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Doctoral Dissertation

STUDY ON LARVAL MASS MORTALITY AND ITS CONTROL IN THE SEED PRODUCTION OF PORTUNID CRABS

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Graduate School of Marine Science and Technology

Tokyo University of Marine Science and Technology

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Table of Content

Abstract……1

Publications……4

Abbreviations……5

1 General Introduction……7
1.1 Status of portunid crabs……8
1.2 Status of portunid crabs in Japan……9
1.3 Seed production of portunid crabs in Japan……10
1.4 Mass mortality occurring during seed production……10
1.5 Thesis objectives and context……12

2 Control of Bacterial Disease……17
2.1 Evaluation of the effects of probiotics in controlling bacterial necrosis symptoms in larvae of the mud crab *Scylla serrata* during mass seed production……18
2.1.1 Summary……18
2.1.2 Introduction……18
2.1.3 Materials and Methods……20
2.1.3.1 Isolation of causative bacteria……20
2.1.3.2 Effects of causative bacteria on larval mortality and necrosis……21
2.1.3.3 Screening of probiotic candidates……22
2.1.3.4 In vitro evaluation of probiotic effects……23
2.1.3.5 Evaluation of probiotic effects in short-term larval rearing experiments……24
2.1.3.6 Evaluation of probiotic effects in pilot-scale larval rearing experiments……25
2.1.3.7 Evaluation of probiotic effects in commercial-scale seed production experiments……25
2.1.3.8 Statistical analysis……26
2.1.4 Results……27
2.1.4.1 Effects of the NY strain on larval mortality and necrosis……27
2.1.4.2 Probiotic potential of candidate strains……28
2.1.4.3 Probiotic effects in short-term larval rearing experiments……29
2.1.4.4 Probiotic effects in pilot-scale larval rearing experiments……32
2.1.4.5 Probiotic effects in commercial-scale seed production experiments……33

2.1.5 Discussion……36

3 Control of Morphological Abnormality……41

3.1 Effects of salinity and dietary n-3 highly unsaturated fatty acids on the survival, development, and morphogenesis of the larvae of laboratory-reared mud crab *Scylla serrata* (Decapoda, Portunidae)……42

3.1.1 Summary……42

3.1.2 Introduction……42

3.1.3 Materials and Methods……43

3.1.3.1 Broodstock and larvae……43

3.1.3.2 Mass culture of larvae for rearing experiments……44

3.1.3.3 Larval rearing experiments……45

3.1.3.4 Biological measurements……46

3.1.3.5 Lipid and fatty acid analyses……47

3.1.3.6 Statistical analysis……47

3.1.4 Results……48

3.1.4.1 Lipid contents and fatty acid profiles of rotifers……48

3.1.4.2 Larval survival……48

3.1.4.3 Developmental period……51

3.1.4.4 Morphogenesis of Z5……51

3.1.4.5 Moulting patterns to MG……53

3.1.5 Discussion……55

3.2 Variations in larval morphology and their relationships to survival during mass seed production by the swimming crab, *Portunus trituberculatus* (Brachyura, Portunidae)……60

3.2.1 Summary……60

3.2.2 Introduction……60

3.2.3 Materials and Methods……62

3.2.3.1 Larval culture methods and survival data……62

3.2.3.2 Morphological measurements of larvae……65

3.2.3.3 Data analysis……67

3.2.4 Results ……68

3.2.4.1 Differences in the survival of the larval stages……68

3.2.4.2 Variations in the larval morphology……68

3.2.4.3 Relationships between larval survival, morphology and culture factors……69

3.2.4.4 Relationships between larval morphologies and culture factors……...……72
3.2.5 Discussion……75
3.3 Eyestalk ablation affects larval morphogenesis in the swimming crab *Portunus trituberculatus* during metamorphosis into megalopae……78
3.3.1 Summary……78
3.3.2 Introduction……78
3.3.3 Materials and Methods……80
3.3.3.1 Broodstock and larvae……80
3.3.3.2 Larval rearing in a 500 L tank……81
3.3.3.3 Larval treatment……82
3.3.3.4 Larval rearing in 1 L beakers……82
3.3.3.5 Morphological measurements……83
3.3.3.6 Data analysis……85
3.3.4 Results……86
3.3.4.1 Larval survival……86
3.3.4.2 Larval developmental velocity……88
3.3.4.3 Morphology of Z4 larvae……88
3.3.4.4 Morphology of fifth instar larvae……90
3.3.5 Discussion and Conclusions……96
3.4 Abnormal morphology in megalopae of the swimming crab, *Portunus trituberculatus* during seed production: causes and prevention……...……102
3.4.1 Summary……102
3.4.2 Introduction……102
3.4.3 Materials and Methods……104
3.4.3.1 Experiment 1: Effects of various feeding schemes on larval morphogenesis……104
3.4.3.2 Experiment 2: Effect of phytoplankton supplementation on larval morphogenesis……105
3.4.3.3 Experiment 3: Effect of countermeasures on the incidence of abnormal larval morphology……107
3.4.3.4 Morphological measurement of larvae……109
3.4.3.5 Data analysis……109

3.4.4 Results……109
3.4.4.1 Experiment 1: Effects of various feeding schemes on larval morphogenesis……109
3.4.4.2 Experiment 2: Effect of phytoplankton supplementation on larval morphogenesis……111
3.4.4.3 Experiment 3: Effect of countermeasures on the incidence of abnormal larval morphology……114
3.4.5 Discussion……115

4 Improvement in nutritional status of *Artemia*……123

4.1 Starvation of *Artemia* in larval rearing water affects post-larval survival and morphology of the swimming crab, *Portunus trituberculatus* (Brachyura, Portunidae)……124
4.1.1 Summary……124
4.1.2 Introduction……125
4.1.3 Materials and Methods……127
4.1.3.1 Experiment 1: Effect of starving rotifers and *Artemia* on larval survival and morphology……127
4.1.3.2 Experiment 2: Effect of starved or non-starved *Artemia* on larval survival and morphogenesis……128
4.1.3.3 Experiment 3: Effect of phytoplankton and rotifer co-existing on starvation of *Artemia*……129
4.1.3.4 Larval morphological measurements……129
4.1.3.5 Chemical analysis……130
4.1.3.6 Data analysis……131
4.1.4 Results……131
4.1.4.1 Experiment 1: Effect of starving rotifers and *Artemia* on larval survival and morphology……131
4.1.4.2 Experiment 2: Effect of starved or non-starved *Artemia* on larval survival and morphogenesis……134
4.1.4.3 Experiment 3: Effect of phytoplankton and rotifer co-existence on starvation of *Artemia*……136
4.1.5 Discussion……137
4.1.6 Conclusions……141

5 Larval culture method to control larval mass mortality……145

5.1 Improved method of culturing the swimming crab *Portunus trituberculatus* larvae to prevent mass mortality during seed production……146

5.1.1 Summary……146

5.1.2 Introduction……146

5.1.3 Materials and Methods……149

5.1.3.1 Experiment 1: Effect of feeding schedule on larval survival and morphology……149

5.1.3.2 Experiment 2: Effect of *Artemia* feeding method on larval survival and morphology……151

5.1.3.3 Morphological measurements of larvae……152

5.1.3.4 Data analysis……152

5.1.4 Results……154

5.1.4.1 Experiment 1: Effect of feeding schedule on larval survival and morphology……154

5.1.4.2 Experiment 2: Effect of *Artemia* feeding method on larval survival and morphology……159

5.1.5 Discussion……164

6 Concluding remarks……169

6.1 Causes of larval mass mortality during the seed production of portunid crabs……170

6.2 Bacterial necrosis disease and its control with probiotics……172

6.3 Mechanism of larval morphogenesis and to the induction of abnormal morphology……173

6.4 Characteristics in nutritional requirement of portunid crab larvae……176

6.5 Starvation of *Artemia* and its relation to larval nutritional deficiency……178

6.6 Culture method to prevent larval mass mortality……179

6.7 Future directions……181

Acknowledgements……184

References……186

Appendix……202
Supplementary data for Chapter 2.1……..……202
Supplementary data for Chapter 3.3……..205
Supplementary data for Chapter 3.4……..……..……208
Supplementary data for Chapter 4.1……..……214

Abstract

Portunid crabs are an important fishery resource with a global production of over 1,000,000 t. These species inhabit coastal or brackish waters and have a high economic value. Because of this, seed production programs have been developed in many countries to both enhance wild stocks and provide seed for aquaculture. The swimming crab *Portunus trituberculatus* and the mud crab *Scylla serrata* are target species for stock enhancement programmes in Japan. During the seed production of these species, rotifers are generally supplied as food throughout the zoeal stages (four instars for *P. trituberculatus* and five instars for *S. serrata*) and *Artemia* are supplied from the late zoeal to megalopal stages. The condition of these live-feeds is maintained by supplementing the larval culture tanks with phytoplankton such as *Chlorella vulgaris* and *Nannochloropsis oculata*. The biggest problem during seed production is low survival to the juvenile crab stage because of frequent larval mass mortality. Larval mass mortality occurs during the zoeal and megalopal stages. The metamorphosis from the zoeal to megalopal stage is an especially critical period. I documented three major causes of mass mortality: 1) bacterial necrosis disease, which causes mortality during zoeal stages, 2) abnormal larval morphology, which induces mortality during metamorphosis into megalopae, and 3) larval nutritional deficiency, which causes mortality during the period from megalopal to first crab stages.

Bacterial necrosis disease is characterized by tissue necrosis of the zoeal spines and appendages and has been frequently observed during seed production of *S. serrata*. Larval mortality occurs in almost all cases within a few days after the occurrence of tissue necrosis. To control this symptom without using antibiotics, I evaluated the effect of supplementation with probiotics both *in vitro* and during mass larval culture experiments. A probiotic bacterial strain that exhibited inhibitory effects against the causative bacterium (family Flavobacteriaceae) was screened from the seed production environment. The supernatant of the sterile medium used to culture the probiotic bacteria inhibited the causative bacterium. This suggests that the inhibitory factor is an extracellular secretion of the bacteria. In large-scale (100 kL) larval culture experiments, the use of probiotics significantly suppressed the incidence of necrosis and improved larval survival during the early culture period. However, this effect disappeared after the fifth zoeal stage, corresponding to a decrease in probiotic bacterial counts in larval culture water. Almost all larvae had died by the first crab stage. The effectiveness of probiotics developed *in vitro* is not yet practical in commercial-scale seed production. Developing a mechanism for the continuous proliferation of probiotic bacteria and continuous secretion of their putative extracellular substance into the larval

culture water are likely the most important needs to consider when developing probiotics for commercial-scale seed production.

To identify the relationship between larval morphological abnormalities and mass mortality during seed production of *P. trituberculatus*, I collected larval survival data and larval morphological samples from 111 seed production trials conducted in Japan. There were two distinct types of morphological abnormality. The first type was observed in last stage zoeae and was characterized by excessively advanced morphological features similar to those of megalopae (e.g., large chelae and pleopods). The second type was observed in megalopae and was characterized by retention of zoeal immature morphological features such as small dorsal spines and telson furcae. Both of these abnormalities induced moulting failure of larvae during metamorphosis into megalopae. The increase in size of last zoeal chelae and retention of the megalopal dorsal spine increased the frequency of megalopae that retained the old exuviae on their carapace and/or appendages. The immature morphology of megalopae had a more potent negative effect on larval survival than did the last zoeal morphology. Thus, I conclude that immature megalopal morphology is the major cause of mass mortality in *P. trituberculatus* seed production in Japanese hatcheries.

To identify the extrinsic factors affecting the morphological abnormalities of last stage zoeae and megalopae, I conducted larval rearing experiments to test the influence of dietary and environmental conditions in *P. trituberculatus* and *S. serrata*. Additionally, to understand the intrinsic mechanism controlling larval morphogenesis, I conducted zoeal eyestalk ablation experiments in *P. trituberculatus*. The morphogenesis of body parts that are enlarged toward metamorphosis into megalopae (e.g., chelae and pleopods) was continuously accelerated throughout the zoeal period under the control of the eyestalk neurosecretory system. The rate of enlargement increased when larvae were provided access to a diet high in energy and nutritional value and favourable environmental conditions (e.g., higher docosahexaenoic acid content in rotifers, higher density of *Artemia* feeding, and higher culture water salinity). To prevent excessive morphogenesis in last stage zoeae, culturists should control the dietary nutritional level, feeding density, and environment to suppress morphogenesis of these body parts below the threshold. Conversely, the morphogenesis of body parts that are resorbed during metamorphosis into megalopae (e.g., dorsal spine and telson furca) was controlled instantaneously at a critical period by the eyestalk neurosecretory system. The critical period was identified at premoult of the penultimate zoeal stage (third zoeal stage for *P. trituberculatus*). The supplementation of phytoplankton such as *Chlorella* and *Nannochloropsis* into the culture water induced the retention of these body parts in megalopae via consumption of rotifers at this critical period, resulting in abnormal morphology of megalopae. The phytoplankton appear to have a factor that disrupts

endocrine control of the resorption of body parts in larvae. To prevent the occurrence of immature morphology in megalopae, culturists should take steps to prevent the indirect larval ingestion of phytoplankton via consumption of rotifers during the critical period.

Poor nutritional status of *Artemia* was also associated with larval mortality during the period from megalopal to first crab stages in *P. trituberculatus*. Newly hatched *Artemia* and starved *Artemia* had low eicosapentaenoic acid (EPA) contents. Larvae that fed on these *Artemia* during the late zoeal period (third and fourth zoeal stages) died during the megalopal period or moulted into unviable first stage crabs. EPA is an essential fatty acid for *P. trituberculatus* larvae. Furthermore, *Artemia* cannot digest phytoplankton that have a rigid cell wall (e.g., *Chlorella* and *Nannochloropsis*). However, these phytoplankton species are commonly supplemented as food for live-foods during seed production in Japanese hatcheries. Thus, it appears likely that *Artemia* may be starving in many instances in the larval culture water during seed production. Enrichment with EPA and supplementation of digestible *Nannochloropsis* (commercially available in Japan), which have physically broken cell walls and contain EPA, into the larval culture water appears to be effective at preventing nutritional deficiency in *Artemia*. Additionally, I found that indirect ingestion of phytoplankton (digestible *Nannochloropsis*) via consumption of *Artemia* did not induce abnormal megalopal morphology in larvae, unlike via consumption of rotifers.

A new larval culture method for *P. trituberculatus* was developed to prevent these multiple causes of mass mortality. To prevent immature morphology of megalopae, larvae were reared only with *Artemia* (i.e., without rotifers). To simplify larval feeding, small newly hatched *Artemia* nauplii were supplied during the first zoeal stage. Then, *Artemia* enriched with digestible *Nannochloropsis* were supplied from the second zoeal stage to provide a source of EPA for larvae. Digestible *Nannochloropsis* were supplemented into the culture tank to prevent *Artemia* starvation and to maintain their nutritional value. To control excessive morphogenesis of last stage zoeae, the *Artemia* feeding density and culture water salinity were reduced. Additionally, the larval culture water was not exchanged, to reduce the potential for contamination with pathogens. These measures resulted in high survival to the first crab stage and demonstrated for the first time that *P. trituberculatus* larvae could be cultured using only *Artemia*.

Publications

Dan S, Hamasaki K (2014) Evaluation of the effects of probiotics in controlling bacterial necrosis symptoms in larvae of the mud crab *Scylla serrata* during mass seed production. Aquaculture International 23, 277–296 (Chapter 2.1)

Dan S, Hamasaki K (2011) Effects of salinity and dietary n-3 highly unsaturated fatty acids on survival, development and morphogenesis of larvae of mud crab *Scylla serrata* (Decapoda, Portunidae) reared in the laboratory. Aquaculture International 19. 323–338 (Chapter 3.1)

Dan S, Kaneko T, Takeshima S, Ashidate M, Hamasaki K (2013) Variations in larval morphology and their relationships to survival during mass seed production of swimming crab *Portunus trituberculatus* (Brachyura, Portunidae). Aquaculture 414–415, 109–118 (Chapter 3.2)

Dan S, Kaneko T, Takeshima S, Ashidate M, Hamasaki K (2014) Eyestalk ablation affects larval morphogenesis in the swimming crab *Portunus trituberculatus* during metamorphosis into megalopae. Sexuality and Early Development in Aquatic Organisms 1: 57–73 (Chapter 3.3)

Abbreviations

Larval moulting stages

Z1; first zoeal stage

Z2; second zoeal stage

Z3; third zoeal stage

Z4; fourth zoeal stage

Z5; fifth zoeal stage

FI; fifth instar stage including supernumerary fifth zoeal stage and megalopal stage

MG; megalopal stage

C1; first crab stage

Morphological measurements

CL; carapace length

CHL; chela length

CHLr; ratio of CHL to CL (%)

CW; carapace width

DSf; frequency of MG retaining dorsal spines (%)

DSL; dorsal spine length

DSLr; ratio of DSL to CL (%)

ED; distance between bilateral eyes

EDr; ratio of ED to CW (%)

FL; Sum of bilateral furcae length on telson

FLr; ratio of FL to CL (%)

MFf; frequency of total (slight and serious) moulting failure (%)

OEf; frequency of serious moulting failure (%)

PL; third pleopod length

PLr: ratio of PL to CL (%)

TELf; frequency of MG retaining furca on the telson (%)

Z5f; frequency of supernumerary fifth stage zoeae

Others

Chlorella; *Chlorella vulgaris*

cfu; colony forming unit

d-Nanno.; digestible *Nannochloropsis oculata*

df; degree of freedom

EAr; nutritionally enriched *Artemia*

dah; days after hatching

DHA; docosahexaenoic acid

EPA; eicosapentaenoic acid

GLM; generalized linear model

GLMM; generalized linear mixed-effects model

HD; high density

LD; low density

LS; low salinity

LSI; larval stage index

MA; Marine ager (solid culture medium)

MB; Marine broth (liquid culture medium)

MDS; moult death syndrome

Nannochloropsis, Nanno.; *Nannochloropsis oculata*

NFS-Na; sodium nifurstyrenate

NHAr; newly hatched *Artemia*

n-3 HUFA; n-3 highly unsaturated fatty acid

n-3 HUFA; n-3 highly unsaturated fatty acid

sd; standard deviation

WE; water exchange

Chapter 1

GENERAL INTRODUCTION

1.1 Status of portunid crabs

The global harvest of brachyuran crabs has increased annually over the past decade and was 1,762,708 t in 2012 (FAO, 2014). Portunid crabs account for a large percentage (60 %, 1,084,778 t) of the harvest (Fig. 1.1). The primary target portunid crab species are the swimming crab *Portunus trituberculatus* (global harvest in 2012: 429,959 t), the mud crab *Scylla serrata* (212,235 t), the blue swimming crab *Portunus pelagicus* (180,138 t), and the blue crab *Callinectes sapidus* (94,207 t). These species inhabit coastal and/or blackish waters that are easily accessed by humans. Because of this, they are subject to intense harvest pressure and are an important source of protein and income in many regions of the world. As a result, the wild fishery resources have been exhausted by over-fishing (Hamasaki et al., 2011; LeVay et al., 2008; Lipcius and Stockhausen, 2002; Liu et al., 2013). To address these declines, stock enhancement efforts involving the release of juvenile crab seeds into natural habitat have been carried out in several regions (Davis et al., 2005; Hines et al., 2008; Lebata et al., 2009; Obata et al., 2006; Okamoto, 2004; Zohar et al., 2008). Additionally, there has been an increase in the aquaculture of portunid crabs with the annual aquaculture production of *P. trituberculatus* in China exceeding 91,050 t in 2010 (Jin et al., 2013). Similarly, the production of *S. serrata*, an important aquaculture species in Southeast Asia, was 158,309 t in 2011 (FAO, 2014).

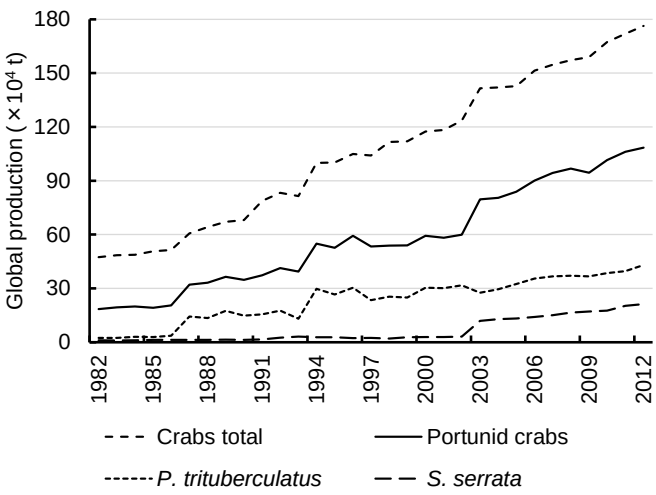

Figure 1.1. Changes in the global harvest of crabs and portunid crabs. Data from FAO (accessed 2014/12/2). Portunid crabs include *Portunus trituberculatus*, *Portunus pelagicus*, *Portunus* spp, *Scylla serrata*, *Scylla olivacea*, *Callinectes sapidus*, *Callinectes danae*, *Charybdis* spp, and other Portunidae, classified in FAO statistics

1.2 Status of portunid crabs in Japan

In Japan, the harvest of portunid crabs, including *P. trituberculatus*, *P. pelagicus*, *Scylla paramamosain*, and *S. serrata*, ranged from 2,319 to 3,354 t in the past 11 years (2002 to 2012), and their monetary value ranged from 2.7 to 4.7 billion yen (23–40 million $US) (Ministry of Agriculture, Forestry and Fisheries, http://www.maff.go.jp/j/tokei/kouhyou/kaimen_gyosei /index.html) (Fig. 1.2). *P. trituberculatus* and *P. pelagicus* inhabit the coastal areas south of Hokkaido and are one of the primary targets of small beam trawl, small set net, and gill net fishers. The mud crabs *S. paramamosain* and *S. serrata* are distributed in the coastal inlets and estuaries south of Honshu Island, and are caught using net cage traps and gill nets. These species sustain the local fisheries because of their high commercial value. In Japan, these species has been selected as targets for stock enhancement programmes. In 2012, a total of 24,557,000, 827,000, and 51,000 juveniles were released for *P. trituberculatus*, *P. pelagicus*, and *S. paramamosain*, respectively (Fisheries Research Agency, 2014). The effectiveness of stocking *P. trituberculatus* was evaluated based on analysis of catch and release statistics. The yield (weight, g) from stocking (yield per release; YPR) ranged from 16.0 to 33.6 g in the Seto Inland Sea, where the majority of releases occurred (Hamasaki et al., 2011; Kayano, 2010). The YPR of the local stock of *S. paramamosain* in Urado Bay, Kochi prefecture, estimated using genetic stock identification based on the mitochondrial DNA D-loop region, was 3.7 g (Obata et al., 2006). Taken together, these observations suggest that the release of juvenile crabs has been effective at sustaining the wild fishery resources.

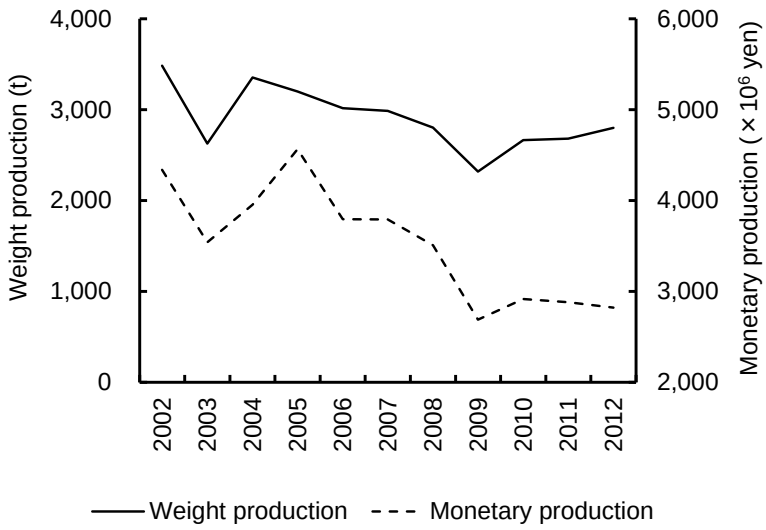

Figure 1.2. Changes in harvest of portunid crabs in Japan. Data from Ministry of Agriculture, Forestry and Fisheries (accessed 14/12/2).

1.3 Seed production of portunid crabs in Japan

Stock enhancement programs and aquaculture are dependent on the artificial production of crab seeds, i.e., seed production. In Japan, methods for seed production of brachyuran crabs were first developed in the 1960s for *P. trituberculatus* (Hamasaki, 1997; Hamasaki et al., 2011). Currently, *P. trituberculatus* seed production occurs in 15 hatcheries and 43,488,000 juveniles were produced in 2014 (Unpublished data). *P. pelagicus* and *S. paramamosain* seed production occurs in 5 and 1 hatcheries, respectively, and 17,580,000 and 70,000 juveniles were produced in 2014. Brachyuran crab larva hatch as a planktonic zoea (Anger, 2001a). *P. trituberculatus* have four zoeal instars and *P. pelagicus*, *S. serrata*, and *S. paramamosain* have five zoeal instars. Subsequent to the zoeal stage, the larva metamorphose into a megalopa (MG) and then moult to a first stage crab (C1). During seed production larvae are cultured from newly hatched first stage zoeae (Z1) to the C1 stage. Because the zoeae are planktotrophic, they are generally fed with a zooplankton rotifer *Brachionus plicatilis* sp. complex and *Artemia* sp. nauplii during seed production. In general, rotifers are supplied throughout the zoeal period and *Artemia* are supplied during the late zoeal (after third zoeal stage, Z3) and megalopal stage depending on their size (Hamasaki, 1997) during *P. trituberculatus* seed production in Japan. To provide an energy source for these live foods, phytoplanktons such as *Chlorella vulgaris*, *Nannochloropsis* sp. and/or diatoms are supplemented into the larval culture water. Using this approach, larvae can be cultured to the C1 stage, though survival is variable and larval mass mortality occurs relatively frequently (Hamasaki, 1997; Hamasaki et al., 2011). The mean survival rate from Z1 to C1 is low (~12 %) during seed production of *P. trituberculatus* (Hamasaki et al., 2011).

1.4 Mass mortality occurring during seed production

The “larval mass mortality” that occurs during seed production of portunid crabs was defined approximately as “simultaneous death >80 % of all larvae within 4 d” by a study group working on seed production technologies for portunid crabs in Japan (Hamasaki, 1997). There are several patterns of larval mass mortality during seed production. Disease is thought to be one of the causes of mass mortality. During seed production of portunid crabs, fungal infection, Vibriosis, and tissue necrosis disease have been reported to cause mass mortality (Hamasaki, 1997; Hamasaki and Hatai, 1993a, b; Hamasaki et al., 2011; Muroga et al., 1994). The occurrence of tissue necrosis disease during the seed production of *S. serrata* induces significant incidences of mass mortality

(Hamasaki et al., 2011). This disease can be prevented by bath treatment with sodium nifurstyrenate (NFS-Na).

Among the 398 seed production trials for *P. trituberculatus* (330 trials), *P. pelagicus* (51 trials), and *S. paramamosain* (17 trials) conducted in 2007 and 2008 by Japanese hatcheries, larval mass mortality occurred in 174 trials (frequency: 43.7 %) (Hamasaki et al., 2011). Among these, bacterial and fungal disease accounted for 26.4 and 7.5 % (46 and 13 cases), respectively, of all cases. The causes of the remaining cases (66.1 %, 115 cases) are unknown. However, symptoms such as moulting failure and inactivity of larvae were observed, according to data from a study group working on seed production technologies for portunid crabs (Hamasaki et al., 2011). These mass mortalities with unknown causes have been known to occur frequently during metamorphosis from last stage zoeae to MG (Hamasaki, 1997; Hamasaki et al., 2011; Yasumoto and Yoshida, 1994). In particular, the phenomenon whereby larvae die during and/or immediately after metamorphosis into MG and retain old exuviae on their carapace and appendages has been termed “moult death syndrome” (MDS) in many brachyuran crabs, particularly in intensively cultured species such as *Mithrax caribbaeus*, *P. pelagicus*, *P. trituberculatus*, *S. serrata*, *Scylla tranquebarica*, and *Ucides cordatus* (Arai et al., 2004; Baylon, 2009; Hamasaki et al., 2002a; Holm et al., 2009; Lárez et al., 2000; Mann et al., 1999; Nurdiani and Zeng, 2007; Rodzuani et al., 2012; Silva et al., 2012; Soundarapandian et al., 2007). In portunid crabs, the occurrence of MDS has been associated with last stage zoeae that have excessively advanced morphological features similar to MG (e.g., large chelae and pleopods) failing to moult into MG (Arai et al., 2004; Hamasaki et al., 2002a). This is also consistent with observations in the non-portunid species *Ucides cordatus* (Silva et al., 2012), suggesting that this phenomenon is possibly common among brachyuran crab species. In portunid crabs, such excessive morphogenesis of last stage zoeae is induced by specific dietary conditions during the zoeal period. The size of last zoeal chelae increased when zoeae were fed rotifers containing higher levels of n-3 highly unsaturated fatty acids (n-3 HUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), or fed *Artemia* at a higher density and earlier in the culture cycle (Arai et al., 2007; Hamasaki et al., 2002b; Suprayudi et al., 2002a, b).

Thus, the roles of disease and abnormal morphology in inducing larval mass mortality are generally understood. However, the cause of tissue necrosis disease of *S. serrata* larvae is not well elucidated as the pathogen is not yet identified, so we are unable to develop prevention measures. During seed production of *P. trituberculatus*, the causes of mortality remain largely

unknown. Additionally, there are no methods to prevent the occurrence of abnormal larval morphology. To achieve stable and high larval survival, there is a need to identify the underlying causes of mass mortality, to develop measures to counteract each cause, and to incorporate these measures into a new culture method that prevents all causes mortality.

1.5 Thesis objectives and context

In Chapter 2, my objectives were to identify the causative pathogen of necrosis disease during the seed production of *S. serrata* and develop a preventative measure that did not involve the use of antibiotics. The use of antibiotics triggers concerns over the potential for development of antibiotic-resistant bacteria, which can have a negative impact not only on the cultured animals but also on humans (Alderman and Hastings, 1998; Huys et al., 2007; Schmidt et al., 2000). An alternative disease prevention method, termed probiotics, involves the use of bacteria that exhibit an inhibitory effect against pathogenic bacteria and has attracted considerable attention in recent years (Balcázar et al., 2006; Fuller, 1989; Gatesoupe, 1999; Irianto and Austin, 2002; Kesarcodi-Watson et al., 2008; Verschuere et al., 2000a). However, very few studies have evaluated the effectiveness of probiotics at preventing of bacterial disease and improving larval survival rates during commercial-scale mass seed production. I identified the causative bacteria that induced tissue necrosis symptoms and mortality of *S. serrata* larvae. Then, using procedures proposed in review papers (e.g., Gomez-Gil et al., 2000; Verschuere et al., 2000a), a probiotic bacterial strain that had an inhibitory effect on the causative bacteria but was innocuous to larvae was screened from the seed production environment. The effectiveness of the probiotic strain which exhibited highest preventative effect in pilot scale (500 L tank) experiments was then evaluated in commercial-scale (100 kL tank) mass seed production.

In Chapter 3, I document research into the causes of abnormal larval morphology. Abnormal morphology of last stage zoeae, characterized by excessively advanced (enlarged) chelae and pleopods, has been observed frequently during seed production in *S. serrata* (Hamasaki et al., 2002a). First, to evaluate the factors affecting last zoeal morphology, I tested the combined effect of salinity and nutritional condition (DHA content in rotifers) on last zoeal morphology in *S. serrata* (Chapter 3.1). To understand the unidentified underlying cause of mass mortality during seed production of *P. trituberculatus*, I evaluated the relationship between rearing conditions, occurrence of abnormal morphology, and larval survival during seed production trials (Chapter 3.2). Information on the larval culture method, larval culture conditions, and survival was

obtained from 111 seed production trials conducted by 12 hatcheries in Japan. Last stage zoeae and MG were also sampled to obtain morphological measurements. Results of this research indicate that there are morphological variations in last stage zoeae and MG, and there is a significant relationship between MG morphology and larval survival. An increase in the occurrence of megalopae retaining small dorsal spines on the carapace and telson furcae (these are characteristic features of zoeae) was associated with a decrease in larval survival. I documented the existence of two types of abnormal morphology in *P. trituberculatus* larvae. The first was in last stage zoeae and was characterized by excessively advanced (enlarged) chelae and pleopods, similar to *S. serrata*. The second was in MG and was characterized by retention of immature morphology of the dorsal spines and telson furcae. The larval metamorphosis of brachyuran crabs is thought to be regulated by the neurosecretory system located in the eyestalks (X-organ sinus grand complex) because bilateral eyestalk ablation of zoeae results in retardation or inhibition of metamorphosis into MG in *Sesarma reticulatum* and *Rhithropanopeus harrisii* (Costlow, 1966a, b; Freeman and Costlow, 1980). However, the effect of eyestalk ablation on the morphogenesis of body parts such as chelae, pleopods, dorsal spines, and telson furcae has not been investigated. Therefore, to understand the relationships between larval morphological abnormalities and the endocrine control of morphogenesis, the effects of zoeal eyestalk ablation on morphogenesis of these body parts were investigated in *P. trituberculatus* (Chapter 3.3). Subsequently, I evaluated which factors induce abnormal morphology of MG by culturing *P. trituberculatus* larvae under different supply conditions of rotifers, *Artemia*, and phytoplankton (Chapter 3.4). In this section, the period (timing) at which a causal factor induced abnormality was also identified, and its effect was compared with the effect of eyestalk ablation. Based on these results, a larval culture method to prevent morphological abnormality of MG was developed.

After developing methods to prevent morphological abnormalities in *P. trituberculatus*, another cause of mass mortality was identified. This mass mortality pattern occurred after metamorphosis into morphologically normal MG, and was frequently observed when phytoplankton were not supplemented into the larval culture water during the late zoeal period. In Chapter 4, the cause of this type of mass mortality was investigated and was hypothesized to be related to nutritional deficiency of zoeae. First, I examined whether starvation of the live foods in the larval culture water (rotifers, *Artemia*, or their combination with supplemented phytoplankton) affected larval survival. I found that *Artemia* starvation induced larval mass mortality. A method of preventing *Artemia* starvation was developed and its effect on larval survival was evaluated.

In Chapter 5, I document a culture method for larval *P. trituberculatus* that mitigates against all causes of mortality identified in this study. This involved prevention of abnormal morphology of MG and *Artemia* starvation. Then, the method of feeding with *Artemia* was improved to prevent the occurrence of abnormal morphology in last stage zoeae. Based on these results, I propose a new method for culturing *P. trituberculatus* larvae that prevents multiple causes of larval mass mortality.

In Chapter 6, I summarise the causes of larval mass mortality during seed production of portunid crabs and their methods of control. Additionally, I discuss the mechanisms controlling larval metamorphosis and the nutritional requirements for this process. Last, I discuss the direction for application of this knowledge and technology in terms of improvement in seed production technologies, in understanding the mechanisms regulating larval metamorphosis, and in larval ecology.

Chapter 2

CONTROL OF BACTERIAL DISEASE

2.1 Evaluation of the effects of probiotics in controlling bacterial necrosis symptoms in larvae of the mud crab *Scylla serrata* during mass seed production

2.1.1 Summary

We aimed to evaluate the practicability of probiotics in controlling larval necrosis symptoms and mass mortality occurred during seed production of the mud crab *Scylla serrata*. We targeted a bacterium in the family Flavobacteriaceae that causes necrosis symptoms of mud crab larvae. Five candidate probiotic bacterial strains, which showed inhibitory effects against the causative bacterium but were innocuous to larvae, were screened from the seed production environment. Sterile culture medium supernatants of the candidate strains inhibited the causative bacterium through the secretion of extracellular factors. These inhibitory effects were largely reduced when the strains were inoculated into seawater, suggesting medium-dependent production of inhibitory factors. The strain that exhibited the highest inhibitory effect on larval necrosis in 7-day larval rearing experiments was selected as a probiotic strain. In large-scale larval rearing experiments, the probiotic treatment significantly suppressed larval necrosis symptoms and improved larval survival in the early rearing period until the fourth zoeal stage. However, the effect disappeared after the fifth (last) zoeal stage, corresponding to a decrease in inoculated probiotic bacterial counts in the larval rearing water. Almost all larvae had died by the first crab stage. The effectiveness of probiotics developed in vitro has not yet proved practical in commercial-scale seed production. The continuous proliferation of probiotic bacteria and extra-cellular production as inhibitory factors in larval rearing water are the key factors in developing probiotics for commercial-scale seed production.

2.1.2 Introduction

At present, the use of antibiotics in aquaculture is restricted in many countries because of concerns over the potential appearance of antibiotic-resistant bacteria, which can have a negative impact not only for the cultured animals but also for humans (Alderman and Hastings, 1998; Huys et al., 2007; Schmidt et al., 2000). As an alternative disease prevention method, biological control (biocontrol), defined as the utilisation of natural enemies to reduce the damage caused by noxious organisms to tolerable levels (DeBach and Rosen, 1991), has been proposed (Maeda et al., 1997; Nogami and Maeda, 1992; Nogami et al., 1997). The concept of biocontrol in aquaculture has

included probiotic approaches, defined as the use of live microorganisms that are beneficial to the health of the host (Balcázar et al., 2006; Fuller, 1989; Gatesoupe, 1999; Irianto and Austin, 2002; Kesarcodi-Watson et al., 2008; Verschuere et al., 2000a).

There have been many studies on the use of probiotics during the larval stages in the rearing of aquatic animals (D'Alvise et al., 2012; Gomez-Gil et al., 2000; Huys et al., 2001; Kesarcodi-Watson et al., 2012; Luis-Villasenõr et al., 2011; Villamil et al., 2010). These studies have succeeded in isolating probiotic bacterial strains that exhibit antagonistic effects towards pathogenic bacteria in vitro. Such probiotic treatments have improved larval survival in small-scale rearing experiments. In addition, there have been several review papers proposing approaches of probiotic procedures in aquaculture involving larval culture (Gomez-Gil et al., 2000; Kesarcodi-Watson et al., 2008; Verschuere et al., 2000a), e.g., Verschuere et al. (2000a) proposed a probiotic procedure consisting of several steps: screening and preselection of pro-biotics involving in vitro axenic and monoxenic tests with a pathogenic bacterium, evaluation of pathogenicity of selected probiotic strains, and final evaluation in mass production. However, very few studies have evaluated the effectiveness of probiotic treatments in improving larval survival rates in commercial-scale mass seed production.

The mud crab species such as *Scylla serrata*, *Scylla paramamosain*, *Scylla olivacea*, and *Scylla tranquebarica* are widely distributed throughout the tropical and temperate zones of the Pacific and Indian Oceans (Keenan, 1999a). Because mud crabs are important local fishery resources with high commercial value and high growth rate, hatchery technology has been studied intensively for the purposes of aquaculture and stock enhancement in many countries (Baylon, 2009; Dan and Hamasaki, 2011; Hamasaki, 2003; Hamasaki et al., 2002a, 2002b, 2011; Keenan, 1999b; Lebata et al., 2009; LeVay et al., 2008; Nghia et al., 2007; Suprayudi et al., 2002a, 2002b, 2004; Wang et al., 2005). However, mass seed production techniques have not been developed fully because larval mortality rates remain very high.

