water depending on experiment, pipette bulbs from both arms were released and water from both arms were flowing to the main leg at the rate of 1.54 to 2.03 ml/minute. The water-flow was set-up at the beginning of every experimental session, by adjusting the flow-controller at the outflow of the system.

The water flow were set at the rate where clear demarcation of the water flowing from control and stimulus end, showed by testing the flow using red food dye.

Figure 4.1 Y-tube bioassay system

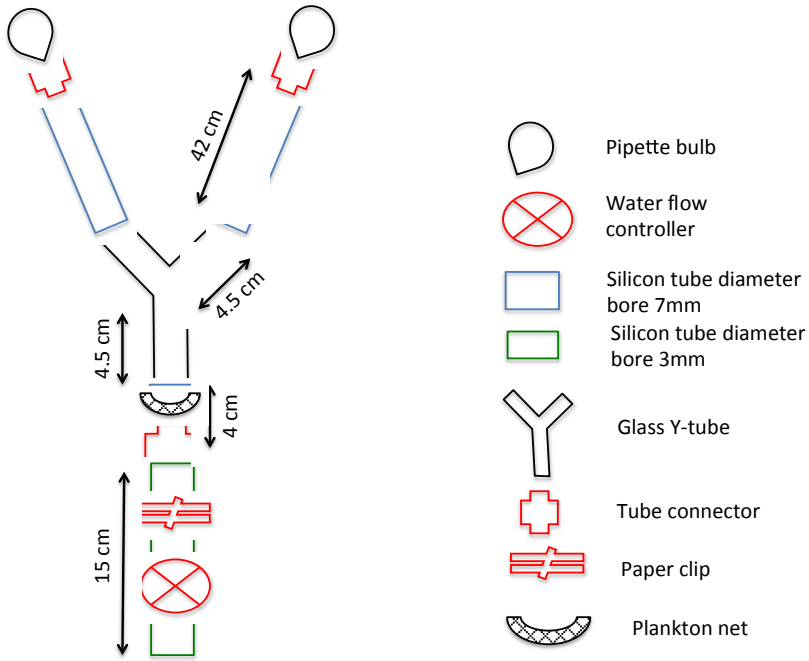
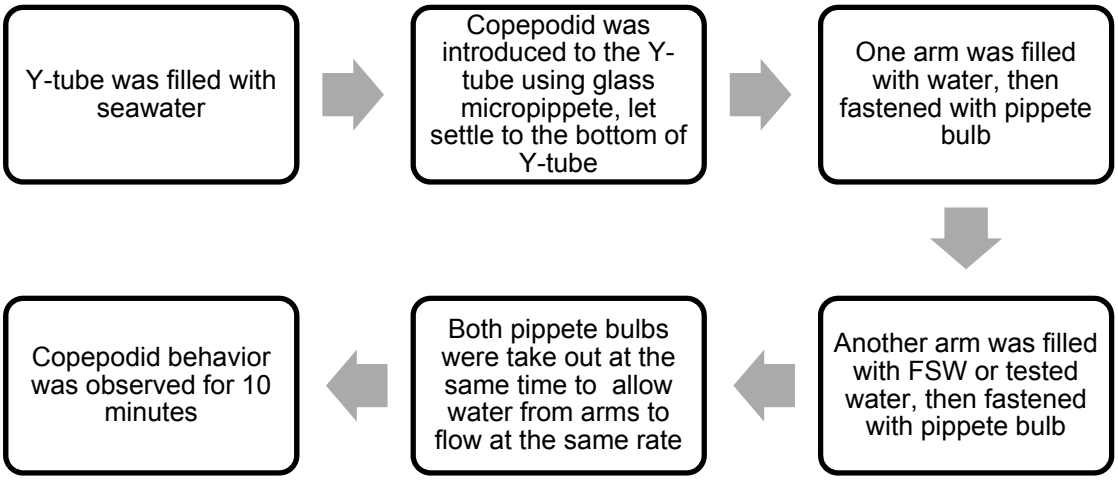


Figure 4.2 Basic procedures for the bioassay experiment using the Y-tube system



4.2.1.3 Behavioral response of copepodids to host-conditioned water

Experimental condition

Experiments were carried out in a small dark chamber to make sure complete darkness. The room temperature where the small dark chamber located was set up to 25 °C. The room was kept dark by closing all windows using black-out curtains. In the chamber, a retort stand was assembled as shown in Figure 4.3. The y-tube system was fastened to the retort stand using sellotape. On top of the retort stand, an aquarium fluorescent lamp was put as the only source of light, that acts as another trigger for the copepodid active swimming behavior. The experiment was carried out based on the work flow in Figure 4.2. Soon before the timing was count using a timer set-up to 10 minutes, the lamp was switch on and the observation started. In every trial, one copepodid was observed at one time for a maximum of 10 minutes. For every treatment, ranges of 14 to 45 copepodids were tested. In total, 428 copepodids were observed.

Figure 4.3 Retort stand used in the experiment

The observation was made based on activity classes described by Bailey et al. (2006). Two activity levels were categorized; “low” activity: the activity within less

than half of the length of main leg; “high” activity: upward swimming more than half length of the leg and if the copepodid swim into any arm. For directional observation, if they chose the control and stimulus arms, these were classified into “control taxis” and “stimulus taxis”, respectively. The copepodid were tested to several stimulations such as fugu-conditioned water, heated fugu-conditioned water and the probe *of T. rubripes* pectoral fin’s full-length cDNA library (see below). Prior to the experiments using stimuli, a control experiment was carried out, observing the activity level of the copepodid in only filtered seawater. Below we described the preparation of the stimulus for every experiment.

4.2.1.4 Preparation of fugu-conditioned water (FCW) and heated FCW

Grass puffer, *T. niphobles* was collected as described above. The fish were kept in a tank (1000 liter) until used for the experiment. For the preparation of FCW, individuals of *T. niphobles* (134 to 210 g, average = 155 g, total number = 7) were isolated in a bucket containing 2 liters seawater for 4 hours prior to the experiment. Aeration was provided during conditioning. The FCW was used immediately after preparation. Water parameters during preparation were recorded by a salinometer; salinity (32±1 psu), water temperature (24±1 °C).

For the preparation of heated-FCW, 500 ml of FCW was filled in a 1000 ml beaker and sealed with saran-wrap to avoid evaporation during heating. A water bath was heated up to 80 °C. The beaker containing the FCW was immersed in the water bath for 20 minutes with continuous shaking to make sure the heating of the FCW equally. Temperature of the FCW in the beaker during heating was not measured. After 20 minutes the heated FCW was let to cool down to room temperature and used for bioassay testing on the same day.

4.2.1.5 Preparation of culture medium of insect cells transfected with full-length cDNA library of the pectoral fins of *T. rubripes*.

The culture medium was prepared in Fisheries Labarotary, of the University of Tokyo, Shizuoka, Japan with the following procedures. Total RNA was isolated from pectoral fins from six individuals of *T. rubripes*. They were pooled and 5 μg total RNA (830 ng per individual) was prepared for cDNA synthesis by GeneRacer™ Kit

(Invitrogen). After amplification of full-length cDNAs by using adapter primers (GeneRacer 3′ and 5′ Primers) with *Sfi*I restriction site, they were digested and ligated into expression vector, pISD2. The resultant cDNA library was then transfected into insect cell, High Five™ (Invitrogen). After transfection, the cells were cultured for three days, and the culture medium was harvested. To remove cell debris, the medium was centrifuged at 15,000 rpm for 30 min at 4 °C, and stored at -20 °C until use.

To observe the dose-dependency of copepod activities under the stimulation of the culture medium, experiments were carried out in several dilution factors (X10, X20, X100, X150, X200, X300). For the dilution, the frozen culture medium was thawed at room temperature. A specific volume of culture medium was mixed in filtered seawater (32 ± 1 psu) to make desired diluted culture medium. The filtered-seawater was autoclaved at 121°C for 20 minutes prior usage. Details of the preparation for each dilution factors are shown in Table 4.1. During the experiment of different dilution factor, one arm of the y-tube were filled with diluted culture medium while in the other arm, filtered seawater were flowing. In these series of experiment, the control experiments were carried out separately, by testing the swimming behavior of copepodid under the stimulation of culture supernatant of cells transfected with empty expression vector. The control culture medium was also diluted with filtered seawater using the same formula as the culture medium of insect cells transfected with full-length cDNA library of the pectoral fins of *T. rubripes*.

Table 4.1 Preparation of diluted series of culture medium of insect cells transfected with full-length cDNA library of the pectoral fins of *T. rubripes*.

Dilution factor	**Culture medium volume (ml)**	**Filtered seawater volume (ml)**	**Total volume (ml)**
X10	1.5	13.5	15
X20	1.5	28.5	30
X100	1.5	148.5	150
X150	1.5	223.5	225
X200	1.5	298.5	300
X300	1.5	448.5	450

4.2.2 Screening of the protein-encoding genes exclusively expressed in the pectoral fins of *T. rubripes*

To screen for the protein-encoding genes exclusively expressed in the pectoral fins of the host *T. rubripes*, SSH PCR techniques was applied. The method allowed the amplification of cDNA fragments of genes exclusively expressed in targeted tissue but not occur in another tissue. The methods involved RNA isolation and mRNA purification from the targeted tissues from puffer, subtraction of cDNAs, testing the efficiency of subtraction using PCR. After confirmation of the efficiency of subtraction process, SSH library was constructed followed by sequencing and further analysis of the sequences of obtained genes. After selecting the genes encoding secreted protein and related substances, PCR-RT analysis were carried out to determine if the genes were highly expressed in pectoral fins but not in the skin.

4.2.2.1 RNA isolation and mRNA purification

Five tiger puffer *T. rubripes* (n=5) were used in this experiment. The fishes were anesthetized in 2-phenoxyethanol before the dissection of the skin and pectoral fins. The skin and pectoral fins tissues then were washed extensively with phosphate buffered saline containing 100 units of heparin and then cut into small pieces before soaked in 10 volumes of RNAlater® (Life Technonogies) for one day at 4 °C with gentle rocking and stored at -20 °C until use.

Total RNA was isolated from approximately 100 mg tissue with 2 ml or 4 ml of RNAiso (Takara) for skin or pectoral fin, respectively, according to the manufacture's instruction. Quality of total RNA was checked by a microchip electrophoresis system, MultiNA (Shimadzu). For both tissues, 50 μg of total RNA from each individual were pooled to minimize the differences in gene expressions caused by individual difference, and mRNA was purified by Oligotex™-dT30 <Super> mRNA Purification Kit (Takara) according to the manufacture's instruction.

4.2.2.2 PCR-select cDNA subtraction

PCR-Select™ cDNA Subtraction Kit (Clontech) were used to construct subtracted cDNA library. From pectoral fins and skin, 1.5 μg of mRNA was prepared as starting

materials for cDNA synthesis. All procedures were carried out according to manufacture's instruction manual.

To perform subtraction in both forward (pectoral fin as tester and skin as driver) and reverse directions, two hybridizations were conducted followed by PCR to selectively amplify cDNAs differentially expressed between the two tissues. Each PCR amplification was conducted using a thermal cycler MyCycler (Bio-Rad) with the procedure and materials as described below;

First PCR amplification

Table 4.2 Materials for first PCR amplification's premix

Materials	**Volume/tube (µl)**
Sterile distilled water (SDW)	13.3
10x Ex Taq Buffer	2.0
dNTP mix	1.6
Ex Taq	0.1
PCR Primer 1 (10 µM)	2.0

Premix was prepared for 6 PCR tubes and for each tube, 1 µl cDNA was added as template. The templates that were used in this PCR were; forward subtracted cDNA, unsubtracted pectoral fin cDNA, reverse subtracted cDNA, and unsubtracted skin cDNA. PCR conditions are shown below.

Table 4.3 Thermal cycler setting (First PCR)

Process	**Temperature**	**Time**	
Initiation	75°C	5 minute	
Denaturation	94 °C	30 seconds	30 cycles
Annealing	66 °C	30 seconds	
Extension	72 °C	1 minute 30 seconds	
Holding	4 °C	∞	

Second PCR amplification

The PCR products from first PCR amplification were then diluted 50 times with SDW and used as a template for the second PCR.

Table 4.4 Materials for second PCR amplification's premix

Materials	Volume/tube (µl)
SDW	13.3
10x Ex Taq Buffer	2.0
dNTP mix	1.6
Ex Taq	0.1
Nested PCR Primer 1 (10 µM)	1.0
Nested PCR Primer 2 R (10 µM)	1.0

The reaction conditions are as follows;

Table 4.5 Thermal cycler setting (Second PCR)

Process	Temperature	Time	
Initiation	94 °C	2 minute	
Denaturation	94 °C	30 seconds	20 cycles
Annealing	68 °C	30 seconds	
Extension	72 °C	1 minute 30 seconds	
Holding	72 °C	10 minutes	
	4 °C	∞	

With 10 µl each PCR product, 2.5 µl loading dye was mixed and loaded into the well of 2% agarose gel. PCR amplicons were resolved by running at 100 mA for 20 min. Bands were visualized by staining with ethidium bromide (EtBr). Sequences of all primers used in this study are summarized in Table 4.6.