In Japan, seed production trials of *S. Serrata* have been conducted since the 1980s for stock enhancement programmes. However, mass mortality occurred in almost all the trials during the zoeal stages. Prior to this, the larvae showed symptoms of tissue necrosis in the dorsal and ventral spines and some appendages. A bath treatment with sodium nifurstyrenate (NFS-Na) was effective in preventing such mass mortality (Hamasaki et al., 2011). The mean survival rate from 28 trials conducted from 1996 to 2003 using 100 kL mass culture tanks without NFS-Na treatment showed only 3.3 % of larvae reaching the last (fifth) zoeal stage (Z5). The larvae survived to the

first crab stage (C1) in only four of the 28 trials (mean survival rate, 0.3 %). By contrast, the mean survival rate from 50 trials using the NFS-Na bath treatment was 38.3 % at Z5, and 38 trials could harvest C1 juveniles (mean survival rate, 10.3 %). Brick (1974) and Nghia et al. (2007) have also demonstrated that the administration of antibiotics such as penicillin-G, polymyxin-B and oxytetracycline significantly improved larval survival during the zoeal stages of *S. serrata* and *S. paramamosain*. Thus, developing alternative measures to decrease larval mortality caused by pathogenic bacteria without using antibiotics is crucial in establishing seed production technology for mud crab species.

The present study aims to develop a control measure of larval mass mortality of *S. serrata* due to bacterial infection without using antibiotics. We performed the probiotic procedures proposed by review papers so far (e.g. Gomez-Gil et al., 2000; Verschuere et al., 2000a) in commercial-scale seed production.

2.1.3 Materials and Methods

The present study was conducted from 2000 to 2004 at the Yaeyama Laboratory of the Seikai National Fisheries Research Institute, Fisheries Research Agency, Ishigaki, Okinawa Prefecture, Japan. First, we isolated a bacterium that causes necrosis and mass mortality of *S. serrata* larvae. Candidate probiotic strains that inhibited the growth of a bacterium but were not harmful to larvae were screened from the seed production environment. The biocontrol abilities of selected probiotic strains for preventing larval necrosis and improving survival were subsequently examined using larval rearing experiments in 500 L pilot-scale tanks. Finally, the effectiveness of the probiotics in mass seed production was evaluated using 100 kL tanks, as outlined below.

2.1.3.1. Isolation of causative bacteria

To isolate bacteria causing larval necrosis, 6–11 zoeae showing symptoms of tissue necrosis in their dorsal and ventral spines or appendages were sampled from four seed production tanks at the Yaeyama Laboratory (Fig. S2.1.1 in the Appendix). To remove bacteria from the surface of the larvae, individuals were blotted with filter papers, dipped into 0.1 % (v/v) benzalkonium chloride solution for 30 s, and then washed in fresh water, chlorinated at 0.1 mg L^{-1}, for 30 s. Each zoea was then homogenised in sterile seawater (34 ppt salinity) using a glass homogeniser. The homogenised samples were serially diluted and plated on marine agar 2216 (MA; Difco Laboratories, Detroit, MI, USA). After 24 to 48 h incubation at 26–28 °C, characteristic bacteria

appeared, showing yellow, smooth-margined, and irregular-shaped colonies (Fig. S2.1.1 in the Appendix). We found this type of colony only from the larvae showing tissue necrosis, but it has not been found from other samples such as seawater, larval rearing water, and symptomless larvae. These colonies were present at levels of 4.74–5.32 log colony-forming units (cfu) larva^{-1}, comprising 34.1–69.5 % of the bacterial community on MA plates (Table S2.1.1 in the Appendix). Pure cultures of this dominant type of bacterial colony were obtained by re-plating on fresh medium. After homogenising the isolated colonies in sterile seawater, the strain was stored at –80 °C in marine broth 2216 (MB; Difco Laboratories) containing 10 % glycerine for further use. This bacterial strain is hereafter referred to as the NY strain. It was classified into the family Flavobacteriaceae (Bernardet et al., 2002) according to the method of Cowan and Steel (Barrow and Feltham, 1993) on the basis of cell morphology, motility, Gram stain reaction, and catalase and oxidase reactions. In addition, the API 20NE rapid identification system (bioMérieux, Marcy L'Etoile, France) supported this classification (Table S2.1.2 in the Appendix).

2.1.3.2 Effects of causative bacteria on larval mortality and necrosis

To evaluate the effects of the NY strain on mortality and necrosis symptoms of mud crab larvae, challenge tests were carried out using individuals from the first, third, and fifth zoeal stages (Z1, Z3, and Z5), the megalopa stage (MG), and the C1 stage at 29 ± 1 °C. The animals were housed in six-well culture plates containing 10 mL of sterile seawater (34 ppt salinity) in each well. The animals were challenged with the NY strain from a MA plate at different doses taking the concentration-dependent effects on larval mortality and necrosis symptoms into consideration. Two or three dose levels were adopted at the designated stage according to the available number of animals (Z1: 4.45, 5.45, and 6.45 log cfu mL^{-1}; Z3: 5.20 and 6.20 log cfu mL^{-1}; Z5: 4.72, 5.72, and 6.72 log cfu mL^{-1}; MG: 5.11 and 6.11 log cfu mL^{-1}; C1: 5.40, 6.40, and 7.40 log cfu mL^{-1}). The control group was not inoculated with the NY strain. The number of animals tested for each challenge dose including the control group was 60, 30, 20, 15, and 20 individuals for the Z1, Z3, Z5, MG, and C1 stages, respectively. After a 1 h incubation, the animals were washed with sterile seawater, then transferred into 500 mL glass beakers, and reared for 46 h at 29 ± 1 °C. Rotifers *Brachionus plicatilis* sp. complex were fed to Z1, Z3, and Z5 larvae at a density of 20 individuals mL^{-1}. Newly hatched *Artemia* nauplii (Utah Strain, INVE, Phichit, Thailand) were also fed to Z3, Z5, MG, and C1 stage individuals at a density of 1.0 individuals mL^{-1}. The death of the animals was confirmed by their lack of reaction to physical stimulation with a water current through a pipetting, and the surviving individuals were counted at 22 and 46 h post-transferral to 500 mL

beakers. The survived larvae were collected and observed under a stereomicroscope when the incubation periods were terminated at 46 h, and the percentages of individuals showing tissue necrosis were calculated.

2.1.3.3 Screening of probiotic candidates

Thirty-five bacterial strains were isolated from rearing water of *S. serrata* larvae (three strains), rotifer culture water (eight strains), and coastal seawater (24 strains) at the Yaeyama Laboratory to screen for probiotic capabilities according to the method of Nogami and Maeda (1992). To enable visual discrimination between candidate strain colonies and those of other bacteria on MA, only strains with characteristic colony morphologies such as unique or unusual colour or shape were selected as candidates. Two rectangular smears of each isolated bacterium to be tested (4 cm long with a 3.5 cm gap between the smears) were made on MA, and a 2 cm-long rectangular smear of the NY strain was made between the two larger smears. The MA plates were incubated at 29 ± 1 °C for 6 days. To determine the inhibitory effect of isolated bacteria on the NY strain, the widths of the NY smears were compared visually against a control MA plate on which three smears of the NY strain were made. Among the 35 tested bacterial strains, six strains capable of inhibiting the growth of NY were selected as probiotic candidates. They were classified into the following families: Aeromonadaceae (strain 18), Alteromonadaceae (strains 7, 12, 25, and 26), and Flavobacteriaceae (strain 16), according to the method of Cowan and Steel (Barrow and Feltham, 1993) on the basis of cell morphology, motility, Gram stain reaction, and catalase and oxidase reactions (Bernardet et al., 2002; Krieg and Holt, 1984) (Table S2.1.2 in the Appendix).

The virulence of the six candidate probiotic strains against *S. serrata* larvae was investigated prior to evaluating their probiotic effects, using newly hatched Z1 individuals. Larvae were placed in six-well culture plates with 10 mL of sterile seawater (34 ppt salinity) in each well (20 individuals well^{-1}), which was inoculated with each candidate strain from MA plates at 5.11–5.85 log cfu mL^{-1}. The larvae in the control group were not inoculated with any bacteria. After 24 h at 29 ± 1 °C, surviving larvae were counted and the survival rate was determined.

2.1.3.4 In vitro evaluation of probiotic effects

Verschuere et al. (2000a) proposed that a common way to screen the candidate probiotics is to perform in vitro antagonism tests, in which pathogens are exposed to the candidate probiotics or their extracellular products. And then, candidate probiotics can be selected based on the production of inhibitory compounds or siderophores, or on the competition for nutrients. According to this, we conducted the following two examinations: a co-incubation experiment using NY strain and probiotic candidates, and evaluation of the effect of probiotic extracellular products on the growth of NY strain.

The NY strain was incubated with each candidate strain in test tubes containing 1 mL of tenfold diluted MB (inoculation dose; NY strain, 4.45 log cfu mL^{-1}; probiotic candidate strains, 1.60–3.48 log cfu mL^{-1}) or sterile seawater (34 ppt salinity) (inoculation dose; NY strain, 5.58 log cfu mL^{-1}; probiotic candidate strains, 5.00–5.38 log cfu mL^{-1}). Following 24 h at 29 ± 1 °C, the cultures were serially diluted and plated on MA, and colonies corresponding to the NY and candidate strains were counted. The specific growth rate of the NY strain was calculated as the natural logarithm of the proportion of the NY strain counts at the end of test period compared with the initial inoculated dose. When the NY strain count was <1.0 log cfu mL^{-1}, we scored the number of the NY strain as zero.

The effects of extracellular secretions from each candidate strain on growth of the NY strain were examined across three experiments. Bacterial strains 7, 12, 16, and 18; strains 7 and 25; and strains 7, 12, and 25 were used in test numbers 1–3, respectively. Each candidate strain was inoculated into 50 mL of MB and incubated with shaking at room temperature for 2 days. The cultures were then centrifuged at 12,009 g for 10 min, and the resulting supernatants were filtered through membrane filters (0.2 μm pore size). The filtered medium of each strain was serially diluted (1/2 in tests 1 and 2, and in twofold increments from 1/2 to 1/64 in test 3) in MB medium in test tubes and then inoculated with the NY strain at 4.00, 4.00, and 5.20 log cfu mL^{-1} in tests 1–3, respectively. MB diluted with an equal volume of sterile seawater was used as the control in each test. After 24 h at room temperature, the culture media were serially diluted, plated on MA, and colonies of the NY strain were counted. The specific growth rate of the NY strain was then calculated.

2.1.3.5 Evaluation of probiotic effects in short-term larval rearing experiments

To examine the prevention abilities of in vitro-selected candidates (strains 7, 12, and 25) for larval necrosis, a short-term larval rearing experiment (7 days after hatching (dah)) was conducted in triplicate for three candidate strains. Newly hatched Z1 individuals were obtained according to the method described by Dan and Hamasaki (2011) (Chapter 3.1). The larvae were stocked in 500 L polyethylene tanks filled with seawater, with the salinity adjusted to 25 ppt using fresh water to reduce the chance of fungal infection (Hamasaki et al., 2011). The seawater was sterilised with chlorine (10 ppm) and neutralised with sodium thiosulfate prior to larval stocking. The water temperature was controlled at the optimum temperature (29 °C) for rearing *S. serrata* larvae (Hamasaki, 2003) using a heater connected to a thermostat. The average stocking number of newly hatched Z1 was 12,808 (8,300–20,600) individuals. The water was not renewed during the larval culture period. Gentle aeration was provided in each tank via an air stone. Rotifers enriched with commercially available concentrated *Chlorella vulgaris* containing n-3 highly unsaturated fatty acids (Super Chlorella V12; Chlorella Industry, Tokyo, Japan) were supplied as food for larvae at densities of 15–20 individuals mL^{-1} once at the beginning of rearing. To maintain the rotifer density in the larval rearing tanks, commercially available concentrated phytoplankton *Nannochloropsis* sp. (Mercian, Tokyo, Japan) was added to the tanks twice a day at a density of 250,000 cells mL^{-1}. Each candidate strain was inoculated into 50 mL of MB and incubated with shaking at room temperature for 2 days. Because it has been suggested that pre-emptive colonisation of probiotic bacteria might extend their reign as pioneer organisms in rearing systems (Atlas and Bartha, 1997; Verschuere et al., 2000a), the incubated candidate bacteria were initially introduced into sterile rearing water together with their culture medium (50 mL of MB) on the first day of larval rearing. From the second day, candidate probiotic bacteria with their culture medium were inoculated every morning (07:00–08:00) prior to the addition of the phytoplankton. In control tanks, larvae were reared without the addition of any bacteria. The viable bacterial counts on MA were enumerated daily as a total bacterial count in the rearing water based on the water samples collected in sterile test tubes at early morning (05:00–07:00), prior to the addition of any bacteria. The numbers of colonies with similar morphologies to the candidate probiotic bacteria were also counted daily on MA and recorded as the approximate numbers of candidate strains in the rearing water 24 h post-inoculation. This counting method could not estimate the accurate number of candidates, but could detect the approximate numbers of candidates quickly and easily; we could successfully detect the candidate bacteria from larval rearing water coincided

with inoculation level early in the larval rearing (see 'Results' section). The numbers of surviving larvae were estimated by the volumetric method based on three 1 L samples taken from the tanks after the water had been agitated to ensure a homogenous suspension of larvae at 3 dah and 7 dah. The survival rate was calculated as the percentage of larvae surviving based on the number of stocking larvae in each tank. The numbers of larvae showing symptoms of tissue necrosis were also estimated daily based on microscopic examination of approximately 30 larvae from each tank, and the infection rate was calculated as the percentage of infected larvae among the sampled larvae.

2.1.3.6 Evaluation of probiotic effects in pilot-scale larval rearing experiments

To evaluate the probiotic effects of the selected strain 7, larval rearing experiments were conducted using individuals from stages Z1 to C1 using 500 L pilot-scale rearing tanks. Treatments with probiotics, without probiotics (control), and following bath treatment with NFS-Na (Ueno Fine Chemicals Industry, Osaka, Japan) at 2 mg L^{-1} at the beginning of the larval rearing period were compared in duplicate across two separate trials (trials 1 and 2). The average stocking numbers of newly hatched Z1 larvae were 12,111 (10,000–13,333) and 6,639 (6,167–8,333) individuals for trials 1 and 2, respectively. Newly hatched *Artemia* nauplii were supplied to the tanks every morning at densities of 1, 2, 3, and 4 individuals mL^{-1} for the Z3, fourth stage zoea (Z4), Z5, and MG stages, respectively. The survival rate of larvae was estimated volumetrically for each larval stage from the second stage zoea (Z2) to MG, and all surviving individuals were counted for C1. The infection rate of larvae with necrosis was estimated daily. Bacterial counts were conducted every 3 days.

2.1.3.7 Evaluation of probiotic effects in commercial-scale seed production experiments

Seed production experiments were conducted in duplicate (trials 1 and 2) using two 100 kL concrete tanks with or without probiotics (strain 7) in each trial. The numbers of newly hatched Z1 stocked in each tank were 1.87×10^6 and 1.23×10^6 individuals in trials 1 and 2, respectively. Probiotic bacteria were inoculated into 50 mL of MB and incubated with shaking at room temperature for 24 h. Then, they were inoculated into 10 L of MB and incubated with oxygenation using an oxygen generator (Ogenator 600; Kinki Sanso, Nishinomiya, Japan) for 2 days. The incubated probiotic bacteria were inoculated into the larval rearing tanks together with their culture medium (10 L of MB) once per day in the morning (07:00–08:00) in trial 1, and twice per day, in the morning (07:00–08:00) and evening (16:00–17:00), in trial 2. The numbers of

surviving larvae were estimated until the MG stage according to the method described by Kogane et al. (2007), and the numbers of C1 individuals were counted as described by Kiyota (1997).

2.1.3.8 Statistical analysis

Statistical analyses were performed using R language (R 3.0.2; R Development Core Term, 2013) with a 5 % significance level. To examine the effects of the addition of the NY strain on the larval mortality, we used a generalised linear model (GLM) (McCullagh and Nelder, 1989; Everitt, 2005; Everitt and Hothorn, 2009), using challenge level of NY strain (0, 4, 5, 6, 7 log cfu mL^{-1}) and larval stage (Z1, Z3, Z5, MG, C1) as categorical explanatory variables. The numbers of dead and live animals during test periods were used as the response variable. Taking the overdispersion of error distribution into consideration, quasi-binomial family (logit link) was adopted in the analysis. The statistical significance of explanatory variables was evaluated with the *F* test using the *Anova* function implemented in the car package (Fox and Weisberg, 2011). The GLM with Gaussian family was also adopted to assess the effect of probiotic addition on growth inhibition of the NY strain in test tubes, using treatments (control or probiotic) and culture medium (diluted MB or sterilised seawater) as categorical explanatory variables. In this analysis, the specific growth rate of the NY strain was used as the response variable.

To evaluate the effect of extracellular secretions (sterile-filtered supernatants of probiotic culture media) on growth of the NY strain, specific growth rates of the NY strain were compared between treatments (twofold dilution of MB with probiotic supernatants or with seawater) using data from tests 1–3 with a Welch's *t* test, without assuming homogeneity in variance between the compared groups. Additionally, Pearson's product-moment correlation coefficient, *r*, was calculated to analyse the relationship between the values of dilution level (1/2, 1/4, 1/8, 1/16, 1/32, 1/64) of probiotic supernatants and specific growth rate of the NY strain in test 3.

In the larval rearing experiments (short-term, pilot-scale, and commercial-scale experiments), the effects of probiotic and antibiotic treatment on larval survival and infection rates were evaluated using a generalised linear mixed-effects model (GLMM) (Everitt, 2005; Everitt and Hothorn, 2009; Zuur et al., 2009). In pilot-scale experiments, statistical analyses were performed for each duplicate rearing trial. In commercial-scale experiments, the data from two trials were treated as replicated treatment. In the analyses of survival rate, the numbers of live and dead animals on 3 dah (Z2) and 7 dah (Z3–Z4) (short-term experiment) or at each larval stage (pilot-scale and commercial-scale experiments) were used as the response variables with the binomial

family (logit link). In the analyses of infection rate, the numbers of infected and non-infected larvae counted daily were used as the response variables with the binomial family (logit link). In these analyses, explanatory variables were larval ages and the categorical fixed factors such as probiotic (strains 7, 12, 25) and control treatments in the short-term experiment, and probiotic (strain 7), control and/or antibiotic treatments in the pilot-scale and commercial-scale experiments. Data on survival and infection rates were collected in the same tank longitudinally. Therefore, taking into account the potential spatial and temporal correlations of the observations, and un-controlled biotic and abiotic factors in different rearing tanks, larval ages and the identity of rearing tank were included in the GLMM as random slope and intercept effects, respectively (Everitt, 2005; Everitt and Hothorn, 2009; Zuur et al., 2009). Moreover, in commercial-scale experiments, two trials were conducted separately. Therefore, the identity of trail was also included in the GLMM as a random intercept effect. The statistical significance of the explanatory variables was evaluated with the Wald χ^2 test (type II) using the *Anova* function implemented in the car package and the *glmer* function in lme4 package (Bates et al., 2012).

2.1.4 Results

2.1.4.1 Effects of the NY strain on larval mortality and necrosis

There was no significant difference in mortality rates among larval stages at 22 h (F = 1.807, df = 4, 9, P = 0.2117) and 46 h (F = 0.9394, df = 4, 9, P = 0.4842), whereas the challenge level of the NY strain significantly affected larval mortality at 22 h (F = 7.137, df = 4, 9, P = 0.0071) and 46 h (F = 4.724, df = 4, 9, P = 0.0249). Mortality rates were lowest at all stages for the control, and none of these individuals showed necrosis (Table 2.1.1). On the other hand, the cumulative mortality and the percentage of individuals showing necrosis increased significantly with increasing challenge doses, suggesting that the NY strain is the causative bacterium of the larval necrosis symptoms occurring during seed production of *S. serrata*.

Table 2.1.1. Cumulative mortality rates of *Scylla serrata* larvae and juveniles challenged with a bacterium (NY strain) isolated from larvae showing necrosis in seed production tanks

Stage	Challenge dose (log cfu mL^{-1})	Cumulative mortality (%)[a]	
		22 h	46 h
Z1	Control	0	0 (0)
	4.45	11.7	51.7 (63.3)
	5.45	38.3	71.7 (76.7)
	6.45	70.0	100
Z3	Control	3.3	10.0 (0)
	5.20	23.3	66.7 (76.7)
	6.20	76.7	93.3 (100)
Z5	Control	0	0 (0)
	4.72	10.0	25.0 (45.0)
	5.72	15.0	70.0 (100)
	6.72	30.0	100
MG	Control	6.7	20.0 (0)
	5.11	6.7	60.0 (60.0)
	6.11	26.7	80.0 (100)
C1	Control	10.0	15.0 (0)
	5.40	0	5.0 (5.0)
	6.40	25.0	40.0 (40.0)
	7.40	15.0	55.0 (55.0)

Z1–Z5, first to fifth stage zoeae; MG, megalopa; C1, first stage crab
cfu, colony-forming units
[a] The percentage of larvae showing necrosis among all surviving larvae in parenthesis

2.1.4.2 Probiotic potential of candidate strains

The Z1 larvae inoculated with strains 7, 12, 16, 18, and 25 showed low mortality rates (5–10 %), similar to the controls (0 %), whereas strain 26 induced 100 % mortality of inoculated larvae (Table S2.1.3 in the Appendix). This strain was excluded from the candidates. The five probiotic candidates (strains 7, 12, 16, 18, and 25) inoculated into tenfold diluted MB decreased the concentration of the NY strain from 4.45 to < 1 log cfu mL^{-1} within 24 h, while their own concentrations increased from 1.60–3.48 to 7.42–8.51 log cfu mL^{-1} after 24 h (Table 2.1.2). In the control tube without probiotic candidates, the NY strain increased to 8.72 log cfu mL^{-1}. In sterile seawater, the NY strain increased from 5.88 to 6.26 log cfu mL^{-1} in the control tube, and

Table 2.1.2. Changes in concentration (log cfu mL^{-1}) of candidate probiotic and NY strain following incubation for 24 h in 10-fold diluted marine broth or sterile seawater

Probiotic strain	Diluted marine broth			Sterile seawater		
	Probiotic inoculation	24 h		Probiotic inoculation	24 h	
		Probiotic	Pathogen		Probiotic	Pathogen
Control[1]	–	–	8.72	–	–	6.26
7	1.60	7.42	<1.00	5.24	6.01	6.11
12	2.89	7.90	<1.00	5.11	6.34	6.15
16	3.15	8.34	<1.00	5.00	5.20	5.73
18	3.48	8.28	<1.00	5.38	6.30	5.15
25	3.36	8.51	<1.00	5.38	6.40	5.86

[1]Inoculated only with NY strain
Values are expressed as log cfu mL^{-1}
<1.00 = not detected
NY strain was inoculated into diluted marine broth and sterile seawater at densities of 4.45 and 5.88 log cfu mL^{-1}, respectively

the probiotic candidates slightly inhibited the growth of the NY strain to 5.15–6.15 log cfu mL^{-1}. The candidate strains increased from 5.00–5.93 to 5.20–6.53 log cfu mL^{-1}. A statistically significant effect on the specific growth rate of the NY strain was detected between control and probiotic treatments (F = 10.868, df = 1, 9, P = 0.0093), and between diluted MB and sterile seawater (F = 8.320, df = 1, 9, P = 0.0180). Thus, probiotic strains inhibited the growth of the NY strain, but the effects were significantly lower in seawater than in diluted MB.

Compared with the control, the addition of twofold diluted sterile-filtered culture supernatants of probiotic strains significantly inhibited the growth of the NY strain in tests 1–3 (t = –17.245, df = 16, P < 0.0001) (Table 2.1.3). In test 3, the specific growth rate of the NY strain significantly decreased with increasing concentration of culture supernatants (r = –0.9741, P < 0.0001). Thus, the extracellular factors secreted by candidate strains into culture media were suggested as an inhibitory action to the growth of the NY strain, and strains 7, 12, and 25, which reduced the concentration of the NY strain to below detectable levels, were selected as probiotic candidates for subsequent short-term larval rearing experiments.

2.1.4.3 Probiotic effects in short-term larval rearing experiments

The bacterial counts of candidate strains 7, 12, and 25 in MB prior to being inoculated into the larval rearing water were 7.00–9.32 (mean, 8.22), 8.11–9.61 (9.14), and 8.18–9.68 (9.21) log cfu mL^{-1}, respectively. Colonies that were morphologically similar to those of candidate strains were only detected in the tanks inoculated with the designated strains, and the levels of strains

Table 2.1.3. Changes in concentration (log cfu mL^{-1}) of NY strain in diluted sterile-filtered supernatants of culture media from candidate probiotic strains

Probiotic strain	Test 1		Test 2		Test 3						
	Concentration[1]	Dilution	Concentration[1]	Dilution	Concentration[1]	Dilution					
		1/2		1/2		1/2	1/4	1/8	1/16	1/32	1/64
Control[2]	–	8.43	–	8.54	–	8.79					
7	8.57	<1.00	8.11	<1.00	8.88	1.18	4.23	7.20	8.56	8.46	8.49
12	9.43	<1.00	–		9.70	<1.00	3.54	5.30	7.58	8.15	8.68
16	9.45	2.62	–		–						
18	9.62	2.77	–		–						
25	–		9.38	<1.00	9.46	<1.00	3.08	5.28	7.91	8.23	8.41

[1]Concentration of each candidate probiotic strain prior to being centrifuged and filtered to eliminate the bacterial cells
[2]Marine broth was diluted with sterile seawater
Values are expressed as log cfu mL^{-1}
<1.00 = not detected
NY strain was inoculated into each medium at concentrations of 4.00, 4.00 and 5.20 log cfu mL^{-1} in tests 1, 2 and 3, respectively

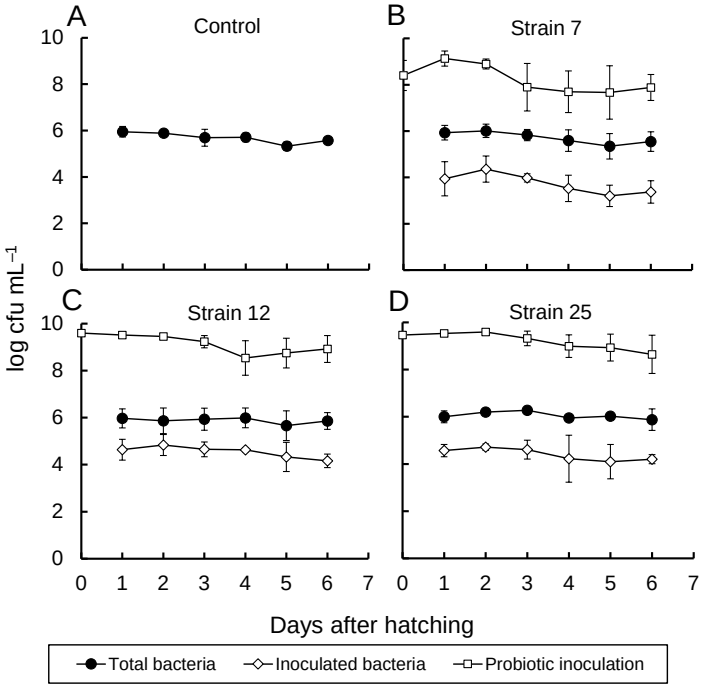

Figure 2.1.1. Short-term (7 day) rearing experiments in the larval rearing water of *Scylla serrata*. Graphs show the colony counts of total bacteria and inoculated bacteria, approximated by the colonies that were morphologically similar to the designated strains, in rearing water treated with control (A) or probiotic strains 7 (B), 12 (C), or 25 (D). The concentration of candidate probiotic bacteria incubated in 50 mL of marine broth (probiotic inoculation) is also shown. Values are the mean ± sd (error bars) of duplicate experiments in trial 2

7, 12, and 25 in the larval rearing water 24 h post-inoculation were 2.70–5.00 (3.73), 3.65–5.34 (4.54), and 3.08–4.92 (4.40) log cfu mL^{-1}, respectively (Fig. 2.1.1). The total bacterial counts in the larval rearing water fluctuated around an average of 5.8 log cfu mL^{-1} throughout the experiments in all tanks. Bacterial colonies similar to the NY strain were isolated at 3.96–5.94 (5.10) log cfu individual^{-1} from 12 zoeae showing necrosis.

The larvae grew to the Z3–Z4 stages by the end of the experiment (7 dah). The survival rate decreased and the necrosis rate increased significantly with larval age (survival rate, $\chi^2 = 47.901$, $df = 1$, $P < 0.0001$; infection rate, $\chi^2 = 23.150$, $df = 1$, $P < 0.0001$) (Fig. 2.1.2). Treatments did not significantly affect the survival rate ($\chi^2 = 4.417$, $df = 3$, $P = 0.2198$). However, a significant effect of treatment on infection rate was detected ($\chi^2 = 54.041$, $df = 3$, $P < 0.0001$), and the lowest infection rate at 7 dah was observed following treatment with strain 7 (5.9 %). Therefore, we selected bacterial strain 7 (Alteromonadaceae) as the probiotic strain for the following experiments.

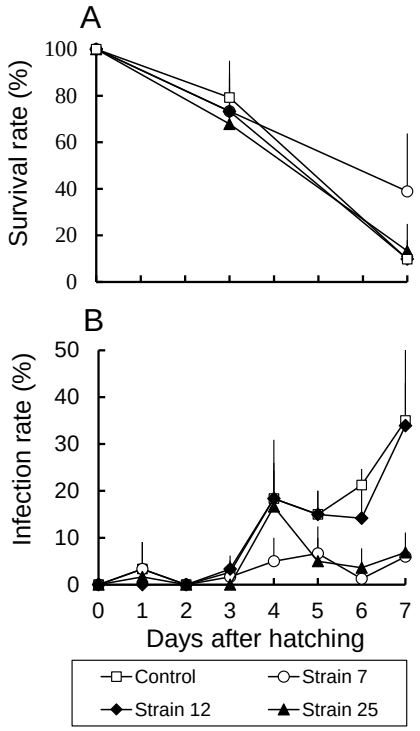

Figure 2.1.2. Survival rate of *Scylla serrata* larvae (A) and infection rate of necrosis symptoms (B) following treatment with control or probiotic strains 7, 12, or 25 in a short-term (7 day) rearing experiment. Values are the mean ± sd (error bars) of triplicate experiments

2.1.4.4 Probiotic effects in pilot-scale larval rearing experiments

Probiotic bacterial strain 7 was added to the larval rearing water in trials 1 and 2 at levels between 6.88–8.98 (8.12) and 7.54–9.15 (8.34) log cfu mL^{-1}, respectively (Fig. 2.1.3). Regardless of daily inoculation, the approximate levels of probiotic strain in the rearing water after 24 h gradually decreased from 4.44 log cfu mL^{-1} at the beginning of rearing to 1.54 log cfu mL^{-1} at 15 dah in trial 1, and from 4.10 to 1.24 log cfu mL^{-1} in trial 2. No bacterial colonies similar to strain 7 were detected from control and NFS-Na bath treatment tanks in either trial. The total bacterial counts in the rearing water fluctuated around an average of 6 log cfu mL^{-1} throughout the experiment in all tanks.

The survival rate decreased with larval age in both trials (trial 1, χ^2 = 32.688, *df* = 1, *P* < 0.0001; trial 2, χ^2 = 26.169, *df* = 1, *P* < 0.0001), and the treatments significantly affected the overall survival rate in both trials (trial 1, χ^2 = 137.497, *df* = 2, *P* < 0.0001; trial 2, χ^2 = 19.877, *df* = 2, *P* < 0.0001) (Fig. 2.1.4A, C). The highest survival rate was recorded in the NFS-Na bath treatment tank throughout the rearing period. The survival rate for the probiotic treatment was between the NFS-Na bath treatment and the control values until the MG stage, but was higher than the control at the Z5 stage. The NFS-Na bath treatment showed 10.2 and 12.5 % survival rates at the C1 stage in trials 1 and 2, respectively, but the control and probiotic treatments

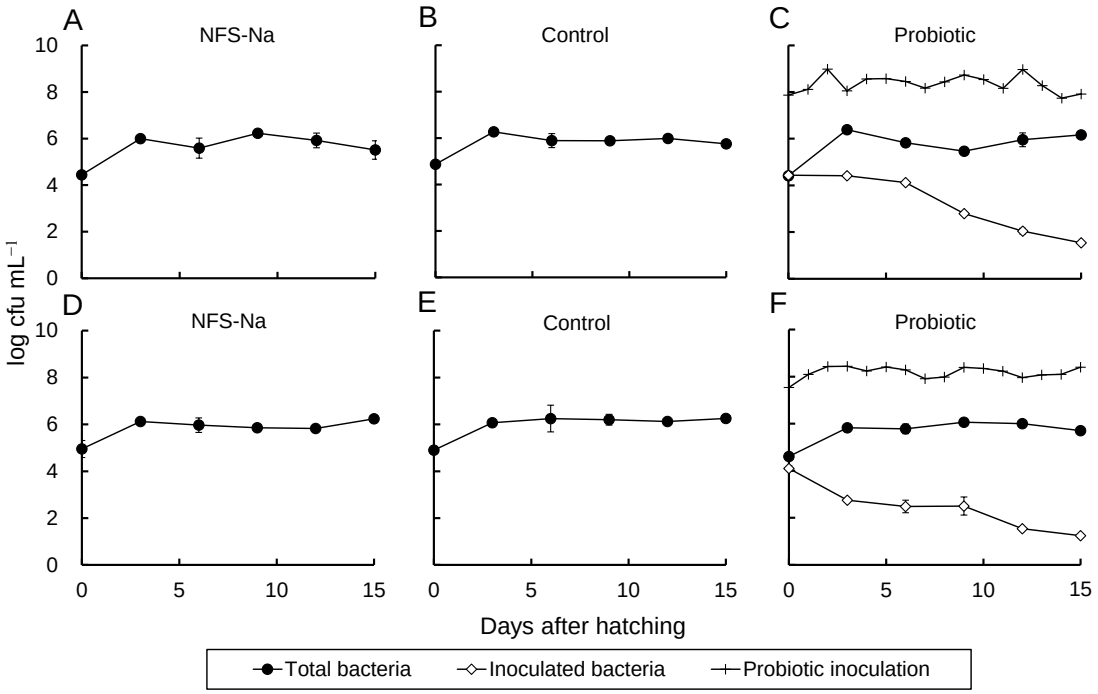

Figure 2.1.3. Concentration of probiotic candidate bacteria (strain 7) incubated in 50 mL of marine broth (probiotic inoculation), and colony counts of total bacteria and inoculated bacteria, approximated by the colonies that were morphologically similar to strain 7, in the larval rearing water of *Scylla serrata*. Groups were treated with NFS-Na, control or probiotic strain 7 in pilot-scale rearing experiments (A–C, trial 1; D–F, trial 2). Values are the mean ± sd (error bars) of duplicate experiments

resulted in <0.5 % survival in both trials. Larvae showing necrosis were not observed in the NFS-Na bath treatment group in either trial, so the NFS-Na bath treatment was not included in the following statistical analysis. The rate of infection with necrosis in the control and probiotic treatment groups significantly increased with larval age in both trials (trial 1, $\chi^2 = 5.220$, $df = 1$, $P = 0.0223$; trial 2, $\chi^2 = 63.013$, $df = 1$, $P < 0.0001$) (Fig. 2.1.4B, D). The infection rate was significantly reduced by probiotic treatment in trial 1 compared with control ($\chi^2 = 8.369$, $df = 2$, $P = 0.0038$). However, in trial 2, a statistically significant difference in the infection rate was not detected between probiotic and control treatments ($\chi^2 = 0.5015$, $df = 2$, $P = 0.4788$).

2.1.4.5 Probiotic effects in commercial-scale seed production experiments

Probiotic bacterial strain 7 was inoculated into the larval rearing water in trials 1 and 2 at concentrations of 7.78–8.81 (8.50) and 8.72–9.69 (9.44) log cfu mL^{-1}, respectively (Fig. 2.1.5A, C). The approximate concentration of strain 7 in the rearing water after 24 h dropped abruptly to an undetectable level at 3 dah in trial 1, and between 3 and 6 dah in trial 2. No bacterial colonies

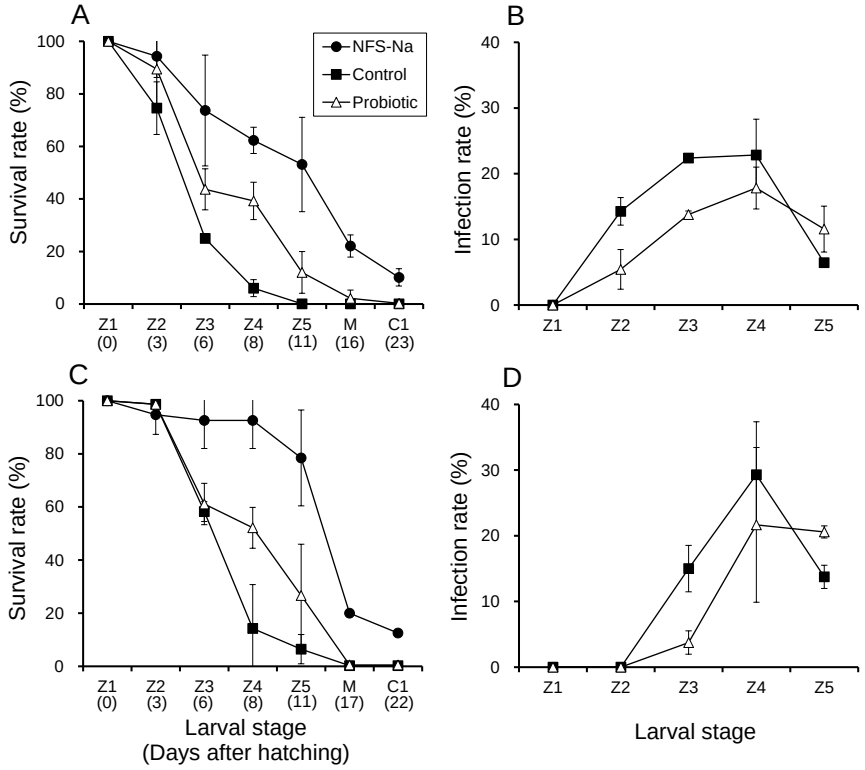

Figure 2.1.4. Survival rate of *Scylla serrata* larvae (A, trial 1; C, trial 2) and infection rate of necrosis symptoms (B, trial 1; D, trial 2) following bath treatment with NFS-Na, control or probiotic strain 7 in pilot-scale rearing experiments. The mean infection rates during each larval stage were calculated based on the daily observations for each tank, and the values of the mean ± sd (error bars) of duplicate tanks are shown for survival rates and infection rates. No larvae infected with bacterial necrosis disease were observed following the NFS-Na bath treatment

similar to strain 7 were detected from control tanks in either trial. The total bacterial counts in the rearing water fluctuated around an average of 6.0 log cfu mL^{-1} throughout the experiment in all tanks in both trials.