Table 4.6 Sequences of primers used in this study

Name	Sequence
PCR Primer 1	CTAATACGACTCACTATAGGGC
Nested PCR primer 1	TCGAGCGGCCGCCCGGGCAGGT
Nested PCR primer 2R	AGCGTGGTCGCGGCCGAGGT
Tr_G3PDH-5′	AGCGCTGGTGCTCGGTATGT
Tr_G3PDH-3′	TGGGGCCGTCCACTGTCTTT
SP6 Promoter	CATACGATTTAGGTGACACTATAG
T7 Promoter	TAATACGACTCACTATAGGG
PS5-1	CTTGCTGGACGAGCACGACACCGTCTAT
PS5-2	CGATTTGCTTCCCTCATCCCTGATCTCC
PS10-1	GGATCAGCCCTCTGAGCCAGAAGCTACG

PS10-2	AACACCTTGATGTCAGGGTCGCTGGAGA
PS11-1	TTCCTGAAGACTACCAGGATGCCCAGGA
PS11-2	CACTCGTGTTGCACTGAGAAGAGAAG
PS13-1	GCGCGGTTATCTTGTCGCGGCAGTAG
PS13-2	GTACAGCTCACCTGCTTCCAATGAAC
PS14-1	GCCCTCACTGGTGCAGGAATCATACTCTTT
PS14-2	ACCTCTGAGGGGCGCTTAGACGATCTGC
PS21-1	GGTCACCGGGTGTCTGAACAAGTGGAAA
PS21-2	GTACGCCGGTGCGCTCTTCATCCAG
PS23-1	GCTTTGCTGAAGCCATACACTCCCCTCA
PS23-2	GGACTCTGTAAAGACAGTGACTGGAGCA
PS34-1	GTGTAGTGATTTCCCTCGGTGCTCTCGATT
PS34-2	GCTGCCCTGGAATCCTAAAGACGTGTGG
PS35-1	GTACATCGCTGGTGCAAATGAGGAAGG
PS35-2	GGGGGTTTTTCCTGGTGTTCAGC
PS38-1	TGCTACACGTTGGCGGAGAACCGTTAAA
PS38-2	GTTGTGACCAGGGTTTTACTCTGAGTGG
PS44-1	CCGGTATAAGTGCAAAACGTGTGTGG
PS44-2	GTACAGACGAGAAGGTCCTGGTGGAGAT
PS63-1	AGCTGAGCTCAAACAGCCACGAGACCAC
PS63-2	CGGCAGCCAAAAGAATGGTGCAAAGAAG
PS66-1	GTACCCGGATGGATGGATGGATGGATG
PS66-2	TAACTGTCCTGGCTGCTCTGCAGTTATG
PS69-1	GGGCTGATATGGTTCCGCTTCCTGGTTT
PS69-2	ACTTCTGCCGTCCCAGTTTGTGCTTGTG
PS82-1	GGCCTTGAAGTTGGACCCGTCTGCTTCT
PS82-2	GTCCTTTCATTCTTCTCGCCGCAGTGGT
PS89-1	TGTCAAGAGCGAGACTCAGGGAGCCATC
PS89-2	GCCAGCTTCCTCACAGTAAAAAGCACTG
PS90-1	CTACCGCCACTGATGCCACTACCACCAC
PS90-2	GGGGTGAACTTGCTGTGAAGGATGCAAA
PS100-1	TTTGGGGAGAATAATGCCGCGGAATAGC
PS100-2	CATGGCAAAGGAAATAAGCGCCGTGAAG

PS102-1	GCAGATGAGCATGCGTCTCTTCAGGA
PS102-2	CTCTTCTGACTCTTTCATGACGCACAGC
PS139-1	CCACACAGATGGGACTCCTCAAAATCAC
PS139-2	GCCTGAGGCTGGTTTACCAGATTGTTGG
PS143-1	GACTACTATGCCTGCAGTGGAATGACTC
PS143-2	GTTCCCAAGCACACTAATGGCGTA
PS147-1	TCCTGACATCCTTGACATCTACGGGTGA
PS147-2	CCGTCAGAAGATGGTTGTAGATGC

4.2.2.3 PCR analysis of subtraction efficiency

Prior to construction of subtracted library, subtraction efficiency was confirmed. Subtracted (both forward and reverse) and unsubtracted secondary PCR products were diluted 10 times with SDW and used as templates for PCR. Materials for premix were as follows:

Table 4.7 Materials for PCR analysis of subtraction efficiency premix

Materials	Volume/tube (µl)
SDW	6.65
10x Ex Taq Buffer	1.00
dNTP mix	0.80
Ex Taq	0.05
Tr G3PDH-5′	0.50
Tr G3PDH-3′	0.50

To each microtube, 9.5 µl premix were added then 0.5 µl template was added. Reaction condition as follows;

Table 4.8 Thermal cycler setting (PCR analysis of subtraction efficiency)

Process	Temperature	Time	
Initiation	94 °C	2 minute	
Denaturation	94 °C	30 seconds	15 cycles
Annealing	55 °C	30 seconds	
Extension	72 °C	30 seconds	
Holding	−15 °C	∞	

PCR amplicons were resolved in 2 % agarose gel by running at 100 mA for 20 min. Bands were visualized by staining with EtBr.

4.2.2.4 Construction of SSH library

Construction of SSH library involved several steps as follows; ligation of secondary PCR products of forward subtracted cDNA into plasmid vector, transformation of *E. coli* competent cells, extraction of the plasmid DNA from *E. coli* and finally purification the plasmid DNA prior to sequence analysis.

Ligation

For ligation, the required materials were as listed in Table 4.9. All the material were mixed sterilized (autoclaved) 1.5 ml centrifuge tube then incubated at 4 °C overnight.

Table 4.9 Materials for ligation

Materials	Volume/tube (µl)
Secondary PCR product	1.0
2X Buffer	2.5
Plasmid vector (pGEM-TEasy,Promega)	1.0
T4 DNA ligase (Promega)	0.5

Transformation

The ligation product then was used for transformation of One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen). The material required for transformation procedures were as follows;

Table 4.10 Materials for transformation

Materials	Volume/tube (µl)
Competent *E. coli* cells	-
Ligation product	5.0
Super Optimal broth with catabolite repression (SOC)	0.5

E. coli cells were thawed on ice, And ligation product was added to the tube containing *E. coli* cells and was tapped very gently. After gentle tapping, the cells were kept on ice for 30 minutes, then heat shock were applied by placing the tube on heat block at 42°C for 30 seconds. The tube was then immediately transferred on ice and kept for 2 minutes and add 450 µl pre-warmed (37°C) SOC to the cells mixture,

followed by inverting. The mixture were then were incubated at 37 °C with shaking at 225 rpm for 1 hour.

Agar plate was warmed and dried by positioning it upside down in an incubator set up to 37°C. The following procedures were carried out in a sterilized laminar flow/biosafety cabinet. 80 µl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (20 mg/ml in dimethylformamide) and 100 µl transformation product were spreaded onto the warmed agar plate using a glass spreader. After spreading, the agar plate were incubated invertly in 37 °C.

After incubation, the *E. coli* will grow into colonies that were visible to the naked eyes. *E. coli* from white colonies were numbered. The *E. coli* colonies were picked up individually and cultured in 1-1.5 ml LB/Ampicillin culture medium at 37 °C with shaking at 200 rpm, overnight. Cultured *E. coli* cells were collected by centrifugation at 15,000 rpm (or maximum rpm) for 1 minute in 1.5 ml tubes before undergoes the alkaline-SDS lysis procedures to extract the plasmid. After the procedures, the extracted plasmid were stored at -20°C until use for sequencing.

4.2.2.5 Sequences analysis

Cycle sequencing was conducted using BigDye® Terminator v3.1 (Applied Biosystems), and nucleotide sequences were determined with 3130 Genetic Analyzer (Applied Biosystems). The materials required for the PCR cycle sequence were as follows;

Table 4.11 Materials for transformation

Materials	Volume/tube (µl)
Premix BigDye® Terminator v3.1	1.0
5x Buffer (Big Dye Terminator)	1.5
Primer SP6 (1.6 µM)	1.0
Primer T7 (1.6 µM)	1.0
Template DNA	0.5

All the listed material above were mixed and exposes to PCR cycle sequence according to the following reaction condition:

Table 4.12 Reaction condition (PCR cycle sequence)

Process	Temperature	Time	
Initiation	96 °C	1 minute	
Denaturation	96 °C	10 seconds	20 cycles

Annealing	50 °C	5 seconds
Extension	60 °C	1 minutes
Holding	4 °C	∞

Prior to sequencing, the PCR product of cycle sequence need to undergoes ethanol precipitation to remove the excessive free fluorosecent dyes. Cycle sequencing was conducted using BigDye® Terminator v3.1 (Applied Biosystems), and nucleotide sequences were determined with 3130 Genetic Analyzer (Applied Biosystems).

For sequences analysis, the raw data were by using Bioedit biological sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html) to remove vector and primer sequences. The locations of obtained DNA fragments in the *T. rubripes* genome were determined by BLAST browser (http://www.fugu-sg.org/blast/) under Fugu Genome Project website (http://www.fugu-sg.org/). The identified locations were examined by Ensemble Fugu genome browser (http://ensembl.fugu-sg.org/Fugu_rubripes_v5/index.html) to determine whether they were located on annotated genes or not. The names and full-length transcript sequences of annotated genes and their assigned gene ontology (GO) terms as listed in the database were recorded. Homologous transcripts were searched by using TBLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and accession numbers and organisms showing the best score were recorded.

4.2.2.6 GO analysis

Collected GO IDs were manually divided into three categories based on their three domains: cellular component, biological process or molecular function. The composition of GO terms in each domain was visualized by GO Terms Classifications Counter tool (Hu et al. 2008). For cellular component GO_slim2 was chosen, while for biological process and molecular function EGAD was chosen for GO classification. Other parameters were left as default values.

4.2.2.6 RT-PCR

From the information gathered through sequences and GO terms analysis, clones encoding enzymes and secreted proteins were sorted. Their gene sequences were used

to design specific primers using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). cDNA of skin and pectoral fins were then synthesized using SuperScript II (Life Technologies) according to the manufacture's instruction. The cDNA synthesis procedures were started by isolating RNA from three *T. rubripes* and then pooled the RNA to 1.6 µg per individual (total 4.8 µg). PCR was conducted in a total volume of 10 µl with 0.5 µl of cDNA diluted 50 times with SDW. Other reagents and reaction condition for PCR were listed below;

Table 4.13 Materials for PCR

Materials	Volume/tube (µl)
SDW	6.65
10x Ex Taq Buffer	1.00
dNTP mix	0.80
Ex Taq	0.05
Template (Substracted cDNA)	0.50
Template (Unsubstracted cDNA)	0.50

Table 4.14 Reaction condition

Process	Temperature	Time		
Initiation	94 °C	2 minute		
Denaturation	94 °C	30 seconds	}	X cycles
Annealing	Y °C	30 seconds		
Extension	72 °C	30 seconds		
Holding	4 °C	∞		

Annealing temperatures and cycle numbers of clones are shown in Table 4.15. Analysis of amplicons was carried out by gel electrophosesis as described above.

Table 4.15 Annealing temperatures and PCR cycle number of clones showing higher expression level in pectoral fin compared to skin

Clone	Annealing temperature (oC)	Cycle number
PS10	63	38
PS11	61	33
PS14	63	33
PS35	57	32
PS67	52	30
PS139	60	34
PS177	54	34

PS186	55	24
PS334	55	30
PS358	54	32

4.3 Results

4.3.1 Bioassay

4.3.1.1 Behavioral response of copepodid to the host-conditioned water

Figure 4.4 shows the activity level of copepodid *C. fugu* under the stimuli of FCW and heated-FCW in comparison to the activity level in plain filtered seawater as a control. In the control experiment, significant percentage of copepodids showed low activity (92.5%) (Chi-square test, $p<0.05$). When tested with FCW in the stimulus arm, 44.4% of copepodids showed high activity level by swimming upward more than half length of the main leg, among them 17.8% moved upward into the stimulus arm where the source of FCW was flowing (Figure 4.5). In the experiment using heated-FCW as a stimulus, the level of activation decreased, with 79.5% of copepodids showed low activity level while 20.5% still actively swimming upward and 2.6% wereable to swim to the stimulus arm (Figure 4.4). The proportion of activity level is significantly different between all treatment (Chi-square test, $p<0.05$). Further analysis shows that the proportion of high activity level shows by copepodids in the stimulation of FCW is significantly different from the activity level observed in control (Fisher's exact test, $p<0.05$) and in the heated FCW test (Fisher's exact test, $p<0.05$). The percentage of copepodids showing directional responses into the stimulus arm containing FCW also significantly higher in comparison to the control and heated FCW tests (Chi-square test, $p<0.05$).