The probiotic treatment significantly reduced the overall infection rate (χ^2 = 65.20, *df* = 1, *P* < 0.0001), but the differences between control and probiotic treatments decreased or were eliminated with increasing infection rates from the Z5 to MG stages in both trials (Fig. 2.1.5B, D). The overall survival rate was also significantly improved by probiotic treatment (χ^2 = 18.990, *df* = 1, *P* < 0.0001). In trial 1, the survival rate decreased with the increasing infection rate and was 13.4 % at the Z5 stage in the control. On the other hand, with the probiotic treatment, the survival rate remained at 85.0 % to the Z4 stage before decreasing, but was still at 41.7 % at the Z5 stage. However, the larval survival in the probiotic treatment trial decreased continuously thereafter until the MG stage. Almost all the larvae died by the end of the experimental period,

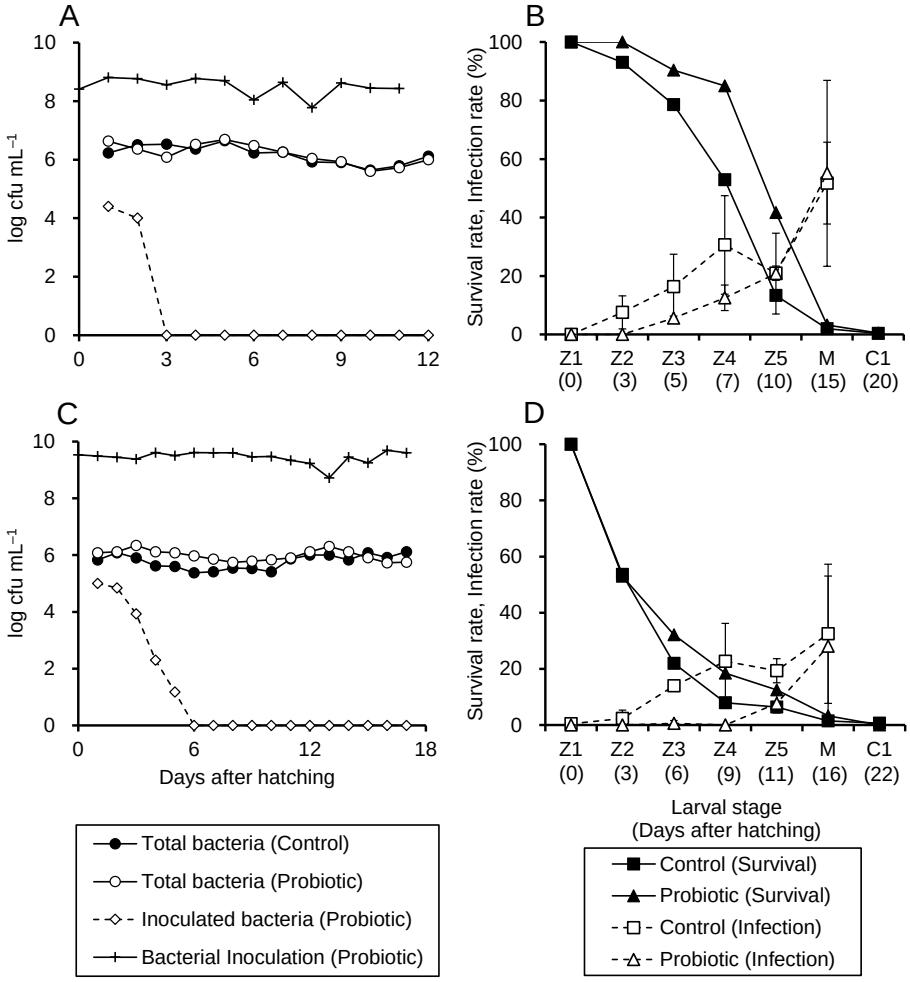

Figure 2.1.5. Concentration of probiotic bacteria incubated in 10 L of marine broth (bacterial inoculation), colony counts of total bacteria, and inoculated bacteria, estimated from the colonies that were morphologically similar to strain 7, in larval rearing water (A, trial 1; C, trial 2), and the survival and necrosis infection rates of *Scylla serrata* larvae (B, trial 1; D, trial 2) following treatment with control or probiotic in commercial-scale seed production experiments. The infection rates are shown as the mean ± sd (error bars) based on the daily observations for each larval stage

and the survival rates were only 0.7 and 1.0 % at the C1 stage for the control and probiotic treatment groups, respectively. In trial 2, the survival rates decreased until the Z2 stage in both treatments (53.7 and 53.0 % in the control and probiotic treatment groups, respectively), but then exhibited similar changes to those observed in trial 1. Thus, the probiotic treatment group showed a higher survival rate than the control until the Z5 stage. However, there was no difference in the final survival rates, being 0.7 and 0.1 % at the C1 stage in the control and probiotic treatment groups, respectively.

2.1.5 Discussion

The NY strain, suggested in the current study as a causative bacterium of tissue necrosis and high levels of mortality for *S. serrata* larvae, was classified as belonging to the family Flavobacteriaceae. Many bacterial species belonging to this family, including *Tenacibaculum maritimum*, *Flavobacterium aquatile*, *Flavobacterium branchiophilum*, *Flavobacterium columnare*, and *Flavobacterium psychrophilum* (formerly classified in the Cytophaga–Flavobacteria–Bacteroides phylum), are known pathogens of many aquatic animal species (Avendanõ-Herrera et al., 2006; Bernardet et al., 2002; Muroga, 2001; Starliper and Schill, 2011; Suzuki et al., 2001).

We successfully isolated five candidate probiotic strains that inhibited the growth of the NY strain from the ambient seed production environment. This is consistent with a report by Verschuere et al. (2000a), which noted that the ability to inhibit the growth of other bacteria is not uncommon for bacteria found in aquaculture environments. The sterile-filtered culture supernatants of the candidate strains inhibited the growth of the NY strain, likely via the production of extracellular substances such as antibiotics, bacteriocins, and siderophores (Pybus et al., 1994; Vandenbergh, 1993; Williams and Vickers, 1986). On the other hand, the inhibitory effects of these strains were largely reduced when inoculated into seawater compared with when they were inoculated into diluted MB. In general, the secretion of compounds by bacteria depends on the composition of the culture medium and the cell density (Mayr-Harting et al., 1972; Miller and Bassler, 2001; Olsson et al., 1992; Preetha et al., 2006). Because the initial cell population densities were higher in the seawater (5.00–5.38 log cfu mL^{-1}) than in the diluted MB (1.60–3.48 log cfu mL^{-1}), the composition of the medium (seawater or diluted MB) probably restricted the secretion of extracellular substances due to a lack of nutrients for these candidate strains.

In the larval rearing and seed production experiments, probiotic treatment successfully suppressed necrosis and improved the survival of larvae compared with the control group until the Z4 stage (≈10 dah). Thus, the probiotic treatment was effective during the early rearing period, even under commercial-scale seed production parameters. However, these probiotic effects disappeared after the Z5 stage. It is reasonable to infer that the reduction in probiotic effects in the later rearing period was related to a decrease in the number of probiotic bacteria in the larval rearing water. Although we did not directly monitor the probiotic bacteria in the larval rearing water using molecular or fluorescence labelling techniques, colonies that were morphologically similar to the probiotic strain were only detected in tanks inoculated with the designated strain, and were not detected from control tanks without probiotic inoculation even in the short-term

larval rearing experiment using three probiotic candidate strains. Therefore, it could be interpreted that our counts of colonies having similar morphology to probiotic strains represented the approximate density of probiotic bacteria in the larval rearing water. The maximum detected level at the beginning of larval rearing and subsequent decreases in probiotic bacterial counts in all rearing experiments (see Figs. 2.1.1, 2.1.3, 2.1.5) imply that the probiotic bacteria could not proliferate in the larval rearing water. Although the probiotic bacteria were added daily together with their culture medium into the larval rearing tanks, the medium was largely diluted to 10,000-fold by the rearing water, and the inability of probiotic bacteria to proliferate in the larval rearing water was therefore similar to in vitro experiments using seawater and diluted MB. Both inhibitory effects of the bacteria against the NY strain and growth of the probiotic candidate bacteria were low in water. Therefore, the inability of the probiotic strain to proliferate appears to be attributed to the lack of nutrients in larval rearing water as culture medium. In other words, to improve the probiotic effect, supplementations of nutrients which are essential for probiotic proliferation and extracellular production may be effective. The use of molecular identification of a candidate probiotic strain may help to identify which nutrients suit its requirements based on the properties of classified taxon of a probiotic strain.

In the present study, we demonstrated that probiotic strains that exhibited high probiotic potential in in vitro experiments could also inhibit larval necrosis symptoms in the seed production experiments. However, this approach is not yet practical for commercial-scale seed production because of low survival at the C1 stage. There are clear environmental differences between in vitro or short-term pilot-scale experiments and commercial-scale seed production. For instance, during seed production, larvae are cultured under monoxenic conditions with rotifer, *Artemia* and phytoplankton, which are supplied as feed according to complex schedules depending on larval growth (Baylon, 2009; Davis et al., 2005; Suprayudi et al., 2002b; Wang et al., 2005). The feeding amount fluctuates daily, because maintenance of optimal feeding density is important for larval survival and growth (Baylon, 2009; Ruscoe et al., 2004; Suprayudi et al., 2002b). These live foods are known to transfer various bacteria, including pathogenic strains, nutrients, and organic matter, such as excrement and debris, to the culture tanks (Nicolas et al., 1989; Olafsen, 2001; Verdonck et al., 1997; Yoshimura et al., 1997). Reflecting these unstable microbial and nutrient inflows, it has been reported that the microflora in larval rearing water undergoes continuous alterations in structural composition (Nakase et al., 2007; Olafsen, 2001; Tomoda et al., 2011). These complex and unstable abiotic and biotic conditions in seed production

differ critically from the well-controlled conditions of in vitro experiments, short-term pilot-scale rearing experiments, and rearing experiments for juvenile animals fed only artificial foods. Although the probiotic strains were screened from the seed production environment and first inoculated into sterile rearing water for pre-emptive colonisation in this study, decrease in probiotic bacterial counts in the larval rearing water indicates the difficulty for probiotic bacteria to expel other bacteria and occupy their niches. In addition, in the larval rearing of crustacean species, old larval exuviae are supplied intermittently into the rearing water. These might function as nutrients and adhesion sites for various bacteria that accumulate in large numbers in mass seed production tanks. Therefore, they might accelerate the growth of native bacteria, suppressing the proliferation of inoculated probiotic bacteria. Interestingly, the abrupt decrease in the numbers of inoculated probiotic bacteria 3 dah in commercial-scale tanks coincided with larval moulting; almost all larvae moulted to the Z2 stage at 3 dah in all larval rearing experiments.

In conclusion, there is a need to further improve the probiotics to control the larval mass mortality due to bacterial infection in commercial-scale seed production. The continuous proliferation of probiotic bacteria and production of inhibitory factors (extracellular substance) in the larval rearing water are considered to be key features to success in probiotics using commercial-scale tanks. The selection of probiotic candidates would be recommended from the point of view of their potency in proliferation and extracellular production in larval rearing water based on the understanding of their own biological properties. In addition, improving the knowledge of mechanisms for the organisation of the bacterial community in larval culture tanks is necessary to apply a lot of in vitro and small-scale probiotic studies accumulated so far to the commercial-scale mass seed production.

Chapter 3

CONTROL OF MORPHOLOGICAL ABNORMALITY

3.1 Effects of salinity and dietary n-3 highly unsaturated fatty acids on the survival, development, and morphogenesis of the larvae of laboratory-reared mud crab *Scylla serrata* (Decapoda, Portunidae)

3.1.1 Summary

We investigated the effects of feeding rotifers containing various levels of n-3 highly unsaturated fatty acids (n-3 HUFA) to *Scylla serrata* larvae at different developmental stages on their survival, development, and morphogenesis when they were cultured at six salinity levels. The first, third, and fifth (last) stage zoeae and megalopae were reared to first stage crabs at salinities of 10, 15, 20, 25, 30, and 35 ppt, with three different feeding regimes of rotifers containing different levels of n-3 HUFA. The larvae successfully developed to the subsequent stages at 20–35 ppt salinity. The highest survival rates to first stage crabs were recorded at 20–25 ppt salinity. The morphological features of the megalopa observed in the last stage zoeae, represented by the ratio of the chela length to carapace length, tended to advance with increasing salinity, indicating higher assimilation efficiency at higher salinities. The megalopal features of the last stage zoeae were enhanced when the larvae were fed rotifers containing higher amounts of docosahexaenoic acid (DHA). As reported previously, final stage zoeal larvae with advanced megalopal features often experienced moult death syndrome (MDS). These results show that when larvae are fed rotifers with high DHA under high salinity conditions, morphogenesis is accelerated, resulting in MDS. Therefore, to evaluate the effects of salinity on larval survival, it is necessary to examine larval morphogenesis in terms of MDS. In conclusion, we recommend that not only survival but also larval morphogenesis should be examined when evaluating the results of rearing experiments with *S. serrata* larvae.

3.1.2 Introduction

The mud crab *Scylla serrata* is widely distributed throughout the tropical to warm temperate zones in the Pacific and Indian Oceans (Keenan, 1999a). This species spends much of its life in brackish waters, especially in mangrove swamps. After mating, the females migrate to offshore areas to spawn and release their larvae for dispersal, and the larvae grow and develop in the sea (Hill, 1994; Hyland et al., 1984). *S. serrata* is an important local fishery resource with a high commercial value and high growth rate, so it has been targeted for aquaculture throughout the Asian region (Chou and Lee, 1997; Keenan, 1999b; Marte, 2003; Trinõ and Rodriguez, 2002;

Wang et al., 2005). This crab has also been selected as one of the target species for stock enhancement programmes, with the release of juveniles into natural habitats (Fushimi, 1983; Ito, 2000; LeVay et al., 2008; Lebata et al., 2009; Oshiro, 1988). Hatchery technology has been studied in many countries to develop aquaculture and stock enhancement programmes for *S. serrata* (Baylon et al., 2004; Davis et al., 2005a, b; Genodepa et al., 2004a, b; Hamasaki, 2003; Hamasaki et al., 2002a, b; Heasman and Fielder, 1983; Holm et al., 2006; Marte, 2003; Quinitio et al., 2001; Rabbani and Zeng, 2005; Ruscoe et al., 2004; Suprayudi et al., 2002a, b, 2004; Wang et al., 2005). However, mass seed production techniques for *S. serrata* have not been fully developed.

In Japan, seed production techniques for *S. serrata* have mainly involved the use of large tanks, with volumes of 100 kL. However, mass mortality has frequently been observed during metamorphosis to the megalopal stage (Hamasaki et al., 2002a), and this phenomenon was referred to as ‘‘moult death syndrome (MDS)’’ by Mann et al. (1999). Hamasaki et al. (2002a) documented that MDS occurred in last stage (fifth stage) zoeae with abnormally advanced morphological features, similar to those of megalopae, such as large chelipeds. Hamasaki et al. (2002b) has also reported that accelerated larval morphogenesis is related to excess dietary n-3 highly unsaturated fatty acids (n-3 HUFA), which are essential fatty acids for the larvae of *S. serrata* (Suprayudi et al., 2002a, 2004).

The larval nutritional status of *S. serrata* larvae should be determined based not only on their diet but also on their prey consumption and assimilation efficiency, which are influenced by abiotic factors such as water temperature and salinity (Anger, 2001e). Water temperature and salinity have been shown to affect larval survival and development in *S. serrata* (Baylon et al., 2001; Hill, 1974; Hamasaki, 2003; Nurdiani and Zeng, 2007; Wang et al., 2005). However, little is known about the combined effects of nutritional and environmental factors on larval morphogenesis, which must be considered if we are to understand MDS during seed production in *S. serrata*. This study was conducted to examine the effects of salinity and dietary n-3 HUFA on the survival, development, and morphogenesis of *S. serrata* larvae.

3.1.3 Materials and methods

3.1.3.1 Broodstock and larvae

Three mature females with carapace lengths of 180, 173, and 164 mm were collected on 6 March 2003, 9 April 2003, and 20 January 2004, respectively, from Iriomote Island, Okinawa

Prefecture, Japan. They were transferred to the Yaeyama Station of the National Center for Stock Enhancement, Fisheries Research Agency, on Ishigaki Island, Okinawa Prefecture, Japan. They were held in rectangular 5 kL fibreglass tanks (3 kL water volume), the bottoms of which were covered with 10–15 cm of coral sand to facilitate egg attachment to the pleopods. Sand-filtered ultraviolet-irradiated seawater was supplied to the tank with a flow-through water system. Vinyl chloride pipes, 20 cm in diameter and 40 cm in length, were cut in half vertically and provided as shelters. The mean water temperature and salinity in the tanks, which were measured each morning, were 22.5–26.8 °C and 34.3–34.9 ppt, respectively.

After spawning, the ovigerous females were reared individually in rectangular 60 L fibreglass tanks with a flow-through water system containing sand-filtered ultraviolet-irradiated seawater. One day before hatching, each ovigerous female was transferred to a cylindrical 1 kL tank containing still water with gentle aeration. The three females hatched their eggs early in the morning on 20 May 2003, 4 August 2003, and 3 May 2004, respectively, and their larvae were used for subsequent rearing experiments 1–3, respectively.

3.1.3.2 Mass culture of larvae for rearing experiments

Newly hatched larvae (Z1) were transferred to a 30 L chamber from the hatching tank, and the number of larvae was estimated using a volumetric method based on three 50 mL samples taken from the chamber after the water had been agitated to ensure a homogeneous suspension of the larvae. The first stage zoeae were then stocked in one 500 L or 1,000 L tank filled with sand-filtered ultraviolet-irradiated seawater for each experiment. The stocking densities of Z1 were in the range of 21.0–31.3 individual L^{-1}. The seawater in the rearing tanks was not renewed during the larval culture period. Gentle aeration was provided to each tank via an air stone. The photoperiod followed the natural condition of 13 h light (~6:00 to ~19:00) and 11 h darkness. The light intensity was allowed to fluctuate naturally, but fluorescent lights were turned on to maintain a minimum light intensity around the rearing facility of ~500 lx between 7:00 and 18:00 The mean ± sd values for water temperature, salinity, dissolved oxygen, and pH, measured once a day during the rearing period in experiments 1–3, were 28.9 ± 0.3 °C, 35.6 ± 0.3 ppt, 6.2 ± 0.1 mg L^{-1}, and 7.9 ± 0.1, respectively.

The S-strain rotifers *Brachionus plicatilis* sp. complex and *Artemia* (Utah Strain, INVE Ltd, Phichit, Thailand) were used as the prey organisms. The rotifers were initially cultured with commercially available concentrated phytoplankton *Chlorella vulgaris*, without n-3 HUFA

(Chlorella Industry Co., Ltd, Tokyo, Japan), which were enriched in cylindrical 100 L tanks at a density of ~1,000 individuals mL^{-1} for 24 h at 29 °C. In experiments 1 and 2, rotifers enriched with 3 mL L^{-1} commercially available concentrated *C. vulgaris* containing n-3 HUFA (Super Chlorella V12; Chlorella Industry Co., Ltd) were used (C-3 rotifers). In experiment 3, rotifers enriched with 2 ml L^{-1} Super Chlorella V12 were used (C-2 rotifers). The rotifers were added to the tank at a density of 20 individuals mL^{-1} once on the first day of Z1. To maintain the rotifer population in the larval rearing tanks, 10 mL of the commercially available concentrated phytoplankton *Nannochloropsis* sp. (Mercian Co., Ltd, Tokyo, Japan) was added to the tanks twice a day throughout the rearing period. *Artemia* cysts were incubated for 24 h at 29 °C, and newly hatched nauplii were added to the tanks once each morning to maintain densities of 1, 2, 3, and 4 individuals mL^{-1} for the third (Z3), fourth (Z4), and fifth stage zoeae (Z5), and megalopae (MG), respectively. Sodium nifurstyrenate (Ueno Fine Chemicals Industry, Ltd, Osaka, Japan) was added to the larval rearing tanks once on the first day of Z1 at a concentration of 2 mg L^{-1} to minimize the effects of bacterial infection.

3.1.3.3 Larval rearing experiments

The larvae were reared from Z1, Z3, Z5, or MG to first stage crabs (C1) in 1 L beakers at six salinity levels under three different feeding regimes (experiments 1–3). Based on previous papers that reported experiments in which *S. serrata* larvae were reared at different salinity levels (Baylon et al., 2001; Hill, 1974; Nurdiani and Zeng, 2007; Wang et al., 2005), six salinity levels of 10, 15, 20, 25, 30, and 35 ppt were selected for the present study. The seawater used to rear the larvae at the designated salinities was prepared daily by diluting sand-filtered ultraviolet-irradiated natural seawater (35 ppt) with distilled fresh water. Salinity was measured with a hand-held refractometer (S/Mill, Atago, Tokyo, Japan). The feeding regimes, with differently enriched rotifers in each experiment, were as follows. In experiment 1, the larvae were fed C-3 rotifers throughout the zoeal stages. In experiment 2, the larvae were fed C-3 rotifers until 2 days after hatching and then fed rotifers enriched with 3 mL L^{-1} commercially available concentrated *Nannochloropsis* sp. (N-3 rotifers) throughout the zoeal stages. In experiment 3, the larvae were fed C-2 rotifers until 2 days after hatching and then fed N-3 rotifers throughout the zoeal stages. The rotifers were given to the larvae at a density of 20 individuals mL^{-1} throughout the zoeal stages in all experiments. *Artemia* nauplii were also given to the larvae from Z3 to the moult to C1 at densities of 1, 2, 3, and 4 individuals mL^{-1} at Z3, Z4, Z5, and MG, respectively.

Newly hatched Z1 from the hatching tanks and newly moulted Z3, Z5, and MG from mass culture tanks were transferred to three beakers (Z1, Z3) or two beakers (Z5, MG) filled with salinity-controlled seawater at each test salinity level. Two sets of beakers were used to determine the survival rates and developmental periods required to reach the subsequent stage. One beaker was used to sample the larvae of Z5 to measure larval morphogenesis when the larvae were reared from Z1 or Z3. Each beaker contained 30 larvae. No acclimatization procedure was used when the larvae were moved from the hatching tanks or mass culture tanks to the beakers. The larval rearing beakers were placed in shallow water baths, and the temperature was controlled at the optimum temperature (29 °C) for rearing *S. serrata* larvae (Hamasaki, 2003) with a heater connected to a thermostat. Slight aeration provided oxygen saturation and sufficient turbulence to prevent the rotifers and *Artemia* from settling. The beakers were located in the laboratory where the mass culture was conducted. Each morning, larvae were transferred with a large-mouthed pipette to newly prepared beakers containing new salinity-controlled seawater and food. After the larvae had been transferred, 10 mg L^{-1} dihydrostreptomycin sulfate (Tamura-seiyaku Co., Ltd, Tokyo, Japan) was added to the rearing water to prevent bacterial proliferation. The larvae were observed daily. The dead larvae were removed from the rearing beakers, and their larval stages were recorded. The survival rate was determined in each rearing beaker as the percentage ratio of the number of larvae that had moulted to the next stage to the initial number of larvae. The mean number of days to reach each larval stage was calculated for the larvae in each rearing beaker. Zoeae and megalopae at different stages were reared in separate beakers to avoid cannibalism. Larval rearing was terminated when all the larvae had moulted to C1 or had died.

3.1.3.4 Biological measurements

Fifth stage zoeae sampled from a rearing beaker at each salinity level and from the mass culture tank were fixed with 10 % neutral formalin for 1 day and then preserved in 70 % ethanol solution. The chela lengths of five Z5 individuals at each salinity level or from the mass culture tank were measured under a microscope as an index of morphogenesis to MG, and the percentage ratio of the chela length to the carapace length (relative chela length) was calculated according to Hamasaki et al. (2002a). The moulting patterns to MG were also classified into one of four types in each rearing beaker according to Hamasaki et al. (2002a), as follows: type A, serious abnormality (larva had not shed its exuvia completely and had died during moulting); type B, partial abnormality (larva had shed its exuvia but was unable to shed the integument of the chelipeds

and/or walking legs); type C, slight abnormality (larva had shed its exuvia, except for the integument of one cheliped); and type D, normal (larva had shed its exuvia completely).

3.1.3.5 Lipid and fatty acid analyses

C-2, C-3, and N-3 rotifers were sampled to analyse their total lipid contents and fatty acid compositions. They were washed with fresh water and then stored at −80 °C until analysis (n = 2 for each rotifer treatment).

The total lipid contents were determined with the chloroform-methanol (2:1, v/v) method, according to Folch et al. (1957). To analyse the fatty acid compositions, the total lipids were saponified with 1 mL of 50 % KOH in 15 mL of ethanol. The saponifiable matter was then esterified with BF3-methanol. The fatty acid methyl esters were diluted in n-hexane and analysed with a gas-liquid chromatograph (GC-17A, Shimadzu, Kyoto, Japan) equipped with a silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness; Supelco Inc., Bellefonte, PA, USA). Helium was used as the carrier gas, and the pressure was adjusted to 80 kPa. The injection port and detector temperatures were 250 and 270 °C, respectively. The column temperature was initially held at 175 °C and then increased at a rate of 1 °C min^{-1} to a final temperature of 225 °C. The individual fatty acids were identified by comparison with commercial standards (Supelco 37 Component FAME Mix, Supelco) and quantified with a C-R8A Chromatopac Data Processor (Shimadzu).

3.1.3.6 Statistical analysis

Differences in the mean values for the fatty acid compositions of the rotifers (n = 2) in the three treatments, their survival rates, numbers of days required to reach each larval stage, and abnormal moulting rates (n = 2) among the salinity groups were tested with a pairwise *t* test (Welch's *t* test) in R language (R Development Core Team, 2009). The level of significance was set at $\alpha = 0.05$. To control for type I errors in pairwise comparisons, the significance level was adjusted using the Holm-Bonferroni method (Holm, 1979). The mean values for the survival rates to MG and C1 were plotted against salinity, and the regression equation was applied to the relationship among the variables using the least squares method. The relationship between salinity and the mean relative chela lengths of Z5 was also evaluated with regression analysis.

Table 3.1.1. Total lipid contents (% dry basis) and major fatty acid compositions (area %) in C-2, C-3, and N-3 rotifers (means ± standard deviations, n = 2)

	C-2	C-3	N-3
Total lipid	10.2 ± 1.30[a]	11.3 ± 0.07[a]	11.3 ± 0.22[a]
Fatty acid			
14:0	2.1 ± 0.28[a]	2.2 ± 0.18[a]	3.6 ± 0.42[a]
16:0	17.2 ± 1.51[a]	18.7 ± 0.75[a]	17.2 ± 0.47[a]
18:0	3.4 ± 0.35[a]	3.1 ± 0.20[a]	3.3 ± 0.14[a]
18:1	2.7 ± 0.27[a]	1.8 ± 1.63[a]	2.6 ± 1.32[a]
18:2n-6	21.9 ± 3.64[a]	25.8 ± 0.37[a]	17.3 ± 0.23[a]
18:3n-3 (LNA)	5.5 ± 0.86[ab]	6.8 ± 0.29[b]	3.9 ± 0.13[a]
20:1	1.4 ± 0.15[a]	1.3 ± 0.13[a]	1.6 ± 0.28[a]
20:4n-6 (ARA)	0.5 ± 0.11[a]	0.5 ± 0.00[a]	1.9 ± 0.12[b]
20:5n-3 (EPA)	3.6 ± 0.47[a]	4.6 ± 0.20[a]	9.3 ± 0.49[b]
22:5n-3	2.2 ± 0.87[a]	1.6 ± 0.12[a]	2.6 ± 0.20[a]
22:6n-3 (DHA)	2.5 ± 0.02[a]	3.2 ± 0.16[b]	nd[*1]
Saturated[*2]	30.6 ± 1.49[a]	32.2 ± 0.07[a]	29.1 ± 1.82[a]
Monounsaturated[*3]	7.5 ± 0.53[a]	6.9 ± 1.60[a]	13.7 ± 1.60[b]
n-3HUFA[*4]	10.0 ± 0.24[a]	11.3 ± 0.53[a]	13.5 ± 0.89[b]

Significant differences were found between groups with different superscripts in the same row (pairwise *t* test, $P < 0.05$)
[1] Not detected
[2] 12:0; 14:0; 15:0; 16:0; 18:0; 20:0; 22:0
[3] 16:1; 18:1; 20:1; 22:1
[4] 20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3

3.1.4 Results

3.1.4.1 Lipid contents and fatty acid profiles of rotifers

The total lipid contents and fatty acid compositions of the C-2, C-3, and N-3 rotifers are shown in Table 3.1.1. The lipid contents of the rotifers did not differ significantly among the groups. However, the proportions of arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3), monounsaturated fatty acids, and n-3 HUFA were significantly higher in the N-3 rotifers than in the C-2 and C-3 rotifers. In contrast, the proportion of docosahexaenoic acid (DHA; 22:6n-3) was significantly higher in the C-3 rotifers than in the C-2 rotifers, or in the N-3 rotifers, which contained no DHA. The proportion of linolenic acid (LNA; 18:3n-3) was also significantly higher in the C-3 rotifers than in the N-3 rotifers.

3.1.4.2 Larval survival

The survival rates to each larval stage are summarized in Table 3.1.2. The larvae reared at 10 ppt salinity did not develop to the subsequent larval stage, but all died within 24 h in all experiments.

Table 3.1.2. Mean survival rates (n = 2) to each larval stage of *Scylla serrata* reared at six salinity levels from four different developmental stages with three different feeding regimes of rotifers containing different levels of DHA

Exp.	Salinity (ppt)	Survival rate (%) to each stage												
		From Z1						From Z3				From Z5		From MG
		Z2	Z3	Z4	Z5	MG	C1	Z4	Z5	MG	C1	MG	C1	C1
1	10	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	15	11.7^{b}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	30.0^{b}	15.0^{b}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	20	98.3^{c}	86.7^{b}	81.7^{b}	71.7^{b}	28.3^{b}	10.0^{a}	81.7^{c}	81.7^{c}	61.7^{c}	13.3^{a}	76.7^{b}	41.7^{b}	68.3^{c}
	25	91.7^{c}	80.0^{b}	68.3^{b}	63.3^{b}	0^{a}	0^{a}	83.3^{c}	76.7^{c}	13.3^{b}	10.0^{a}	88.3^{b}	33.3^{b}	70.0^{c}
	30	90.0^{c}	80.0^{b}	65.0^{b}	65.0^{b}	0^{a}	0^{a}	83.3^{c}	75.0^{c}	26.7^{b}	1.7^{a}	91.7^{b}	41.7^{b}	56.7^{c}
	35	95.0^{c}	83.3^{b}	80.0^{b}	73.3^{b}	0^{a}	0^{a}	88.3^{c}	88.3^{c}	6.7^{a}	0^{a}	86.7^{b}	30.0^{b}	41.7^{b}
2	10	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	15	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	15.0^{b}	11.7^{a}	0^{a}	0^{a}	0^{a}	0^{a}	65.0^{b}
	20	75.0^{b}	61.7^{b}	53.3^{b}	53.3^{b}	48.3^{b}	25.0^{b}	76.7^{c}	76.7^{b}	70.0^{b}	31.7^{c}	81.7^{b}	41.7^{b}	61.7^{b}
	25	86.7^{bc}	75.0^{b}	63.3^{b}	61.7^{b}	51.7^{b}	30.0^{b}	68.3^{c}	63.3^{b}	61.7^{b}	33.3^{c}	91.7^{b}	46.7^{b}	71.7^{b}
	30	95.0^{c}	66.7^{b}	60.0^{b}	60.0^{b}	50.0^{b}	16.7^{ab}	76.7^{c}	65.0^{b}	48.3^{b}	30.0^{c}	91.7^{b}	26.7^{b}	75.0^{b}
	35	78.3^{b}	63.3^{b}	46.7^{b}	41.7^{b}	20.0^{b}	5.0^{ab}	81.7^{c}	68.3^{b}	43.3^{b}	10.0^{b}	95.0^{b}	36.7^{b}	73.3^{b}
3	10	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	15	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	63.3^{b}	48.3^{b}	41.7^{b}	1.7^{a}	0^{a}	0^{a}	20.0^{b}
	20	95.0^{b}	95.0^{b}	93.3^{b}	91.7^{b}	88.3^{b}	28.3^{a}	95.0^{c}	93.3^{c}	91.7^{c}	56.7^{b}	65.0^{b}	31.7^{b}	58.3^{bc}
	25	100^{b}	93.3^{b}	85.0^{b}	80.0^{b}	68.3^{ab}	43.3^{a}	98.3^{c}	88.3^{bc}	80.0^{bc}	55.0^{b}	96.7^{c}	73.3^{b}	91.7^{c}
	30	93.3^{b}	86.7^{b}	81.7^{b}	73.3^{b}	45.0^{ab}	23.3^{a}	100^{c}	88.3^{bc}	81.7^{c}	46.7^{b}	90.0^{bc}	68.3^{b}	71.7^{c}
	35	96.7^{b}	95.0^{b}	90.0^{b}	85.0^{b}	30.0^{ab}	5.0^{a}	100^{c}	83.3^{bc}	41.7^{bc}	18.3^{ab}	95.0^{c}	73.3^{b}	71.7^{c}

Significant differences were found between groups with different superscripts in the same column in each experiment (pairwise *t* test, $P < 0.05$). Z1–Z5, first to fifth stage zoeae; MG, megalopa; C1, first stage crab

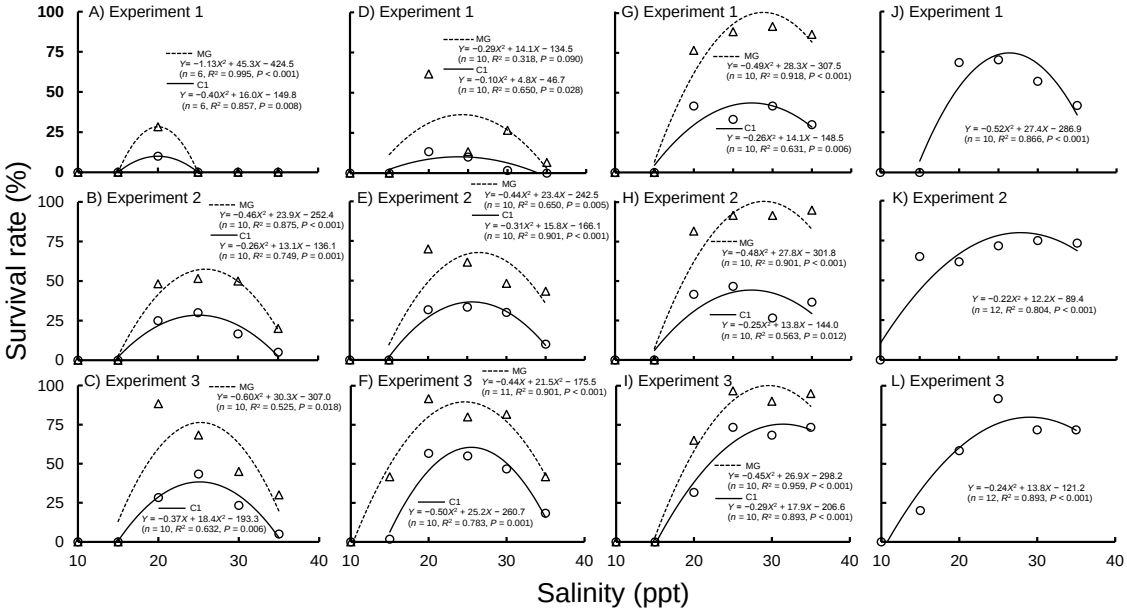

Figure 3.1.1. Relationship between salinity and mean survival rate to megalopa (MG) and first stage crab (C1) of *Scylla serrata* reared at six salinity levels (10, 15, 20, 25, 30, and 35 ppt) with three different feeding regimes of rotifers containing different levels of DHA (experiments 1–3) from the first (A–C), third (D–F), and fifth (last) stage zoeae (G–I), and megalopa (J–L). The curves were drawn from quadratic equations estimated with the least squares method

When rearing started from Z1, 11.7 % of the larvae reared at 15 ppt salinity developed to Z2 in experiment 1, but not in experiments 2 or 3. At 20–35 ppt salinity, no statistically significant differences in the survival rates to Z5 were detected in any experiment, whereas the survival rates to MG and C1 varied among the experiments. In experiment 1, mass mortality occurred during the metamorphosis to MG, and none of the larvae, except those at 20 ppt salinity, moulted to MG. In experiments 2 and 3, the survival rates to MG and C1 at 25–35 ppt salinity tended to decrease with increasing salinity. Regression analyses showed that the highest survival rates to MG and C1 occurred at 20.0 and 20.0 ppt salinity, respectively, in experiment 1; at 25.9 and 25.1 ppt, respectively, in experiment 2; and at 25.3 and 25.1 ppt, respectively, in experiment 3 (Fig. 3.1.1A–C).

When rearing commenced from Z3, some of the larvae reared at 15 ppt salinity developed to Z5 (experiments 1 and 2) or C1 (experiment 3). However, the survival rates at 15 ppt salinity tended to be significantly lower than those at 20–35 ppt. Between 20 and 35 ppt salinity, the survival rates to MG and C1 tended to decrease with increasing salinity, especially at 35 ppt. The salinities with the highest survival rates to MG and C1 were calculated with regression equations to be 24.3 and 23.8 ppt, respectively, in experiment 1; 26.5 and 25.6 ppt, respectively, in

experiment 2; and 24.6 and 25.5 ppt, respectively, in experiment 3, although the equation was not statistically significant for MG in experiment 1 (Fig. 3.1.1D–F).

When rearing commenced from Z5, the larvae reared at 15 ppt salinity did not moult to MG in any experiment. At 20–35 ppt salinity, no statistically significant differences in the survival rates to MG and C1 were detected in any experiment, except the survival rate to MG at 20 ppt salinity in experiment 3, which was lower than those at 25 and 35 ppt. With regression analysis, the highest survival rates to MG and C1 occurred at 28.8 and 27.3 ppt salinity, respectively, in experiment 1; at 28.9 and 27.3 ppt, respectively, in experiment 2; and at 29.6 and 31.4 ppt, respectively, in experiment 3 (Fig. 3.1.1G–I).

When rearing commenced from MG, the larvae moulted to C1 at 20–35 ppt salinity in experiment 1 and at 15–35 ppt in experiments 2 and 3. In experiment 1, the survival rate at 35 ppt salinity was significantly lower than at 20–30 ppt salinity. In experiment 2, no statistically significant differences in the survival rates were found at 15–35 ppt salinity. In experiment 3, the survival rates at 15 and 20 ppt salinity tended to be lower than those at 25–35 ppt salinity. Regression analysis indicated that the highest survival rates to C1 occurred at 26.4, 27.8, and 29.1 ppt salinity in experiments 1–3, respectively (Fig. 3.1.1J–L).

3.1.4.3 Developmental period

The developmental periods (days) to reach each larval stage are shown in Table 3.1.3. The longest developmental periods were recorded for larvae reared at 15 ppt salinity, if the larvae were able to develop at this salinity level, and in most cases, the differences were statistically significant. Although longer developmental periods to C1 tended to be observed at 20 and 35 ppt salinity, the differences in days between the salinity levels were small and not statistically significant.

3.1.4.4 Morphogenesis of Z5

The ratios of chela length to carapace length of Z5 larvae reared from Z1 or Z3 are plotted against the salinity levels in Fig. 3.1.2. The relative chela length tended to increase with increasing salinity in all experiments, although relative stable values were observed between 25 and 35 ppt salinity in experiment 1. Moreover, the relative chela lengths were much smaller in larvae reared from Z3 than in larvae reared from Z1 in experiment 1. The relative chela lengths of Z5 reared in the mass culture tanks were 38.6 ± 1.8 %, 38.3 ± 1.6 %, and 37.3 ± 1.3 % in experiments 1–3, respectively, showing no significant differences between the experiments.

Table 3.1.3. Mean numbers of days (n = 2) to each larval stage of *Scylla serrata* reared at six salinity levels from four different developmental stages with three different feeding regimes of rotifers containing different levels of DHA

Exp.	Salinity* (ppt)	Days to each stage												
		From Z1						From Z3				From Z5		From MG
		Z2	Z3	Z4	Z5	MG	C1	Z4	Z5	MG	C1	MG	C1	C1
1	15	5.8^{b}	–	–	–	–	–	5.2^{b}	7.6^{b}	–	–	–	–	–
	20	3.1^{a}	6.4^{a}	9.2^{a}	12.1^{a}	16.1	23.5	3.0^{a}	5.5^{a}	9.4^{a}	17.4^{a}	4.0^{a}	11.7^{a}	7.6^{a}
	25	3.0^{a}	6.4^{a}	9.4^{a}	12.3^{a}	–	–	2.4^{a}	5.4^{a}	9.2^{a}	17.2^{a}	3.9^{a}	11.7^{a}	7.3^{a}
	30	3.1^{a}	6.6^{a}	9.5^{a}	12.1^{a}	–	–	2.3^{a}	5.3^{a}	9.0^{a}	16.0^{a}	4.0^{a}	11.7^{a}	7.2^{a}
	35	3.1^{a}	6.8^{a}	9.8^{a}	12.7^{a}	–	–	2.4^{a}	5.4^{a}	9.0^{a}	–	4.0^{a}	12.7^{a}	7.9^{a}
2	15	–	–	–	–	–	–	3.6^{b}	6.0^{c}	–	–	–	–	6.1^{a}
	20	3.2^{a}	5.2^{a}	7.6^{a}	10.6^{a}	14.4^{a}	21.5^{a}	2.6^{a}	5.7^{bc}	9.5^{a}	16.5^{a}	3.9^{b}	11.0^{a}	5.9^{a}
	25	3.0^{a}	5.0^{a}	7.5^{a}	10.4^{a}	14.3^{a}	21.8^{a}	2.4^{a}	5.3^{ab}	9.3^{a}	17.0^{a}	3.4^{a}	11.3^{a}	5.5^{a}
	30	3.0^{a}	5.1^{a}	7.8^{a}	10.8^{a}	14.8^{a}	22.2^{a}	2.3^{a}	5.3^{ab}	9.3^{a}	16.2^{a}	3.3^{a}	10.9^{a}	5.6^{a}
	35	3.1^{a}	5.5^{a}	8.4^{a}	11.0^{a}	14.7^{a}	23.0^{a}	2.2^{a}	5.1^{a}	9.1^{a}	17.5^{a}	3.2^{a}	11.5^{a}	6.0^{a}
3	15	–	–	–	–	–	–	6.5^{b}	9.4^{b}	11.6^{a}	21.0^{a}	–	–	8.1^{a}
	20	3.0^{a}	5.9^{ab}	8.9^{b}	11.7^{b}	15.5^{a}	22.4^{a}	3.1^{a}	5.9^{a}	9.9^{a}	16.9^{a}	4.0^{a}	10.6^{a}	7.6^{a}
	25	3.0^{a}	5.7^{a}	8.4^{a}	11.0^{a}	14.7^{a}	21.4^{a}	3.0^{a}	5.6^{a}	9.5^{a}	16.6^{a}	3.9^{a}	10.9^{a}	7.3^{a}
	30	3.1^{a}	5.6^{a}	8.5^{a}	10.7^{a}	14.9^{a}	21.8^{a}	2.8^{a}	5.3^{a}	9.1^{a}	16.6^{a}	3.9^{a}	11.0^{a}	7.2^{a}
	35	3.1^{a}	6.1^{b}	9.0^{a}	11.7^{b}	15.7^{a}	24.8^{a}	2.8^{a}	5.7^{a}	9.6^{a}	17.6^{a}	3.8^{a}	11.3^{a}	7.7^{a}

Significant differences were found between groups with different superscripts in the same column in each experiment (pairwise *t* test, $P < 0.05$). Z1–Z5, first to fifth stage zoeae; MG, megalopa; C1, first stage crab

* No larvae survived to the next stage at 10 ppt salinity

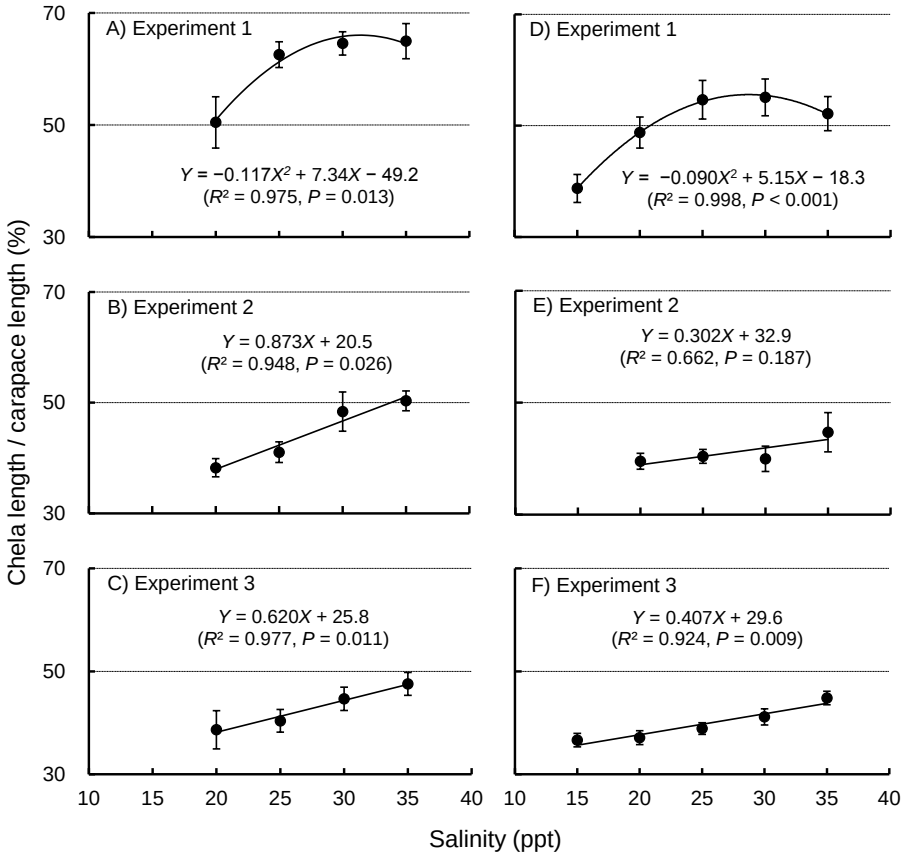

Figure 3.1.2. Relationship between salinity and ratio of chela length to carapace length of fifth stage zoeae of *Scylla serrata* reared from first (A–C) and third stage zoeae (D–F) at six salinity levels with three different feeding regimes of rotifers containing different levels of DHA (experiments 1–3). Vertical bars indicate standard deviations

3.1.4.5 Moulting patterns to MG

The frequencies of the moulting types to MG are summarized in Table 3.1.4. Individuals showing abnormal moulting (types A–C) were few when the larvae were reared from Z5, and the frequencies of abnormal moulting when the larvae were reared from Z1 tended to be higher than those when they were reared from Z3. When the larvae were reared from Z1 or Z3, the frequencies of abnormal moulting tended to increase with increasing salinity, except at 15 ppt salinity in all experiments, and the frequencies tended to be higher in experiment 1 than in experiments 2 and 3.

Table 3.1.4. Mean frequencies (n = 2) of each moulting type that occurred during metamorphosis to megalopae of *Scylla serrata* reared at six salinity levels from four different developmental stages with three different feeding regimes of rotifers containing different levels of DHA

Exp.	Salinity[*1] (ppt)	Frequency of each moulting type (%)[*2]											
		From Z1				From Z3				From Z5			
		A	B	C	D	A	B	C	D	A	B	C	D
1	15	–	–	–	–	100^{b}	0^{a}	0^{a}	0^{a}	–	–	–	–
	20	55.4^{a}	7.2^{a}	14.3^{a}	23.0^{b}	24.3^{a}	19.1^{b}	26.7^{a}	29.9^{a}	0^{a}	0^{a}	0^{a}	100^{a}
	25	100^{b}	0^{a}	0^{a}	0^{a}	52.3^{a}	25.0^{b}	6.8^{a}	15.9^{a}	0^{a}	0^{a}	0^{a}	100^{a}
	30	100^{b}	0^{a}	0^{a}	0^{a}	60.2^{ab}	12.4^{ab}	17.9^{a}	9.5^{a}	0^{a}	0^{a}	0^{a}	100^{a}
	35	100^{b}	0^{a}	0^{a}	0^{a}	90.8^{b}	4.2^{a}	2.9^{a}	2.1^{a}	0^{a}	0^{a}	0^{a}	100^{a}
2	15	–	–	–	–	100^{b}	0^{a}	0^{a}	0^{a}	–	–	–	–
	20	3.6^{a}	0^{a}	0^{a}	96.4^{c}	4.2^{a}	2.1^{a}	4.2^{a}	89.6^{b}	9.1^{a}	7.4^{a}	11.0^{a}	72.5^{a}
	25	14.6^{a}	0^{a}	13.7^{a}	71.7^{bc}	0^{a}	0^{a}	2.9^{a}	97.1^{b}	12.7^{a}	1.9^{a}	3.8^{a}	81.6^{a}
	30	14.9^{a}	7.9^{a}	18.8^{a}	58.4^{b}	0^{a}	2.4^{a}	7.1^{a}	90.5^{b}	8.5^{a}	0^{a}	0^{a}	91.5^{a}
	35	65.0^{b}	8.3^{a}	15.0^{a}	11.7^{a}	15.9^{a}	6.3^{a}	9.6^{a}	68.2^{b}	3.3^{a}	0^{a}	6.8^{a}	89.8^{a}
3	15	–	–	–	–	80.2^{b}	3.1^{a}	3.1^{a}	13.5^{a}	–	–	–	–
	20	3.4^{a}	0^{a}	0^{a}	96.6^{b}	0^{a}	0^{a}	0^{a}	100^{b}	0^{a}	0^{a}	0^{a}	100^{a}
	25	8.6^{a}	0^{a}	3.6^{a}	87.9^{ab}	7.4^{ab}	1.9^{a}	1.9^{a}	88.7^{b}	1.7^{a}	0^{a}	0^{a}	98.3^{a}
	30	16.4^{a}	0^{a}	7.1^{a}	76.5^{ab}	6.0^{ab}	2.1^{a}	8.0^{a}	83.9^{b}	0^{a}	0^{a}	0^{a}	100^{a}
	35	66.2^{b}	5.9^{a}	5.9^{a}	22.0^{a}	48.1^{b}	14.9^{a}	6.5^{a}	30.6^{a}	1.7^{a}	0^{a}	0^{a}	98.3^{a}

Significant differences were found between groups with different superscripts in the same column in each experiment (pairwise *t* test, $P < 0.05$)
Z1–Z5, first to fifth stage zoeae; MG, megalopa; C1, first stage crab
[1] No larvae survived to the megalopal stage at 10 ppt salinity
[2] A serious abnormality; B partial abnormality; C slight abnormality; D normal

3.1.5 Discussion

In this study, low salinity levels of 10–15 ppt were found to be inappropriate for rearing the larvae of *S. serrata* because the survival rates were low and the developmental periods prolonged, as previously shown for this species (Baylon et al., 2001; Hill, 1974; Nurdiani and Zeng, 2007). This study demonstrates that at 15 ppt salinity, larvae reared from Z3 and MG showed a greater capacity to survive and develop to the following stage than did larvae reared from Z1 or Z5. The low tolerance for low salinity in the first and last zoeal stages has also been observed in the larvae of other crustacean decapod species (Anger, 1991, 1996). Anger (1996) hypothesized that the undeveloped osmoregulatory system in Z1 and imminent metamorphosis in last stage zoeae are the causes of this phenomenon.

The survival rates of larvae decreased with the mass mortality caused by abnormal moulting during metamorphosis to MG at 20–35 ppt salinity in all experiments. Thus, the phenomenon of larval mass mortality during metamorphosis to MG constituted MDS. Hamasaki et al. (2002a) reported that when Z5 larvae of *S. serrata* show morphological features similar to those of megalopae, such as large chelipeds and pleopods with plumose setae, MDS results. Here, we evaluated the relationship between the relative chela length of Z5 reared at 20–35 ppt salinity and the frequency of the most serious abnormal moulting, type A. As shown in Fig. 3.1.3, the frequency of abnormal moulting increased, producing a sigmoidal curve, with increasing relative chela length, especially at ratios greater than ~45 %. The relationship between these variables was similar to that observed by Hamasaki et al. (2002a). Therefore, it is clear that in the present study, MDS was caused by morphologically advanced Z5.

Whereas n-3 HUFA, such as EPA and DHA, are known to be essential fatty acids for *S. serrata* larvae (Suprayudi et al. 2002a, 2004), Hamasaki et al. (2002b) demonstrated that larval morphogenesis was accelerated according to the n-3 HUFA content of the prey rotifers and that DHA had a greater effect than EPA in accelerating larval morphogenesis. In the present study, different feeding regimes with rotifers with different fatty acid profiles were used in each experiment. In experiment 1, the larvae were fed C-3 rotifers, with the highest concentration of DHA (3.2 %), throughout the zoeal stages at each salinity level. In experiments 2 and 3, the larvae were fed C-3 rotifers and C-2 rotifers (DHA, 2.5 %), respectively, until 2 days after hatching, after which they were fed N-3 rotifers, which lack DHA. Therefore, it can be inferred that the larvae in

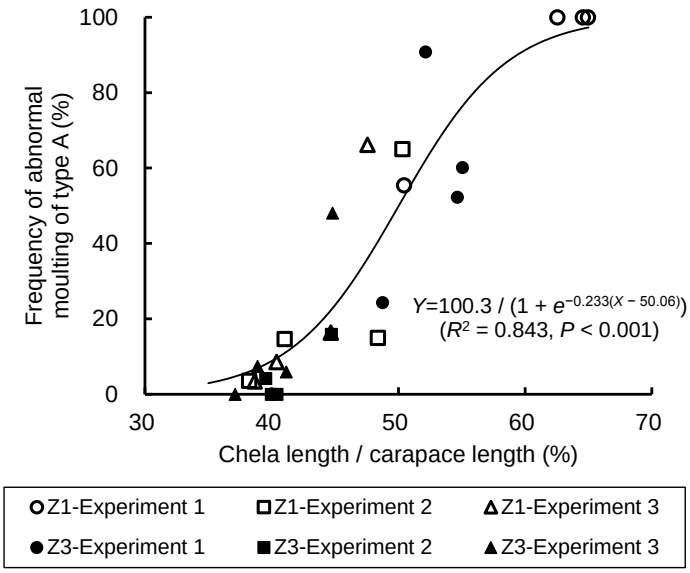

Figure 3.1.3. Relationship between the ratio of chela length to carapace length of fifth stage zoeae of *Scylla serrata* reared at 20–35 ppt salinity and the frequency of type A (most serious) abnormal moulting during metamorphosis to megalopae. Z1, reared from first stage zoeae; Z3, reared from third stage zoeae

experiment 1 assimilated the highest amount of DHA. The relative chela length of the larvae was highest, and the survival rate was lowest in experiment 1. Therefore, we deduce that the nutritional condition (DHA) of the prey affects larval survival and moulting by controlling the morphogenesis of the larvae of *S. serrata*, as suggested by Hamasaki et al. (2002b). The relative chela length was much smaller in the larvae reared from Z3 than in those reared from Z1 at each salinity level in experiment 1. This phenomenon can be explained by the nutritional condition (DHA) of both the prey and the larvae. The newly moulted Z3 used for the rearing experiment in the beakers had been produced in mass culture tanks. In the beakers, the larvae were fed C-3 rotifers (DHA, 3.2 %) throughout the zoeal stages. In contrast, in the mass culture tanks, C-3 rotifers were supplied to the larvae once on the first day of Z1, after which the rotifer population was maintained on *Nannochloropsis* phytoplankton lacking DHA. Dan and Koiso (2008) reported that the fatty acid composition of rotifers changes to that of the supplemented phytoplankton in the seed production tank. Therefore, it can be postulated that the relative chela length of the larvae reared from Z3 decreased because of the reduced DHA content of the rotifers, attributable in turn to the supplementation of *Nannochloropsis* to the mass culture tank. This could also explain the small relative chela length of the larvae reared until Z5 in the mass culture tank.

The relative chela length of Z5 increased significantly with increasing salinity. Anger et al. (1998, 2000) and Anger (2001e) suggested that unfavourable environmental conditions, such as reduced salinity, depress the larval food intake, their conversion efficiency of dietary nutrients, or both. Consequently, larval survival and development are depressed under sub-optimal salinity conditions. This phenomenon was interpreted as a partitioning of energy between osmoregulation and growth (Anger, 2001e; Anger et al., 1998, 2000). Torres et al. (2002) also reported that the larvae of *Homarus gammarus* and *Cancer pagurus* exhibited very low lipid and protein contents when exposed to low salinity (20 and 25 ppt) compared with those of larvae exposed to higher salinity (32 ppt). The same explanation may be applied to the salinity effect on the *S. serrata* larvae in our experiments. The larvae reared at lower salinity levels assimilate less dietary nutrient (DHA) than the larvae reared at higher salinity levels. Such a relatively poorly nourished condition might negatively affect the morphogenesis of larvae reared at lower salinity levels. In other words, we can infer that lower salinity levels are inappropriate for *S. serrata* larvae in terms of their morphogenesis. Conversely, the morphogenesis of larvae reared at higher salinity levels, under good nutritional conditions, would advance and induce MDS. Suprayudi et al. (2002b) reported that advanced larval morphogenesis was triggered by an excessive density of prey *Artemia*, which seems to support our hypothesis.

In this study, we found that a higher DHA content in the rotifers at higher salinity levels (representing the normal salinity condition of seawater) accelerated larval morphogenesis in *S. serrata* and that larval mass mortality ultimately occurred when larval morphogenesis advanced beyond an upper limit of the relative chela length. If we had only examined the survival rates of larvae reared from Z1 or Z3, we would have concluded that the appropriate rearing salinity was around 20–25 ppt, which produced the highest survival rates to C1. However, the appropriate rearing salinity for *S. serrata* larvae should be increased to 30–35 ppt under appropriate dietary conditions. Therefore, we recommend that not only survival but also larval morphogenesis should be examined when evaluating the results of rearing experiments with *S. serrata* larvae.

MDS during metamorphosis to MG by morphologically advanced last stage zoeae has also been reported in the seed production of *Portunus trituberculatus*, *Portunus pelagicus*, *Scylla paramamosain*, and *Scylla tranquebarica* (Arai et al., 2004, 2007; Baylon, 2009; Motonaga et al., unpublished data; Takano et al., 2004), indicating that MDS is a common phenomenon among portunid species under artificial rearing conditions. Our results suggest that larval morphogenesis is largely affected by both dietary factors, such as DHA, and environmental conditions, such as

salinity and that both these elements in combination exert a complex and synergistic effect on larval survival. To develop reliable seed production technologies for portunid crabs, it is important to control MDS by controlling larval morphogenesis, for which the optimal combination of nutritional and environmental conditions must be determined. In future studies, both the effects of other nutritional and environmental factors on larval morphogenesis and the hormonal regulation of larval morphogenesis and metamorphosis must be examined to clarify the mechanisms underlying the development of MDS.

3.2 Variations in larval morphology and their relationships to survival during mass seed production by the swimming crab, *Portunus trituberculatus* (Brachyura, Portunidae)

3.2.1 Summary

The aim of this study was to clarify the underlying cause of the mass mortality that occurs during seed production by the swimming crab, *Portunus trituberculatus*, an important fisheries and aquaculture resource. We sampled fourth stage (last stage) zoeae and megalopae to make morphological measurements and obtained information on the larval culture methods, larval culture conditions and survival rates from 111 seed production trials conducted by 12 hatcheries in Japan between 2009 and 2010. We examined the relationships between the larval culture factors, morphological variations and survival rates. The survival rate declined more significantly from the fourth stage zoeae to the megalopae (52.9 %) and from the megalopae to the juvenile crabs (43.9 %) than between any other successive zoeal stages (80.5–85.4 %). Morphological variations were observed in the megalopae; i.e., some larvae retained a dorsal spine or furcae on their telsons, which are zoeal morphological features. The larval survival rates from the first stage zoeae to the juvenile crabs and from the fourth stage zoeae to the megalopae decreased significantly with increasing zoeal features in the megalopae, whereas survival was not affected by the megalopal morphological features observed in the fourth stage zoeae or any of the culture factors. The carapace length of the fourth stage zoeae decreased significantly with later hatching date and increasing water temperature. The carapace length was the only factor that correlated positively with the survival rate from the megalopae to the juvenile crabs, which suggests that nutrient accumulation during the zoeal stages affects survival after the megalopal stage. Our study identified a relationship between the presence of immature megalopae that retained zoeal morphological features and mass larval mortality from the last stage zoeae to the megalopae, which provides new insights into the causes of mass larval mortality in a brachyuran crab.

3.2.2 Introduction

The swimming crab, *Portunus trituberculatus*, is found throughout the temperate zones of the western Pacific Ocean (FAO, 2014). This species is an important fisheries resource, with a global production of 385,000 t in 2010 (FAO, 2014). The swimming crab farming has also expanded rapidly in east China during the past decade, where its aquaculture production exceeded 80,000 t

in 2006 (Wu et al., 2010). To improve and sustain depleted stocks, this species has been a target species for stock enhancement programmes in Japan since the 1960s. Recently, about 40 million juveniles were produced annually in Japanese hatcheries, and the released juveniles contributed to fisheries production in local seas (Hamasaki et al., 2011). Mass seed production is usually conducted in large tanks. However, mass mortality is frequent during seed production, and 20–30 % of trials fail to harvest juvenile crabs (Hamasaki et al., 2011). Thus, the survival rate is relatively low (national average = 11.2 %) (Hamasaki et al., 2011) compared with other marine species targeted for stock enhancement programmes in Japan, such as the red sea bream *Pagrus major* (54.6–67.8 %) (Kamoshida et al., 2006) and the Japanese flounder *Paralichthys olivaceus* (41.7 %) (Takahashi, 1998). This means that numerous trials (142–188 trials annually) are required to produce sufficient numbers of juveniles for release (Hamasaki et al., 2011).

It has been reported that bacterial and fungal diseases are possible causes of the mass mortality events that occur during swimming crab seed production. However, the causes of the majority (62 %) of mass mortalities are unknown, although abnormal larval morphologies have been suggested as an alternative cause (Hamasaki et al., 2011). Larvae with morphological abnormalities are frequent during seed production using this species, especially during the last zoeal stage and the megalopal stage (Arai et al., 2004; Yasumoto and Yoshida, 1994). For example, Arai et al. (2004) reported that morphologically advanced fourth stage (last stage) zoeae failed to shed their exuviae completely during their metamorphosis into megalopae. These fourth stage zoeae had similar morphological features to megalopae, such as large chelipeds and long pleopods with natatory setae. The morphological abnormalities of megalopae were categorized according to the degree of old exoskeleton retention on their bodies, and megalopae with serious abnormalities eventually died. This mortality phenomenon is known as the “moult death syndrome” (MDS) in other brachyuran species, such as *Portunus pelagicus* (Maheswarudu et al., 2008), *Scylla paramamosain* (Takano et al., 2004), *Scylla serrata* (Dan and Hamasaki, 2011; Hamasaki et al., 2002a; Mann et al., 1999), *Scylla tranquebarica* (Baylon, 2009) and *Ucides cordatus* (Silva et al., 2012). In addition to the morphological abnormalities observed in the last zoeal stage of the swimming crab, we noticed morphological abnormalities in the megalopae, including the presence of a small dorsal spine and furcae on the telson, which were similar to those seen in the zoeae. However, it has never been tested whether these morphological abnormalities are related to larval survival during the mass seed production of this species.

The aim of the present study was to clarify the underlying causes of the low survival rates during swimming crab seed production. We sampled fourth stage zoeae and megalopae to make morphological measurements and obtained information on the larval culture methods, larval culture conditions and survival rates during seed production trials conducted by 12 hatcheries in Japan. We then analyzed the relationships between the larval culture factors, morphologies and survival rates. Our results provide new insights into the causes of mass larval mortality during the intensive production of a brachyuran crab.

3.2.3 Material and methods

3.2.3.1 Larval culture methods and survival data

We surveyed 55 and 56 seed production trials conducted in 12 hatcheries during 2009 and 2010, respectively. Table 3.2.1 summarizes the overall larval culture methods, such as the production tanks, water exchange and feeding regimens. The seed production trials were conducted using 25–200 kL tanks, and most used large (151–200 kL) tanks (47.7 %). In all trials, larvae were reared with a continuous flow water system or daily intermittent water exchange, and the water exchange rate tended to increase with larval growth. The maximum daily water exchange rates were 20–260 % and 30–400 % during the zoeal and megalopal periods, respectively, although most trials used 20–50 % and 51–100 %. In general, the larvae were fed the rotifer *Brachionus plicatilis* species complex, *Artemia*, formula feeds, frozen copepods and minced frozen mysids, depending on the larval stage. It is known that n-3 highly unsaturated fatty acids (n-3 HUFA), such as eicosapentaenoic acid and docosahexaenoic acid, are essential fatty acids for swimming crab larvae (Hamasaki et al., 1998; Takeuchi et al., 1999a, 1999c). The rotifers were enriched with n-3 HUFA before feeding in most trials (97.3 %), although fewer trials used n-3 HUFA enriched *Artemia* (32.4 %) compared with non-enriched hatched nauplii (67.6 %). The rotifer density was maintained at >5 individuals mL^{-1}, although rotifers were not added until after the third and fourth zoeal stages in 6 and 18 trials, respectively, even when the density was lower than the required density. *Artemia* were supplied after the third zoeal stage in many trials (81.1 %), but after the second zoeal stage in some trials (18.9 %). Formula feeds were supplied in 88.4 % of trials, mainly after the third zoeal stage (37.8 %). *Artemia* were supplied during the megalopal stage in all trials, and other foods such as formula feeds, frozen copepods and minced frozen mysids were supplied simultaneously. A combination of *Artemia*, formula feed, copepods

Table 3.2.1. Summary of the larval culture methods used in 111 swimming crab, *Portunus trituberculatus*, seed production trials conducted in 12 Japanese hatcheries during 2009 and 2010

Culture method	Number of trials	Frequency (%)
Water volume (kL)		
25–50	18	16.2
51–100	28	25.2
101–150	12	10.8
151–200	53	47.7
Maximum water exchange rate during zoeal period (%/d)		
20–50	55	49.5
51–100	41	36.9
101–150	11	9.9
151–200	0	0.0
201–260	4	3.6
Maximum water exchange rate during megalopal period (%/d)[1]		
30–50	30	27.3
51–100	40	36.4
101–150	35	31.8
151–200	1	0.9
201–400	4	3.6
Enrichment of rotifers with n-3 HUFA[2]		
Enriched	108	97.3
Not enriched	3	2.7
Enrichment of *Artemia* with n-3 HUFA[2]		
Enriched	36	32.4
Not enriched	75	67.6
Minimum density of rotifers maintained (individuals/mL)		
5–10	83	74.8
11–15	28	25.2
Zoeal stage when *Artemia* feeding commenced[3]		
Z2	21	18.9
Z3	90	81.1
Zoeal stage when formula feeding commenced[3]		
Z1	19	17.1
Z2	15	13.5
Z3	42	37.8
Z4	11	9.9
Not feeding	24	21.6
Food provided to megalopae other than *Artemia*[1, 3]		
Formula feeds	5	4.5
Formula feeds + copepods[4]	39	35.5
Formula feeds + mysids[5]	20	18.2
Formula feeds + copepods + mysids	42	38.2
Mysids	4	3.6
Phytoplankton addition[3]		
Chlorella vulgaris[6]	35	31.5
Diatoms[7]	8	7.2
Nannochloropsis sp.	20	18.0
Chlorella + *Nannochloropsis*	13	11.7
Diatoms + *Nannochloropsis*	30	27.0
Tetraselmis sp.	5	4.5

[1] Summary of 110 trials, excluding one trial in which rearing was stopped during the megalopal period because of complete mortality
[2] *Nannochloropsis* sp., n-3 HUFA-fortified baker's yeast and *Chlorella vulgaris*, or other n-3 HUFA-containing materials were used as enrichment materials
[3] The addition or feed amount varied among trials
[4] Commercially available frozen copepods were used in all trials
[5] Commercially available frozen mysids were used in all trials
[6] Commercially available condensed n-3 HUFA-fortified *Chlorella vulgaris* was used in all trials
[7] Commercially available condensed *Chaetoceros calcitrans*, *C. gracilis* or natural diatoms cultured in each hatchery were used

and mysids was most common (38.2 %). Phytoplankton were added to the rearing tanks to maintain the density by providing nutrients for the live food. Four types of phytoplankton, i.e., n-3 HUFA-fortified commercially available *Chlorella vulgaris*, diatoms, *Nannochloropsis* sp. and *Tetraselmis* sp., were added to the rearing water, either individually or in combination, as food for the rotifers and *Artemia*. The diatoms included commercially available *Chaetoceros calcitrans*, *Chaetoceros gracilis* and natural diatoms cultured in each hatchery. n-3 HUFA-fortified *C. vulgaris* was used most frequently (31.5 %).

The temperature of the larval culture water was generally kept around 23–25 °C because the natural water temperature was below ~ 25 °C, and the mean temperature during production trials ranged between 22.5 °C and 28.6 °C (n = 111; mean = 24.9 °C). The daily pH value of the rearing water was also used as a water quality criterion in 41 trials, and the minimum pH ranged between 7.54 and 8.03.

The larval hatching date ranged from 28 April to 18 September. The numbers of larvae stocked in the seed production tanks were calculated based on the brood weights of ovigerous females, according to the equation proposed by Hamasaki (1996). The stocking density was 7800–69,400 larvae kL^{-1} (n = 111; average = 28,000 kL^{-1}). The number of surviving larvae was estimated using the volumetric method until the megalopal stage, according to the method described by Kogane et al. (2007). The larvae moulted during the night, and the surviving larvae were counted on the next day after moulting. Counts were made during the hour after dusk in the fourth zoeal and megalopal stages, because the larvae were not distributed homogeneously in the tank during the day-time. The juvenile crabs were harvested mainly at the first crab stage, although 40.3 % of the trials included second stage crabs. The number of harvested crabs was estimated using a weighing method whereby the total harvested weight was converted to the number of crabs based on the average number of crabs per unit weight, which was counted in small samples of harvested crabs during each trial. The number of newly hatched larvae and harvested juvenile crabs was estimated in all trials. The numbers of surviving larvae at all larval stages were estimated based on 52 trials (26 trials each year during 2009 and 2010) conducted in six hatcheries, and the numbers of surviving fourth stage zoeae and megalopae were estimated based on 58 trials (31 and 27 trials during 2009 and 2010, respectively) conducted in eight hatcheries.

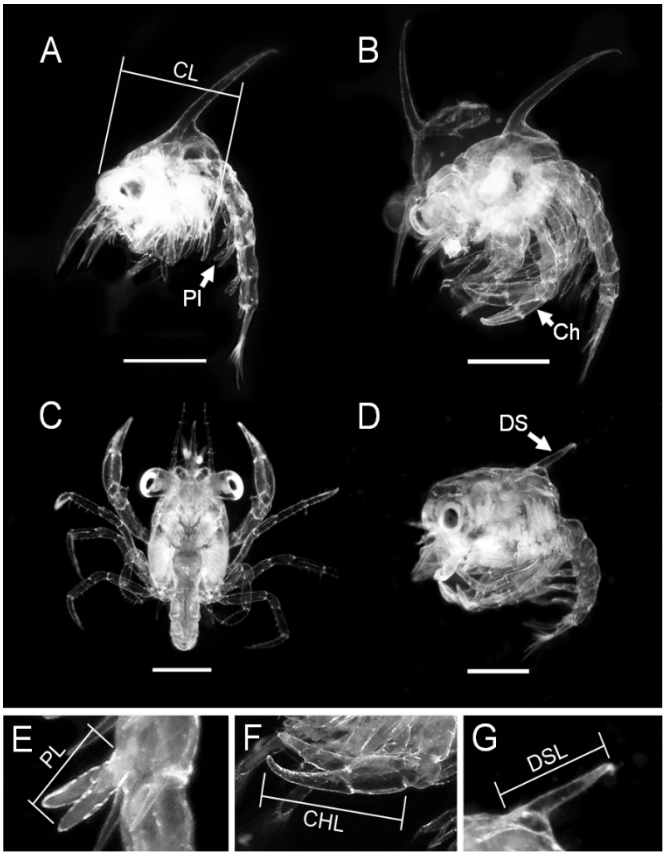

Figure 3.2.1. Photographs of fourth stage zoeae and megalopae observed during seed production using the swimming crab, *Portunus trituberculatus*. A, normal fourth stage zoea; B, morphologically advanced abnormal fourth stage zoea with large chelipeds and pleopods; C, normal megalopa; D, morphologically abnormal immature megalopa with a small dorsal spine on the carapace; E, third pleopod of a fourth stage zoea; F, cheliped of a fourth stage zoea; G, retained dorsal spine on a megalopa. Abbreviations: Pl, pleopod; Ch, cheliped; DS, dorsal spine. Morphological measurements: CL, carapace length; PL, pleopod length; CHL, chela length; DSL, dorsal spine length. Scale bars = 1 mm

3.2.3.2 Morphological measurements of larvae

Samples of fourth stage zoeae and megalopae were collected from the culture tanks in the day-time after moulting. They were fixed with 10 % neutral formalin for one day and preserved in 70 % ethanol solution until the morphological measurements were made.

The lengths of the carapace (CL), chela (CHL) and third pleopod (PL) were measured in the fourth stage zoeae using a microscope (Fig. 3.2.1A, E, F). In addition to the absolute lengths of the chela and pleopods, the ratios of the chela length (relative chela length: CHLr) and the

Table 3.2.2. Abbreviations of the morphological measurements analysed in fourth stage zoeae and megalopae of the swimming crab, *Portunus trituberculatus*

Abbreviations	Morphological traits
Fourth stage zoeae	
CL	Carapace length (mm)
CHL	Chela length (μm)
CHLr	Ratio of CHL to CL (%)
PL	Third pleopod length (μm)
PLr	Ratio of PL to CL (%)
Megalopae	
DSL	Retained dorsal spine length (μm)
DSf	Frequency of retained dorsal spines (%)
TELf	Frequency of abnormal telsons retaining furcae (%)
OEf	Frequency of megalopae with old exuviae (%)

See Figs 3.2.1 and 3.2.2 for details

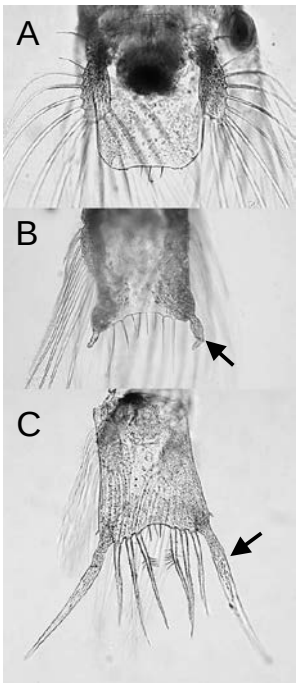

Figure 3.2.2. Photographs of the telsons of megalopae observed during seed production using the swimming crab, *Portunus trituberculatus*. A, normal telson; B, abnormal telson with vestigial furcae; C, severely abnormal telson with large furcae, similar to those of zoeae. For all of the morphological observations, types B and C were considered to be abnormal telsons. The arrows indicate furcae

pleopod length (relative pleopod length: PLr) to the carapace length were calculated (as percentages) as indices of the megalopal features of the fourth stage zoeae, according to the methods of Hamasaki et al. (2002a) and Arai et al. (2004). The morphological measurements were made using 10–20 (average = 13.0) and 29–30 (average = 30.0) individual fourth stage zoeae for each trial during 2009 and 2010, respectively. The length of the dorsal spine retained on the carapace of the megalopa (DSL) was measured using a microscope (Fig. 3.2.1G), and the frequencies of megalopae retaining the dorsal spine (DSf) and furcae on their telsons (TELf) were calculated (Fig. 3.2.2B, C). The frequency of megalopae with old exuviae on their carapace and/or appendages (OEf) was also calculated. OEf has been used as an index of the degree of MDS and is known to be related to lower survival during seed production using the swimming crab and the mud crab, *S. serrata* (Arai et al., 2004; Dan and Hamasaki, 2011; Hamasaki et al., 2002a; Suprayudi et al., 2002b). In total, 27–50 megalopae

(average, 39.9) were measured in each trial during 2009 and 30 in each trial during 2010. The abbreviations used for the morphological measurements are provided in Table 3.2.2.

3.2.3.3 Data analysis

Differences in the survival rates of the larval stages were tested with a pairwise *t*-test using R (R 2. 15; R Development Core Term, 2012) based on Welch's *t* test, without assuming homogeneity of variance in the groups compared. The level of significance was set to $\alpha = 0.05$. To control for type I errors during pairwise comparisons, the significance level was adjusted using the Holm-Bonferroni method (Holm, 1979).

We used a generalized linear mixed-effects model (GLMM) (Zuur et al., 2009) to explore the relationships between larval survival (response variable) and the morphological measurements (for the fourth stage zoeae: CL, CHL, CHLr, PL and PLr; for the megalopae: DSL, DSF, TELf and OEf) (explanatory variables). The hatching season and larval culture conditions, such as the water temperature, water quality and stocking density, are known to affect larval survival and growth (Andrés et al., 2007; Anger, 2001d; Hamasaki et al., 2006). Therefore, larval culture factors such as the hatching date (the number of days after 1 April), water temperature, lowest pH value and the stocking density of larvae, which were quantified during the seed production trials, were also included as explanatory variables in the GLMM. To identify the causes of the mass mortalities that occurred during metamorphoses into megalopae and into juvenile crabs, as well as the survival rate from the first stage zoeae to juvenile crabs (throughout the whole seed production process), the survival rates were assessed from the fourth stage zoeae to the megalopae, and from the megalopae to juvenile crabs. The estimates of dead and live animals during these periods were used as the response variables in the GLMM with the binomial “family” in R. The survival of larvae was also affected by various factors such as disease, the feeding schedules and amounts, the nutritional quality of food and cannibalism (Hamasaki and Hatai, 1993a, b; Muroga et al., 1989; Takeuchi et al., 1999a, 1999b; Yasunobu et al., 2001). In the present study, information was obtained on the feeding schedules and the designated density of prey organisms, although the actual levels in the tanks were unknown, and other factors could not be quantified. Therefore, to include the potential effects of these factors in the analysis, the identity of each seed production trial was included in the models as a random effect. To detect the effects of the explanatory variables, the model selection was based on Akaike's information criterion (AIC), where the best model had the lowest AIC value (Akaike, 1973; Burnham and Anderson, 2002). Previous studies have demonstrated a relationship between the fourth stage zoeal morphology and

the OEf in the megalopae (Arai et al., 2004, 2007), but it is not known whether megalopal morphology similar to that found in zoeae is related to the fourth stage zoeal morphology and MDS. Therefore, we also examined the relationships between OEf and DSf in the megalopae (response variables), the other larval morphological measurements and the larval culture factors (explanatory variables) using GLMM with the binomial "family" in R. The GLMM parameters (with standard errors; *z*-values with probabilities) were estimated using the *glmmML* function implemented in the glmmML package (Broström and Holmberg, 2011) in R. To explore the factors that affected zoeal growth, the relationships between the CL of fourth stage zoeae (response variable) and larval culture factors (explanatory variables) were also examined using a linear mixed-effects model (LMM) with the Gaussian "family" in R. The LMM parameters were estimated using the *lme* function implemented in the nlme package (Pinheiro and Bates, 2000) in R.

3.2.4 Results

3.2.4.1 Differences in the survival of the larval stages

Fig. 3.2.3 shows the mean survival rates between successive larval stages in the seed production trials conducted during 2009 and 2010. The survival rates were significantly lower in the transitions from fourth stage zoeae to megalopae (52.9 %) and from megalopae to juvenile crabs (43.9 %) compared with other successive stages (first to second stage zoeae = 85.4 %; second to third stage zoeae = 81.4 %; third to fourth stage zoeae = 80.5 %) ($P < 0.0001$).