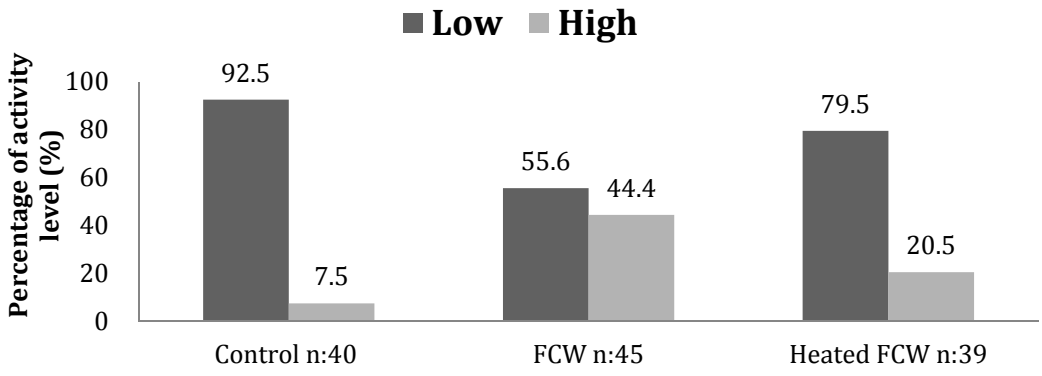


Figure 4.3 Activity level of copepodid *C. fugu* under the stimulation of control, FCW and heated-FCW

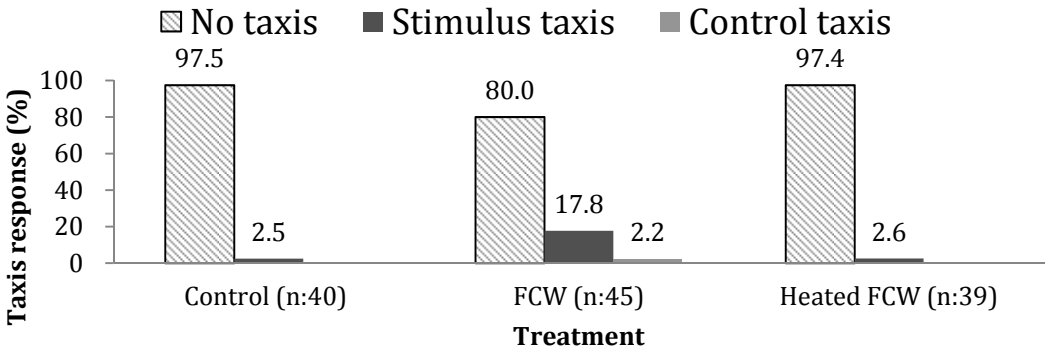


Figure 4.4 Taxis response of copepodid *C. fugu* under the stimulation of control, FCW and heated-FCW

4.3.1.2 Behavioral response of copepodid to the master probe

Copepodids were exposed to the diluted culture medium of a series of X10, X20, X100, X150, X200 and X300 dilution factors. Under the stimulation of diluted culture medium, percentages of copepodids showing high activity level ranged from 57 to 100% (Figure 4.6). In the X10 dilution, all tested copepodids (100%) showed high activity level. The sequence of dilution factor according to descending percentage of high activity level of copepodid was as follows; X10 (100%), X150 (93%), X100 (85%), X200 (63%), X300 (57%) and X20 (57%). The proportions of activity level of copepodid in all dilution factors are significantly different (Chi-square test, $P < 0.001$).

For observation of taxis response among copepodids in this experiment, the percentages of copepodids swimming actively into the stimulus arm ranged from 6.7

to 36.7% (Figure 4.7). The highest percentage of positive taxis response was recorded under the stimulation using X150 (36.7%), followed by X10 (33.3%), X20 (28.6%), X100 (21.2%), X200 (13.3%) and X300 (6.7%). The percentages of copepodid shows directional swimming into the stimulus arms are significantly different (Chi-square test, $p<0.05$).

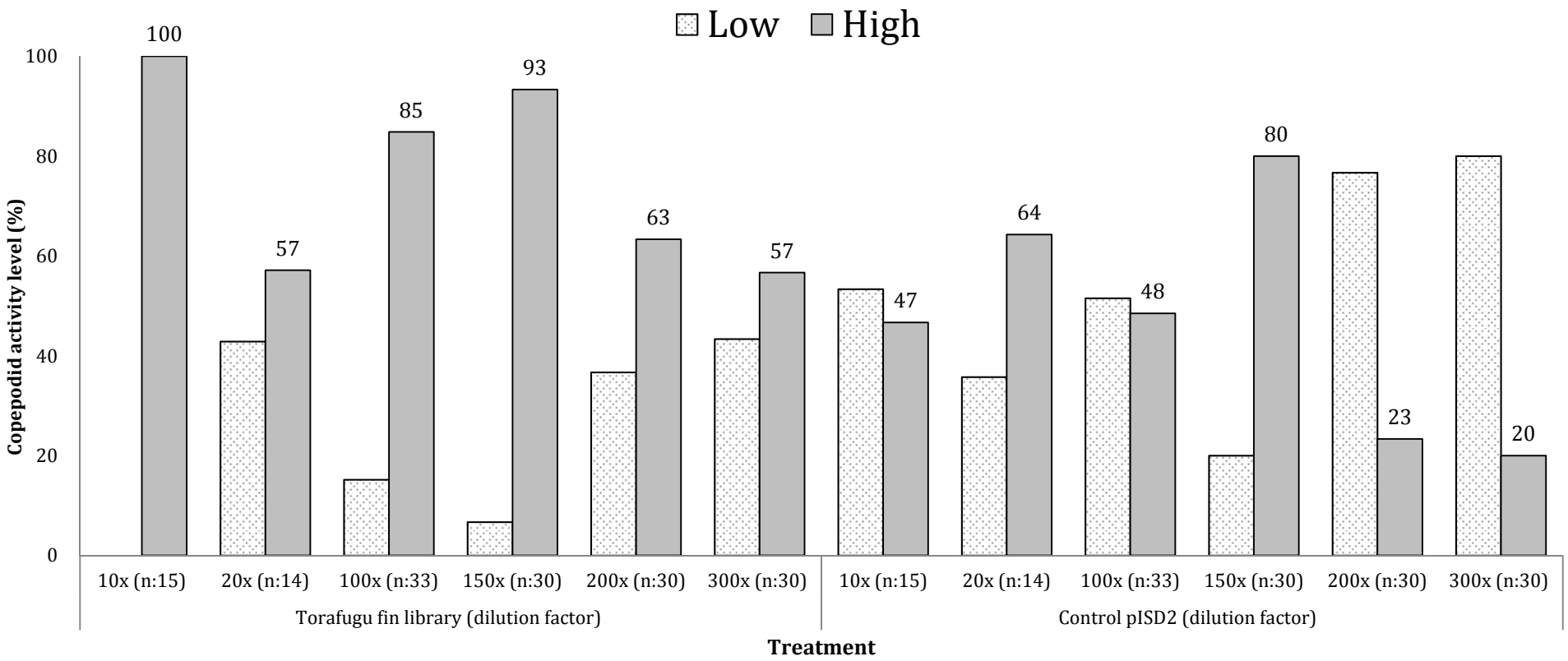


Figure 4.5 Activity level of copepodid *C. fugu* under the stimulation of diluted series of probe solution from the *T. rupripes* pectoral fin's gene library

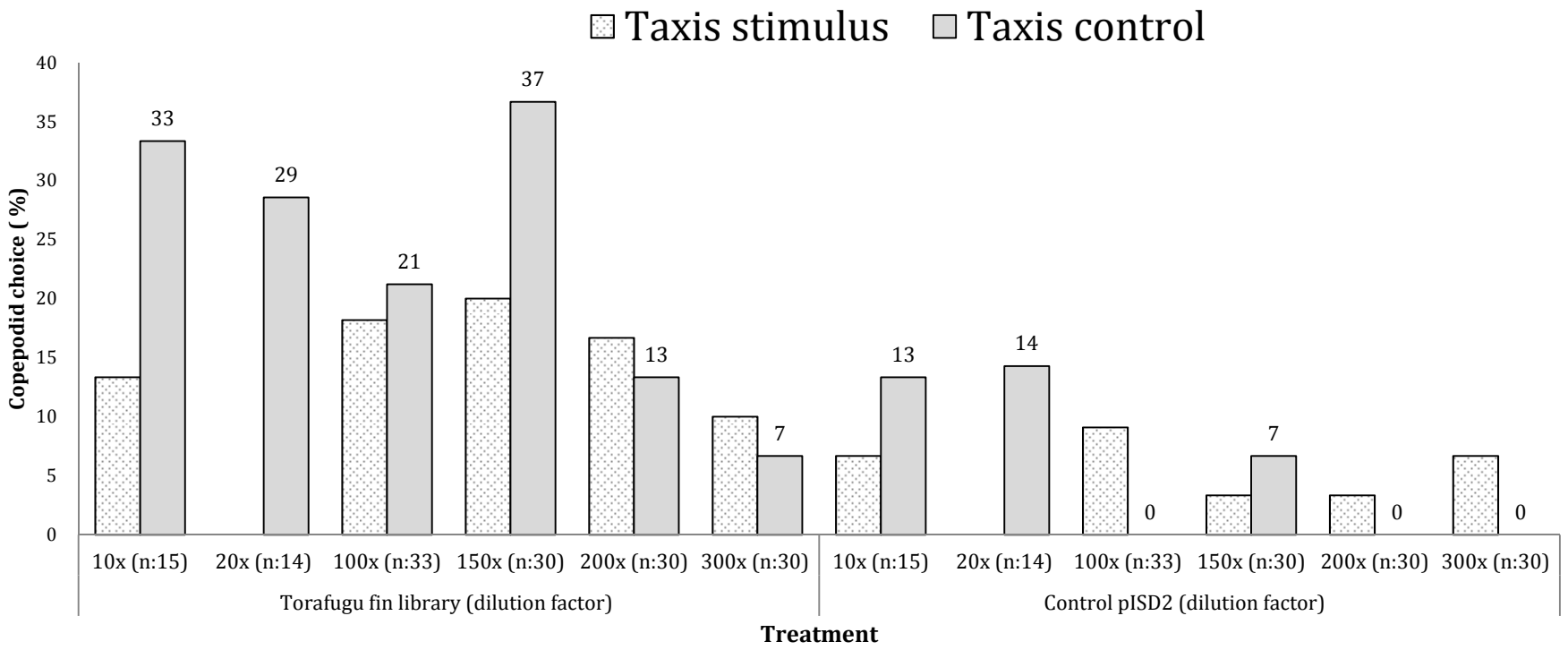


Figure 4.6 Taxis response of copepodid *C. fugu* under the stimulation of diluted series of probe solution from the *T. rupripes* pectoral fin's gene library

4.3.2 Screening of the protein-encoding genes exclusively expressed in the pectoral fins of *T. rubripes*

4.3.2.1 PCR Analysis of Subtraction Efficiency

At first subtraction efficiency was estimated by PCR with G3PDH specific primers. As shown in Figure 4.8, bands of G3PDH were detected when unsubtracted (both pectoral fin and skin) secondary PCR products were used as templates, while no band was detected in the case of subtracted (both forward and reverse) secondary PCR products were used as templates. Since G3PDH is one of the housekeeping genes and we had confirmed that the expression level is roughly equal in pectoral fin and skin, this result indicates that subtraction was done efficiently.

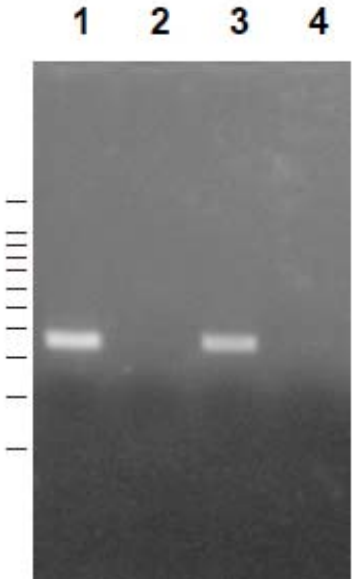


Figure 4.8 PCR Analysis of Subtraction Efficiency. PCR amplicon with G3PDH specific primers were resolved in 2 % agarose gel electrophoresis, and bands were visualized by staining with EtBr. Templates for PCR were secondary PCR products of unsubtracted pectoral fin (*lane 1*), forward subtracted (pectoral fin minus skin, *lane 2*), unsubtracted skin (*lane 3*) and reverse subtracted (skin minus pectoral fin, *lane 4*). Molecular markers are shown at the *left* by bards. Sizes are 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000 and 1,500 bp.