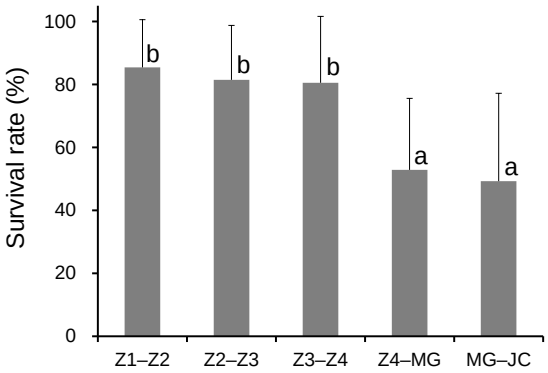

Figure 3.2.3. Mean survival rates in each larval period from the first stage zoeae to juvenile crabs during seed production using the swimming crab, *Portunus trituberculatus*. The data represent the mean values from 52 trials conducted in six hatcheries during 2009 and 2010. Larval stage: Z1–Z4, first to fourth stage zoeae; MG, megalopa; JC, juvenile crabs (mixture of first and second stage crabs). The vertical bars indicate standard deviations. The different letters indicate significant differences ($P < 0.05$) between periods

3.2.4.2 Variations in the larval morphology

There were variations in the morphologies of the fourth stage zoeae and megalopae. In the fourth stage zoeae, CL ranged from 1.33 to 1.59 mm (mean ± sd, 1.49 ± 0.05 mm), CHL from

346 to 797 μm (563 ± 68.9 μm), CHLr from 25.7 % to 51.1 % (37.7 ± 4.0 %), PL from 229 to 493 μm (359 ± 40.8 μm) and PLr from 17.1 % to 32.1 % (24.1 ± 2.3 %). According to Arai et al. (2004), the OEf increases with CHLr, especially when CHLr >37 %. In this study, CHLr was >37 % in 59 of 111 trials, i.e., a frequency of 53.2 %. In the megalopae, DSL ranged from 0.0 to 547.8 μm (35.0 ± 67.9 μm), and the retention of spines was observed in 105 trials (frequency = 94.6 %). DSf ranged from 0.0 % to 100.0 % (38.9 ± 27.8 %). Megalopae that retained furcae on their telsons were observed in 53 trials (frequency, 47.7 %), and TELf ranged from 0.0 % to 100.0 % (17.1 ± 29.4 %). Megalopae with old exuviae were observed in 38 trials (frequency, 34.2 %), and OEf ranged from 0.0 % to 100.0 % (9.9 ± 23.2 %).

3.2.4.3 Relationships between larval survival, morphology and culture factors

Fig. 3.2.4 shows the relationships between the larval survival, morphological measurements and culture factors. The results of the GLMM analysis are summarized in Table 3.2.3.

The larval culture factors had no significant effects on the survival rates during the transition from the first stage zoeae to juvenile crabs, from the fourth stage zoeae to megalopae and from megalopae to juvenile crabs, according to the GLMM ($P > 0.0796$) (Fig. 3.2.4A).

The coefficients of all the explanatory variables (morphological measurements) of the fourth stage zoeae for the survival rate during the transition from the first stage zoeae to juvenile crabs were not statistically significant, according to the GLMM ($P > 0.101$) (Fig. 3.2.4B), whereas those for the megalopae were significantly related to the survival rate ($P < 0.003$) (Fig. 3.2.4C). All of the coefficients were negative, and the survival rate tended to decrease as the morphological measurements and frequency values increased for the megalopae. The lowest AIC was obtained in the model in which DSL was the explanatory variable.

The relationships between the survival rate during the transition from fourth stage zoeae to megalopae and the morphological measurements had similar trends to those detected in the transition from first stage zoeae to juvenile crabs. The coefficients for the morphological measurements related to the megalopae were all statistically significant ($P < 0.03$) (Fig. 3.2.4C), whereas those for the fourth stage zoeae were not significant ($P > 0.159$) (Fig. 3.2.4B), thereby indicating that the mortality during this period was affected by the megalopal morphology, rather than by the fourth stage zoeal morphology. All of the coefficients were negative, and the survival rate tended to decrease as the morphological measurements and frequencies increased for the megalopae. The AIC was lowest in the model in which DSf was the explanatory variable.

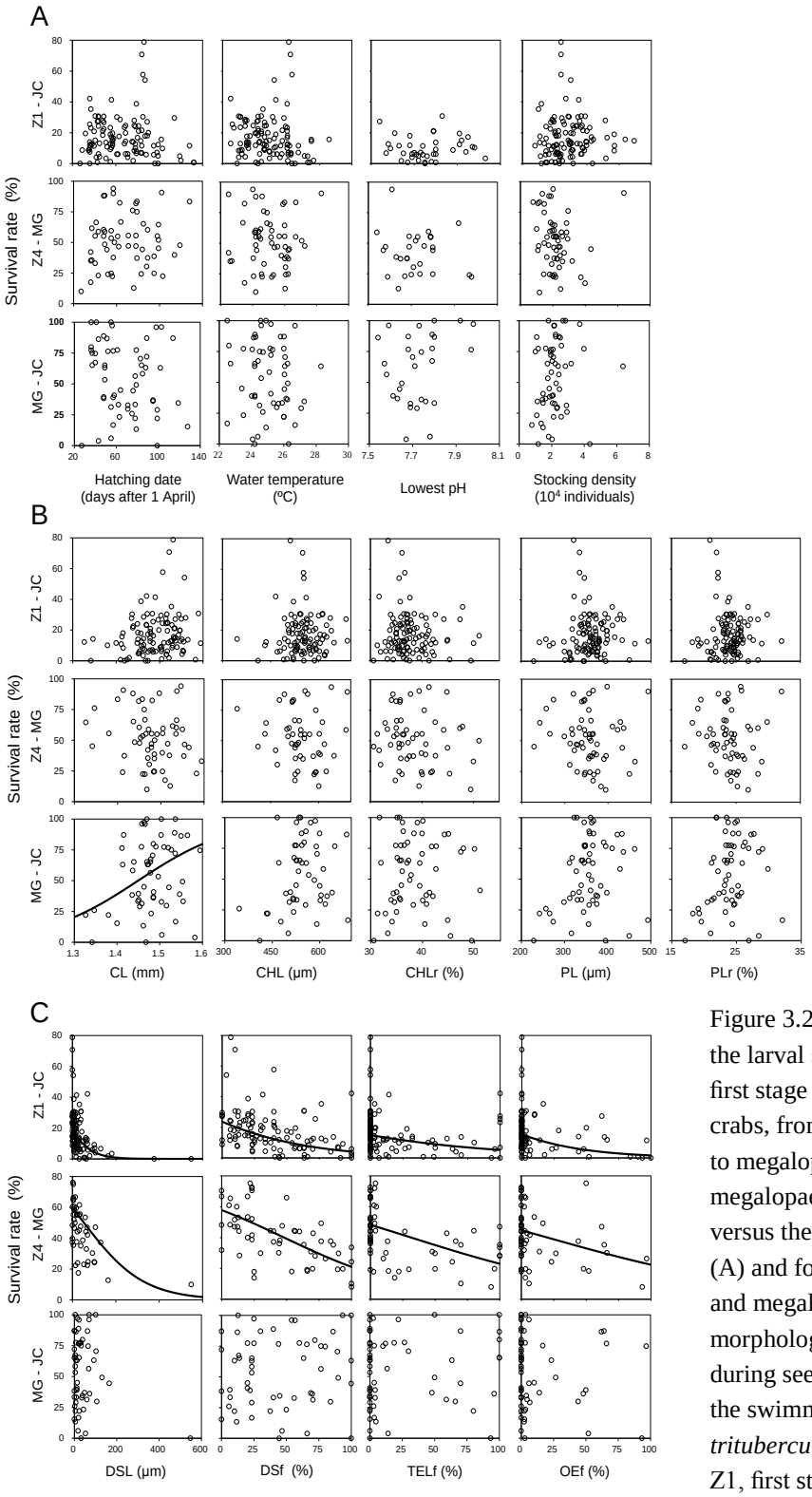

Figure 3.2.4. Distributions of the larval survival rates from first stage zoeae to juvenile crabs, from fourth stage zoeae to megalopae and from megalopae to juvenile crabs versus the larval culture factors (A) and fourth stage zoeal (B) and megalopal (C) morphological measurements during seed production using the swimming crab, *Portunus trituberculatus*. Larval stages: Z1, first stage zoeae; Z4, fourth stage zoeae; MG, megalopae; JC, juvenile crabs (mixture of first and second stage crabs). The abbreviations of the morphological measurements are defined in Table 3.2.2. Solid lines show the logistic curves that had statistically significant coefficients ($P < 0.05$), which were estimated using generalized linear mixed-effects models

Table 3.2.3. Estimated coefficients and Akaike's information criteria (AICs) for the generalized linear mixed-effects models used to examine the effects of larval morphologies and culture factors (explanatory variables) on the larval survival rates (response variables) during seed production using the swimming crab, *Portunus trituberculatus*. *Δi* indicates the difference in the AIC between a given model and the model with the lowest AIC, for each response variable. Explanatory variables with higher AIC values (*Δi* > 100) are not shown in the table

Response variables	Explanatory variables		Coefficient			AIC	*Δi*
			Estimated	SE	*P*		
Survival rate from Z1 to JC	Hatching date[1]		–0.0076	0.0048	0.1100	551.6	49.7
	Z4	CHL	0.0004	0.0017	0.8330	554.1	52.2
		CHLr	–0.0171	0.0291	0.5580	553.8	51.9
		PL	0.0047	0.0028	0.1010	551.4	49.5
	MG	DSL	–0.0166	0.0026	< 0.0001	501.9	0.0
		DSf	–0.0197	0.0038	< 0.0001	528.9	27.0
		TELf	–0.0116	0.0039	0.0027	545.3	43.4
		OEf	–0.0239	0.0050	< 0.0001	531.6	29.7
Survival rate from Z4 to MG	Hatching date[1]		0.0050	0.0057	0.3860	279.3	24.2
	Water temperature		–0.0020	0.1135	0.9860	280.1	25.0
	Lowest pH[2]		–0.9785	1.4090	0.4870	–	–
	Stocking density		–0.0843	0.1555	0.5880	288.0	32.9
	Z4	CL	–2.9030	2.4830	0.2420	278.7	23.6
		CHL	–0.0022	0.0015	0.1590	278.1	23.0
		CHLr	–0.0357	0.0269	0.1840	278.3	23.2
		PL	–0.0019	0.0027	0.4880	279.6	24.5
		PLr	–0.0211	0.0492	0.6690	279.9	24.8
	MG	DSL	–0.0073	0.0017	< 0.0001	263.0	7.9
		DSf	–0.0199	0.0036	< 0.0001	255.1	0.0
		TELf	–0.0133	0.0035	0.0002	267.2	12.1
		OEf	–0.0118	0.0056	0.0342	275.8	20.7
Survival rate from MG to JC	Hatching date[1]		–0.0150	0.0100	0.1350	286.3	2.2
	Water temperature		–0.2315	0.1978	0.2420	287.1	3.0
	Lowest pH[2]		5.3390	3.0460	0.0796	–	–
	Stocking density		0.1876	0.2728	0.4920	288.0	3.9
	Z4	CL	9.0870	4.2910	0.0342	284.1	0.0
		CHL	0.0019	0.0028	0.4840	288.0	3.9
		CHLr	0.0172	0.0484	0.7220	288.4	4.3
		PL	0.0082	0.0047	0.0818	285.5	1.4
		PLr	0.1376	0.0847	0.1040	285.9	1.8
	MG	DSL	–0.0050	0.0041	0.2140	286.8	2.7
		DSf	0.0068	0.0078	0.3840	287.7	3.6
		TELf	0.0074	0.0069	0.2830	287.3	3.2
		OEf	–0.0077	0.0105	0.4640	287.9	3.8

Larval stages: Z1, first stage zoeae; Z4, fourth stage zoeae; MG, megalopae; JC, juvenile crabs (mixture of first and second stage crabs). The abbreviations of the morphological traits are defined in Table 3.2.2

[1] Days after 1 April

[2] The number of trials with available pH data was lower (30 trials) than the number with other explanatory variables, so the AIC value was not compared with the other explanatory variables

For the survival rate during the transition from megalopae to juvenile crabs, the coefficient was only significant for CL in the fourth stage zoeae ($P = 0.034$) (Fig. 3.2.4B), and the value was positive. The AIC was also minimal in the model in which the CL of the fourth stage zoeae was used as the explanatory variable.

3.2.4.4 Relationships between larval morphologies and culture factors

Fig. 3.2.5 shows the relationships between megalopal morphologies (OEf and DSf) and larval culture factors, between megalopal morphologies and the other morphological measurements, and between the CL of the fourth stage zoeae and culture factors. The results of the GLMM (OEf and DSf) and LMM (CL) analyses are summarized in Tables 3.2.4 and 3.2.5, respectively.

The larval culture factors were not significantly correlated to OEf and DSf in the megalopae ($P > 0.1290$) (Fig. 3.2.5A).

In the analysis of OEf, the coefficients of CHL, CHLr and PL for the fourth stage zoeae, and DSL, DSf and TELf for the megalopae, were statistically significant ($P < 0.0065$) (Fig. 3.2.5B, C), thereby indicating that the MDS during the metamorphosis into megalopae was related to the fourth stage zoeal and megalopal morphologies. All of the coefficients were positive, and the AIC was lowest in the model in which the CHL of the fourth stage zoeae was used as the explanatory variable.

The analysis of DSf in the megalopae showed that the coefficients of the other morphological measurements related to the megalopae had strong positive relationships ($P < 0.0001$), whereas the morphological measurements of the fourth stage zoeae had no effects on the frequency ($P > 0.074$), with the exception of CL, which had a weak but significant positive relationship ($P = 0.039$) (Fig. 3.2.5B, C). The lowest AIC was observed in the model in which the DSL of the megalopae was used as the explanatory variable.

The analysis of CL in the fourth stage zoeae showed that the coefficients of the hatching date and water temperature had a significant negative relationship ($P < 0.0004$) (Fig. 3.2.5A). The AIC was lowest in the model in which the hatching date was used as the explanatory variable.

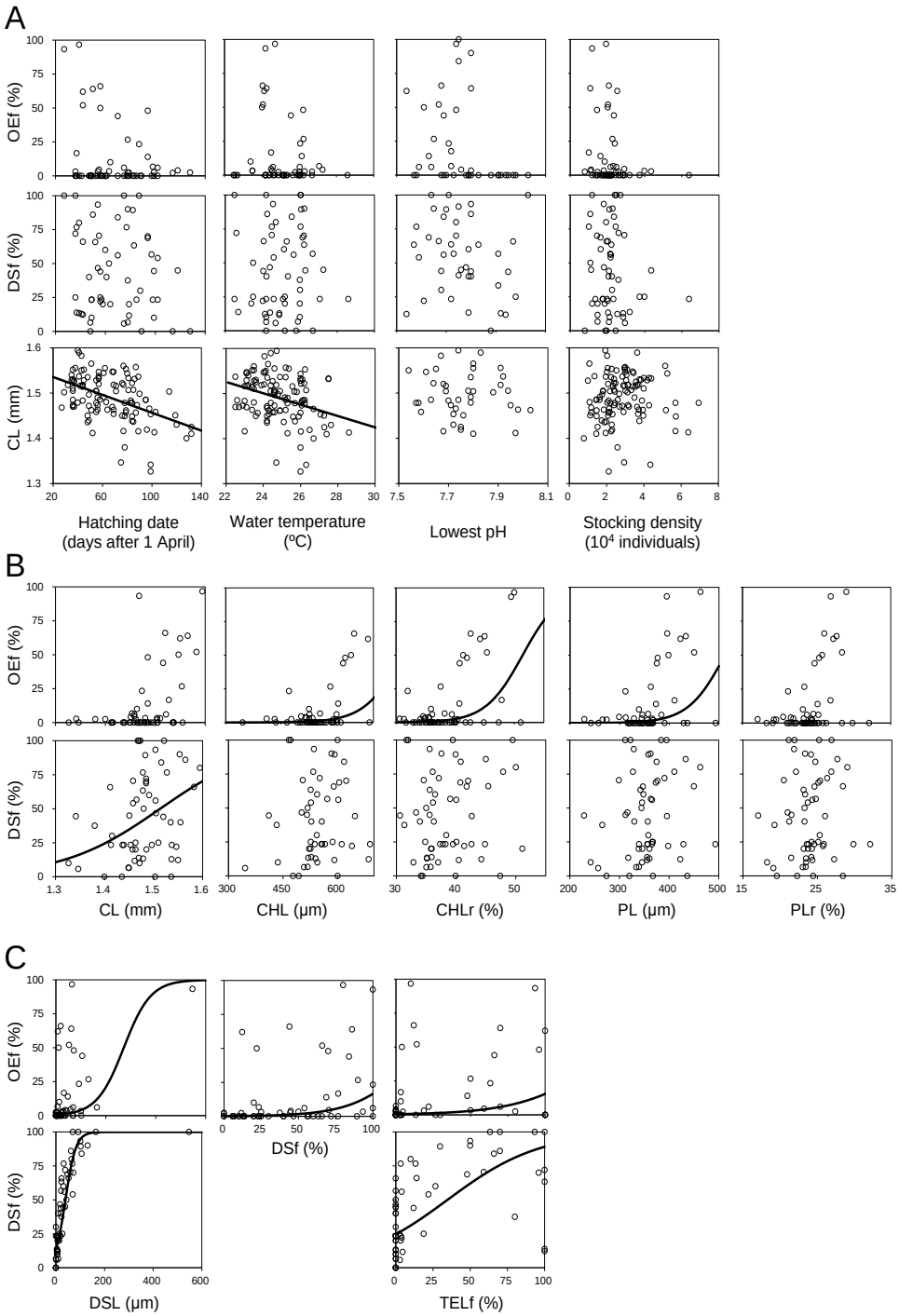

Figure 3.2.5. Distributions of larval morphologies versus the larval culture conditions (A) and fourth stage zoeal (B) and megalopal (C) morphological measurements during seed production using the swimming crab, *Portunus trituberculatus*. The abbreviations of the morphological measurements are defined in Table 3.2.2. Solid lines show the linear correlations with statistically significant coefficients ($P < 0.05$), which were estimated using linear mixed-effects models of the carapace lengths of fourth stage zoeae, and the logistic curves estimated using generalized linear mixed-effects models of the megalopal morphology

Table 3.2.4. Estimated coefficients and Akaike's information criteria (AICs) for the generalized linear mixed-effects models used to examine the effects of larval morphologies and culture factors (explanatory variables) on the frequency of megalopae retaining a dorsal spine and the frequency of megalopae retaining old exuviae (response variables) during seed production using the swimming crab, *Portunus trituberculatus*. *Δi* indicates the difference in the AIC between a given model and the model with the lowest AIC, for each response variable. Explanatory variables with higher AIC values (*Δi* > 100) are not shown in the table

Response variables	Explanatory variables		Coefficient			AIC	*Δi*
			Estimated	SE	*P*		
OEf	Hatching date[1]		–0.0112	0.0208	0.5910	165.6	16.0
	Z4	CHL	0.0195	0.0044	< 0.0001	149.6	0.0
		CHLr	0.3174	0.0797	0.0001	152.7	3.1
		PL	0.0269	0.0086	0.0017	157.4	7.8
	MG	DSL	0.0185	0.0053	0.0005	154.2	4.6
		DSf	0.0492	0.0148	0.0009	155.0	5.4
		TELf	0.0359	0.0132	0.0065	158.7	9.1
DSf	Hatching date[1]		–0.0178	0.0117	0.1290	240.2	83.6
	Water temperature		–0.0211	0.2251	0.9250	242.5	85.9
	Lowest pH[2]		–2.2120	2.1540	0.3040	–	–
	Stocking density		–0.1098	0.3080	0.7220	242.4	85.8
	Z4	CL	9.9440	4.8250	0.0393	238.4	81.8
		CHL	0.0042	0.0031	0.1760	240.7	84.1
		CHLr	0.0571	0.0547	0.2970	241.4	84.8
		PL	0.0060	0.0055	0.2750	241.3	84.7
		PLr	0.0679	0.0987	0.4920	242.0	85.4
	MG	DSL	0.0468	0.0040	< 0.0001	156.6	0.0
		TELf	0.0322	0.0067	< 0.0001	221.8	65.2

Larval stages: Z1, first stage zoeae; Z4, fourth stage zoeae; MG, megalopae. The abbreviations of the morphological traits are defined in Table 3.2.2

[1] Days after 1 April

[2] The number of trials with available pH data was lower (41 trials) than the number with other explanatory variables, so the AIC value was not compared with the other explanatory variables

Table 3.2.5. Estimated coefficients and Akaike's information criteria (AICs) in the linear mixed-effects models used to examine the effects of the larval culture factors (explanatory variables) on the carapace lengths of fourth stage zoea (response variable) during seed production using the swimming crab, *Portunus trituberculatus*. *Δi* indicates the difference in the AIC between a given model and the model with the lowest AIC, for each response variable. Explanatory variables with higher AIC values (*Δi* > 100) are not shown in the table

Response variables	Explanatory variables	Coefficient			AIC	*Δi*
		Estimated	SE	*P*		
CL	Hatching date[1]	–0.0010	0.0002	< 0.0001	–367.5	0.0
	Water temperature	–0.0126	0.0035	0.0004	–352.0	15.5
	Lowest pH[2]	–0.0396	0.0652	0.5469	–	
	Stocking density	0.0017	0.0041	0.6755	–339.5	28.0

CL: carapace length of fourth stage zoeae

[1] Days after 1 April

[2] The number of trials with available pH data was lower (41 trials) than the number with other explanatory variables, so the AIC value was not compared with the other explanatory variables

3.2.5 Discussion

In the present study, the survival rates were significantly lower during the transitions from fourth stage zoeae to megalopae and from megalopae to juvenile crabs compared with the survival during the transitions between other zoeal stages. Hamasaki (1997) also reported that the survival rates decreased from the fourth stage zoeae to the first stage crabs during seed production using this species, which caused acute mass mortality, i.e., the simultaneous death of >80% of the larvae.

Mass mortalities are frequent during the metamorphosis into megalopae in swimming crab seed production (Arai et al., 2004; Yasumoto and Yoshida, 1994) and are often accompanied by morphological abnormalities in the megalopae, which are unable to shed their exuviae completely (Arai et al., 2004). Mass mortality during the metamorphosis into megalopae is known as MDS and has been reported in other brachyuran species (Baylon, 2009; Dan and Hamasaki, 2011; Hamasaki et al., 2002a; Mann et al., 1999; Silva et al., 2012; Takano et al., 2004), which suggests that it may be a common phenomenon during the larval culture of crabs. It has been demonstrated that the megalopal morphological characteristics of the last stage zoeae, such as CHLr, can be used to predict the degree of MDS (Arai et al., 2004; Dan and Hamasaki, 2011; Hamasaki et al., 2002a; Silva et al., 2012; Takano et al., 2004). In the present study, the OEf of megalopae had negative effects on the survival rates from the first stage zoeae to juvenile crabs and from the fourth stage zoeae to megalopae. The CHL and the CHLr of the fourth stage zoeae were also predictors of OEf, and they had lower AIC values than the other morphological measurements and larval culture factors, according to the GLMM analysis. This suggests that advanced megalopal morphological features in the fourth stage zoeae cause MDS during the metamorphosis into megalopae. These results are consistent with previous reports.

There was great variation in the morphologies of the megalopae and fourth stage zoeae. However, the megalopal morphology had stronger effects on survival during the transition from the fourth stage zoeae to the megalopae than did the morphology of the fourth stage zoeae. The survival rate tended to decrease when the megalopae retained morphological features of the zoeal stage, such as the dorsal spine and furcae on the telson, which suggests that this immature megalopal morphology is abnormal. Larval samples were collected on the next day after moulting, and the larval survival rates were estimated during the night-time. Most of the abnormal immature megalopae died within a few days after their metamorphosis. Thus, the immature morphological abnormalities of the megalopae may be the major cause of the mass mortality during the metamorphosis into the megalopal stage in recent swimming crab seed production trials in

Japanese hatcheries. The reduction in survival during the metamorphosis into megalopae also reduced the survival rate from the first stage zoeae to juvenile crabs (throughout the entire seed production process) with increasing megalopal morphological abnormalities.

Compared with the morphological variations of zoeal larvae, which have been described well, especially in the later zoeal stages (Anger, 2001a), very few studies have reported variations in the megalopal morphology. It has been reported that abnormally advanced morphological features in the last stage zoeae of *S. serrata*, such as CHLr, are related to the excess intake of essential fatty acids, i.e., n-3 HUFA (Dan and Hamasaki, 2011; Hamasaki et al., 2002b; Suprayudi et al., 2002a, 2004; Chapter 3.1). n-3 HUFA are known to be essential fatty acids in the swimming crab (Hamasaki et al., 1998; Takeuchi et al., 1999a, c) and are considered to accelerate zoeal morphogenesis (Arai et al., 2004, 2007). In the present study, there was no correlation between the megalopal features in the fourth stage zoeal morphology and the zoeal features in the megalopal morphology, suggesting that excess dietary n-3 HUFA, which induces abnormality in fourth stage zoeae, may not be a cause of abnormal immaturity in the megalopae. Furthermore, the positive correlation between DSf in the megalopae and CL in the fourth stage zoeae suggests that abnormal megalopal immaturity is not caused by culture factors that suppress larval growth, such as nutritional deficiencies, whereas it may be related to factors that accelerate somatic growth during the zoeal stages. Costlow (1966a, 1966b) reported that eyestalk extirpation during the early zoeal stages of the crabs *Rhithropanopeus harrisii* and *Sesarma reticulatum* inhibited their metamorphosis into megalopae and led to supernumerary zoeal stages. Freeman and Costlow (1983) suggested that the larval metamorphosis of a crab was regulated by a moult-inhibiting hormone and/or a metamorphosis-inhibiting factor, which was produced in and secreted by the X-organ sinus gland complex in the eyestalk. Recently, we found that extirpation of the zoeal eyestalk of the swimming crab produced megalopae with similar morphological features to those of the abnormal megalopae observed in the present study (see Chapter 3.3). Further research is required to clarify the larval culture factors that produce morphologically immature megalopae by disrupting the hormonal regulation related to the X-organ sinus gland complex.

The survival rate during the transition from megalopae to juvenile crabs was significantly lower than that during transitions between various zoeal stages. In contrast to the survival rate from the fourth stage zoeae to megalopae, abnormal morphologies in the fourth stage zoeae and megalopae had no effects on survival during this period. However, higher CL values in the fourth stage zoeae improved their survival after the megalopal stage. In general, the body size of larvae

increases when the culture conditions are optimal and with increasing hatching size (Anger, 2001d). Therefore, the larval body size was used as an approximate indicator of the environmental and nutritional conditions in the larval cultures, which showed that the accumulation of nutrients and/or energy during the zoeal period was important for survival during the metamorphosis from megalopae into juvenile crabs. Among the larval culture factors investigated in the present study, later hatching dates and higher water temperatures significantly reduced the CL in the fourth stage zoeae. This agrees with a previous study, which demonstrated that the size of the first stage zoeae of the swimming crab declined with increasing water temperature and later brooding times throughout the hatching season (Hamasaki et al., 2006; Kurata, 1983). This suggests that the level of nutrients accumulated may be lower in seed production trials conducted late in the hatching season and/or with high water temperatures. Based on these results, it is reasonable to suggest that the reduced survival during the transition from megalopae into juvenile crabs was caused by a lack of nutrient accumulation during the zoeal stages.

In this study, we demonstrated that the mortalities during the transitions from fourth stage zoeae to megalopae and from megalopae to juvenile crabs were related to the abnormal immaturity of the megalopal morphology and the smaller body size of the fourth stage zoeae, probably because they failed to accumulate sufficient nutrients during the zoeal stages. Thus, our findings highlight the importance of examining the larval morphology and provide new insights into the causes of mass larval mortality during the larval culture of brachyuran crabs. To overcome mass larval mortality, further research is required to understand the causes of morphological immaturity in the megalopae from an endocrinological perspective.

3.3 Eyestalk ablation affects larval morphogenesis in the swimming crab *Portunus trituberculatus* during metamorphosis into megalopae

3.3.1 Summary

To understand the role of the eyestalk neurosecretory system in regulation of larval morphogenesis, we performed eyestalk ablation on swimming crab *Portunus trituberculatus* larvae at various times during zoeal development. We measured the length of the chelae and pleopods, which become enlarged during development, and the dorsal spine and telson furcae, which are resorbed during metamorphosis in the final (fourth) stage zoeae and subsequent larval stages, including a supernumerary fifth stage zoeae and megalopae (fifth instar larvae). The length of the chela and pleopod of fourth stage zoeae decreased when the bilateral eyestalks were ablated earlier during development. Eyestalk ablation had little effect on the zoeal dorsal spine and furcae. In fifth instar larvae, the effects of eyestalk ablation changed radically depending on the time when the ablation was performed, and a critical period during the premoult of the third zoeal stage was identified. Ablation before this period caused retention of a large dorsal spine and furcae and resulted in moult to the supernumerary fifth zoeal stage. Ablation after this period allowed larvae to metamorphose into normal megalopae. Ablation during this period resulted in megalopae with immature morphology, whereby the larvae retained small dorsal spines and telson furcae. The results demonstrated that the eyestalk neurosecretory system most likely regulates larval morphogenesis during metamorphosis in 2 ways: the morphogenesis of body parts that are enlarged are continuously controlled throughout the zoeal stages, whereas the resorption of body parts is controlled instantaneously at a critical point during the premoult of the third zoeal stage.

3.3.2 Introduction

The larval development of brachyuran crabs is generally accompanied by metamorphosis during which individuals undergo a distinct morphological change from a planktonic zoea to a crab-like megalopa (Anger, 2001a). Although larval metamorphosis is a major event in crab life history, little is known about the mechanisms regulating this metamorphosis. In contrast, the endocrine systems controlling the moulting cycle and sexual maturity have been intensively studied in adult decapod crustaceans (Hopkins, 2012; Keller, 1992). Our understanding of crustacean endocrinology has largely been developed based on investigations into the role of the eyestalk (Hopkins, 2012). The eyestalk consists, in part, of an X-organ and sinus gland, both of

which are important parts of the neurosecretory system. In crab larvae, to our knowledge, only 3 studies have examined the effects of eyestalk ablation in zoeae on larval development in *Sesarma reticulatum* and *Rhithropanopeus harrisii* (Costlow, 1966a, b; Freeman and Costlow, 1980). These studies demonstrated that bilateral eyestalk ablation of zoeae resulted in retardation or inhibition of metamorphosis into megalopae depending on the timing of ablation. Eyestalk ablation during early zoeal stages resulted in the occurrence of a supernumerary additional zoeal stage. Larval eyestalk ablation of snapping shrimp *Alpheus heterochaelis*, American lobster *Homarus americanus*, and shrimp *Palaemon macrodactylus* also resulted in delayed or inhibited metamorphosis when ablation occurred before a critical point in the zoeal stage (Charmantier and Aiken, 1987; Charmantier et al., 1988; Gross and Knowlton, 1997, 1999, 2002; Knowlton, 1994; Little, 1969; Snyder and Chang, 1986). Moreover, some of these studies noted that eyestalk ablation induced the development of morphologically distinct megalopae (postlarvae) that were characterised as larval-postlarval intermediates because they exhibited both zoeal and megalopal features of the carapace (shape), appendages and telson. For example, *R. harrisii* megalopae retained a small dorsal spine on the carapace and *H. americanus* postlarvae retained long lateral spines on the telson (Charmantier and Aiken, 1987; Costlow, 1966a; Gross and Knowlton, 2002; Knowlton, 1994). Taken together, these observations suggest that the larval metamorphosis of decapod crustaceans is regulated by the neurosecretory system located in the eyestalks.

In recent years, the large-scale larval culture of commercially important brachyuran crabs has expanded globally because of the increase in demand for crab seed for purposes of aquaculture and stock enhancement (Castine et al., 2008; Cheng et al., 2008; Davis et al., 2005; Hamasaki and Kitada, 2008; Hamasaki et al., 2011; Jinbo et al., 2013; Keenan and Blackshaw, 1999; Kogane et al., 2007: Wang et al., 2005; Zmora et al., 2005). Accordingly, there has been an increase in the number of rearing trials to improve larval rearing techniques. As a result, there has been a concomitant increase in the number of reports documenting the existence of variations in larval morphology and a relationship with larval mass mortality during seed production (Arai et al., 2004; Dan and Hamasaki, 2011; Dan et al., 2013; Hamasaki et al., 2002a, b, 2011; Takano et al., 2004; Silva et al., 2012). Arai et al. (2004) reported that, during seed production of the swimming crab *Portunus trituberculatus*, morphologically advanced fourth stage (last stage) zoeae failed to shed their exuviae completely during their metamorphosis into megalopae and eventually died. These final stage zoeae had morphological features similar to megalopae, such as large chelipeds and long pleopods with natatory setae. This mortality phenomenon is known as ‘moult death

syndrome' (Mann et al., 1999), and it has also been documented in other brachyuran crabs such as *Scylla paramamosain*, *Scylla serrata*, and *Ucides cordatus* (Dan and Hamasaki, 2011; Hamasaki et al., 2002a, b; Mann et al., 1999; Silva et al., 2012; Takano et al., 2004). Furthermore, our previous study demonstrated that there were significant variations in the morphology of *P. trituberculatus* megalopae cultured in 12 hatcheries in Japan. In this instance, the occurrence of morphologically immature megalopae that retained zoeal morphological features, such as a small dorsal spine and telson furcae, was related to larval mass mortality in this species (Dan et al., 2013; Chapter 3.2). Such immature morphology of megalopae has also been reported in other brachyuran crab species such as *Aratus pisonii*, *Paramola petterdi*, *Pinnaxodes mutuensis*, and *Pinnotheres pisum* under artificial rearing conditions (Atkins, 1955; Konishi, 1981; Warner, 1968; Williamson, 1965). These observations suggest that morphological variations in final stage zoeae and megalopae are relatively common among crab species, and abnormality in morphogenesis has been a bottleneck limiting the hatchery production of commercially important brachyuran crabs.

To determine the cause of abnormal morphogenesis in brachyuran crab larvae, there is a need to improve our knowledge of the control mechanisms for larval morphogenesis, in particular the regulation of metamorphosis into megalopae by the eyestalk neurosecretory system. Therefore, we conducted eyestalk ablation on *P. trituberculatus* larvae at various times during zoeal development. To quantify the effects of treatment on larval morphogenesis, we measured the dimensions of larval body parts that are enlarged during metamorphosis (e.g., the chelae and pleopods; megalopal features) and those that are resorbed during metamorphosis (e.g., dorsal spine and telson furcae; zoeal features). Our results provide new insights into the causes of abnormal morphogenesis of brachyuran crab larvae under artificial rearing conditions.

3.3.3 Materials and Methods

3.3.3.1 Broodstock and larvae

A total of 12 ovigerous female *Portunus trituberculatus* were captured by small beam-trawls between July 24 and 25, 2011 in the Seto Inland Sea off Asakuchi, Okayama, Japan. They were transferred to the Tamano laboratory of the National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, Tamano, Okayama, Japan, on July 25 at 11:00 h, and stocked in separate cages in a 1.7 kL fibreglass-reinforced plastic (FRP) tank. Sand-filtered

and ultraviolet-irradiated seawater was supplied to the tank with a flow-through water system. Three females (carapace width: 179, 206 and 210 mm) carrying eggs which could be predicted to hatch within a day were selected by microscopic observation of egg subsamples according to Hamasaki et al. (2003). At 16:00 h, each female was transferred to a cylindrical 600 L FRP tank containing still seawater sterilised with chlorine (5 ppm) and neutralised with sodium thiosulfate. The water temperature was maintained at 25 °C, and aeration was provided via an air stone. As expected, these females hatched their eggs during the night, July 25, and their larvae were used for the experiments outlined below beginning the next morning.

3.3.3.2 Larval rearing in a 500 L tank

A subsample of the first stage zoeae (Z1) that hatched from each female were transferred from the hatching tank to a 30 L chamber, and the number of larvae was determined using a volumetric method based on three 50 mL samples taken from the chamber after the water had been agitated to ensure a homogeneous distribution of the larvae. A mixture of equal numbers of Z1 larvae from each of the 3 females was stocked and reared in a cylindrical 500 L polyethylene tank filled with sand-filtered seawater sterilised with chlorine (10 ppm) and neutralised with sodium thiosulfate prior to larval stocking. The stocking density of Z1 was 8,000 individuals per 500 L. Sodium nifurstyrenate (2 mg L^{-1}, Ueno Fine Chemicals Industry) was added at the beginning of the larval rearing period to prevent bacterial infection. The seawater in the rearing tanks was not renewed during the larval culture period. The salinity of the rearing water was 33 ppt at the beginning and the end of the larval rearing. Gentle aeration was provided via an air stone. The photoperiod mimicked natural conditions: 14 h light (05:00 to 19:00 h) and 10 h dark. The mean ± sd light intensity at 13:00 h was 8477 ± 6710 lx. The water temperature was maintained by an air conditioner in the room in which the tank was placed. The mean ± sd values for water temperature, dissolved oxygen, and pH measured once a day during the rearing period were 25.6 ± 0.1 °C, 8.00 ± 0.34 mg L^{-1} and 8.06 ± 0.16, respectively.

The rearing tank was supplemented once on the day of stocking with rotifer *Brachionus plicatilis* species complex (25 individuals mL^{-1}) that was cultured with commercially available concentrated phytoplankton *Nannochloropsis* sp. (Yanmarine K-1, Chlorella Industry). To maintain the rotifer population in the larval rearing tank, *Nannochloropsis* sp. was added to the tank twice a day to maintain a density of 200,000 cells mL^{-1} throughout the rearing period. *Artemia* cysts (Utah Strain, Kitamura) were hatched daily for 24 h at 26 °C, and newly hatched nauplii were added to the tank once each morning to maintain densities of 0.5 and 1.0 individuals

mL^{-1} for the third stage zoeae (Z3) and fourth stage zoeae (Z4), respectively. Every day, 20 larvae were sampled from the tank and observed using a stereo microscope. The larval moulting stage and the developmental stage within the moulting cycle was determined according to the criteria as described by Anger (2001b).

3.3.3.3 Larval treatment

P. trituberculatus have four zoeal stages (Z1 to Z4) and the stalked eyes begin to develop when larvae moult to the second zoeal stage (Z2). Therefore, eyestalk ablation was performed on Z2 to Z4 larvae. The larvae were collected from the 500 L tank, and the bilateral eyestalks were ablated using two thin needles with handles under a stereo microscope. Simultaneously, the larval moulting stage and the developmental stage within the moulting cycle were verified. The eyestalk-ablated larvae were stocked in 1 L plastic beakers, then reared until the Z4 larvae had moulted to the next stage, which is termed the fifth instar stage in this study. To test the potential effects of physical damage associated with eyestalk ablation, a comparative treatment was included in which two-thirds of the dorsal spines of larvae were ablated using the same operating technique as with eyestalk ablation. Additionally, to control for the effects of loss of vision in the eyestalk-ablated individuals, a group of intact larvae were reared in 1 L beakers in complete darkness (0 lx: darkness control). Lastly, a control group (handling) consisting of intact larvae that were transferred from the 500 L tank to 1 L beakers was also prepared. All treatments were conducted on the first day of the Z2 (2 day after hatching [dah]), Z3 (4 dah), and Z4 (6 dah) stages. These larvae were classified as being in the postmoult period (Stage B, in sensu Anger, 2001b). To evaluate the effect of timing of eyestalk ablation within the moulting cycle across the entire zoeal period on larval morphogenesis, eyestalk ablation was also conducted in the premoult period (Stage D1 to D2) of Z2 (3 dah) and Z3 (5 dah) larvae, and in the intermoult period (Stage C, 7 dah) and the premoult period (Stage D2 to D3, 8 dah) of Z4 larvae.

3.3.3.4 Larval rearing in 1 L beakers

A total of 25 larvae were stocked in each 1 L beaker and larval rearing was conducted in quadruplicate for each treatment group. The beakers containing the control, dorsal spine ablation, and eyestalk ablation treatment groups were placed in shallow water baths, and the temperature was maintained at 25.6 ± 0.4 °C (mean ± sd) by a heater connected to a thermostat. The water temperature of the darkness control was maintained at 25.5 °C using an incubator. The beakers were lightly aerated to provide oxygen and sufficient turbulence to prevent the rotifers and

Artemia from settling. All the beakers except for those of the darkness control were located in the laboratory where the 500 L tank was placed. Thus, the photoperiod was the same as that described for the 500 L tank. Each morning, larvae were transferred with a large-mouthed pipette to newly prepared beakers containing freshly sterilised seawater and food. To prevent bacterial proliferation, dihydrostreptomycin sulphate (50 mg L^{-1}, Tamura Seiyaku) was added to the sea-water. The rotifers cultured with *Nannochloropsis* sp. were fed to the larvae at a density of 50 individuals mL^{-1} throughout the zoeal stages. Newly hatched *Artemia* nauplii were also provided to the larvae at densities of 0.5 and 1.0 individuals mL^{-1} during the Z3 and Z4 stages, respectively. The number of live and dead larvae and the larval moulting stage were determined daily. As the concentration of the *Artemia* supplementation varied among the moult stages, the individuals at different moult stages within a group/replicate were reared in separate beakers.