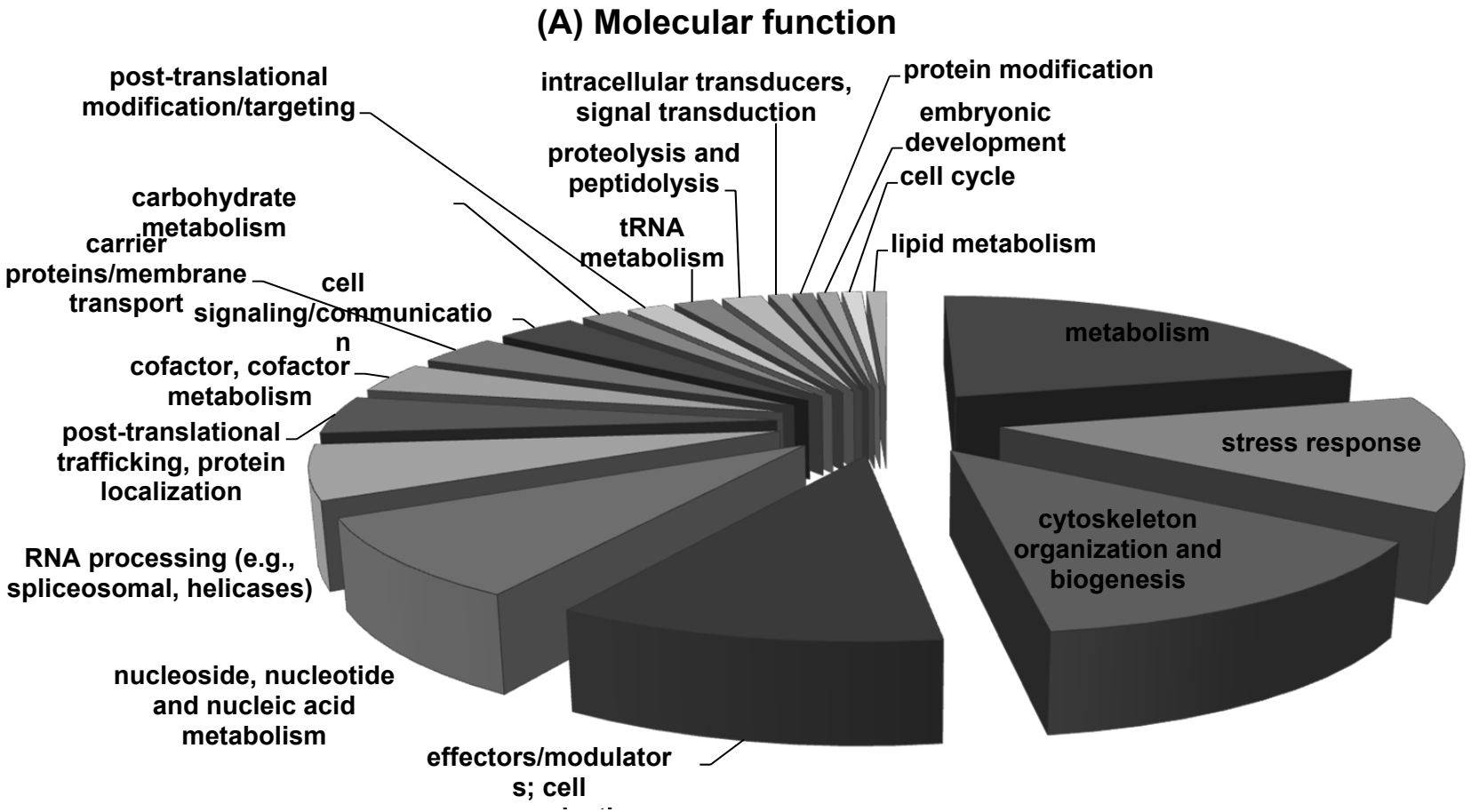
4.3.2.2 Screening of genes expressed higher in pectoral fin compared to skin

SSH library was constructed by inserting secondary PCR amplicon of forward subtracted cDNA into pGEM-T Easy vector. A total of 392 clones were sequenced, and the number of non-redundant sequences is 276. Of those 135 were located on 118 annotated genes while 141 were at the positions where no genes are annotated. The result of TBLASTN search using sequences of full-length transcripts from annotated genes as queries revealed that 99 showed the highest score to translated proteins including hypothetical ones from *T. rubripes* and the remaining showed the highest score to those from other species. Those transcripts include 47 sequences coding secreted proteins or enzymes.

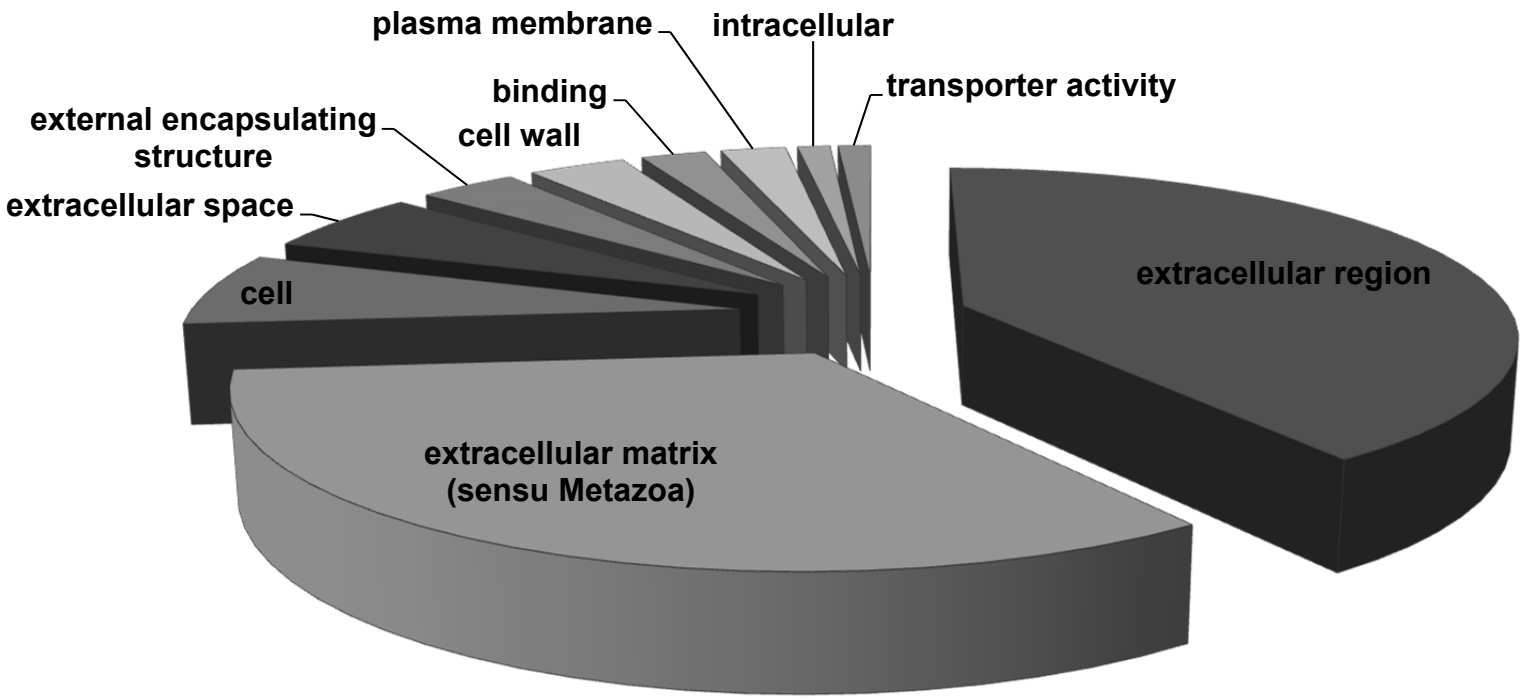
4.3.2.3 GO analysis of the genes from pectoral fin SSH Library

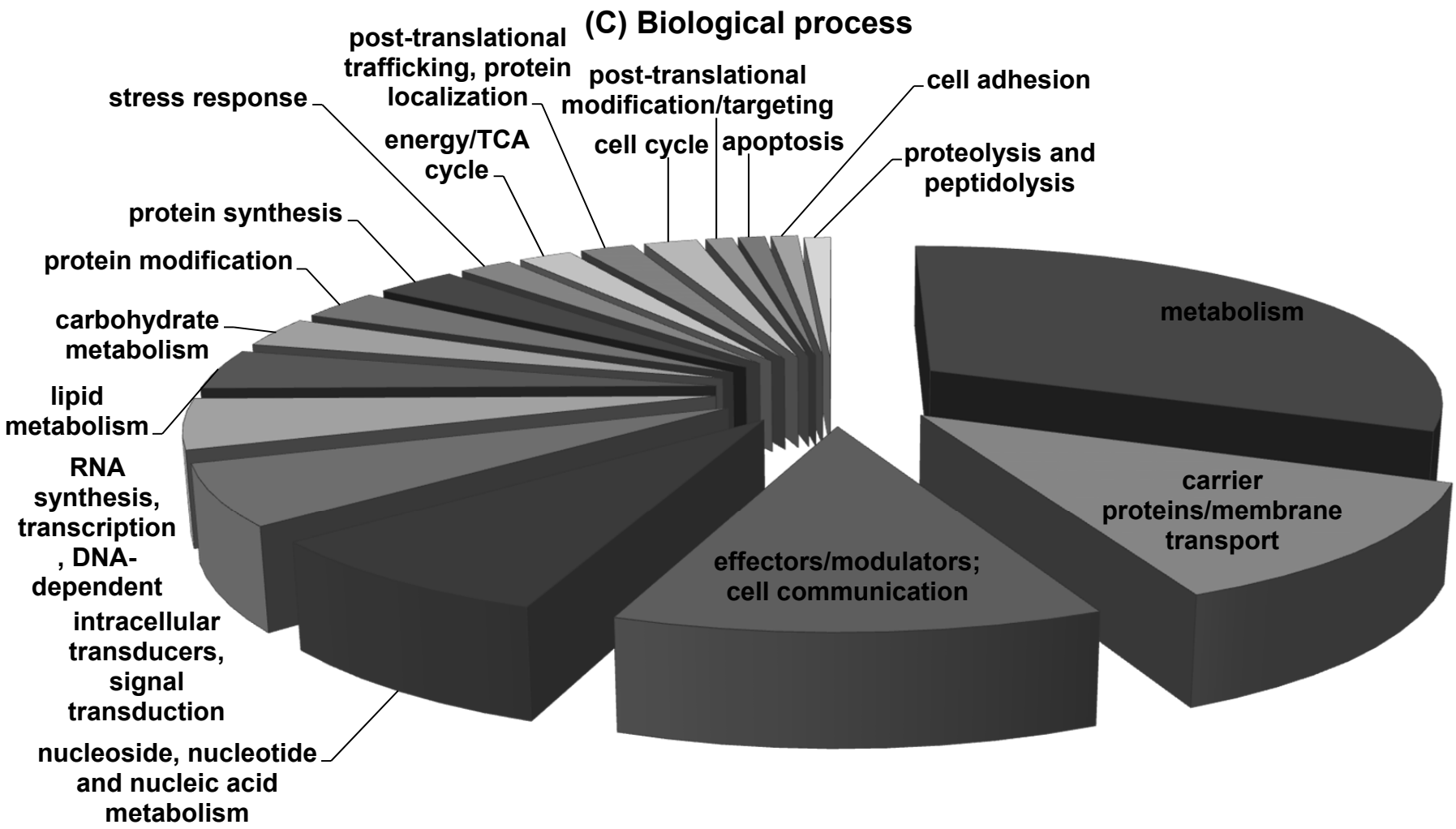
Figure 4.9 shows the composition of ESTs annotation according to three terms of the gene ontology analysis: cellular component (Figure 4.9A), molecular function (Figure 4.9B), and biological process (Figure 4.9C). Concerning the molecular function, the most represented categories are those of protein binding (19), sequence-specific DNA binding transcription factor activity (10), zinc ion binding (8), ATP binding (7) and structural constituent of ribosome (6). The other molecular functions were represented at lower extent. In the category of cellular component, the most represented were intracellular (19 %), membrane (16 %), integral component of membrane (11 %) followed by ribosome (8 %) and nucleus (7 %). Other cellular components represent at lower percentage ranging from 1-4 %. In view of biological process, 10 % of the analyzed genes involved in regulation of transcription. Other biological processes that could be involved by genes identified in this study are proteolysis, translation, metabolic process, and also oxidation-reduction process.

Figure 4.9 GO term distribution of ESTs specific to pectoral fin SSH cDNA library. GO categories are provided in the (A) molecular function, (B) cellular components and (C) biological process vocabularies.



(B) Cellular component




(C) Biological process
post-translational trafficking, protein localization
post-translational modification/targeting
cell adhesion
stress response
energy/TCA cycle
cell cycle
apoptosis
proteolysis and peptidolysis
protein synthesis
protein modification
carbohydrate metabolism
lipid metabolism
RNA synthesis, transcription, DNA-dependent
intracellular transducers, signal transduction
nucleoside, nucleotide and nucleic acid metabolism
metabolism
carrier proteins/membrane transport
effectors/modulators; cell communication

4.3.2.4 RT-PCR

There are two possible chemoattractant of *C. fugu*: protein or non-protein small molecules. Secreted proteins expressed exclusively in pectoral fin and enzymes related to synthesis of small molecules and/or modifications of secreted proteins such as glycosylation are likely to be strong candidate factors determining tissue specificity of *C. fugu* copepodid. We therefore examined gene expression levels of 47 genes encoding secreted proteins and enzymes by RT-PCR. As the result, 10 of them (PS10, 11, 14, 35, 67, 139, 177, 186, 334 and 358) were indeed expressed higher in pectoral fin compared to skin (Fig. 4.9). The amounts of PCR amplicon of G3PDH were almost the same in both tissues, indicating that the amplification reaction was successful (Fig. 4.10).

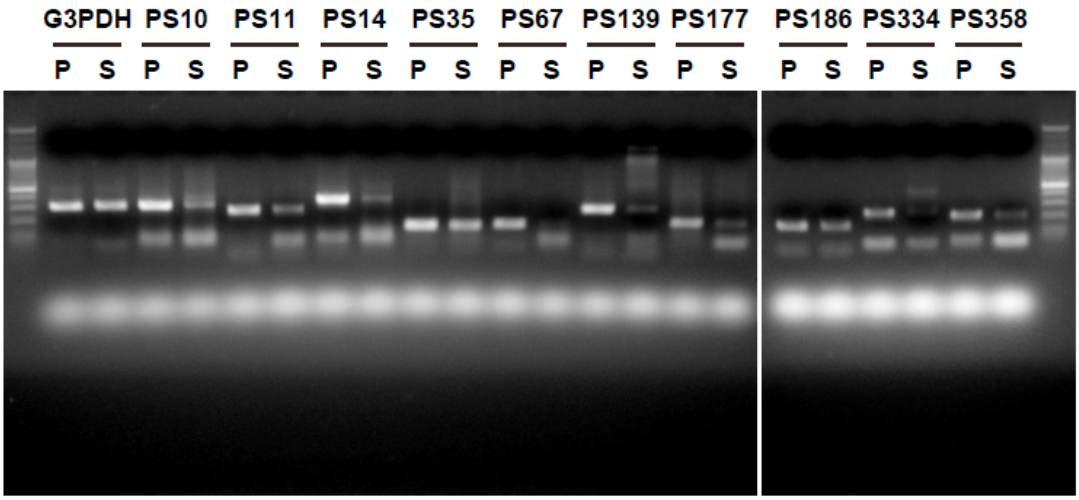


Figure 4.10 Substracted PCR efficiency test. (PS10, 11, 14, 35, 67, 139, 177, 186, 334 and 358)

4.4 Discussion

Behavioral response of copepodid to the puffer-conditoned water

In control experiment, copepodid were exposed to filtered-seawater, resulting in low activity, where they only swim and move at the base of the y-tube leg. According to Mordue & Birkett (2005), without the present of host, infective copepodid swim in a manner to conserve energy, and the result of the present study is in good agreement with their report. Although some small percentage of the copepodid swam actively in control experiment, the behavior might be due to their normal response to the water flow, since copepodid was known to shows upstream swimming against water current (Bron et al. 1993). Swimming activity increased when FCW was introduced to the system. They started swimming upward and swirling in the y-tube arena. After several attempts of upward jump and spiraling movement, active copepodid were usually able to swim into the arm containing the stimulus water. In a comparable study carried out on *L. salmonis*, the infective copepodid of the species also being activated by the stimulation of salmon-conditioned water and the solid-phase extraction extract of salmon-conditioned water (Bailey et al. 2005).

The experiment then was extended using heated-FCW with the inference that the chemoattractant might be in a form of protein. Activation and directional swimming were significantly reduced, however a small percentage of copepodid still showing active swimming behavior, suggesting that the chemicals involve as chemoattractant is/are protein(s) which work in combination with other type of chemical substance(s) that cannot be destroyed using heat. Chemical cues that mediating host and site specificity is very complex (Whittington, 2000). According to Buchmann (1998), more than a single receptor is needed for successful attachment to a host cell even in the case of virus, therefore, it is likely that more factors are operating for exhibiting host-specificity in the case of metazoan parasites since they are one of the more complex creatures.

Behavioral observation was continued further by testing the culture medium supernatant of insect cells transfected with *T. rubripes* pectoral fins full-length cDNA

library. The medium is assumed to contain numerous kinds of secreted proteins expressed in pectoral fins, which might be functioning as semiochemical in host and/or site recognition of copepodid of *C. fugu*. Percentages of high activity were 100, 85, 63 and 57 % at X10, X100, X200 and X300, respectively, showing dose-dependent reaction tendency, however, at X20 and X150 the percentages were 57 and 93 %, respectively. Such fractulation might be caused due to individual variation of copepodid activity level or it might be because the dilution factor used in this experiment is still to low contributing to overdose effect. It should be noted that at a maximum x300 dilution factor, copepodid still have the tendency to actively swim once the stimulation is detected. Thus it might be possible to dilute the medium more to observe whether the medium shows activity or not, which is advantageous for screening of attractant in the future research. The taxis response data (Figure 4.2) however, shows that copepodid may swim randomly, either towards the arm containing the culture medium or towards the control arm with filtered seawater. The random choice of copepodid might be influence by the factor(s) other than chemicals cue such as strong light and changing water current. To stabilize the y-tube assay system, optimization of factors, e.g, intensity and angle of light source, and controlling water current should be considered.

Screening of the protein-encoding genes exclusively expressed in the pectoral fins of *T. rubripes*

To date, researches regarding chemoattractant or semiochemical in parasitic copepods are more focused on the mechanism of host-specificity in comparison to site specificity. Previous reports are available for *L. salmonis* (Devine et al. 2000; Ingvarsdóttir et al. 2002b; Bailey et al. 2006) *Caligus rogercresseyi*, Boxshall & Bravo 2000 (J. Pino-Marambio et al. 2007) and *Lernaeocera branchialis* (Brooker et al. 2012). For *L. salmonis*, two chemical substances; isophorone and 6-methyl-5-hepten-2-one were identified as the chemoattractant responsible for the host-recognition of the salmon-specific louse (Ingvarsdóttir et al. 2002b; Bailey et al. 2006). These are small, lipophilic organic molecules, with physical properties similar to the semiochemical used in terrestrial systems (Mordue & Birkett, 2009).

In this study, so far ten genes encoding secreted protein and enzymes were shortlisted as the candidate genes that may act as the attractant being recognize by infective copepodid *C. fugu*. Even thought there is no previous study supporting that

copepod has the ability to detect it host using protein or protein-related substances, the theory need to be investigated. Another fish parasite, the monogenean are known to response to host glycoproteins, proteins and carbohydrates (Buchmann & Lindenstrom 2002). Moreover, copepods, particularly harpacticoid copepod, *Tigripous* sp. has been shown to detect protein and glycoprotein on the surface of female during mate recognition (Kelly & Snell 1998; Kelly et al. 1998; Ting et al. 2000). Thus, proteins and the related substances might still be strong candidates of the attractant responsible for the tissue and host specificity exhibited by copepodid of *C. fugu*.

Among the candidate genes, six sequences are encoding proteins that exist as a component of extracellular matric (ECM). Those genes are PS10, PS11, PS14, PS67, PS139 and PS358. ECM might be a good candidate of the attractant since skins of fish is continuously regenerating and old cells are continuously discarded to the environment. It is likely, ECM and its component are also being discarded together in the process could result in functioning as attractant for the copepodid. PS10 and PS14 are collagenase 3-like (mmpf1) and matrix metalloproteinase-9, respectively. Both are matrix metalloproteinase (MMP) family members, which regulates physiological remodelling processes such as tissue repair (Murphy and Nagase 2008). Thrombospondin-4-B is the gene PS11 and PS67 show the highest identity, which is supposed to be an adhesive glycoprotein mediating cell-to-cell and cell-to-matrix interactions found in zebrafish (Adolph 2002). Closest homologous protein of PS139 is MAM domain-containing protein 2, of which function is unknown. PS358 is identified as fibronectin, which is a multifunctional, extracellular matrix glycoprotein (Hynes and Yamada 1982).

PS35, S177, PS186 and PS334 are identified as heme-binding protein 2, hyaluronan and proteoglycan link protein 1, lipocalin and keratin, respectively. It is not clear that those proteins exist at the surface of fish body based on previous reports mainly from mammalians, but they are still candidate for the future study.

4.5 Conclusion

In the present study, behavioral observation using Y-tube bioassay showed that copepodid of *C. fugu* positively responded to the stimulation of fugu-conditioned water by actively swimming upward and toward the arm containing stimulus water. The active and directional swimming activity was reduced after the FCW was heated, suggesting that at least some of the semiochemical candidate(s) might be in a form of water-soluble protein. In the future, negative control using non-puffer fish-conditioned water could be tested to confirm if the results we have now shows that *C. fugu* only will activated by substances released from puffer-fish. Copepodids showed activation and directional response when they were stimulated with a series of diluted culture medium that may contain the secreted proteins expressed in pectoral fins of *T. rupripes*. Ten gene sequences encoding secreted protein or enzymes had been confirmed to be expressed higher in the fins in comparison to skin. The shortlisted secreted protein sequences are strong candidates of the chemoattractant involves in host and/or site recognition of copepodid *C. fugu* to pufferfish host, however further investigation to gather more candidates and to confirm their attractant activity is necessary to find actual genes responsible for host/tissue specificity of *C. fugu*.

Table 4.4 Genes identified from the *T. rupripes* pectoral fin suppression subtractive hybridization cDNA library

Clone	Accession number	Animal resource and homologous gene	*E* value	GO ID	GO term
PS3	XM_003967442	*Takifugu rubripes* dnaJ homolog subfamily A member 4-like	0	GO:0006457	Protein folding
				GO:0031072	Heat shock protein binding
				GO:0051082	Unfolded protein binding
PS4	XM_003963139	*Takifugu rubripes* protein RER1-like	8.00E-142		
PS5	XM_003962526	*Takifugu rubripes* collagen alpha-1(X) chain-like	0	GO:0005578	Proteinaceous extracellular matrix
				GO:0005201	Extracellular matrix structural constituent
PS9	XM_003973101	*Takifugu rubripes* phosphatidylinositol 5-phosphate 4-kinase type-2 gamma-like	0		
PS10	NM_001280032	*Takifugu rubripes* collagenase 3-like (mmpf1)	0	GO:0006508	Proteolysis
				GO:0031012	Extracellular matrix
				GO:0004222	Metalloendopeptidase activity
				GO:0008270	Zinc ion binding
PS11	XM_003975142	*Takifugu rubripes* thrombospondin-4-B-like	0	GO:0005509	Calcium ion binding
PS13	XM_003977576	*Takifugu rubripes* LON peptidase N-terminal domain and RING finger protein 1-like	0	GO:0005515	Protein binding
				GO:0008270	Zinc ion binding
PS14	NM_001037870	*Takifugu rubripes* matrix metalloproteinase-9 (mmp-9)	0	GO:0006508	Proteolysis
				GO:0008152	Metabolic process
				GO:0005578	Proteinaceous extracellular matrix

				GO:0031012	Extracellular matrix
				GO:0004222	Metalloendopeptidase activity
				GO:0005509	Calcium ion binding
				GO:0008233	Peptidase activity
				GO:0008237	Metallopeptidase activity
				GO:0008270	Zinc ion binding
				GO:0016787	Hydrolase activity
				GO:0046872	Metal ion binding
PS21	XM_003964575	*Takifugu rubripes* sodium/myo-inositol cotransporter 2-like	0	GO:0006810	Transport
				GO:0055085	Transmembrane transport
				GO:0016020	Membrane
				GO:0005215	Transporter activity
PS22	XM_003975346	*Takifugu rubripes* twisted gastrulation protein homolog 1-like	2.00E-142		
PS23	XM_003977252	*Takifugu rubripes* nucleoprotein TPR-like	5.00E-72		
PS28	XM_003966742	*Takifugu rubripes* arylsulfatase J-like	0	GO:0008152	Metabolic process
				GO:0008484	Sulfuric ester hydrolase activity
PS32	XM_003962280	*Takifugu rubripes* fos-related antigen 2-like	6.00E-175	GO:0006355	Regulation of transcription, DNA-templated
				GO:0005634	Nucleus
				GO:0003677	DNA binding
				GO:0003700	Sequence-specific DNA binding transcription factor activity
				GO:0043565	Sequence-specific DNA binding