For morphological measurements, five recently moulted Z4 larvae and all fifth instar larvae were sampled from each replicate group, fixed with 10 % neutral formalin for 24 h and then preserved in a 70 % ethanol solution.

3.3.3.5 Morphological measurements

We selected the morphological traits which indicate the significant changes related to metamorphosis from zoeal to megalopal stages during normal morphogenesis, e.g., enlargement of chelae and pleopods, and resorption of a dorsal spine and telson furcae (Table 3.3.1). Therefore, we measured the length of chela (CHL) and the third pleopod (PL) in Z4, and the CHL in fifth instar larvae using a microscope (Fig. 3.3.1A, B). These values were used as indices of morphological enlargement during the metamorphosis into megalopae (MG) (Table 3.3.1). Additionally, we measured dorsal spine length (DSL) and total length of the bilateral telson furcae (FL) in Z4 and fifth instar larvae (Fig. 3.3.1C, F). These values were used as indices of morphological resorption during metamorphosis into MG. To exclude the effect of differences in somatic growth among treatments, the ratios of CHL, PL, DSL and FL to the carapace length (CL) were also calculated and are reported as CHLr, PLr, DSLr and FLr, respectively. Because the dorsal spine and the telson furcae disappear completely in normal MG, the proportion (frequency) of fifth instar larvae that retained the dorsal spines (DSf) and furcae on their telsons (TELf) was calculated and used as an index of incomplete metamorphosis. The morphological

Table 3.3.1. Abbreviations used to represent the morphological measurements taken from fourth stage zoeae and fifth instar larvae (supernumerary fifth stage zoeae and megalopae) of the swimming crab *Portunus trituberculatus*, and their normal changes during metamorphosis into megalopae. See Fig. 3.3.1 for further details

Abbreviation	Morphological trait	Changes during metamorphosis
CL	Carapace length (μm)	–
CHL	Chela length (μm)	Increase
CHLr	Ratio of CHL to CL (%)	Increase
PL	Third pleopod length (μm)	Increase
PLr	Ratio of PL to CL (%)	Increase
DSL	Dorsal spine length (μm)	Resorbed
DSLr	Ratio of DSL to CL (%)	Decrease
FL	Sum of bilateral furcae length on telson (μm)	Resorbed
FLr	Ratio of FL to CL (%)	Decrease
DSf	Percentage of fifth instar larvae that retain dorsal spines	Normally zero
TELf	Percentage of fifth instar larvae that retain furcae on the telson	Normally zero
Z5f	Percentage of fifth stage zoeae relative to the number of fifth instar larvae	Normally zero

measurements for CHL, PL and DSL and the shape of telson are described in more detail in Chapter 3.2 (Dan et al., 2013). In some eyestalk ablation treatment replicates, some fifth instar larvae exhibited morphological features typical of zoeae (e.g., laterally compressed carapace, long dorsal and rostrum spines, maxillipeds that function to aid swimming, non-functional chelipeds and walking legs, and forked telson) (Gore, 1985) (Fig. 3.3.1D). We identified these larvae as a supernumerary zoeal stage (fifth stage zoeae, Z5) according to Costlow (1966a). We then calculated the frequency of Z5 to the total number of fifth instar larvae (Z5f). As the carapace shape of Z5 was completely identical to that of Z4 and individuals frequently lost or bent the tip of their long rostrum spine, the CL of Z5 was measured according to the method used for Z4 (Fig. 3.3.1A, D). The abbreviations used for the morphological measurements are provided in Table 3.3.1.

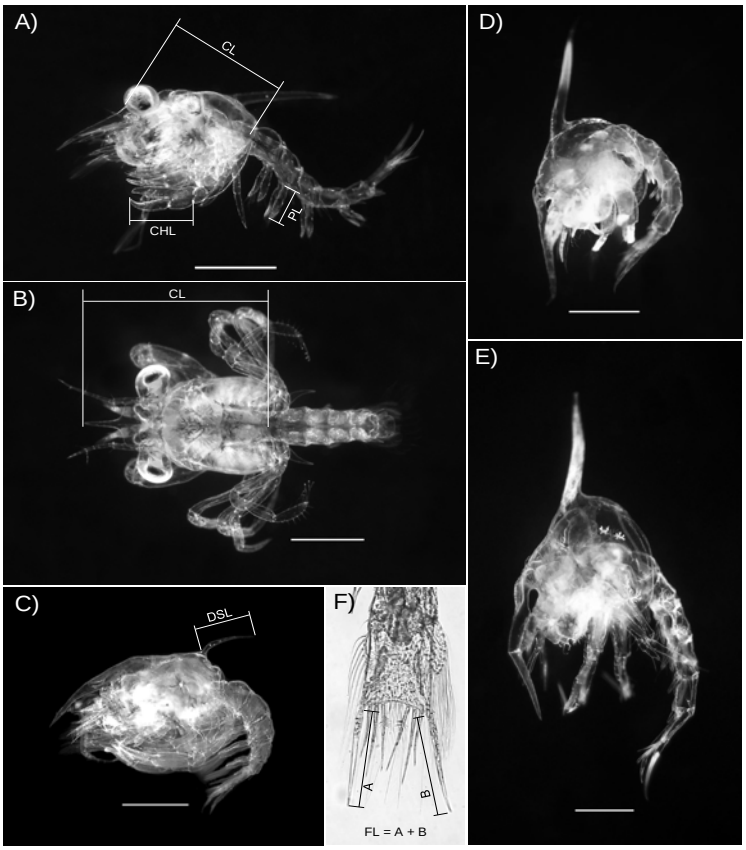

Figure 3.3.1. Photographs of zoeae and megalopae of the swimming crab *Portunus trituberculatus* showing schematic diagrams of morphological measurements. (A) Fourth stage zoea; (B) megalopa; (C) morphologically immature megalopa induced by bilateral eyestalk ablation during premoult of the third zoeal stage; (D) supernumerary fifth stage zoea induced by bilateral eyestalk ablation during the postmoult of the second zoeal stage; (E) sixth stage zoea moulted from a supernumerary fifth stage zoea; (F) telson of megalopa retaining furcae. CL: carapace length; CHL: chela length; PL: pleopod length; DSL: dorsal spine length; FL: total furcae length. Scale bars = 1 mm

3.3.3.6 Data analysis

Statistical analyses were performed using R (R 3.0.2; R Development Core Team, 2013) with a 5 % significance level. To examine the effects of treatments (control, darkness, dorsal spine ablation and eyestalk ablation) on survival and developmental velocity of larvae treated at the postmoult stage in Z2, Z3 and Z4, we applied a generalised linear model (GLM) (Everitt and Hothorn, 2009; McCullagh and Nelder, 1989) with the *glm* function. To test for differences in survival, the cumulative numbers of live and dead animals to reach each larval moulting stage were used as the response variable with the quasi-binomial family (logit link), to account for the overdispersion of error distribution. To avoid the errors attributed to zero data in the analyses using the quasi-binomial family, the groups showing 100 % survival were excluded from the analyses. In the analyses of larval developmental velocity, the number of days to reach each moulting stage by each larva was used as the response variable with the Poisson family (logarithmic link). In these analyses, the explanatory variable was treatment (control, darkness, dorsal spine ablation and eyestalk ablation). The statistical significance of the explanatory variables was evaluated with an F test (quasi-binomial family) or the Wald χ^2 test (Poisson family) using the

Anova function (Type II) implemented in the car package (Fox and Weisberg, 2011). Furthermore, differences between treatments were evaluated with the Tukey method using the *glht* function implemented in the multcomp package (Hothorn et al., 2008).

The effect of the treatments on morphology was also analysed using the GLM. The morphological measurements were used as response variables with the Gaussian family (identical link) (CL, CHL, CHLr, PL, PLr, DSL, DSLr, FL and FLr) or quasi-binomial family (logit link) (Z5f, DSf and TELf), to account for the overdispersion of error distribution. In these analyses, the explanatory variables were larval age and the categorical fixed factors (treatments). Because the dorsal spine and telson furcae were absent in almost all fifth instar larvae from the control, darkness, and dorsal spine ablation treatments, the DSL and FL values were very low (they moulted to normal megalopae). Thus, we did not compare the DSLr and FLr of the fifth instar larvae among treatments. Furthermore, data for the CL and CHLr of Z5 from the eyestalk ablation treatment were not included in the analyses for the fifth instar larvae, because the method of measuring CL differed between Z5 and MG (Fig. 3.3.1).The significance of the explanatory variables was evaluated with an *F* test and differences between treatments were tested using the *glht* function.

To evaluate the effects of timing of eyestalk ablation at various points in the moulting cycle throughout the entire zoeal period on morphogenesis, larval age was used as the explanatory variable in the GLM. In this analysis, morphological measurements were used as response variables and the significance of the explanatory variable was evaluated using an *F* test. As the method of measuring CL differed between Z5 and MG in the fifth instar larvae (Fig. 3.3.1), the data for CL, CHLr, PLr, DSLr and FLr from Z5 and MG were analysed separately.

3.3.4 Results

3.3.4.1 Larval survival

The larvae developed successfully through their fifth instar stages in all treatments tested (Fig. 3.2.2). When eyestalks were ablated at the Z2-postmoult stage, the treatments had a significant effect on survival to all moulting stages (to reach Z3, $F = 10.87$, $df = 3, 12$, $P = 0.0010$; to reach Z4, $F = 10.92$, $df = 3, 12$, $P = 0.0010$; to reach fifth instar stage, $F = 8.103$, $df = 3, 12$, $P = 0.0032$), and survival to the fifth instar stage was significantly lower in the eyestalk ablation group (8.8 ± 2.1 individuals; mean ± sd) than in other treatment groups (control, 14.0 ± 0.0 individuals;

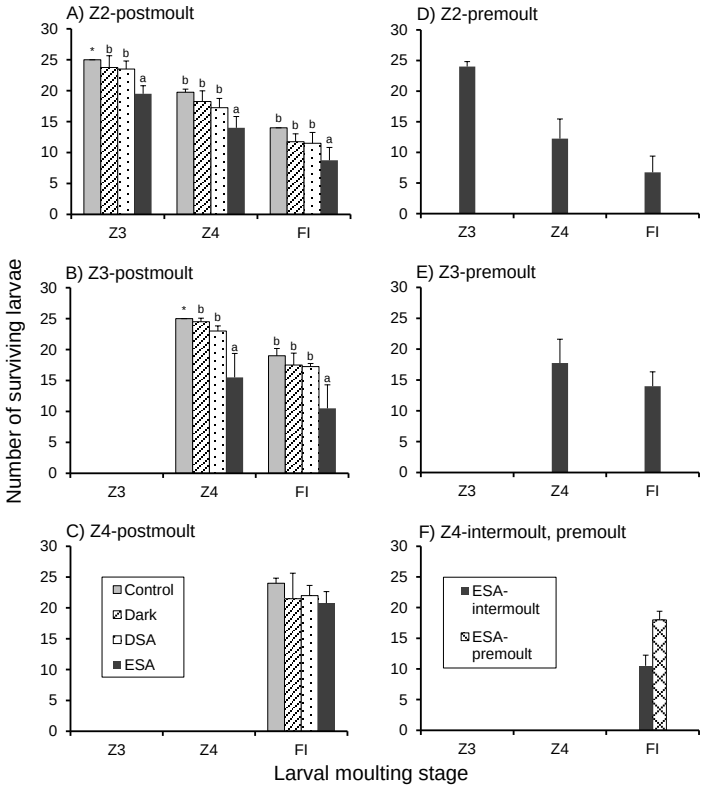

Figure 3.3.2. Number of surviving *Portunus trituberculatus* larvae that reach each larval moulting stage following treatment during the postmoult of the (A) second (Z2), (B) third (Z3) or (C) fourth (Z4) zoeal stages, premoult of the (D) second or (E) third zoeal stages, or (F) intermoult or premoult of the fourth zoeal stage. Vertical bars indicate the standard deviations of 4 replicates in each treatment group. Significant differences between the control (Control), darkness (Dark), dorsal spine ablation (DSA), and eyestalk ablation (ESA) treatment groups are indicated by a different letter ($P < 0.05$). FI: fifth instar stage including fifth zoeal and megalopal stages. Asterisks indicate the groups with 100 % survival which were excluded from the analyses to avoid errors

darkness, 11.8 ± 1.3 individuals; dorsal spine ablation, 11.5 ± 1.7 individuals) (Fig. 3.3.2A). When eyestalks were ablated at the Z3-postmoult stage, the treatments also had a significant effect on survival to all moulting stages (to reach Z4, $F = 31.71$, $df = 3, 12$, $P < 0.0001$; to reach fifth instar stage, $F = 11.52$, $df = 3, 12$, $P = 0.0008$), and survival to the fifth instar stage was again significantly lower in the eyestalk ablation group (10.5 ± 3.8 individuals) than in other treatment groups (control, 19.0 ± 1.2 individuals; darkness, 17.5 ± 1.9 individuals; dorsal spine ablation, 17.3 ± 0.5 individuals) (Fig. 3.3.2B). When eyestalks were ablated at the Z4-postmoult stage, treatment did not affect survival ($F = 1.655$, $df = 3, 12$, $P = 0.2292$), and the mean number of surviving larvae in each treatment group ranged from 20.8 to 24.0 individuals (Fig. 3.3.2C). Overall, the highest and the lowest survival was in the control and eyestalk ablation groups, respectively. In the eyestalk ablation treatment groups, the mean number of surviving fifth instar larvae following ablation treatment at the Z2-, Z3- and Z4-premoult, and Z4-intermoult stages ranged from 6.8 to 18.1 individuals (Fig. 3.3.2D−F) and was similar to the range in the postmoult treatments (Fig. 3.3.2A−C). Thus, eyestalk ablation reduced larval survival.

3.3.4.2 Larval developmental velocity

When different treatments were conducted during the Z2-postmoult, they did not have a significant effect on the cumulative developmental time required to reach the Z3 stages (χ^2 = 6.563, *df* = 3, *P* = 0.0872), but did have significant effects on the time needed to reach the Z4 and fifth instar stages (to reach Z4, χ^2 = 8.753, *df* = 3, *P* = 0. 0328; to reach fifth instar stage, χ^2 = 13.32, *df* = 3, *P* = 0.0040). The developmental time required to reach the fifth instar stage was significantly shorter in the control and eyestalk ablation group (control; 10.6 ± 0.14 dah, eyestalk ablation; 10.5 ± 0.60 dah) than in the darkness group (12.8 ± 0.26 dah), and there was no significant difference between the dorsal spine ablation group (11.4 ± 0.23 dah) and other groups (Table 3.3.2). When treatments were carried out during the Z3-postmoult, they did not significantly affect developmental time (to reach Z4, χ^2 = 0.1042, *df* = 3, *P* = 0.9913; to reach fifth instar stage, χ^2 = 5.736, *df* = 3, *P* = 0.1252). The time needed to reach the fifth instar stage ranged from 9.6 to 11.1 dah. When treatments were performed during the Z4-postmoult, they had no significant effect on the developmental time required to reach the fifth instar stage (χ^2 = 0.9132, *df* = 3, *P* = 0.8222), which ranged from 9.5 to 9.9 dah. The developmental time needed to reach the fifth instar stage in the eyestalk ablation groups treated at Z2-, Z3- and Z4-premoult, and Z4-intermoult were 9.9 ± 0.48, 9.3 ± 0.14 9.0 ± 0.06 and 9.7 ± 0.14 dah, respectively. Thus, eyestalk ablation did not affect the larval developmental velocity.

3.3.4.3 Morphology of Z4 larvae

The mean values of measurements in each group are summarised in Tables S3.3.1 and S3.3.2 in the Appendix, and the comparisons between treatments or larval ages at treatments are shown in Figs. 3.3.3 and 3.3.4. The treatments significantly affected the CL, CHL, CHLr, PL and PLr values of Z4 larvae (CL, *F* = 10.42, *df* = 3, 27, *P* = 0.0001; CHL, *F* = 26.68, *df* = 3, 27, *P* < 0.0001; CHLr, *F* = 29.04, *df* = 3, 27, *P* < 0.0001; PL, *F* = 22.73, *df* = 3, 27, *P* < 0.0001; PLr, *F* = 23.96, *df* = 3, 27, *P* < 0.0001). The CL values were significantly smaller in individuals from the darkness and eyestalk ablation treatment groups relative to the other treatment groups (Fig. 3.3.3A). The CHL, CHLr, PL and PLr values were lower in eyestalk-ablated larvae than in the other treatment groups (Fig. 3.3.3B, C, F, and G). The larval age (Z2- and Z3-postmoult) when treatments were conducted did not significantly affect the CL (*F* = 3.862, *df* = 1, 27, *P* = 0.060), but did have a significant effect on CHL, CHLr, PL and PLr (CHL, *F* = 5.987, *df* = 1, 27, *P* = 0.0212; CHLr,

Table 3.3.2. Cumulative developmental time required to reach each moulting stage of *Portunus trituberculatus* larvae treated during the postmoult of the second (Z2), third (Z3), or fourth (Z4) zoeal stages, premoult of Z2 or Z3, or intermoult and premoult of Z4. Values are mean ± sd. Significant differences were found between treatments with different superscripts in the same column within the same treatment (multiple comparisons with the Tukey method, $P < 0.05$). dah: days after hatching, FI: fifth instar stage including fifth zoeal and megalopal stages

Time at treatment	Treatment	Cumulative developmental time (dah)		
		Z3	Z4	FI
Z2-postmoult (2 dah)	Control	4.2 ± 0.16	7.3 ± 0.15[a]	10.6 ± 0.14[a]
	Darkness	4.9 ± 0.07	8.5 ± 0.20[b]	12.8 ± 0.26[b]
	Dorsal spine ablation	4.9 ± 0.06	8.0 ± 0.06[ab]	11.4 ± 0.23[ab]
	Eyestalk ablation	4.8 ± 0.26	7.5 ± 0.25[ab]	10.5 ± 0.60[a]
Z2-premoult (3 dah)	Eyestalk ablation	4.2 ± 0.12	7.1 ± 0.13	9.9 ± 0.48
Z3-postmoult (4 dah)	Control		6.9 ± 0.06	10.2 ± 0.10
	Darkness		7.0 ± 0.03	11.1 ± 0.10
	Dorsal spine ablation		7.0 ± 0.04	10.2 ± 0.08
	Eyestalk ablation		7.0 ± 0.05	9.6 ± 0.12
Z3-premoult (5 dah)	Eyestalk ablation		6.0 ± 0.00	9.3 ± 0.14
Z4-postmoult (6 dah)	Control			9.8 ± 0.15
	Darkness			9.9 ± 0.10
	Dorsal spine ablation			9.9 ± 0.05
	Eyestalk ablation			9.5 ± 0.33
Z4-intermoult (7 dah)	Eyestalk ablation			9.7 ± 0.14
Z4-premoult (8 dah)	Eyestalk ablation			9.0 ± 0.06

$F = 5.433$, $df = 1, 27$, $P = 0.0275$; PL, $F = 9.686$, $df = 1, 27$, $P = 0.0044$; PLr, $F = 9.382$, $df = 1, 27$, $P < 0.0001$). The differences between larval ages were minor within the control, darkness, and dorsal spine ablation groups (Fig. 3.3.3A−C, F, G). However, CL, CHL, PL, CHLr and PLr increased significantly as the age at eyestalk ablation increased (Fig. 3.3.3A−C, F, G) (see statistical values in Fig. 3.3.4A−C, F, G). The treatments also significantly affected the DSL and DSLr values (DSL, $F = 132.65$, $df = 3, 27$, $P < 0.0001$; DSLr, $F = 136.30$, $df = 3, 27$, $P < 0.0001$), which were lowest in the dorsal spine ablation group and highest in the eyestalk ablation group (Fig. 3.3.3D, H). The larval age at which treatments were conducted had no effect on the DSL and DSLr (DSL, $F = 0.9332$, $df = 1, 27$, $P = 0.3426$; DSLr, $F = 2.219$, $df = 1, 27$, $P = 0.1480$) (Fig. 3.3.3D, H). Similarly, the timing of eyestalk ablation had no effect on DSL and DSLr (Fig. 3.3.4D, H). The treatments had a significant effect on FLr but not FL (FL, $F = 0.7048$, $df = 3, 27$, $P = 0.5575$; FLr, $F = 0.4.587$, $df = 3, 27$, $P = 0.0101$). The dorsal spine ablated larvae had smaller

FLr values than larvae from other treatment groups (Fig. 3.3.3E, I). The larval age at treatment had a significant effect on the FL and FLr values (FL, F = 7.178, df = 1, 27, P = 0.0124; FLr, F = 6.367, df = 1, 27, P = 0.0178): the larvae treated at Z3-postmoult had slightly higher FL scores but lower FLr values than the larvae treated at Z2-postmoult (Fig. 3.3.3E, I). The timing of eyestalk ablation did not significantly affect the FL (Fig. 3.3.4E), whereas a delay in eyestalk ablation was associated with a slight but significant decrease in FLr (Fig. 3.3.4I). Thus, there were distinct differences in the effects of eyestalk ablation on the morphological character traits of Z4 larvae. The morphogenesis of chelae and pleopods, which become enlarged towards MG, was consistently suppressed by eyestalk ablation, and this effect became more prominent when ablation was performed earlier. Conversely, eyestalk ablation had little effect on the dorsal spine and telson furcae, which disappear during metamorphosis into MG.

3.3.4.4 Morphology of fifth instar larvae

The mean values of measurements in each group are summarised in Tables S3.3.3, S3.3.4 and S3.3.5 in the Appendix, and the comparisons between treatments or larval ages at treatments are shown in Figs. 3.3.5, 3.3.6 and 3.3. 7. The treatments significantly affected all the measurements (CL, CHL, CHLr, DSL, FL, DSf, TELf) of fifth instar larvae (CL, F = 7.659, df = 3, 35, P = 0.0005; CHL, F = 29.057, df = 3, 43, P < 0.0001; CHLr, F = 4.556, df = 3, 35, P < 0.0001; DSL, F = 24.838, df = 3, 43, P < 0.0001; FL, F = 25.975, df = 3, 43, P < 0.0001; DSf, F = 10.417, df = 3, 43, P < 0.0001; TELf, F = 15.371, df = 3, 43, P < 0.0001). The CL was lower in the darkness treatment group than in the other treatments (Fig. 3.3.5A). The eyestalk ablated larvae had higher DSL, FL, DSf and TELf values and lower CHL and CHLr values than the other treatment groups, particularly when ablation was conducted during the postmoult of Z2 and Z3 (Fig. 3.3.5B−G). The effects of larval age at treatment on the CHL, CHLr, DSL, FL and TELf were significant (CHL, F = 13.77, df = 1, 43, P = 0.0006; CHLr, F = 5.453, df = 1, 35, P = 0.0254; DSL, F = 8.009, df = 1, 43, P = 0.0070; FL, F = 14.03, df = 1, 43, P = 0.0005; TELf, F = 43.37, df = 1, 43, P < 0.0001), but the differences between larval ages (Z2-, Z3- and Z4-postmoult) were small in the control, darkness and dorsal spine ablation groups compared with the eyestalk ablation group (Fig. 3.3.5B−G). Larval age at which treatments were conducted had no effect on CL and DSf (CL, F = 0.4696, df = 1, 35, P = 0.4977; DSf, F = 0.7006, df = 1, 43, P = 0.4072).

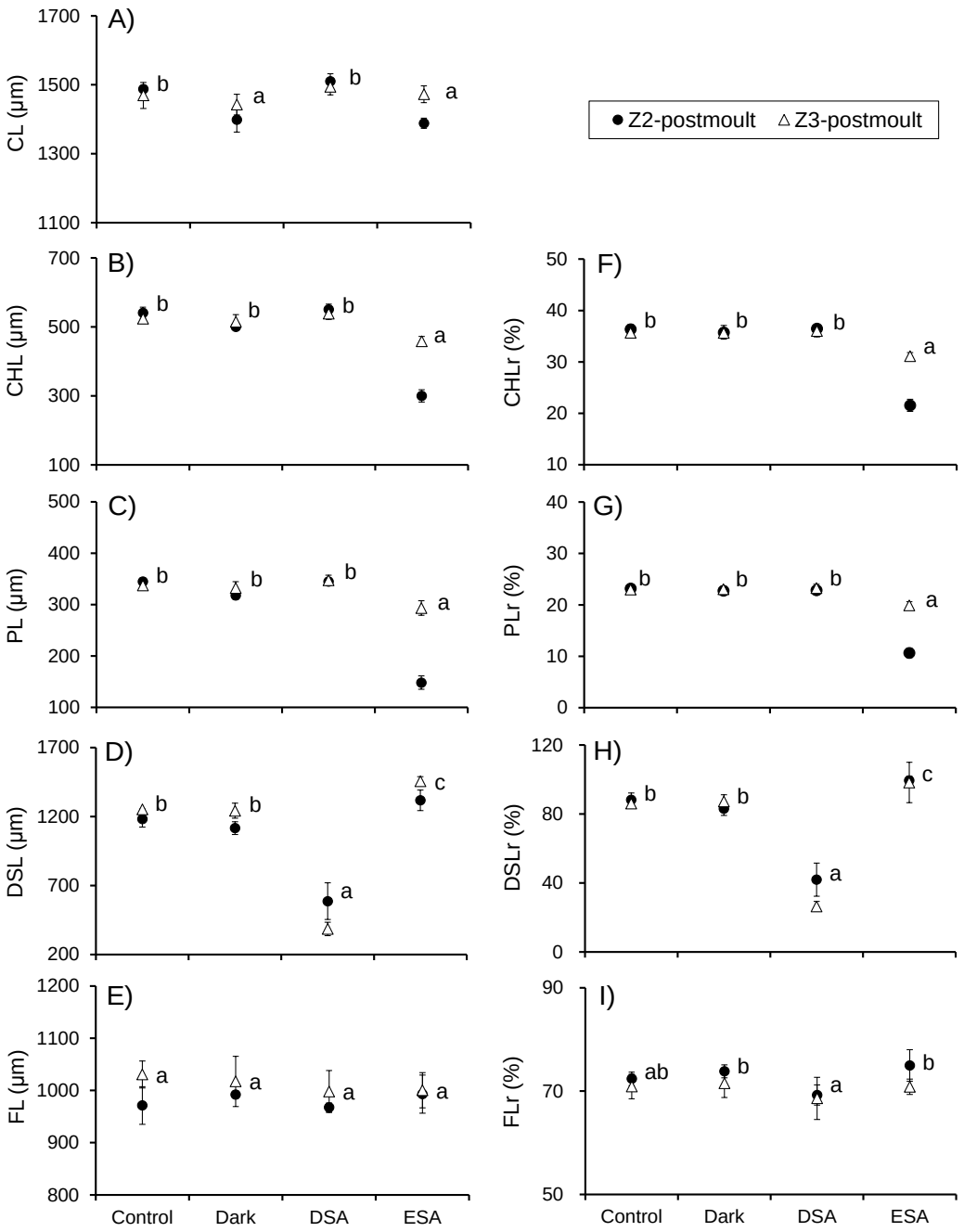

Figure 3.3.3. Differences in (A) carapace length (CL), (B) chela length (CHL), (C) pleopod length (PL), (D) dorsal spine length (DSL), (E) telson furcae length (FL), (F) relative chela length (CHLr), (G) relative pleopod length (PLr), (H) relative dorsal spine length (DSLr), and (I) relative telson furcae length (FLr) in fourth stage zoeae of *Portunus trituberculatus* between the control (Control), darkness (Dark), dorsal spine ablation (DSA) and eyestalk ablation (ESA) treatment groups. Vertical bars indicate the standard deviations of the 4 replicates in each treatment group. Significant differences among treatments are indicated by a different letter ($P < 0.05$)

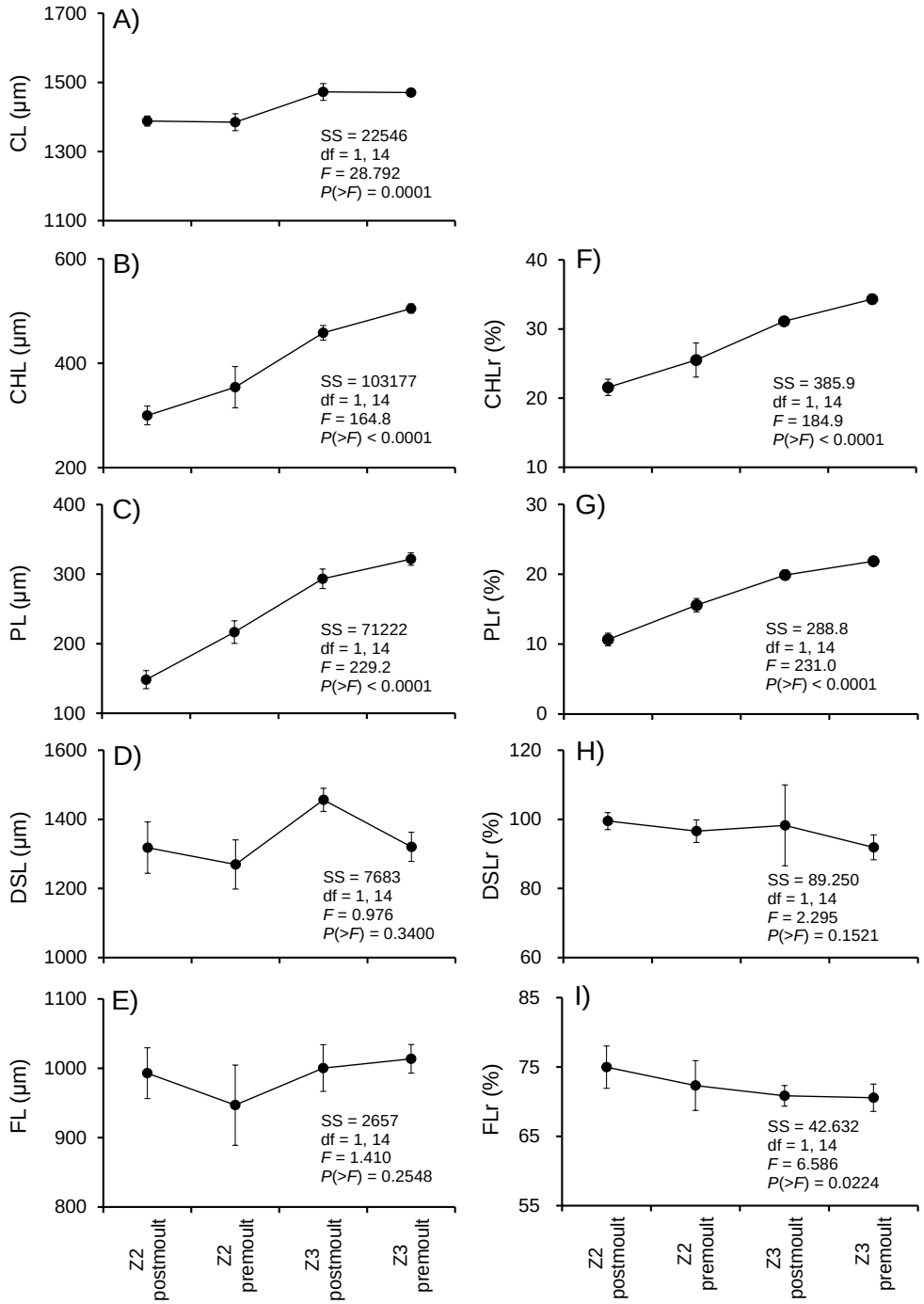

Figure 3.3.4. Changes in (A) carapace length (CL), (B) chela length (CHL), (C) pleopod length (PL), (D) dorsal spine length (DSL), (E) telson furcae length (FL), (F) relative chela length (CHLr), (G) relative pleopod length (PLr), (H) relative dorsal spine length (DSLr), and (I) relative telson furcae length (FLr) of fourth stage zoeae of *Portunus trituberculatus* following eyestalk ablation at various times. Vertical bars indicate the standard deviations of the 4 replicates in each treatment group. The results of analysis of deviance by an *F* test (Type II) to evaluate the effects of time of eyestalk ablation are shown for each measurement

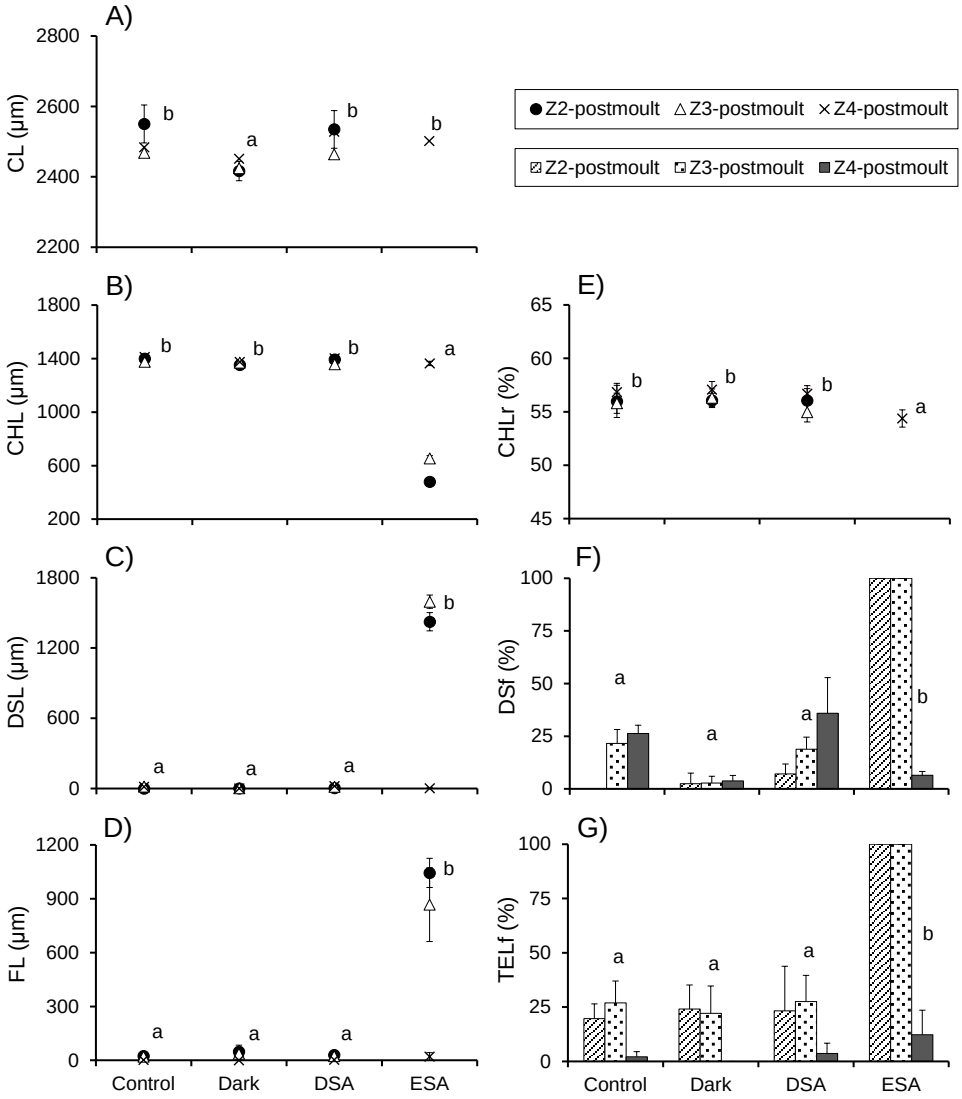

Figure 3.3.5. Differences in (A) carapace length (CL), (B) chela length (CHL), (C) dorsal spine length (DSL), (D) telson furcae length (FL), (E) relative chela length (CHLr), (F) frequency of retained dorsal spine (DSf) and (G) frequency of retained furcae on the telson (TELf) of fifth instar *Portunus trituberculatus* larvae between the control (Control), darkness (Dark), dorsal spine ablation (DSA) and eyestalk ablation (ESA) treatment groups. Vertical bars indicate the standard deviations of the 4 replicates in each treatment group. Significant differences among treatments are indicated by a different letter ($P < 0.05$)

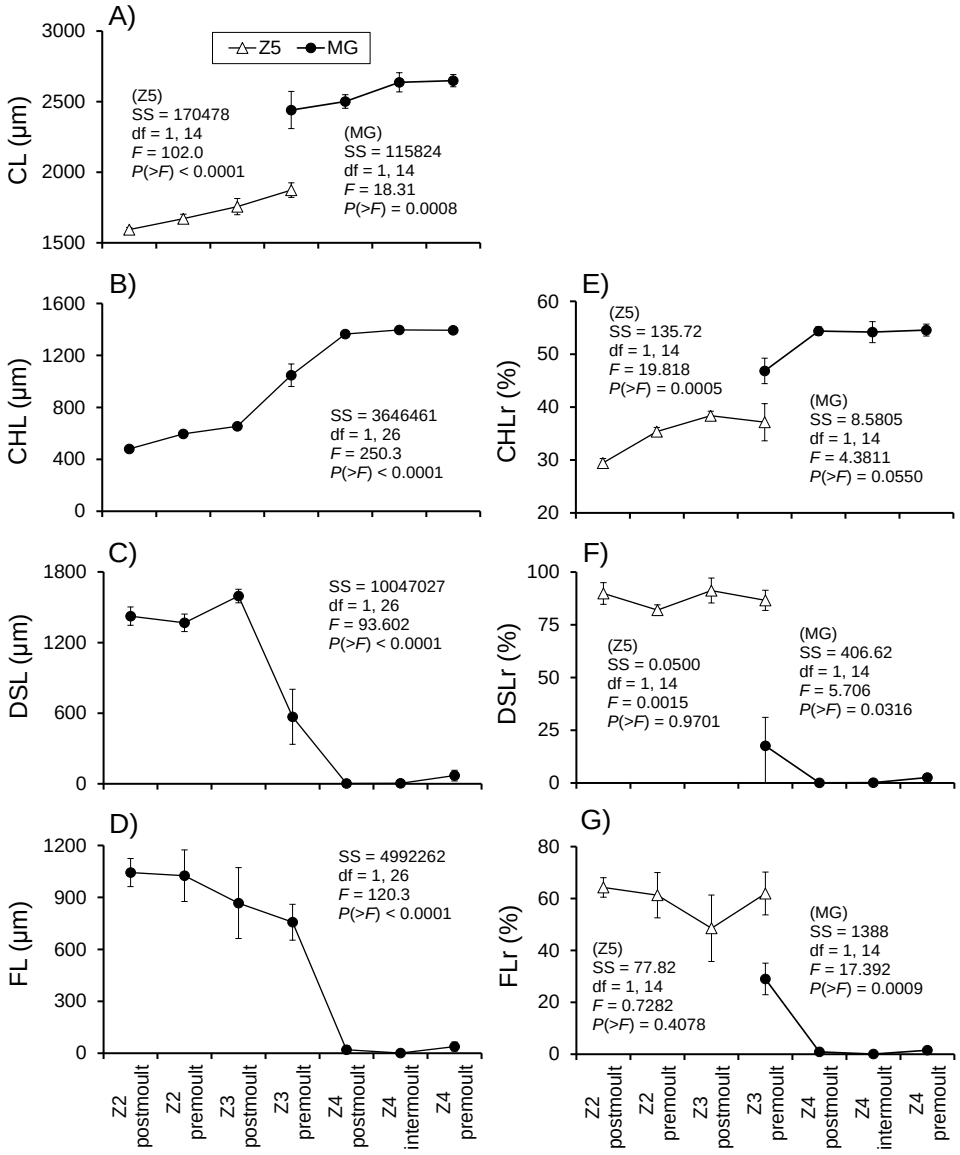

Figure 3.3.6. Changes in (A) carapace length (CL), (B) chela length (CHL), (C) dorsal spine length (DSL), (D) telson furcae length (FL), (E) relative chela length (CHLr), (F) relative dorsal spine length (DSLr), and (G) relative telson furcae length (FLr) of fifth instar *Portunus trituberculatus* larvae following eyestalk ablation at various times. Vertical bars indicate the standard deviations of the 4 replicates in each treatment group. The results of an analysis of deviance by an *F* test (Type II) to evaluate the effects of timing of eyestalk ablation are shown for each measurement. Because the method of measuring carapace length differed between fifth stage zoeae (Z5) and megalopae (MG) (see Fig. 3.3.1), the data for CL, CHLr, PLr, DSLr and FLr for Z5 and MG were analysed separately

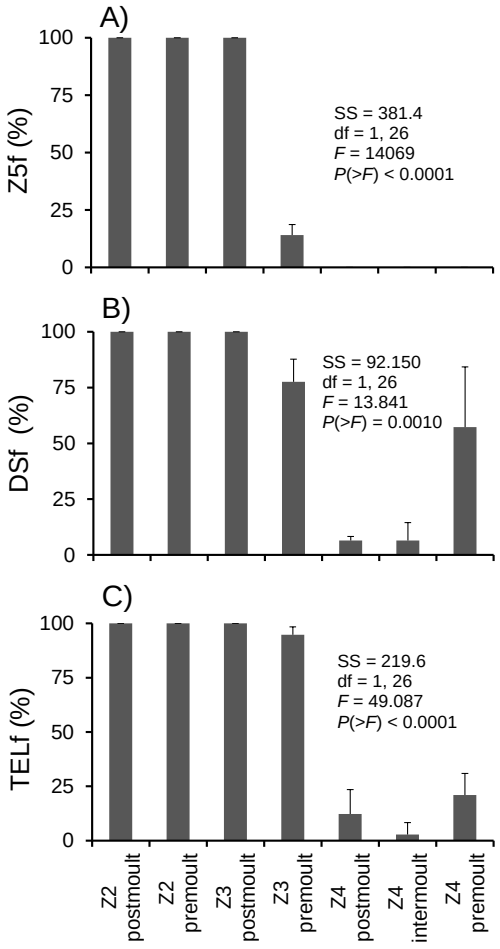

Figure 3.3.7. Changes in the frequency of occurrence of (A) supernumerary fifth stage zoea (Z5f), (B) retained dorsal spine (DSf), and (C) retained furcae on the telson (TELf) in fifth instar *Portunus trituberculatus* larvae following eyestalk ablation at various times. Vertical bars indicate the standard deviations of the 4 replicates in each treatment group. The results of an analysis of deviance by an *F* test (Type II) to evaluate the effects of timing of eyestalk ablation on the measurements are shown for each morphological trait

All larvae in the eyestalk ablation treatment group moulted to Z5 when the eyestalks were ablated prior to the postmoult of Z3 (Figs. 3.3.1D, 3.3.6A and 3.3.7A). In a preliminary rearing experiment using eyestalk-ablated zoeae, we confirmed that these Z5 larvae moulted to the next instar stage and had zoeal morphology (sixth stage zoeae, Z6) (Fig. 3.3.1E). In Z5 larvae produced by eyestalk ablation before the Z3-premoult, the CL and CHLr tended to be larger as ablation was delayed, and DSLr and FLr were not significantly affected by the timing of ablation (Fig. 3.3.6A, E−G). The effects of eyestalk ablation on Z5 larvae were consistent with those observed in Z4 larvae. When the eyestalks were ablated at the premoult of Z3, the frequency of MG was higher than that of Z5 (Fig. 3.3.7A), but these MG had zoeal morphological features (as shown in Fig. 3.3.1C), such as small chela, a dorsal spine and telson furcae (Fig. 3.3.6E−G). Conversely, all larvae successfully moulted into MG when the eyestalks were ablated after the postmoult of Z4 (Fig. 3.3.7A), and few zoeal features were retained in these MG (Fig. 3.3.6B−G). The effects of timing of ablation on CHL, DSL, FL, DSf and TELf were all statistically significant (Figs.