				GO:0046983	Protein dimerization activity
PS33	XM_003964476	*Takifugu rubripes* ATP-dependent DNA helicase Q5-like	0.00E+00		
PS34	XM_003970870	*Takifugu rubripes* macrophage colony-stimulating factor 1 receptor 2-like	0.00E+00	GO:0006468	Protein phosphorylation
				GO:0007169	Transmembrane receptor protein tyrosine kinase signaling pathway
				GO:0016020	Membrane
				GO:0016021	Integral component of membrane
				GO:0000166	Nucleotide binding
				GO:0004672	Protein kinase activity
				GO:0004713	Protein tyrosine kinase activity
				GO:0004714	Transmembrane receptor protein tyrosine kinase activity
				GO:0004872	Receptor activity
				GO:0005515	Protein binding
				GO:0005524	ATP binding
				GO:0016301	Kinase activity
				GO:0016740	Transferase activity
PS35	XM_003964829	*Takifugu rubripes* heme-binding protein 2-like	2.00E-85		
PS36	XM_004086350	*Oryzias latipes* melanoma-associated antigen G1-like	1.00E-77		
PS38	XM_003976842	*Takifugu rubripes* sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1-like	6.00E-68		
PS44	XM_003972421	Takifugu rubripes annexin A5-like	0.00E+00	GO:0050819	Negative regulation of coagulation

				GO:0005509	Calcium ion binding
				GO:0005544	Calcium-dependent phospholipid binding
PS47	XM_003976115	*Takifugu rubripes* nuclear factor NF-kappa-B p100 subunit-like	0.00E+00	GO:0006355	Regulation of transcription, DNA-templated
				GO:0005634	Nucleus
				GO:0003700	Sequence-specific DNA binding transcription factor activity
				GO:0005515	Protein binding
PS49	XM_003971292	*Takifugu rubripes* GPI transamidase component PIG-S-like	0.00E+00		
PS52	XM_003964155	*Takifugu rubripes* inhibitor of nuclear factor kappa-B kinase subunit alpha-like	0.00E+00	GO:0006468	Protein phosphorylation
				GO:0004672	Protein kinase activity
				GO:0004674	Protein serine/threonine kinase activity
				GO:0005524	ATP binding
PS55	XM_003964058	*Takifugu rubripes* enoyl-CoA hydratase, mitochondrial-like	0.00E+00	GO:0008152	Metabolic process
				GO:0003824	Catalytic activity
PS63	XM_005472136	*Oreochromis niloticus* pleckstrin homology-like domain, family B, member 2 (phldb2), transcript variant X1	0.00E+00	GO:0005515	Protein binding
PS65	XM_003969008	*Takifugu rubripes* tRNA (cytosine(34)-C(5))-methyltransferase-like	0.00E+00		
PS66	XM_005750936	*Pundamilia nyererei* membrane-spanning 4-domains subfamily A	4.00E-15	GO:0016021	Integral component of membrane

		member 3-like			
PS67	XM_003975142	*Takifugu rubripes* thrombospondin-4-B-like	0.00E+00		
PS69	XM_005733456	Pundamilia nyererei sodium- and chloride-dependent betaine transporter-like	8.00E-56	GO:0006584	Catecholamine metabolic process
				GO:0006836	Neurotransmitter transport
				GO:0055114	Oxidation-reduction process
				GO:0016021	Integral component of membrane
				GO:0004500	Dopamine beta-monooxygenase activity
				GO:0005328	Neurotransmitter:sodium symporter activity
PS70	XM_003962817	*Takifugu rubripes* AT-rich interactive domain-containing protein 4A-like	0.00E+00		
PS78	XM_003967112	*Takifugu rubripes* dickkopf-related protein 3-like	0.00E+00		
PS81	XM_003963842	*Takifugu rubripes* cell division cycle 5-like protein-like	0.00E+00		
PS82	XM_003964272	*Takifugu rubripes* collagen alpha-1(I) chain-like, transcript variant 1	0.00E+00	GO:0005515	Protein binding
PS85	XM_003963677	*Takifugu rubripes* thioredoxin-dependent peroxide reductase, mitochondrial-like	0.00E+00		
PS89	XM_003979464	*Takifugu rubripes* IgM heavy chain constant region	0.00E+00	GO:0019028	Viral capsid
				GO:0004674	Protein serine/threonine kinase activity
				GO:0005515	Protein binding

PS90	XM_003963019	*Takifugu rubripes* intermediate filament protein ON3-like	0.00E+00	GO:0045095	Keratin filament
				GO:0005198	Structural molecule activity
PS95	XM_003967999	*Takifugu rubripes* leukocyte cysteine proteinase inhibitor 1-like	1.00E-67	GO:0005622	Intracellular
				GO:0004866	Endopeptidase inhibitor activity
PS99	XM_005811897	*Xiphophorus maculatus* microcephalin-like	2.00E-179	GO:0005622	Intracellular
PS100	XM_003961332	*Takifugu rubripes* ras-related C3 botulinum toxin substrate 1-like	1.00E-128	GO:0006813	Potassium ion transport
				GO:0007264	Small gtpase mediated signal transduction
				GO:0005525	GTP binding
				GO:0008324	Cation transmembrane transporter activity
PS102	XM_005730890	*Pundamilia nyererei* zinc finger CCCH domain-containing protein 13-like	0.00E+00		
PS121	XM_003962686	*Takifugu rubripes* ALK tyrosine kinase receptor-like	0.00E+00	GO:0006468	Protein phosphorylation
				GO:0007169	Transmembrane receptor protein tyrosine kinase signaling pathway
				GO:0016020	Membrane
				GO:0004672	Protein kinase activity
				GO:0004713	Protein tyrosine kinase activity
				GO:0004714	Transmembrane receptor protein tyrosine kinase activity
				GO:0005515	Protein binding
				GO:0005524	ATP binding

PS125	XM_003964150	*Takifugu rubripes* homeobox protein MSH-C-like	1.00E-155	GO:0006355	Regulation of transcription, DNA-templated
				GO:0005634	Nucleus
				GO:0003700	Sequence-specific DNA binding transcription factor activity
				GO:0005515	Protein binding
				GO:0043565	Sequence-specific DNA binding
PS135	XM_005801268	*Xiphophorus maculatus* heme-binding protein 1-like	8.00E-131		
PS137	XM_003965667	*Takifugu rubripes* ensconsin-like	2.00E-132		
PS138	XM_003971742	*Takifugu rubripes* heat shock protein HSP 90-beta-like	0.00E+00	GO:0006457	Protein folding
				GO:0006950	Response to stress
				GO:0005524	ATP binding
				GO:0051082	Unfolded protein binding
PS139	XM_003965130	*Takifugu rubripes* MAM domain-containing protein 2-like	0.00E+00	GO:0016020	Membrane
PS142	XM_003977764	*Takifugu rubripes* E3 UFM1-protein ligase 1-like	0.00E+00	GO:0005996	Monosaccharide metabolic process
				GO:0006184	GTP catabolic process
				GO:0006351	Transcription, DNA-templated
				GO:0006355	Regulation of transcription, DNA-templated
				GO:0006412	Translation
				GO:0006413	Translational initiation
				GO:0006606	Protein import into nucleus
				GO:0006886	Intracellular protein transport

	GO:0007131	Reciprocal meiotic recombination
	GO:0016032	Viral process
	GO:0016114	Terpenoid biosynthetic process
	GO:0019385	Methanogenesis, from acetate
	GO:0045892	Negative regulation of transcription, DNA-templated
	GO:0046649	Lymphocyte activation
	GO:0055114	Oxidation-reduction process
	GO:0000794	Condensed nuclear chromosome
	GO:0005622	Intracellular
	GO:0005634	Nucleus
	GO:0005643	Nuclear pore
	GO:0005737	Cytoplasm
	GO:0005785	Signal recognition particle receptor complex
	GO:0005840	Ribosome
	GO:0005852	Eukaryotic translation initiation factor 3 complex
	GO:0009986	Cell surface
	GO:0016021	Integral component of membrane
	GO:0000287	Magnesium ion binding
	GO:0003677	DNA binding
	GO:0003700	Sequence-specific DNA binding transcription factor activity

				GO:0003735	Structural constituent of ribosome
				GO:0003743	Translation initiation factor activity
				GO:0003887	DNA-directed DNA polymerase activity
				GO:0003924	Gtpase activity
				GO:0004872	Receptor activity
				GO:0005047	Signal recognition particle binding
				GO:0005525	GTP binding
				GO:0008565	Protein transporter activity
				GO:0016853	Isomerase activity
				GO:0030976	Thiamine pyrophosphate binding
				GO:0043565	Sequence-specific DNA binding
				GO:0046429	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase activity
				GO:0046983	Protein dimerization activity
				GO:0048029	Monosaccharide binding
PS143	XM_003973317	*Takifugu rubripes* sodium- and chloride-dependent GABA transporter 2-like	0.00E+00	GO:0006836	Neurotransmitter transport
				GO:0016021	Integral component of membrane
				GO:0005328	Neurotransmitter:sodium symporter activity
PS145	XM_003964933	*Takifugu rubripes* heat shock 70 kDa protein 1-like	3.00E-133	GO:0006935	Chemotaxis
				GO:0006950	Response to stress

				GO:0007165	Signal transduction
				GO:0016020	Membrane
				GO:0004888	Transmembrane signaling receptor activity
				GO:0005524	ATP binding
PS147	XM_003974305	*Takifugu rubripes* receptor-type tyrosine-protein phosphatase S-like	0.00E+00	GO:0006470	Protein dephosphorylation
				GO:0016311	Dephosphorylation
				GO:0004725	Protein tyrosine phosphatase activity
				GO:0005515	Protein binding
				GO:0016791	Phosphatase activity
PS156	XM_003973071	*Takifugu rubripes* 60S acidic ribosomal protein P2-like	2.00E-27	GO:0006412	Translation
				GO:0005622	Intracellular
				GO:0005840	Ribosome
				GO:0003735	Structural constituent of ribosome
PS160	XM_003966814	*Takifugu rubripes* mitogen-activated protein kinase kinase kinase kinase 3-like	8.00E-79		
PS165	XM_003976174	*Takifugu rubripes* nucleolar GTP-binding protein 1-like	0.00E+00	GO:0007264	Small gtpase mediated signal transduction
				GO:0005525	GTP binding
PS166	XM_003965807	*Takifugu rubripes* cathepsin S-like	0.00E+00	GO:0006508	Proteolysis
				GO:0004197	Cysteine-type endopeptidase activity
				GO:0008234	Cysteine-type peptidase activity

PS168	XM_004070229	*Oryzias latipes* angiopoietin-related protein 7-like	0.00E+00		
PS172	XM_003977933	*Takifugu rubripes* matrix metalloproteinase-14-like	0.00E+00	GO:0006508	Proteolysis
				GO:0016020	Membrane
				GO:0031012	Extracellular matrix
				GO:0004222	Metalloendopeptidase activity
				GO:0008270	Zinc ion binding
PS177	XM_003975122	*Takifugu rubripes* hyaluronan and proteoglycan link protein 1-like	0.00E+00	GO:0007155	Cell adhesion
				GO:0005515	Protein binding
				GO:0005540	Hyaluronic acid binding
PS178	AB437703	*Takifugu* sp. S4 mitochondrial gene for cytochrome b	3.00E-36	GO:0022904	Respiratory electron transport chain
				GO:0005739	Mitochondrion
				GO:0016020	Membrane
				GO:0009055	Electron carrier activity
				GO:0016491	Oxidoreductase activity
PS179	XM_003970951	*Takifugu rubripes* heat shock 70 kDa protein 4-like	0.00E+00	GO:0005524	ATP binding
PS181	XP_003968865	60S ribosomal protein L11-like [*Takifugu rubripes*]	5.00E-125	GO:0006412	Translation
				GO:0005622	Intracellular
				GO:0005840	Ribosome
				GO:0003735	Structural constituent of ribosome
PS186	XM_003967852	*Takifugu rubripes* lipocalin-like	8.00E-154	GO:0006629	Lipid metabolic process
				GO:0006810	Transport
				GO:0005215	Transporter activity
				GO:0005488	Binding

				GO:0005550	Pheromone binding
PS189	XM_003976864	*Takifugu rubripes* lysosomal protective protein-like	0.00E+00	GO:0006508	Proteolysis
				GO:0004177	Aminopeptidase activity
				GO:0004185	Serine-type carboxypeptidase activity
PS191	XM_003970799	*Takifugu rubripes* centromere protein V-like	4.00E-61		
PS199	XM_003971618	*Takifugu rubripes* inositol-trisphosphate 3-kinase B-like	0.00E+00	GO:0006366	Transcription from RNA polymerase II promoter
				GO:0005665	DNA-directed RNA polymerase II, core complex
				GO:0003677	DNA binding
PS204	XM_003974422	*Takifugu rubripes* elongation factor 1-delta-like, transcript variant 2	7.00E-136	GO:0006414	Translational elongation
				GO:0005853	Eukaryotic translation elongation factor 1 complex
				GO:0003746	Translation elongation factor activity
PS207	XM_003969868	*Takifugu rubripes* fibronectin type III and SPRY domain-containing protein 2-like	0.00E+00	GO:0005515	Protein binding
PS208	XM_003972994	*Takifugu rubripes* asporin-like	0.00E+00		
PS210	XM_003966663	*Takifugu rubripes* zinc finger E-box-binding homeobox 2-like, transcript variant 2	0.00E+00	GO:0006355	Regulation of transcription, DNA-templated
				GO:0005622	Intracellular
				GO:0003700	Sequence-specific DNA binding transcription factor activity

				GO:0008270	Zinc ion binding
				GO:0043565	Sequence-specific DNA binding
PS211	XM_003962424	*Takifugu rubripes* protein archease-like	4.00E-115		
PS214	XM_003968183	*Takifugu rubripes* 60S ribosomal protein L22-like 1-like	8.00E-68		
PS220	XM_003962519	*Takifugu rubripes* fermitin family homolog 2-like, transcript variant 1	0.00E+00	GO:0005856	Cytoskeleton
				GO:0005515	Protein binding
PS227	AY651247	Sparus aurata osteopontin-like	2.00E-35	GO:0001503	Ossification
				GO:0007155	Cell adhesion
PS229	XM_005456599	Oreochromis niloticus disks large homolog 5-like	0.00E+00	GO:0042981	Regulation of apoptotic process
				GO:0005622	Intracellular
				GO:0005200	Structural constituent of cytoskeleton
				GO:0005515	Protein binding
PS230	XM_003978247	*Takifugu rubripes* mitochondrial import inner membrane translocase subunit TIM14-like	8.00E-64	GO:0006457	Protein folding
				GO:0031072	Heat shock protein binding
				GO:0051082	Unfolded protein binding
PS235	XM_003963691	*Takifugu rubripes* kinesin light chain 1-like	5.00E-79		
PS241	XM_003974002	*Takifugu rubripes* elongation factor 2-like	0.00E+00	GO:0003924	Gtpase activity
				GO:0005525	GTP binding
PS243	XM_003963443	*Takifugu rubripes* glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic-like	0.00E+00	GO:0005975	Carbohydrate metabolic process

	GO:0006072	Glycerol-3-phosphate metabolic process
	GO:0006813	Potassium ion transport
	GO:0008152	Metabolic process
	GO:0046168	Glycerol-3-phosphate catabolic process
	GO:0055114	Oxidation-reduction process
	GO:0005737	Cytoplasm
	GO:0009331	Glycerol-3-phosphate dehydrogenase complex
	GO:0003824	Catalytic activity
	GO:0004367	Glycerol-3-phosphate dehydrogenase [NAD+] activity
	GO:0005488	Binding
	GO:0008324	Cation transmembrane transporter activity
	GO:0016491	Oxidoreductase activity
	GO:0016614	Oxidoreductase activity, acting on CH-OH group of donors
	GO:0016616	Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor
	GO:0042803	Protein homodimerization activity
	GO:0050662	Coenzyme binding

				GO:0051287	NAD binding
PS245	XM_003966487	*Takifugu rubripes* neurobeachin-like protein 1-like	0.00E+00	GO:0006355	Regulation of transcription, DNA-templated
				GO:0005622	Intracellular
				GO:0003700	Sequence-specific DNA binding transcription factor activity
				GO:0005515	Protein binding
				GO:0043565	Sequence-specific DNA binding
PS246	XM_003970872	*Takifugu rubripes* A disintegrin and metalloproteinase with thrombospondin motifs 2-like	0.00E+00	GO:0006508	Proteolysis
				GO:0005578	Proteinaceous extracellular matrix
				GO:0004222	Metalloendopeptidase activity
				GO:0008237	Metallopeptidase activity
				GO:0008270	Zinc ion binding
PS248	XM_003972298	*Takifugu rubripes* actin-related protein 2/3 complex subunit 1B-like	0.00E+00		
PS258	XM_003965192	*Takifugu rubripes* nuclear pore complex protein Nup214-like	0.00E+00	GO:0006813	Potassium ion transport
				GO:0016020	Membrane
				GO:0005249	Voltage-gated potassium channel activity
PS264	XM_005805657	*Xiphophorus maculatus* ceramide synthase 5-like	4.00E-175	GO:0006355	Regulation of transcription, DNA-templated
				GO:0003700	Sequence-specific DNA binding

					transcription factor activity
				GO:0043565	Sequence-specific DNA binding
PS265	XM_003964325	*Takifugu rubripes* 40S ribosomal protein SA-like	0.00E+00	GO:0006412	Translation
				GO:0005622	Intracellular
				GO:0005840	Ribosome
				GO:0003735	Structural constituent of ribosome
PS266	XM_005936874	*Haplochromis burtoni* transport and Golgi organization protein 2 homolog	5.00E-156		
PS269	XM_003968449	*Takifugu rubripes* serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform-like	0.00E+00		
PS271	XM_003962289	*Takifugu rubripes* protein tyrosine phosphatase type IVA 2-like, transcript variant 1	3.00E-121	GO:0006470	Protein dephosphorylation
				GO:0016311	Dephosphorylation
				GO:0004725	Protein tyrosine phosphatase activity
				GO:0016791	Phosphatase activity
PS272	XM_003455230	*Oreochromis niloticus* protein S100-A13-like	5.00E-45		
PS278	NM_001173845	*Salmo salar* Cyclic AMP-dependent transcription factor ATF-5 (atf5)	0.00E+00	GO:0006355	Regulation of transcription, DNA-templated
				GO:0003700	Sequence-specific DNA binding transcription factor activity
				GO:0043565	Sequence-specific DNA binding
PS281	XM_003962482	*Takifugu rubripes* grainyhead-like protein 1 homolog, transcript variant 1	0.00E+00		

PS283	XM_003977829	*Takifugu rubripes* P2Y purinoceptor 1-like	0.00E+00	GO:0007186	G-protein coupled receptor signaling pathway
				GO:0007596	Blood coagulation
				GO:0035589	G-protein coupled purinergic nucleotide receptor signaling pathway
				GO:0070493	Thrombin receptor signaling pathway
				GO:0016021	Integral component of membrane
				GO:0004945	Angiotensin type II receptor activity
				GO:0004974	Leukotriene receptor activity
				GO:0004982	N-formyl peptide receptor activity
				GO:0005515	Protein binding
				GO:0015057	Thrombin receptor activity
				GO:0016494	C-X-C chemokine receptor activity
				GO:0045028	G-protein coupled purinergic nucleotide receptor activity
PS287	XM_003974496	*Takifugu rubripes* angiopoietin-related protein 2-like	0.00E+00	GO:0007165	Signal transduction
				GO:0005102	Receptor binding
PS290	XM_003974540	*Takifugu rubripes* protein SET-like	4.00E-160		
PS292	XM_003967080	*Takifugu rubripes* NEDD4-like E3 ubiquitin-protein ligase WWP2-like, transcript variant 1	0.00E+00	GO:0006464	Cellular protein modification process
				GO:0005622	Intracellular
				GO:0005515	Protein binding

				GO:0016881	Acid-amino acid ligase activity
PS293	XM_003965929	*Takifugu rubripes* glyceraldehyde 3-phosphate dehydrogenase 2-like	0.00E+00		
PS295	XM_003968017	*Takifugu rubripes* glycogenin-1-like	0.00E+00		
PS298	XM_004545199	*Maylandia zebra* angiogenic factor with G patch and FHA domains 1-like	3.00E-163	GO:0006334	Nucleosome assembly
				GO:0000786	Nucleosome
				GO:0005622	Intracellular
				GO:0003676	Nucleic acid binding
				GO:0003677	DNA binding
				GO:0005515	Protein binding
PS300	XM_003971754	*Takifugu rubripes* RNA-binding protein 25-like	4.00E-127	GO:0003676	Nucleic acid binding
PS302	XM_005733039	*Pundamilia nyererei* serine/arginine-rich splicing factor 6-like	4.00E-95	GO:0006412	Translation
				GO:0005622	Intracellular
				GO:0005840	Ribosome
				GO:0003676	Nucleic acid binding
				GO:0003735	Structural constituent of ribosome
PS303	XM_005729008	*Pundamilia nyererei* GPI-anchor transamidase-like	5.00E-43	GO:0006508	Proteolysis
				GO:0004197	Cysteine-type endopeptidase activity
PS305	XM_003974130	*Takifugu rubripes* AP-3 complex subunit delta-1-like, transcript variant 2	0.00E+00	GO:0006508	Proteolysis
				GO:0004197	Cysteine-type endopeptidase activity
PS311	XM_003965802	*Takifugu rubripes* cytosolic 5'-nucleotidase III-like	0.00E+00		

PS316	XM_003975841	*Takifugu rubripes* myocilin-like	0.00E+00	GO:0016020	Membrane
				GO:0004930	G-protein coupled receptor activity
PS318	XM_003968131	*Takifugu rubripes* annexin A13-like	0.00E+00	GO:0005509	Calcium ion binding
				GO:0005544	Calcium-dependent phospholipid binding
PS319	XM_003976137	*Takifugu rubripes* synaptophysin-like	3.00E-165	GO:0006810	Transport
				GO:0008021	Synaptic vesicle
				GO:0016020	Membrane
				GO:0005215	Transporter activity
PS320	XM_003962122	*Takifugu rubripes* collagen alpha-2(V) chain-like	5.00E-126		
PS325	XM_003978351	*Takifugu rubripes* tumor suppressor candidate 3-like	7.00E-152		
PS334	NM_001011879	*Takifugu rubripes* keratin	0.00E+00	GO:0045095	Keratin filament
				GO:0005198	Structural molecule activity
PS341	XM_003974099	*Takifugu rubripes* elongation of very long chain fatty acids protein 4-like	0.00E+00	GO:0016021	Integral component of membrane
PS346	XM_005468470	*Oreochromis niloticus* nucleosome-remodeling factor subunit BPTF-like	0.00E+00	GO:0005515	Protein binding
				GO:0008270	Zinc ion binding
PS352	XM_003964395	*Takifugu rubripes* 26S proteasome non-ATPase regulatory subunit 11-like	0.00E+00		
PS357	XM_003969430	*Takifugu rubripes* aminopeptidase N-like	0.00E+00	GO:0008237	Metallopeptidase activity
				GO:0008270	Zinc ion binding
PS358	XR_172528	*Takifugu rubripes* fibronectin-like	2.00E-134	GO:0005576	Extracellular region

PS360	XM_003979409	*Takifugu rubripes* translation initiation factor eIF-2B subunit gamma-like	0.00E+00	GO:0006355	Regulation of transcription, DNA-templated
				GO:0006412	Translation
				GO:0008654	Phospholipid biosynthetic process
				GO:0009058	Biosynthetic process
				GO:0009103	Lipopolysaccharide biosynthetic process
				GO:0055114	Oxidation-reduction process
				GO:0005622	Intracellular
				GO:0005840	Ribosome
				GO:0016020	Membrane
				GO:0003700	Sequence-specific DNA binding transcription factor activity
				GO:0003735	Structural constituent of ribosome
				GO:0008233	Peptidase activity
				GO:0008715	CDP-diacylglycerol diphosphatase activity
				GO:0016491	Oxidoreductase activity
				GO:0016779	Nucleotidyltransferase activity
				GO:0046872	Metal ion binding
PS361	XR_172544	*Takifugu rubripes* plectin-like	0.00E+00		
PS363	XM_003963168	*Takifugu rubripes* serine/threonine-protein kinase receptor R3-like	0.00E+00		
PS372	XM_003966850	*Takifugu rubripes* 40S ribosomal protein S4, X isoform-like	0.00E+00		