3.3.6B−D and 3.3.7B, C). Our data suggest that metamorphosis to MG accompanying the resorption of a dorsal spine and telson furcae was inhibited by eyestalk ablation before the postmoult of Z3, and eyestalk ablation in the premoult of Z3 induced immature morphology in the MG.

3.3.5 Discussion and Conclusions

Eyestalk ablation reduced larval survival in *Portunus trituberculatus*. Because a number of hormones that play important physiological roles are secreted from eyestalks (e.g., crustacean hyperglycaemic hormone, moult-inhibiting hormone, mandibular organ-inhibiting hormone and vitellogenesis-inhibiting hormone) (Hopkins, 2012; Keller, 1992; Liu and Laufer, 1996), the absence of the eyestalk is expected to result in physiological abnormalities and lowered survival. However, in the present study, at least 42 % of eyestalk-ablated larvae survived to the fifth instar stage, suggesting that the absence of eyestalks is not lethal for crab larvae. In *Rhithropanopeus harrisii*, *Homarus americanus* and *Alpheus heterochaelis*, the carapace length of MG or postlarva was increased by eyestalk ablation at an early zoeal stage (Costlow, 1966a; Snyder and Chang, 1986; Gross and Knowlton, 1999). However, in this study, the CL was smaller in ablated Z4 individuals than in the control group. Because the CL of Z4 individuals that were reared in darkness was also smaller, the small CL in eyestalk-ablated larvae can be inferred to have been caused by their loss of vision. Andrés et al. (2010) demonstrated that constant darkness reduced the somatic growth of *Portunus pelagicus* larvae, by decreasing the number of prey encounters due to the low swimming activity of larvae in the dark. Thus, the reduction in food intake attributed to the loss of vision (darkness condition) may have affected the somatic growth of ablated larvae. Unfavourable dietary conditions result in the occurrence of the additional supernumerary zoeal stage (Minagawa and Murano, 1993; Zeng et al., 2004). However, the occurrence of supernumerary Z5 following early eyestalk ablation in the present study is not thought to be caused by lower food intake due to their loss of vision, because the larvae that were reared in the dark were able to moult into normal megalopae despite the small CL during the Z4 and MG stages. The duration of larval development was also not different between ablated and control larvae. Thus, the effects of eyestalk ablation on larval morphogenesis could be evaluated without being confounded by retardation or acceleration of growth.

Our study is the first to demonstrate the effects of eyestalk ablation on zoeal morphogenesis in decapod crustacean larvae. Our results clearly showed that the morphogenesis of chelae and

pleopods of Z4 larvae was suppressed by eyestalk ablation, and the effect was stronger when the treatment was applied earlier in development. The chelae and pleopods become enlarged as the larvae metamorphose into MG, suggesting that zoeal morphogenesis toward the megalopal stage is also regulated by the endocrine cells located in the eyestalks. Based on the timing of effects we observed, the signal from the eyestalks accelerates zoeal morphogenesis from early on in the zoeal stages, beginning no later than the postmoult of Z2. Though the larvae of *P. trituberculatus* normally start to develop rudimentary chelae and pleopods at the Z3 stage, the regulation of this morphogenesis begins prior to this, early in zoeal development. Furthermore, we observed a linear increase in CHLr and PLr as the timing of ablation was delayed, suggesting that the eyestalk neurosecretory system exerts continuous control throughout zoeal development. In contrast, eyestalk ablation had little effect on the dorsal spine or furcae on the telson of Z4 larvae, both of which disappear during metamorphosis into MG. This suggests that the resorption of these body parts is not under continuous regulation by the eyestalk neurosecretory system.

We observed a transition in the effects of eyestalk ablation on the morphology of the fifth instar larvae related to the time of ablation. Ablation prior to the premoult of Z3 clearly inhibited metamorphosis into MG. Ablation at this point (Z3-premoult) resulted in an intermediate morphology between zoeae and MG, with individuals retaining a small dorsal spine and furcae on the telson but having a megalopal-shaped carapace. These observations strongly suggest that the premoult of Z3 is a critical point with respect to the control of metamorphosis by the endocrine system in eyestalks. Costlow (1966a) demonstrated that metamorphosis into MG was inhibited when the eyestalk was ablated prior to the end of Z3 in *R. harrisii*, a species that normally terminates the zoeal stage at Z4. Similarly, eyestalk ablation prior to Z2 inhibited metamorphosis and resulted in the occurrence of a supernumerary Z4 in *Sesarma reticulatum*, a species that normally terminates the zoeal stage at Z3 (Costlow, 1966b). The critical time for *A. heterochaelis* and *H. americanus*, species that normally have three larval (zoeal) stages, was during the premoult of the second larval stage (Charmantier and Aiken, 1987; Charmantier et al., 1988; Gross and Knowlton, 2002; Knowlton, 1994). Thus, it appears to be a common occurrence among decapod crustacean species that the metamorphosis into postlarva is inhibited when the eyestalks are ablated before the premoult during the penultimate zoeal stage. The occurrence of larvae with morphology intermediate between zoeae and MG as a result of eyestalk ablation at this critical point can be interpreted as the outcome of incomplete metamorphosis owing to the lack of endocrine control by the neurosecretory system located in the eyestalks. Taken further, this

suggests that an endocrine signal is secreted from the eyestalks at precisely this time. The larvae were able to complete the metamorphosis into MG when the eyestalks were ablated immediately after the moult to Z4. Thus, the morphogenesis accompanying resorption of the dorsal spine and telson furcae appears to have already been initiated prior to the beginning of the last zoeal stage (Z4). Given this, the regulation of metamorphosis involving resorption by the eyestalk neurosecretory system appears to be instantaneous, likely controlled by a short-term surge in hormone/s.

We demonstrated that the neurosecretory system in the eyestalks most likely regulates larval morphogenesis in two ways. The morphogenesis of the chelae and pleopods is controlled continuously throughout the zoeal stages, whereas resorption of the dorsal spine and telson furcae is controlled instantaneously at a critical point during the premoult of Z3. An important factor in understanding this regulatory system is that the former is characterised by enlargement and the latter by resorption. Because the enlargement of body parts and appendages requires a large amount of energy, it may be advantageous to spread the energy demand over a longer period. In this sense, it is reasonable that larvae would enlarge their chelae and pleopods as much as possible when nutritional conditions allow during the zoeal stage, before metamorphosis, even though the chelae and pleopods are not functional during the zoeal stages. This strategy likely reduces the energy burden during metamorphosis and raises the success rate of metamorphosis. Conversely, the dorsal spine and telson furcae play important roles in preventing predation and facilitating swimming during zoeal stages (Morgan, 1989), but their resorption does not place energy demands on the individual. The larvae require these body parts during the zoeal stages, but they must then be quickly resorbed during metamorphosis. The two pathways by which the eyestalk neurosecretory system regulated morphogenesis in our study fit well with these complex requirements.

n-3 highly unsaturated fatty acids (n-3 HUFA) are essential fatty acids for larvae of *Scylla serrata* and *P. trituberculatus* (Hamasaki et al., 1998; Suprayudi et al., 2002a, 2004; Takeuchi et al., 1999a, c). Zoeal morphogenesis of the chelae and pleopods in *S. serrata* and *P. trituberculatus* can be accelerated by altering the dietary intake of n-3 HUFA, such as docosahexaenoic acid and eicosapentaenoic acid which are contained in rotifers (Arai et al., 2007; Dan and Hamasaki, 2011; Hamasaki et al., 2002b; Chapter 3.1). Zoeal morphogenesis is also affected by the salinity of the rearing water; the CHLr of the last zoeae of *S. serrata* was high when held at the optimal salinity but low at lower salinities (Dan and Hamasaki, 2011; Chapter 3.1). Thus, when zoeae experience good nutritional and environmental conditions (e.g., n-3 HUFA and salinity), they can divert more

energy into the morphogenesis of chelae and pleopods. In other words, the eyestalk neurosecretory system controls the partitioning of energy between somatic growth and morphogenesis towards MG, depending on the nutritional and environmental conditions. Interestingly, when larvae are supplied with high levels of n-3 HUFA under optimal rearing conditions during seed production, the chelae and pleopods become excessively enlarged, which results in mass mortality because of incomplete moulting into MG. The old exuviae cannot be shed because of the size of the chelae and walking legs, a phenomenon that is termed 'moult death syndrome' (Arai et al., 2004; Dan and Hamasaki, 2011; Hamasaki et al., 2002a; Mann et al., 1999; Silva et al., 2012; Takano et al., 2004; Chapter 3.1). Thus, the cause of moult death syndrome may relate to the regulation of morphogenesis by the eyestalk neurosecretory system as a normal response to the rearing condition. To prevent moult death syndrome, culturists should control dietary intake such that energy inputs remain below a certain threshold, to prevent excess growth (Hamasaki et al., 2002b).

We concluded that the abnormal morphology of MG (i.e., retention of immature zoeal traits) during seed production of *P. trituberculatus* was the major cause of mass mortality (Dan et al., 2013; Chapter 3.2). This is consistent with our current observation of immature morphology of *P. trituberculatus* MG following eyestalk ablation during the premoult of Z3; MG retained both a small dorsal spine and furcae on the telson. The resorption of these parts is thought to be instantaneously regulated by the eyestalk neurosecretory system during the premoult of Z3. Therefore, we speculate that the abnormalities occurring during seed production are related to disruption of the regulatory system in the eyestalks at this critical period. Recently, we found that the supplementation of phytoplankton such as *Chlorella vulgaris* and *Nannochloropsis* sp. into larval rearing water to provide food for rotifers was associated with increases in abnormal morphology of *P. trituberculatus* MG, specifically during the premoult of Z3 (see Chapter 3.4), which provides further support for the critical period hypothesis.

The role of the eyestalk neurosecretory system in morphogenesis during metamorphosis has been documented based on changes in phenotype. However, the endocrine system controlling this phenomenon is poorly understood. In adult crustaceans, the moulting cycle is regulated by the secretion of moult-inhibiting hormone from the X-organ sinus gland complex in the eyestalks and by ecdysteroid, which is produced in the Y-organ and regulated in turn by moult-inhibiting hormone (Hopkins, 2012; Keller, 1992; Nakatsuji et al., 2009). In addition to moult-inhibiting hormone and ecdysteroid, it is thought that the secretion of juvenile hormone (methyl farnesoate)

from the mandibular organ and mandibular organ-inhibiting hormone from the X-organ sinus gland complex play a role in regulating larval metamorphosis (Abdu et al., 1998a; Anger, 2001b; Freeman and Costlow, 1984; Gross and Knowlton, 2002). A number of studies have evaluated the effects of these hormones on metamorphosis by injection into the larval body or administration into larval rearing water and tissue culture medium (Abdu et al., 1998a, b; Charmantier et al., 1988; Freeman and Costlow, 1983, 1984; Snyder and Chang, 1986). However, the mechanisms by which these hormones exert their effects remain poorly understood. To document the actions of these hormones in larvae, there is a need for a highly sensitive analysis (e.g., gene expression analysis using PCR) linked to quantified morphological traits. We suggest that a species such as *P. trituberculatus* that exhibits significant morphological change during metamorphosis, having both enlarged and resorbed traits, may be suitable for further study.

3.4 Abnormal morphology in megalopae of the swimming crab, *Portunus trituberculatus* during seed production: causes and prevention

3.4.1 Summary

The occurrence of morphologically immature megalopae, which retain zoeal features such as dorsal spines and furcae of telson, is closely correlated with larval mass mortality during seed production of the swimming crab *Portunus trituberculatus* in Japanese hatcheries. To determine the cause of immature megalopal morphology, zoeae were reared with various supplementary schedules and density of diets (rotifer, *Artemia*, and phytoplanktons including *Chlorella vulgaris* and *Nannochloropsis oculata*) (experiment 1). To assess the relationship between immature morphology and endocrine control, the effect of causative dietary factor was compared with the effect of eyestalk ablation at various timing during zoeal development (experiment 2). In experiment 1, megalopal immature morphology was more distinct in *Chlorella*-supplemented groups than in *Nannochloropsis*-supplemented groups. High density *Chlorella* supplementation was associated with the highest incidence of immaturity and resulted in larval mass mortality. In experiment 2, the premoult of the third zoeal stage was identified as a critical period at which *Chlorella* supplementation led to the highest incidence of immaturity. This critical period coincided with the critical period at which larval metamorphosis was regulated by the eyestalk neurosecretory system. Our results suggested that the occurrence of immature megalopal morphology under culture conditions is most likely caused by phytoplankton (especially, *Chlorella*) supplementation, which disrupts the endocrine regulation. Based on our results, in experiment 3, we successfully prevented the occurrence of immature megalopal morphology in 500 L tanks by excluding the influence of phytoplankton before the critical period (i.e., discontinuing phytoplankton supplementation and supplying rotifer cultured with non-phytoplankton materials).

3.4.2 Introduction

The swimming crab, *Portunus trituberculatus*, is distributed throughout the temperate western Pacific Ocean, primarily in the coastal waters of Japan, Korea, and China (FAO, 2014). This species is the basis of an economically important fishery with global production of 429,959 t in 2012 (FAO, 2014). Seed production is conducted in hatcheries throughout Asia to produce juvenile crabs for the purposes of stock enhancement and aquaculture (Hamasaki et al., 2011; Jin et al., 2013; Lee et al., 2013; Liu et al., 2013). However, survival to the juvenile stage has been

variable and low because of frequent mass mortality of larvae (Dan et al., 2013; Hamasaki et al., 2011).

We previously evaluated the relationships between larval survival, larval morphology, and a range of culture factors (e.g., water quality and larval stocking density) using data collected from 111 seed production trials conducted in 12 hatcheries in Japan (Dan et al., 2013; Chapter 3.2). Among the factors we tested, the variation in megalopal morphology appeared to be the dominant factor affecting larval survival, while the other factors had little or no effect. We found that the occurrence of morphologically immature megalopae which retained zoeal morphological features (e.g., small dorsal spines and telson furcae) was associated with the occurrence of larval mass mortality in this species; these morphologically immature megalopae died within a few days after their metamorphosis (Dan et al., 2013; Chapter 3.2).

In general, the variability in larval morphological development can be viewed as the response of intrinsic plasticity (endocrine control) to extrinsic (environmental) factors (Anger, 2001a, 2003, 2006). In crustaceans, variability in larval morphology, i.e. larval morphogenesis, is thought to be controlled by the neurosecretory system located in the eyestalks, which consists, in part, of an X-organ and sinus gland (Costlow, 1966a, 1966b; Webster and Dircksen, 1991). To investigate the role of the eyestalk neurosecretory system on larval morphogenesis of *P. trituberculatus*, we conducted bilateral eyestalk ablation at various times during zoeal development in a subsequent study. We demonstrated that the effects of eyestalk ablation on megalopal morphology changed radically depending on the timing of ablation. Based on this, we identified a critical period during the premoult of the third zoeal stage (Dan et al., 2014; Chapter 3.3). Ablation before this period caused retention of a large dorsal spine and telson furcae and resulted in moult to the supernumerary fifth zoeal stage. Ablation after this period allowed larvae to metamorphose into normal megalopae. Ablation at this critical period, however, resulted in the production of immature megalopae with small dorsal spines and telson furcae, similar to the immature morphology observed in megalopae during seed production. These observations suggest that the morphogenesis accompanying metamorphosis into megalopal stage is regulated by the eyestalk neurosecretory system, and that the occurrence of immature morphology during seed production may be attributed to inhibition or disruption of morphogenesis regulation by the eyestalk neuro-secretory system.

Extrinsic factors that affect larval morphology and its development have been documented in many brachyuran crab species and include extreme temperatures, suboptimal salinity, and

unsuitable diet (Anger, 2001a, 2003; Arai et al., 2007; Dan and Hamasaki, 2011; Giménez and Anger, 2005; Hamasaki et al., 2002; Hamasaki, 2003; Minagawa and Murano, 1993; Pestana and Ostrensky, 1995; Zeng et al., 2004). In principal, we attempted to maintain the water temperature, water quality, and salinity at optimal levels during seed production. Therefore, the water temperature and pH of the larval rearing water likely did not affect the megalopal morphology during seed production of *P. trituberculatus* (Dan et al., 2013; Chapter 3.2). The salinity during seed production of this species was relatively constant among trials; almost all trials use unadjusted seawater of which salinity fluctuates between 30 and 35 ppt in Japanese coastal waters. Thus, these factors (temperature, pH, and salinity) do not appear to explain the occurrence of megalopal immature morphology during seed production. Arai et al. (2007) evaluated the effect of diet on the morphology of last (fourth) stage zoeae. The authors reported that the megalopal feature in last stage zoeae (e.g., size of chelae and pleopods) was influenced by the dietary intake of docosahexaenoic acid (DHA) contained in rotifers being given as food. n-3 highly unsaturated fatty acids (n-3 HUFA) including DHA are essential fatty acids for larvae of *P. trituberculatus* (Takeuchi et al., 1999a, c). However, the effect of diet on megalopal morphology remains poorly understood.

There is a need to better understand the factors in culture that influence megalopal morphology to prevent the occurrence of immature morphology. To address this, we evaluated the effect of a range of dietary conditions; e.g., feeding density of rotifers and *Artemia* and concentration of phytoplankton (*Chlorella vulgaris* and *Nannochloropsis oculata*) that were typical of conditions in commercial culture. Additionally, we evaluated the effects of phytoplankton supplementation at various times during zoeal development on larval morphogenesis. We then compared the effects of phytoplankton supplementation with those of zoeal eyestalk ablation. Based on our results, we recommend measures to prevent the occurrence of immature morphology in megalopae.

3.4.3 Materials and Methods

3.4.3.1 Experiment 1: Effects of various feeding schemes on larval morphogenesis

Newly hatched larvae (first stage zoeae, Z1) were obtained from a female with 159 mm carapace width (CW) on July 28, 2008 following the method prescribed in Chapter 3.3 (Dan et al., 2014). The larvae were stocked in 12 polyethylene tanks (500 L seawater; temperature, ~27 °C; salinity, 33 ppt) at a mean density of 9,951 individuals 500 L^{-1} (range: 9,500 to 10,500).

The tanks were divided into six treatment groups including 1) control, 2) Chlorella, 3) high density (HD) Chlorella, 4) HD rotifer, 5) HD Artemia, and 6) a combination of HD Chlorella, HD rotifer, and HD Artemia (duplicate for each group). These treatments were designed to represent the range of live foods (rotifer *Brachionus plicatilis* and *Artemia* sp.) and feeding regimes typically used during seed production of *P. trituberculatus* in Japanese hatcheries as detailed in Table 3.4.1 (Dan et al., 2013; Chapter 3.2). Commercially available condensed *Nannochloropsis* sp. containing eicosapentaenoic acid (EPA) in its cells (Yanmarine K-1, Chlorella Industry Co. Ltd, Tokyo, Japan) was supplemented in the control, HD rotifer, and HD Artemia groups. Commercially available condensed *Chlorella vulgaris* fortified with DHA and EPA in its cells (Super Chlorella V12; Chlorella Industry Co. Ltd., Tokyo, Japan) was added to the Chlorella, HD Chlorella and combination groups. The larval rearing procedures were the same as those described in Chapter 3.3 (Dan et al., 2014). Twenty larvae were sampled daily from each tank and their larval moulting stage was determined by observation under a stereomicroscope. Larval stage index (LSI) was calculated according to the method of Villegas and Kanazawa (1980), and Millamena and Quinitio (2000) as: LSI = (absolute value × number of larvae)/20. Absolute value were assigned as 1, 2, 3, 4, or 5 corresponding to Z1, second stage zoeae (Z2), third stage zoeae (Z3), fourth stage zoeae (Z4), and megalopae (MG), respectively. The numbers of surviving larvae were estimated by the volumetric method for each stage from Z2 to MG, and all surviving individuals were counted at the first crab stage (C1). Twenty recently moulted Z4 and 50 recently moulted MG were sampled from each tank for morphological measurements.

3.4.3.2 Experiment 2: Effect of phytoplankton supplementation on larval morphogenesis

The larvae were initially cultured in a 500 L tank as the control group in experiment 1; a mixture of equal numbers of Z1 larvae from three females (175, 222, and 238 mm CW) was stocked and reared at a density of 11,000 individuals 500 L^{-1} (~25 °C) on May 14, 2012. The larvae were collected from a 500 L tank, and then, given the designated treatment at various periods/time during zoeal development. The larval moulting stage and the developmental stage within the moulting cycle were verified using a stereomicroscope as described by Anger (2001b).

Table 3.4.1. Protocols for rearing swimming crab *Portunus trituberculatus* larvae in experiments 1 and 3 using 500 L tanks

Treatment	Water exchange		Rotifer addition[1]		Phytoplankton supplementation[2]			*Artemia* addition (ind. mL^{-1})			
	Time	Rate (%)	Density (ind. mL^{-1})	Timing	Species	Density (10^4 cell mL^{-1})	Duration	Z2	Z3	Z4	MG
Experiment 1											
Control	Stagnant	0	20	Z1	*Nanno.*	20	Z1 - MG	0	0.5	1.0	4.0
Chlorella			20		*Chlorella*	10		0	0.5	1.0	4.0
HD Chlorella			20		*Chlorella*	50		0	0.5	1.0	4.0
HD rotifer			45		*Nanno.*	20		0	0.5	1.0	4.0
HD Artemia			20		*Nanno.*	20		2.5	5.0	10.0	20.0
Combination			45		*Chlorella*	50		2.5	5.0	10.0	20.0
Experiment 3											
Nanno.	Stagnant	0	32	Z1	*Nanno.*	20	Z1 - MG	0	0.5	1.0	2.0
Nanno. WE	Z3-postmoult	80		Z1 and Z3	*Nanno.*	20	Z1 - Z2				
Chlorella	Stagnant	0		Z1	*Chlorella*	9.5	Z1 - MG				
Chlorella WE	Z3-postmoult	80		Z1 and Z3	*Chlorella*	9.5	Z1 - Z2				

Moulting stage: Z1, first zoeal stage; Z2, second zoeal stage; Z3, third zoeal stage; Z4, fourth zoeal stage; MG, megalopal stage.

[1] Rotifers were enriched with commercially available material consisting of phytoplanktons containing docosahexaenoic acid (DHA) (Marine Gloss, Marinetech Co., Ltd., Aichi, Japan) in experiment 1 and with commercially available frozen baker's yeast containing DHA (Asuka, Asuka pharmaceutical Co., Ltd., Tokyo, Japan) in experiment 3. In Nanno. WE and Chlorella WE treatments in experiment 3, rotifers were added on the first day of larval rearing and after water exchange on the first day of Z3.

[2] Commercially available condensed *Nannochloropsis oculata* (*Nanno.*) containing eicosapentaenoic acid (EPA) (Yanmarine K-1, Chlorella Industry Co., Ltd, Tokyo, Japan) and commercially available *Chlorella vulgaris* containing EPA and docosahexaenoic acid (Super Chlorella V12, Chlorella Industry Co., Ltd, Tokyo, Japan) were used. *Chlorella* density (10 or 9.5 ×10^4 cells mL^{-1}) was adjusted approximately the same amount as for *Nannochloropsis* density of 20×10^4 cells mL^{-1} based on dry weight. In Nanno. WE and Chlorella WE treatments in experiment 3, phytoplankton supplementation was discontinued after water exchange on the first day of Z3.

We prepared three treatment groups: 1) control, 2) Chlorella, and 3) eyestalk ablation as detailed in Table 3.4.2. In the Chlorella group, the larvae were reared with *Chlorella vulgaris* supplementation at a concentration of 5,000,000 cells mL^{-1} during five designated treatment periods in 1 L beakers. Subsequent to *Chlorella* supplementation, the larvae were reared until MG in 1 L beakers without *Chlorella* supplementation. In the eyestalk ablation group, the bilateral eyestalks of larvae were ablated at six different moulting stage and developmental stage within the moulting cycle according to the method of Chapter 3.3 (Dan et al., 2014). The control groups consisted of intact larvae that were transferred from the 500 L tank into 1 L beakers at five different stages. The larvae in the control and eyestalk ablation groups were reared until MG in 1 L beakers without phytoplankton supplementation. Thirty larvae were stocked in each 1 L beaker (~25 °C) (quadruplicate for each group) and reared according to the method in Chapter 3.3 (Dan et al., 2014), but *Artemia* nauplii were not provided to the larvae because *Artemia* nauplii are unable to digest *Chlorella* (Sick, 1976), of which the supplementary effects were under evaluation in this experiment. We collected five recently moulted Z4 larvae and all MG larvae from each replicate group for morphological measurement. In the eyestalk ablation group, supernumerary fifth stage zoeae (Z5) (Costlow, 1966a, b; Dan et al., 2014; Chapter 3.3) that had moulted from Z4 were counted and all sampled for morphological measurement.

3.4.3.3 Experiment 3: Effect of countermeasures on the incidence of abnormal larval morphology

We prepared four treatment groups: 1) Nannochloropsis, 2) Nannochloropsis with water exchange (WE), 3) Chlorella, and 4) Chlorella WE as detailed in Table 3.4.1. The Z1 hatched from respective three females (158, 195, and 212 mm CW) on June 9, 2012 were stocked in four 500 L tanks with the three different broods serving as replicates (triplication of each group, total 12 tanks). The mean stocking density of Z1 was 9,708 individuals 500 L^{-1} (range: 8,167 to 12,833). Larvae were reared at ~25 ºC according to experiment 1. In the Nannochloropsis and Chlorella groups, the larval rearing water was not renewed during the culture period from Z1 to C1, and phytoplanktons (*Nannochloropsis oculata* or *Chlorella vulgaris*) were supplemented into rearing water throughout the culture period. In the Nannochloropsis WE and Chlorella WE groups, 80 % (400 L) of the larval culture water was exchanged at the postmoult of Z3 (five days after hatching), and phytoplankton supplementation was discontinued after the water exchange.

Table 3.4.2. Treatment schedules for rearing swimming crab *Portunus trituberculatus* larvae in experiment 2

Treatment group	Treatment timing	Z1			Z2			Z3			Z4		
		Post[1]	Inter[1]	Pre[1]	Post	Inter	Pre	Post	Inter	Pre	Post	Inter	Pre
		0[2]	1	2	3	4	5	6	7	8	9	10	
Control	Z1 postmoult	[3]											
	Z1 premoult			[3]									
	Z2 premoult					[3]							
	Z3 premoult								[3]				
	Z4 intermoult										[3]		
Chlorella	Z1 postmoult-Z1 intermoult	[4]	[5]										
	Z1 premoult-Z2 postmoult			[4]	[6]								
	Z2 premoult-Z3 postmoult					[4]	[6]						
	Z3 premoult-Z4 postmoult								[4]	[6]			
	Z4 postmoult-Z4 premoult										[4]	[7]	
Eyestalk ablation	Z2 postmoult				[8]								
	Z2 premoult					[8]							
	Z3 postmoult						[8]						
	Z3 premoult								[8]				
	Z4 postmoult									[8]			
	Z4 intermoult										[8]		

Moulting stage: Z1, first zoeal stage; Z2, second zoeal stage; Z3, third zoeal stage; Z4, fourth zoeal stage
[1] Developmental stage within the moulting cycle: Post, postmoult; Inter, intermoult; Pre, premoult
[2] Days after hatching
[3] Larvae were transferred from a 500 L tank and then reared in 1 L beakers
[4] Larvae were transferred from a 500 L tank and then reared in 1 L beakers with *Chlorella* supplementation. After the treatment period, the larvae were reared until megalopal stage in 1 L beakers without *Chlorella* supplementation
[5] *Chlorella* supplementation was terminated at one day after hatching
[6] *Chlorella* was supplemented until first day after subsequent moulting
[7] *Chlorella* was supplemented until moulting to megalopal stage
[8] Larvae were sampled from a 500 L tank and both eyestalks were ablated, and then they were reared in 1 L beakers

3.4.3.4 Morphological measurement of larvae

We measured the lengths of the carapace (CL) and chela (CHL) in Z4 using a microscope in all experiments, and the ratio of the CHL (relative chela length, CHLr) to the CL were calculated (as percentages) as indices of the megalopal feature of the Z4. The length of the dorsal spine (DSL) retained on the carapace of MG and Z5 was measured, and the frequency of these larvae retaining the dorsal spine (DSf) and furcae on their telsons (TELf) were calculated in all experiments. In experiment 2, the total length of the bilateral furcae of the telson (FL) in MG and Z5 was also measured and the frequency of Z5, which were induced by eyestalk ablation, to the total number of larvae moulted from Z4 (Z5f) were also calculated. All measurement methods are described in Chapter 3.2 and 3.3 (Dan et al., 2013, 2014).

3.4.3.5 Data analysis

Statistical analyses were performed using R (R 3.0.2; R Development Core Term, 2013) with a 5 % significance level. We used a generalised linear model (GLM) (McCullagh and Nelder, 1989; Everitt and Hothorn, 2009) and a generalised linear mixed-effects model (GLMM) (Everitt, 2005; Everitt and Hothorn 2009; Zuur et al., 2009) to evaluate the effects of treatment on larval survival, rate of development, and morphological measurements as detailed in Table S3.4.1 in the Appendix.

In experiment 2, the larvae were cultured in a 500 L tank with phytoplankton (*Nanno-chloropsis*) supplementation before being treated with *Chlorella* supplementation in 1 L beakers. Therefore, to visually evaluate the effect of *Chlorella* supplementation by excluding the basal effects of phytoplankton (*Nannochloropsis*) supplementation, the residuals of the body parts measurements were calculated and plotted by subtracting the measurements of control group from those of concurrently treated *Chlorella* supplemented groups. The differences in the values between concurrently treated control and *Chlorella* groups were evaluated with Welch's *t* test. In this analysis, the DSf and TELf values were transformed to arcsine data.

3.4.4 Results

3.4.4.1 Experiment 1: Effects of various feeding schemes on larval morphogenesis

The supplementation with a high concentration of *Chlorella* induced larval mass mortality during metamorphosis into MG; all the larvae died in the HD Chlorella and combination groups during the period from Z4 to MG (Table 3.4.3 and Fig. S3.4.1A in the Appendix). The develop-

Table 3.4.3. Mean survival rates of swimming crab *Portunus trituberculatus* larvae in each treatment in experiments 1 and 3

Treatment		Z1-Z4	Z1-MG	Z1-C1	Z4-MG	MG-C1
Experiment 1						
Control		24.7 (11.2)	5.4 (0.8)[a]	1.7 (2.4)	25.2 (14.8)	28.5 (40.4)
Chlorella		35.3 (2.5)	4.2 (2.5)[a]	2.2 (0.5)	12.1 (8.1)	61.6 (25.5)
HD Chlorella		29.6 (2.7)	0 0	0.0 (0.0)	0.0 (0.0)	–
HD rotifer		23.5 (9.8)	11.3 (0.1)[ab]	9.1 (3.0)	52.8 (22.3)	80.9 (26.9)
HD Artemia		42.9 (9.5)	21.8 (9.0)[b]	3.3 (2.6)	49.7 (10.1)	19.3 (19.8)
Combination		52.2 (2.4)	0 0	0.0 (0.0)	0.0 (0.0)	–
Treatment effect	*F*	3.908	7.886	3.144	4.352	2.411
	df	3, 6	3, 4	3, 4	3, 4	3, 4
	P(>*F*)	0.0637	0.0373	0.1487	0.0947	0.2073
Experiment 3						
Nanno.		81.2 (23.7)	10.1 (7.9)	2.2 (1.1)	12.3 (8.3)	31.4 (25.0)
Nanno. WE		86.3 (19.7)	27.9 (1.1)	11.4 (7.5)	33.9 (10.0)	41.3 (28.7)
Chlorella		81.3 (14.7)	14.4 (10.9)	5.2 (4.5)	18.3 (14.8)	34.3 (3.7)
Chlorella WE		75.5 (21.4)	21.8 (15.3)	14.8 (17.2)	33.7 (26.6)	72.9 (46.9)
Treatment effect	*F*	0.1003	1.989	1.526	1.281	0.6099
	df	3, 8	3, 8	3, 8	3, 8	3, 8
	P(>*F*)	0.9576	0.1943	0.2809	0.3451	0.6272

Values in parentheses represent the standard deviations of the mean. Significant differences were found between treatments with different superscripts in the same column (comparison of generalized linear model with Tukey method, $P < 0.05$). To avoid errors associated with zero data in analyses with a quasi-binomial distribution, the groups with 0 % survival rate were excluded from the analysis in experiment 1. Z1, first zoeal stage; Z4, fourth zoeal stage; MG, megalopal stage; C1, first crab stage, Chlorella, *Chlorella vulgaris*; Nanno., *Nannochloropsis oculata*

mental rates (LSI) were significantly faster in the groups in which *Artemia* were supplied at high density (HD Artemia and combination groups) than in other treatment groups (Fig. S3.4.1B in the Appendix).

The CL of Z4 was smaller in the groups for which *Chlorella* was supplied at a high concentration (HD Chlorella and combination; Fig. 3.4.1A). However, the megalopal features in Z4, represented by CHL and CHLr, were distinct in the *Chlorella* supplemented groups (Chlorella, HD Chlorella, and combination; Fig. 3.4.1B). We observed morphologically immature MG that retained the dorsal spine and telson furcae in all treatment groups, though the frequency of occurrence varied among treatments (Fig. 3.4.1C, D). The immature morphology in MG was distinct in the *Chlorella* supplemented groups (Chlorella, HD Chlorella, and combination) than

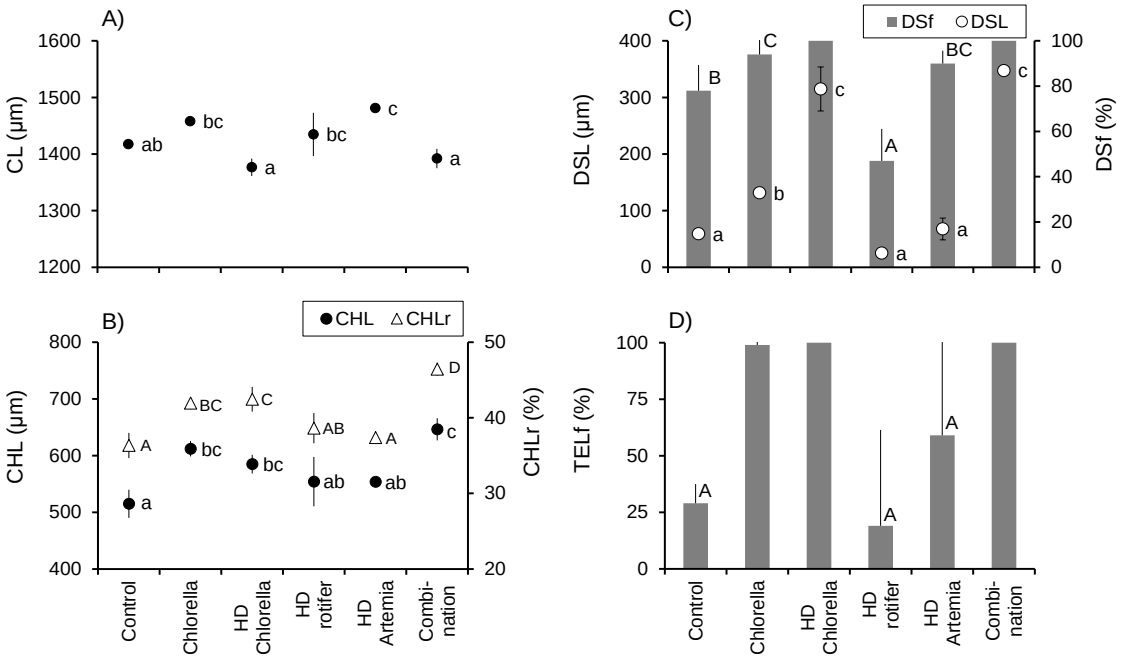

Figure 3.4.1. Effect of diet (experiment 1) on carapace length (CL) (A), chela length (CHL) and relative chela length (CHLr) (B) in fourth stage zoeae, and dorsal spine length (DSL) and frequency of dorsal spine retention (DSf) (C), and frequency of telson furcae retention (TELf) (D) in megalopae of *Portunus trituberculatus*. Vertical bars represent the standard deviation of the duplicate groups in each treatment group. Treatment effect: CL, $F = 8.966$, $df = 5, 6$, $P = 0.0094$; CHL, $F = 7.756$, $df = 5, 6$, $P = 0.0135$; CHLr, $F = 16.21$, $df = 5, 6$, $P = 0.0020$; DSL, $F = 122.8$, $df = 5, 6$, $P < 0.0001$; DSf, $F = 19.15$, $df = 3, 4$, $P = 0.0078$; TELf, $F = 7.382$, $df = 3, 4$, $P = 0.0416$. Differences among treatments are indicated by a different letter ($P < 0.05$). See Table 3.4.1 for detail of treatments

in the other *Nannochloropsis* supplemented groups (control, HD rotifer, and HD Artemia). The *Chlorella* supplementation at a high concentration induced most severe immaturity of MG.