PS376	XM_003968818	*Takifugu rubripes* elongation factor 1-alpha-like	0.00E+00		
PS379	NM_053776	*Rattus norvegicus* DnaJ (Hsp40) homolog, subfamily C, member 2 (Dnajc2)	0.00E+00	GO:0000746	Conjugation
				GO:0001503	Ossification
				GO:0006184	GTP catabolic process
				GO:0006355	Regulation of transcription, DNA-templated
				GO:0006606	Protein import into nucleus
				GO:0006810	Transport
				GO:0006886	Intracellular protein transport
				GO:0007049	Cell cycle
				GO:0007155	Cell adhesion
				GO:0030198	Extracellular matrix organization
				GO:0045893	Positive regulation of transcription, DNA-templated
				GO:0005576	Extracellular region
				GO:0005634	Nucleus
				GO:0005643	Nuclear pore
				GO:0005737	Cytoplasm
				GO:0005785	Signal recognition particle receptor complex
				GO:0016020	Membrane
				GO:0016021	Integral component of membrane

				GO:0016529	Sarcoplasmic reticulum
				GO:0043189	H4/H2A histone acetyltransferase complex
				GO:0003677	DNA binding
				GO:0003700	Sequence-specific DNA binding transcription factor activity
				GO:0003924	Gtpase activity
				GO:0005047	Signal recognition particle binding
				GO:0005102	Receptor binding
				GO:0005215	Transporter activity
				GO:0005488	Binding
				GO:0005515	Protein binding
				GO:0005516	Calmodulin binding
				GO:0005525	GTP binding
				GO:0008565	Protein transporter activity
				GO:0031072	Heat shock protein binding
PS389	XM_003976988	*Takifugu rubripes* E3 ubiquitin-protein ligase TRIP12-like	0.00E+00	GO:0006464	Cellular protein modification process
				GO:0005622	Intracellular
				GO:0016881	Acid-amino acid ligase activity

CHAPTER 5

General Discussion

In this present study, I try to understand the fundamentals of parasitism by investigating three different fields. In chapter 2 and 3, I am focusing my research on *Peniculus minuticaudae* Shiino, 1956 (Pennellidae) infecting threadsail filefish, *Stephanolepis cirrhifer* (Temminck & Schlegel, 1850). While in chapter 4, the investigation to understand the mechanism underlying the host and site recognition were extended using another well-researched and economically important species, the *Caligus fugu*, infecting puffer fishes. Therefore, in this final chapter, I will try to discuss all of the aspects that are being investigated in this study, in relation to the preferences of these parasites that specifically infect the fins of the hosts.

In chapter 2, the complete life cycle of *P. minuticaudae* has been described. The report contributes to the knowledge on the life cycle of the fourth genus of pennellid copepod. Previously, the complete life cycle of pennellid copepod were only recorded for three species which are *Lernaeocera branchialis* (Linnaeus, 1767) (Sproston, 1942; Brooker et al. 2011), *Lernaeenicus sprattae* (Sowerby, 1806) (Schram, 1979) and *Cardiodectes medusaeus* (Wilson C.B., 1908) (Ho, 1966; Perkins 1983).

From these reports, the life cycle of pennellids can be divided into four categories based on the number of host and the presence or absence of nauplii (Table 5.1). The combinations are stated as follows: (1) *L. branchialis* (two hosts, nauplii present); (2) *C. medusaeus* (two hosts, no nauplii); (3) *L. sprattae* (single host, nauplii present); (4) *P. minuticaudae* (single host, no nauplii). In *L. sprattae*, adult females change their infection sites from fins and body surface to the eyes of the host for oviposition, whereas in *P. minuticaudae*, all stages including ovigerous females were infecting the fins of the host. Among Pennellidae, bloodsucker parasites may usually have two hosts, while histophagous taxa does not change its host and/or attachment sites.

Table 5.1 Comparison of hatching stages, host(s) and attachment site(s) of parasitic copepod in Family Pennellidae

Species	Hatching stage	Host(s)		Attachment site(s)	
		Intermediate host	Definitive host	Developmental stages	Post-metamoprhic females
L. branchialis	Nauplii I	Pleuronectidae	Gadidae	Gills	Gills penetrating to the heart
C. medusaeus	Nauplii I	Pelagic gastropods Cavolinidae	Myctophidae	Gills	Body surface penetrating to the heart
L. sprattae	Copepodid	Clupeidaie		Fins	Eyes
P. minuticaudae	Copepodid	Mostly Monacanthidae		Fins	

Some pennellids especially at the reproductive stage (post-metamorphic adult females), are known to infect the eyes of their hosts. For example, *L. sprattae* on sprat (Anstensrud & Schram, 1988) and *Phrixocephalus cincinnatus* Wilson C.B., 1908 on flounder (Kabata, 1969; 1974; Woo & Bruno, 2011). Among these examples, *L. sprattae* were known to switch their infection site; from the fins during developmental stages, to the eyes for adult females (Schram, 1979). Adult females will continue metamorphing and producing eggs after anchoring themselves deeply into the eyes (Schram, 1979; Woo & Bruno, 2011). The site-switching infection might be explained by concluding a study by Shariff (1981) on the distribution and abundance of the adult female *Lernaea piscinae* Harding, 1950 growing on big head carp. After three weeks of infection by *L. piscinae*, only the adult female infecting the eyes was retained, while the remaining females infecting the body surface were eliminated. It was suggested that copepod might stay on the eyes because eyes are an immunologically privileged site on the body of the host (Shariff, 1981; Woo & Bruno, 2011).

The infection site of various adult females pennellids showed that they often choose a site that allows them to feed on the blood of the host. *Lernaeenicus hemirhamphi* Kirtisinghe, 1932 burrow deeply seeking any organ with rich blood supply (Natarajan & Nair, 1973). Besides the eyes, pilchard *L. sprattae* also pierce the heart occasionally (Rousset & Raibaut, 1989). *L. branchialis* penetrates near the

ventral end of the third or fourth arch; developing anchor and mouthpart in the wall of either the bulbus arteriosus (Kabata, 1979) or ventral aorta (Khan, 1988). *Lernaeolophus sultanus* (Milne Edwards, 1840) attached to the fish palate, anchoring the eye socket (Grabda, 1972). *Lernaeolophus aceratus* (Ho & Honma, 1983) attached to the gill cavity and embedded its head between the liver and vertebral column (Ho & Honma, 1983). *Haemobaphes diceraus* Wilson, 1917 and *C. medusaeus* penetrate directly into the blood vessels of the host (Grabda, 1975; Perkins, 1983). While *Peroderma cylindricum* (Heller, 1865) embeds into the kidney of its pilchard host (Woo & Bruno, 2011).

Adult female pennellids generally feed on the blood and lymph of fish from haemorrhage and inflammation within the granuloma (Woo & Bruno, 2011). The blood is more nutritious in comparison to the epithelial tissues or mucous of the host (). Therefore, it is clear that the site-switching among adult females pennellids is to obtain more nutritious food and nutrient for reproduction purposes. According to Woo & Bruno (2011), adaptation to a specific site increases the fitness of the parasite in a particular site over the parasite's fitness in some other site. Fitness can be defined as the success of contributing genetic information to subsequent generations (Bush et al. 2001).

Site specificity is common among parasitic copepods. In this study, I am investigating two parasitic copepods from two different families, which sharing a similarity where at least part of the life cycle stages of these parasites prefer fins as their infection site. All life stages of *P. minuticaudae* are site-specific to the fins (Ismail et al. 2013) with highest preference to the pectoral fin of the host. The infective copepodid and developing chalimi of *Caligus fugu* were exclusively infecting the fins of puffer fishes (Ohstuka et al. 2009). Table 5.2 listed the parasitic copepods from various families, which at least some part of their life cycle preferring fins as their infection site.

Site specificity is a restriction where the parasites choose and prefer a particular site or habitat on the host (Woo & Bruno, 2011). Why do they prefer the fins.

Table 5.2. List of some parasitic copepods that preferred fins as an attachment site

Family	Species	Host	Stage of life cycle attached to the fins	References
Caligidae	*Lepeophtheirus salmonis*	Salmonid fishes	Copepodid and chalimus	Wootten et al. (1982)
	Lepeophtheirus pectoralis	Flounder, plaice	All stages	Boxshall (1976; 1977)
	Caligus elongatus	*Salmo salar,*	Adult female	Hogans & Trudeau (1989)
	Caligus epidemicus	*Oreochromis mossambicus Acanthopagrus australis*	Copepodid and chalimus	Lin & Ho (1993), Roubal (1994)
	Caligus fugu	Pufferfishes	Copepodid and chalimus	Ohtsuka et al. (2009)
Pennellidae	*Peniculisa wilsoni*	*Diodon histrix*	Adult female	Radhakrishnan & Nair (1981)
	Lernaeenicus sprattae	Pilchard and sprat	Copepodid and chalimus	Schram (1979)
	Salmincola edwardsii	*Salvelinus fontinalis*	Copepodid and chalimus	Black et al. (1983)
	Peniculus minuticaudae	*Monacanthidae*	All stages	Okawachi et al. (2012); Ismail et al. (2013)
Ergasilidae	*Neoergasilus japonicus*	*Pimephales promelas*	Adult	Hayden & Rogers (1998)

Bron (1993) suggested that rather than selection, the preferences of copepodid to a particular area is determined by the ability of the copepodid to attach initially and subsequently remain attached. The suitability of a given area for settlement maybe depends upon the nature of the epidermis that varies over the host body, and the exposure of water current at different sites. In a study carried out upon the attachment of *L. salmonis* on three different salmonid hosts by Johnson &Albrightz (1992), copepodids were found to have infected fins, body surface and gills of the hosts. However, after certain period of infection, many copepodids were eliminated from the gills but remained on the fins. The fins shows from moderate to mild response towards the feeding activity of copepodid, with the responses characterised by erosion of the epidermis in the vicinity of the mouth cone, lack of dermal reaction and normal to mild hyperplastic epithelium in the vicinity of the frontal filament. In contrast, gills showed extensive epithelial hyperplasia and well-developed inflammatory response. In this study, copepodid attached to the gills also enduring slow developmental rate in comparison to the copepodid attached to the fins. It is suggested that this phenomenon was a result of different tissue response and immunity. The copepodid may have preference to the fins where the immune system is less pronounces in comparison to the gills (Johnson &Albrightz, 1992).

Sukhdeo and Sukhdeo (1994) suggested that the main advantage for site-specificity is the ability for a particular parasite to maintain its relative stability and survival. Specificity of a particular habitat enables parasites to refine their recognition to relatively few but unique features of their preferred habitat. He also elaborated the concept of site-specificity based on three observations; (1) The parasites have the ability to predict and identify their preferred habitat/site, (2) parasites have the ability to predict the behaviour of the hosts, which may leads to the identification of their preferred habitat/site and (3) migrating to other site and maintain the position to that particular site that may become a fixed behaviour of parasite during the course of evolution.

Whittington et al. (2000) suggested that parasites and their host enduring the coevolution to become closely adapted so that the predictability of host biology (e.g. their behaviour, physiology and biochemistry) can be exploited by parasites. This coevolution enhances the fitness of a parasite because the parasite only needs to evolve to adapt, maintain their position and combat the immunity for one, or a limited spectrum of, host species or particular organ.

In chapter 4, I try to investigate the site-specificity for infective stage of *C. fugu*, the copepodid to the fins of puffer fishes. The experiments that have been carried out are a very small step towards achieving the aim to fully understand the mechanism involves in site-specificity of copepodid *C. fugu* to the fins. Behavioural study that has been carried out in this study shows that copepodid *C. fugu* has the ability of responding to the chemical cues in the host-conditioned water. In addition, by applying heat to the host-conditioned water, it left us with a clue that the chemical involves in the experiment might be in a form of protein. However, it is a long way ahead and more trials are required in order to identify the exact chemical substance(s) that may act as chemoattractant for *C. fugu* site recognition. However, from the behavioural observation of copepodid *C. fugu* in the y-tube bioassay experiments, it is worth to know that the copepodids does have the ability to response to the stimulation of host-conditioned water. This response might be the key to the questions that will be further investigated in the future.

Conclusion

Parasites and parasitic copepod now became a problem in the growing aquaculture industry. The aquaculture is one of the ways of generating income for a country and also seems to satisfy the needs of growing human population. In natural condition, parasite and fish may only live in a peaceful symbiotic relationship. As we know, parasites can rarely kill their hosts, as they need to maintain their habitat and food source. However, the aquaculture systems changed the situation, where the groups of hosts were confined in a crowded place, giving the opportunity to the parasites to enhance their fitness and reproduction ability. When they were finally became a major problem, not only to the fish's host but also to human, which is also a potential problem to the whole system of ecology. At this stage, parasite management is crucial and need to be carefully planned. Fundamental understanding of the whole aspect of parasites is essential towards achieving a successful parasite management. In this three years research, only a small part of a very small parasite has been explored. Leaving a whole bunch of questions to remain unanswered and rising a thousand more questions that are yet to be investigated. I believe this is just a beginning of my interesting life, exploring the complex field of parasitology.

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