These data indicate that *Chlorella* supplementation was associated with the occurrence of immature morphology in MG in a concentration dependent manner, and that retention of immature morphology in MG caused larval mass mortality during metamorphosis into MG.

3.4.4.2 Experiment 2: Effect of phytoplankton supplementation on larval morphogenesis

The treatments had a significant effect on the survival of larvae treated during the Z1- and Z2-premoult and Z4-postmoult and intermoult stages, but had no significant effect during the Z1-postmoult and Z3-premoult stages (Fig. S3.4.2 in the Appendix). The treatments had no significant effect on developmental rates (Fig. S3.4.3 in the Appendix).

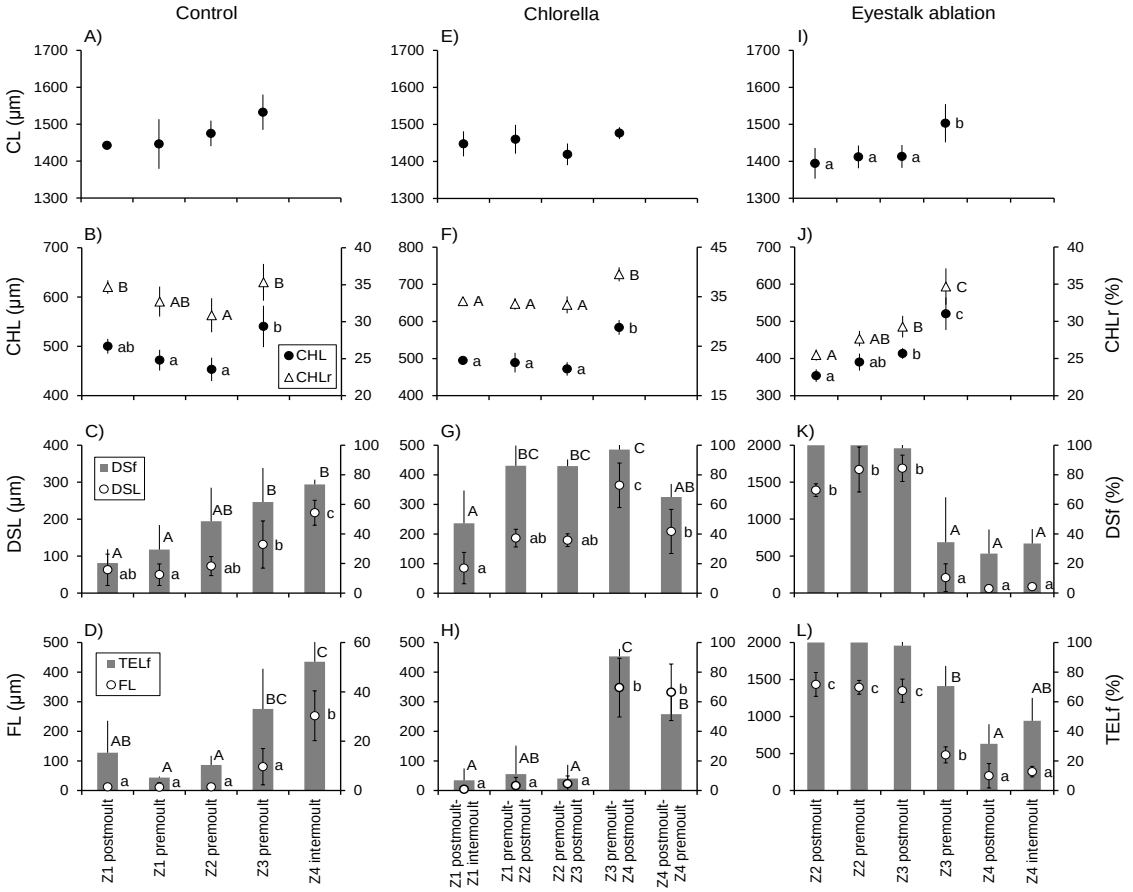

Figure 3.4.2. Effect of control treatment (A−D), *Chlorella vulgaris* supplementation (E−H), and bilateral eyestalk ablation (I−L) in experiment 2 on carapace length (CL) (A, E, I), chela length (CHL) and relative chela length (CHLr) (B, F, J) in fourth stage zoeae, and dorsal spine length (DSL) and frequency of dorsal spine retention (DSf) (C, G, K), and furcae length of telson (FL) and frequency of telson furcae retention (TELf) (D, H, L) in larvae moulted from fourth stage zoeae of *Portunus trituberculatus*. Vertical bars represent the standard deviations of the four replicate groups in each treatment group. See Table S3.4.2 in the Appendix for the statistical evaluation of the treatment effect. Differences among treatments are indicated by a different letter ($P < 0.05$)

In the control group, the megalopal features in Z4 (CHL and CHLr) were lower in the Z2-premoult treated group (Fig. 3.4.2B), but the immature morphology in MG (DSL, DSf, FL, and TELf) increased with a delay in the transfer of larvae from the 500 L tank (Fig. 3.4.2C, D). In the Chlorella group, the megalopal features in Z4 was larger in the larvae treated from Z3-premoult to Z4-postmoult (Fig. 3.4.2F), and the immature morphology in MG was distinct in larvae treated from Z3-premoult to Z4-postmoult (Fig. 3.4.2G, H). In the comparison between the Chlorella and control groups, the megalopal features in Z4 were slightly large when the *Chlorella* was supplemented from Z3-premoult to Z4-postmoult (Fig. 3.4.3B), while the immature morphology in MG was most aggravated when the *Chlorella* was supplemented from Z3-premoult to Z4-postmoult

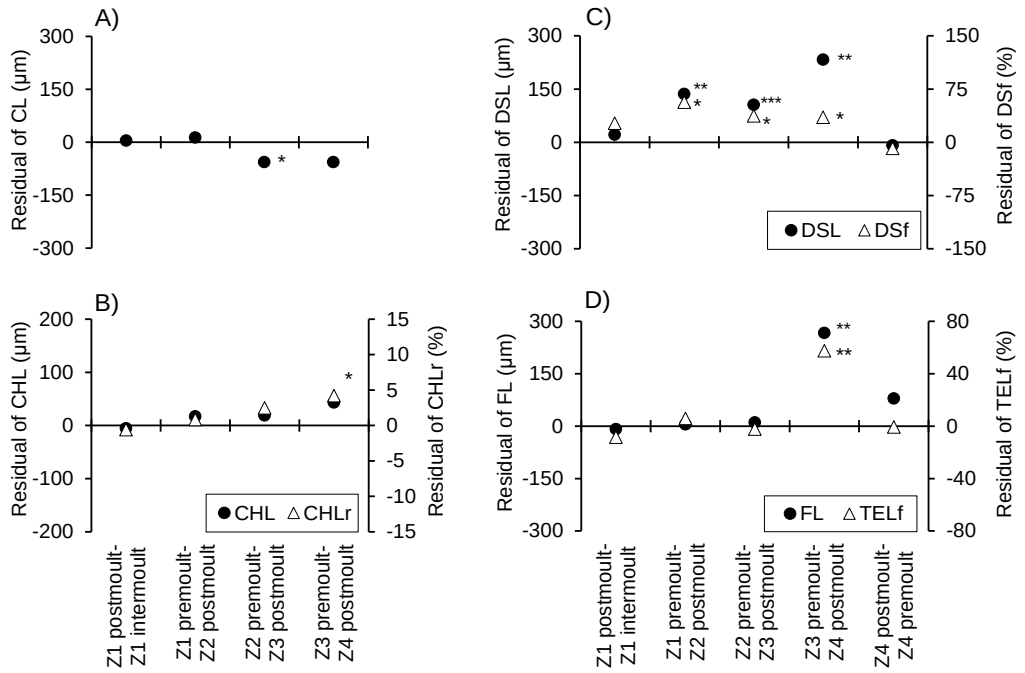

Figure 3.4.3. Effect of *Chlorella vulgaris* supplementation in experiment 2 on carapace length (CL) (A), chela length (CHL) and relative chela length (CHLr) (B) in fourth stage zoeae, and dorsal spine length (DSL) and frequency of dorsal spine retention (DSf) (C), and furcae length of telson (FL) and frequency of telson furcae retention (TELf) (D) in larvae moulted from fourth stage zoeae of *Portunus trituberculatus*. Values were calculated by subtracting the mean of the control group from those of concurrently treated *Chlorella*-supplemented groups. Differences between treatments are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

(Fig. 3.4.3C, D). In the eyestalk ablation group, the megalopal features in Z4 were larger as eyestalk ablation timing delayed (Fig. 3.4.2J). Eyestalk ablation before Z3-premoult inhibited metamorphosis to MG and resulted in occurrence of supernumerary Z5 larvae showing the normal zoeal morphology with dorsal spines and telson furcae (Fig. 3.4.2K, L, Fig. 3.4.4). In contrast, all the larvae of which the eyestalks were ablated after the Z4-postmoult were able to moult to MG, and the eyestalk ablation at Z3-premoult induced a small number of Z5 larvae (Fig. 3.4.4) and immature morphology of the megalopal telson (Fig. 3.4.2L).

Thus, the effect of eyestalk ablation on megalopal morphology could be clearly separated at Z3-premoult as a boundary, and the Z3-premoult was identified as a critical period at which *Chlorella* supplementation led to the highest incidence of immature morphology in MG.

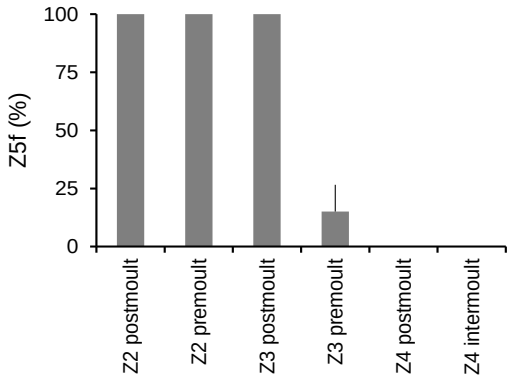

Figure 3.4.4. Changes in the frequency of occurrence of supernumerary fifth stage zoeae (Z5f) to the total number of larvae moulted from fourth stage zoeae of *Portunus trituberculatus* of which the eyestalks were ablated at various times during the zoeal period in experiment 2. Vertical bars represent the standard deviation of the four replicate groups in each treatment group.

3.4.4.3 Experiment 3: Effect of countermeasures on the incidence of abnormal larval morphology

The survival rates were high until to reach Z4 in all groups (range: 75.5 to 86.3 %), but they largely declined during metamorphosis into megalopae (from Z4 to MG) (Table 3.4.3 and Fig. S3.4.4A in the Appendix). Although survival rates during metamorphosis dispersed and statistical significant difference was not detected, the groups with water exchange and discontinuing phytoplankton supplementation (Nannochloropsis WE and Chlorella WE groups) exhibited relatively high survival rates (33.9 and 33.7 %) compared with Nannochloropsis and Chlorella groups (12.3 and 18.3 %) with non-water exchange and continuous phytoplankton supplementation. Overall survival from Z1 to C1 also tended to be higher in Nannochloropsis WE and Chlorella WE groups (11.4 and 14.8 %) compared with Nannochloropsis and Chlorella groups (2.2 and 5.2 %). The treatment had no effect on larval developmental rates (Fig. S3.4.4B in the Appendix). The treatment did not significantly affect the morphological measurements of Z4 (Fig. 3.4.5A, B), but did have a significant effect on those of MG (Fig. 3.4.5C, D). The occurrence of immature morphology of MG was suppressed in the Nannochloropsis WE and Chlorella WE groups. In the groups with continuous phytoplankton supplementation, the immature morphology (DSL) in MG was more aggravated by *Chlorella* supplementation.

Thus, overall, water exchange and discontinuing phytoplankton supplementation could prevent the occurrence of immature morphology of MG and improve larval survival.

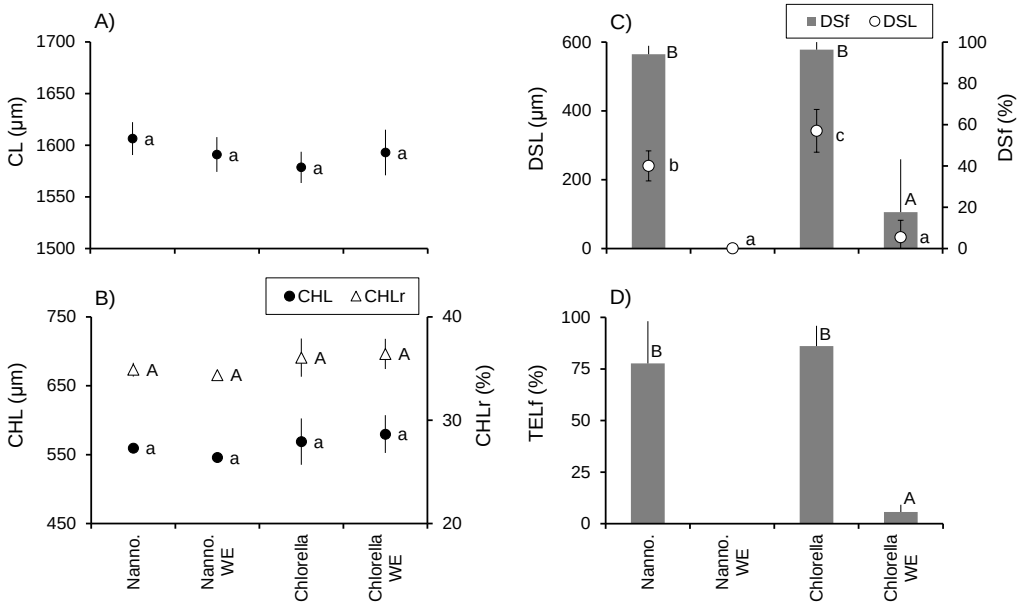

Figure 3.4.5. Effect of water exchange and phytoplankton supplementation in experiment 3 on carapace length (CL) (A), chela length (CHL) and relative chela length (CHLr) (B) in fourth stage zoeae, and dorsal spine length (DSL) and frequency of dorsal spine retention (DSf) (C), and frequency of telson furcae retention (TELf) (D) in megalopae of *Portunus trituberculatus*. Vertical bars represent the standard deviation of the triplicate groups in each treatment group. Treatment effect: CL, $F = 1.262$, $df = 3, 8$, $P = 0.3507$; CHL, $F = 1.228$, $df = 3, 8$, $P = 0.3613$; CHLr, $F = 1.900$, $df = 3, 8$, $P = 0.2081$; DSL, $F = 39.47$, $df = 3, 8$, $P < 0.0001$; DSf, $F = 17.20$, $df = 2, 6$, $P = 0.0033$; TELf, $F = 23.55$, $df = 2, 6$, $P = 0.0014$. Differences among treatments are indicated by a different letter ($P < 0.05$)

3.4.5 Discussion

Although the morphological variation in MG was closely correlated with larval mass mortality during seed production of *P. trituberculatus* in Japanese hatcheries (Dan et al., 2013; Chapter 3.2), the cause of the morphological variation in MG has been poorly understood. In experiment 1, the degree of retention of the dorsal spine and telson furcae in MG was higher in the *Chlorella*-supplemented groups than in the *Nannochloropsis*-supplemented groups. The immature morphology observed in *Chlorella*-supplemented groups is consistent with that observed during seed production. Furthermore, high density *Chlorella* supplementation induced severe immature morphology and resulted in total mortality of larvae immediately after metamorphosis to MG. Additionally, continuous supplementation of *Chlorella* and *Nannochloropsis* throughout the

zoeal duration in experiment 3 induced the occurrence of immature morphology in MG, though to a greater extent in the *Chlorella*-supplemented group than in *Nannochloropsis*-supplemented group. These observations suggest that both *Chlorella vulgaris* and *Nannochloropsis oculata* have factor(s) inducing megalopal immature morphology that lead to larval mass mortality. Furthermore, *Chlorella vulgaris* appears to exert a more potent effect than *Nannochloropsis oculata*.

During the seed production trials conducted in Japanese hatcheries, phytoplankton such as *Chlorella vulgaris*, *Nannochloropsis* sp., Diatoms (*Chaetoceros calsitrans*, *Chaetoceros gracilis*, and natural diatoms), and *Tetraselmis* sp. were supplemented into the larval rearing water in all trials to maintain the live food populations (Dan et al., 2013; Chapter 3.2). Thus, there is a high probability that the immature megalopal morphology observed during seed production was also induced by phytoplankton supplementation. The larvae of brachyuran crabs, including portunid crabs, are not able to ingest phytoplankton directly (Hamasaki, 1997; Hassan et al., 2011; McConaugha, 1985). Thus, the phytoplankton derived nutrients and inhibitory factors are taken indirectly via consumption of live foods, primarily rotifers (*Artemia* nauplii also cannot digest *Chlorella* and *Nannochloropsis*) (Sick, 1976). The extent of phytoplankton nutrient uptake by rotifers is primarily influenced by the enrichment environment before they are introduced into the larval rearing tanks (Dan and Hamasaki, 2011; Kobayashi et al., 2008; Matsunari et al., 2012; Chapter 3.1) and by the species and concentration of phytoplankton that are supplemented into the larval rearing tanks (Dan and Koiso, 2008; Hamasaki et al., 2011; Reitan et al., 1997). Because these enriching and supplementing factors varied among seed production trials (Dan et al., 2013; Chapter 3.2), we speculate that this explains the variation in megalopal morphology that was observed among trials during *P. trituberculatus* seed production.

In experiment 2, the immature morphology of MG was largely retained when *Chlorella* was supplied during the period from Z3-premoult to Z4-postmoult. Conversely, the megalopal morphology was not affected when *Chlorella* supplementation began at the Z4-postmoult stage (see comparison of Chlorella group with control group in Fig. 3.4.3). Thus, *Chlorella* supplementation appears to have the greatest effect on megalopal morphology during the Z3-premoult period. In the control group in which larvae were transferred from a *Nannochloropsis*-supplemented 500 L tank into 1 L beakers and reared without phytoplankton-supplementation, the occurrence of immature morphology in MG increased as larval transfer from the 500 L tank was delayed and was highest when larvae were transferred after the Z3-premoult. This suggests that *Nannochloropsis* supplementation also induce immature megalopal morphology during the

critical Z3-premoult period. Resorption of the dorsal spine and telson furcae during metamorphosis is controlled instantaneously by the eyestalk neurosecretory system at a critical period during the Z3-premoult (Dan et al., 2014; Chapter 3.3). Bilateral eyestalk ablation before this period is associated with retention of a large dorsal spine and telson furcae and resulted in moult to the supernumerary Z5. Conversely, ablation after this period allows larvae to moult into normal MG. Ablation during this critical period results in immature morphology of MG, whereby they retain small dorsal spines and telson furcae. This is consistent with our observations following eyestalk ablation in experiment 2 in the current study. Here, phytoplankton-induced megalopal immature morphology can be interpreted as inhibition of the resorption of the dorsal spine and telson furcae. Thus, we speculate that phytoplankton-supplementation appears to affect the endocrine control exerted by the eyestalk neurosecretory system at this critical period. Interestingly, the critical period at which phytoplankton induce immature morphology coincides with the critical period of morphogenesis regulation by the eyestalk neurosecretory system; both were at the Z3-premoult.

There are two potential mechanisms underlying the occurrence of phytoplankton-induced immature megalopal morphology. Phytoplankton supplementation may affect the nutritional status of larvae thereby resulting in physiological abnormality. Alternatively, phytoplankton and/or live foods that feed on the phytoplankton may possess a teratogenic factor(s) [chemical(s)] that inhibits or disrupts endocrine control by the eyestalk neurosecretory system. We believe that nutrition is unlikely to explain the effect as following reasons. Larvae of *P. trituberculatus* are able to moult to normal MG by way of a supernumerary zoeal stage (Z5) even under unfavourable dietary conditions (Hamasaki, 1997). Additionally, excess live food supplementation did not induce the megalopal immature morphology (HD rotifer and HD Artemia groups in experiment 1). The dietary DHA has been known to accelerate the morphogenesis of megalopal features in Z4 of *P. trituberculatus* (Arai et al., 2007). In experiment 1, supplementation of *Chlorella* containing DHA accelerated megalopal features in Z4 and induced immature morphology in MG. Thus, the immature morphology in MG also seems to be affected by dietary DHA. However, the incidence of megalopal features in Z4 and immature morphology in MG was not always linked in the seed production trials (Dan et al., 2013; Chapter 3.2), and supplementation with phytoplankton only induced distinct immature morphology of MG at a critical period in experiment 2. Moreover, in contrast to the absorption of zoeal features (dorsal spine and telson furcae) during metamorphosis into MG that is controlled instantaneously by the eyestalk

neurosecretory system at a critical period during the Z3-premoult, we previously demonstrated that the morphogenesis of megalopal features (chelae and pleopods) in Z4 is controlled continuously by the eyestalk neurosecretory system throughout the zoeal stages because the length of the chela and pleopod of Z4 decreased as the bilateral eyestalks were ablated earlier during development (Dan et al., 2014; Chapter 3.3). This was also confirmed in experiment 2 in the present study. Because the enlargement of body parts and appendages may require a large amount of energy, it is reasonable that larvae would advance their morphogenesis towards MG as much as possible when nutritional conditions (e.g., DHA) allow during the zoeal stage. Conversely, resorption of body parts may not place energy demands on the individual (Dan et al., 2014; Chapter 3.3). Thus, the dietary nutrition appears to have no or little effect on immature morphology in MG, but significant on megalopal features in Z4.

The bloom-forming phytoplanktons such as coccolithophorid, diatoms, and dinoflagellate produce chemicals such as saxitoxin, phycotoxin, domoic acid, and oxylipins. These chemicals reduce the reproductive success of grazing copepods via a reduction of hatching success and production of abnormal nauplii, leading to a decrease in the population of grazing zooplanktons (Caldwell, 2009; Ianora et al., 2004; Miralto et al., 1999; Van Donk et al., 2011). Furthermore, in terrestrial habitats, many plant species produce phytoecdysones, which have ecdysone-like effects and disrupt the endocrine control of insect larvae causing reduced reproductive success, and leading to control of grazing insect populations (Miralto et al., 1999; Van Donk et al., 2011). Although such a chemical defence has not been reported in *Chlorella* and *Nannochloropsis*, it is reasonable to expect that they may have evolved such a defence given they are exposed to risk of predation by grazer animals, including crustaceans, in the wild.

During in vitro tissue culture, resorption of the zoeal dorsal spine of the crab *Rhithropanopeus harrisii* can be accelerated by the addition of ecdysone (20-Hydroxyecdysone) into the culture medium (Freeman and Costlow, 1983, 1984). Similarly, the addition of crustacean juvenile hormone (methyl farnesoate) into larval culture water appears to induce immature morphology in prawn *Macrobrachium rosenbergii* post larvae (Abdu et al., 1998a, b). However, the endocrine system controlling the larval morphogenesis accompanying metamorphosis remains poorly understood (Anger, 2006). Interestingly, the production of these hormones is controlled by the eyestalk neurosecretory system in adult crustacean; ecdysone is produced in the Y-organ and regulated by moult-inhibiting hormone secreted from the X-organ sinus grand complex in the eyestalk. Likewise, methyl farnesoate is produced in the mandibular organ and regulated by

mandibular organ-inhibiting hormone that is secreted by the X-organ sinus grand complex (Hopkins, 2012; Keller, 1992; Nagaraju, 2007; Nakatsuji et al., 2009). Given the overlap in the critical period of endocrine control by eyestalk neurosecretory system and the putative teratogenicity of phytoplankton-supplementation, we speculate that phytoplankton may affect the endocrine pathways associated with the eyestalk neurosecretory system. Further study is needed to evaluate whether phytoplankton possess chemicals that induce immature megalopal morphology, and whether there is an interaction between the endocrine control of larval morphogenesis and chemical defence by phytoplankton. This would serve to improve not only larval culture technology, but also our understanding of the regulatory mechanism of larval metamorphosis and interspecific relationships between phytoplankton and crustacean species.

Based on the results of experiments 1 and 2, we evaluated the effects of elimination of phytoplankton before the critical period during the Z3-premoult in experiment 3 as a countermeasure for preventing megalopal immature morphology. We exchanged the majority (80 %) of the larval culture water for fresh seawater at the Z3-postmoult to eliminate the phytoplankton and subsequently supplemented the system with rotifers that were enriched with n-3 HUFA fortified baker's yeast. This measure was successful in preventing immature megalopal morphology, with little or no immature morphology being observed. In contrast, the MG of the control groups that had continuous *Nannochloropsis* and *Chlorella* supplementation retained immature morphology. Thus, megalopal morphology can be controlled during mass larval culture by taking preventative measures during the critical period.

Despite our success at preventing the occurrence of immature megalopal morphology in experiment 3, the survival rates to C1 were still low. For example, the survival from Z1 to C1 was 11.4 ± 7.5 % (mean ± sd) and 14.8 ± 17.2 % in the Nannochloropsis WE and Chlorella WE groups, respectively, although these value were relatively high compared with non-treated groups (Nannochloropsis group; 2.2 ± 1.1 %, Chlorella group; 5.2 ± 4.5 %). This suggests that there are other underlying factors reducing larval survival. Discontinuation of phytoplankton-supplementation results in the starvation of rotifers that remain in the larval culture tank. Furthermore, *Artemia* nauplii may starve in the larval culture tank whether *Chlorella* and *Nannochloropsis* are supplemented or not because they cannot digest those phytoplanktons (Sick, 1976). Starvation of live foods may have the unintended side effect of causing nutritional deficiency in the larvae and result in low survival after MG. Further research is needed to improve larval rearing methods,

with a focus on maintaining the nutritional value of live foods at a sufficient level while preventing megalopal immature morphology.

Chapter 4

IMPROVEMENT IN NUTRITIONAL STATUS OF *ARTEMIA*

4.1 Starvation of *Artemia* in larval rearing water affects post-larval survival and morphology of the swimming crab, *Portunus trituberculatus* (Brachyura, Portunidae)

4.1.1 Summary

Survival of the swimming crab, *Portunus trituberculatus*, during the post-larval period from the megalopal to the first crab stage was low compared with successive larval stages in seed production trials conducted by Japanese hatcheries. To clarify the cause for the low survival, we explored whether rotifers, *Artemia*, or phytoplankton have negative effects during this period. The effects of the causative factor on larval survival and morphology were verified, and measures to overcome low survival were investigated. We conducted three experiments. In experiment 1, larvae were reared with different types of food (rotifers or newly hatched *Artemia*) with or without *Nannochloropsis oculata* supplementation during the late zoeal period (from the third to fourth zoeal stages). After moulting to megalopae, all larvae were fed newly hatched *Artemia*. The somatic sizes of the last stage (fourth stage) zoeae and megalopae were large, and developmental velocity was faster in the *Artemia*-fed groups than that in the rotifer-fed groups. However, *Artemia* feeding induced unviable and morphologically abnormal first stage crabs with close-set eyes, regardless of *Nannochloropsis* supplementation. In experiment 2, third stage zoeae were reared to first stage crabs with variously treated *Artemia* such as newly hatched, 48 h starved, and *Artemia* enriched with commercially available digestible *Nannochloropsis* with physically broken cell walls to confirm the negative effects of feeding *Artemia*. Larvae reared with the newly hatched and starved *Artemia* containing lower eicosapentaenoic acid (EPA) content exhibited low survival (1.9–3.5 %) from the megalopal to the first crab stage, whereas larvae reared with *Artemia* enriched with digestible *Nannochloropsis* containing higher EPA content achieved higher survival (40.7 %). In experiment 3, newly hatched *Artemia* were cultured under various conditions such as supplementation with untreated *Nannochloropsis*, digestible *Nannochloropsis*, or without phytoplankton to investigate what caused the *Artemia* to starve. The treatments supplemented with untreated *Nannochloropsis* and without phytoplankton suppressed *Artemia* growth and resulted in total mortality 4 days after hatch. Supplementing with digestible *Nannochloropsis* maintained *Artemia* growth and resulted in higher survival, suggesting that early *Artemia* nauplii could not digest *Nannochloropsis*, which resulted in starvation. Our results indicate that *Artemia* starve in larval rearing water regardless of supplementation with *Nannochloropsis*; however, *Nannochloropsis* are usually added to seed production tanks. *Artemia*

starvation during the late zoeal period exerted a negative carry-over effect during the post-larval period. We recommend nutritionally enriching *Artemia* with EPA and supplementing larval rearing water with digestible *Nannochloropsis* during the late zoeal stage.

4.1.2 Introduction

The swimming crab, *Portunus trituberculatus*, is an economically important crab with global production of 429,959 t in 2012 (FAO, 2014). This crab is found throughout the temperate western Pacific Ocean in the coastal waters of Japan, Korea, and China (FAO, 2014). This wild resource has been depleted by overfishing; thus, stock enhancement programs have been implemented in Japan, Korea, and China. At present, more than 30 million and 10 million juveniles are released annually into the wild in Japan and Korea, respectively (Hamasaki et al., 2011; Lee et al., 2013; Liu et al., 2013). This crab has become one of the most important aquaculture species in east China, as aquaculture production exceeded 91,050 t in 2010 (Jin et al., 2013). Therefore, seed is produced in hatcheries throughout Asia for stock enhancement and aquaculture. However, survival to the juvenile stage has been low and varied because of frequent mass larval mortality (Dan et al., 2013; Hamasaki et al., 2011; Chapter 3.2).

During Japanese hatchery seed production of *P. trituberculatus* from 2009 to 2010, the larval survival rate declined more significantly from the last (fourth) zoeal stage to the megalopal stage and from the megalopal stage to the juvenile crab stage, including first and second stage crabs, than between any other successive zoeal stage (Dan et al., 2013; Chapter 3.2). The occurrence of morphologically immature megalopae with zoeal features, such as a small dorsal spine and furcae of telson, has been associated with decreased survival during the last zoeal to the megalopal stages; these morphologically immature megalopae die within a few days after metamorphosis (Dan et al., 2013; Chapter 3.2). The immature morphology of the megalopae is induced by phytoplankton, such as *Chlorella vulgaris* and *Nannochloropsis oculata*, supplemented in larval rearing water as food for rotifers (Chapter 3.4). The critical period at which the phytoplankton induce immature megalopae was identified as premoult of the third zoeal stage (Dan et al., 2014; Chapter 3.4). Based on these results, the occurrence of immature megalopal morphology was preventable by excluding phytoplankton before a critical period; i.e., exchanging the majority of larval culture water before the critical period, discontinuing phytoplankton supplementation, and alternative feeding with rotifers enriched with a non-phytoplankton source after water exchange (Chapter 3.4).

Thus, the cause of the mass mortality that occurs from the last zoeal stage to the megalopal stage has become clear, and controlling measures have been developed. However, the cause of low survival from the megalopal stage to the juvenile crab stage remains uncertain. Although we used these measures and succeeded in preventing abnormal morphology in megalopae, post-larval survival to reach the first crab stage remains low (Chapter 3.4), suggesting that other underlying factors are retarding larval survival. We found that larval survival after the megalopal stage was reduced in almost all cases when larvae were reared without phytoplankton supplementation after the third zoeal stage to prevent the immature megalopal morphology. Because the absence of phytoplankton in larval rearing water may lead to starvation of rotifers and *Artemia*, this raises the possibility that the nutritional status of these live foods during the late zoeal period affects the post-larval survival after the megalopal stage. In addition, *Artemia* nauplii cannot digest some species of phytoplanktons such as *Chlorella conductrix* (Sick, 1976), and this indicates that *Artemia* may starve according to the phytoplankton species supplemented into the larval rearing water.

A number of reports have demonstrated the effects of different nutritional enrichment formulations and live food feeding schedules (rotifers and *Artemia*) on survival and development of brachyuran crab larvae (Arai et al., 2007; Baylon, 2009; Davis et al., 2005; Hamasaki et al., 1998; Kobayashi et al., 2000a; Minagawa and Murano, 1993; Suprayudi et al., 2002a, b, 2004; Takeuchi et al., 1999a, b, c). The effects of starvation in the absence of live food have also been studied extensively in terms of their ecological adaptation to the natural habitat (Anger, 1987; Anger and Dawirs, 1981, 1982; Calado et al., 2010; Figueiredo et al., 2008; Giménez and Anger, 2005; Giménez et al., 2004; Guerao et al., 2012). However, little attention has been paid to the nutritional status (including starvation) of live food after being supplied to larval rearing water and its effects on larval survival, development, and morphogenesis. Live food is generally supplied to retain a particular density the following day to avoid starvation of cultured animals.

To address this issue, we examined whether starving the live food (rotifers, *Artemia*, and their combinations with supplemented *Nannochloropsis*) in larval rearing water during the late zoeal period (from third to fourth zoeal stages) affected survival, rate of development, and morphogenesis throughout the larval and post-larval stages (experiment 1). Based on our results suggesting that starving *Artemia* have a negative impact on post-larval morphology and survival, we confirmed the effects of variously treated (newly hatched [NH], starved, and non-starved) *Artemia* on larvae in a subsequent experiment (experiment 2). Finally, we investigated what

factors lead to starvation of *Artemia* retained in the larval rearing water (experiment 3). Our results highlight the potential effects of starving *Artemia* on survival and development and its carry-over effect on post-larval stages for the first time. Based on our results, we recommend improving measures to prevent starvation of *Artemia* by illustrating its effect on larval and post-larval survival and morphology.

4.1.3 Materials and Methods

We conducted two larval rearing experiments with *P. trituberculatus* in 2013 (experiments 1 and 2) and a culture experiment with *Artemia* in 2014 (experiment 3).

4.1.3.1 Experiment 1: Effect of starving rotifers and *Artemia* on larval survival and morphology

First stage zoeae (Z1) that hatched from three females with carapace widths (CW) of 163, 174, and 190 mm were equally stocked in a cylindrical 500 L tank (total, 9,800 larvae) and cultured at 25 °C and 33 ppt salinity following the method described in Chapter 3.3 (Dan et al., 2014). Then, they were given the designated treatment in cylindrical 30 L tanks the day before moulting to the third zoeal stage (Z3). About 20 larvae were sampled daily from the tank, and their moulting stage was determined by observation under a stereomicroscope. They reached premoult (D3 stage according to Anger (2001b)) of the second zoeal stage (Z2) 4 days after hatch (dah) and were transferred to 30 L tanks.

We prepared four treatment groups (triplicate, total of 12 tanks), including 1) Rotifer, 2) Rotifer + Nannochloropsis, 3) Artemia, and 4) Artemia + Nannochloropsis. A total of 400 Z2 larvae were stocked in each 30 L tank (24.8 °C, 32.5 ppt). The seawater in the rearing tanks was not renewed during the culture period (to reach first crab stage [C1]). Sodium nifurstyrenate (2 mg L^{-1}) was added at the beginning of larval rearing to prevent bacterial proliferation. Rotifers enriched with Baker's yeast fortified with docosahexaenoic acid (DHA) (Asuka Pharmaceutical Co., Ltd, Tokyo, Japan) were supplied to the tanks on the day of stocking (31.0 individuals mL^{-1}) in the rotifer-fed groups. Newly hatched (NH) *Artemia* nauplii (Utah Strain, Kitamura Co., Ltd., Kyoto, Japan) that hatched daily over 24 h at 25 °C were added once each morning to maintain a density of 2.0 individuals mL^{-1} in the *Artemia*-fed groups. *Nannochloropsis oculata* (Yanmarine K-1, Chlorella Industry Co., Ltd, Tokyo, Japan; hereafter, *Nannochloropsis*) was added to the tank twice daily to maintain a density of 200,000 cells mL^{-1} until moulting to the megalopal stage

(MG) in the *Nannochloropsis*-supplemented groups. After moulting to MG, only NH *Artemia* nauplii were added to maintain a density of 4.0 individuals mL^{-1} in all treatment groups.

Thirty larvae were sampled daily from each tank, and the larval moulting stage was visually determined according to Hamasaki (1997), and then the larvae were returned to the tank. The larval stage index (LSI) was calculated according to the method of Villegas and Kanazawa (1980) and Millamena and Quinitio (2000) to provide an index of the larval development rate as: LSI = (absolute value × number of larvae)/30. Absolute values were assigned 1, 2, 3, 4, or 5 corresponding to Z1, Z2, Z3, fourth stage zoeae (Z4) and MG, respectively. The number of surviving larvae was estimated by the volumetric method based on three 300 mL samples taken from the tanks with each Z3 to MG larval stage, and all individuals moulted to C1 were counted. Thirty recently moulted Z4 and MG larvae were sampled from each tank for morphological measurements, fixed in 10 % neutral buffered formalin for 24 h, and preserved in 70 % ethanol.

4.1.3.2 Experiment 2: Effect of starved or non-starved *Artemia* on larval survival and morphogenesis

A total of 10,167 larvae from three females (162, 172, and 209 mm CW) were initially cultured in a 500 L tank (25.5 °C, 33 ppt) as in experiment 1. They were given the designated treatments using plastic 1 L beakers on the morning they moulted to Z3 (5 dah).

We prepared three treatment groups (quadruplicate, total of 12 beakers): 1) NH Artemia, 2) starved Artemia, and 3) digestible Nannochloropsis (d-Nannochloropsis) Artemia. Thirty Z3 larvae were stocked in each 1 L beaker (25.2 °C, 33.3 ppt) and reared according to the method described in Chapter 3.3 (Dan et al., 2014). *Artemia* were hatched over 24 h at 25 °C (NH Artemia group), and they were cultured for 48 h at 25 °C at 200 individuals mL^{-1} in 10 L tanks without (starved Artemia group) or with commercially available digestible *Nannochloropsis* (Marine Alfa, Marinetech Co. Ltd, Aichi, Japan) at 30,000,000 cells mL^{-1} (d-Nannochloropsis Artemia group). This digestible *Nannochloropsis* is commercially available in Japan and has a characteristic that all cell walls are physically broken. These *Artemia* were supplied at a density of 2.0 individuals mL^{-1} into the beakers for all treatment groups every morning. We collected five recently hatched MG larvae and all C1 from each replicate beaker for morphological measurements. The *Artemia* supplied daily were sampled to analyse their dry weight, total lipid content, and fatty acid composition.

4.1.3.3 Experiment 3: Effect of phytoplankton and rotifer co-existing on starvation of *Artemia*

NH *Artemia* were stocked in aerated 10 L tanks at a density of 2.0 individuals mL^{-1} and cultured for 5 days to simulate survival and growth of *Artemia* in *P. trituberculatus* larval rearing water. We prepared seven treatment groups (triplicate, total of 21 tanks): 1) control, 2) Nannochloropsis, 3) Nannochloropsis + low density rotifer (LR), 4) Nannochloropsis + high density rotifer (HR), 5) d-Nannochloropsis, 6) d-Nannochloropsis + LR, and 7) d-Nannochloropsis + HR. The control group *Artemia* were cultured without supplementation. *Nannochloropsis* (Yanmarine K-1), and digestible *Nannochloropsis* (Marine Alfa) was added to the tank twice per day to maintain a density of 200,000 cells mL^{-1} in the *Nannochloropsis*-supplemented groups and d-*Nannochloropsis*-supplemented groups, respectively. Rotifers were stocked at a density of 10 and 20 individuals mL^{-1} once on the first day of cultivation in the LR and HR groups, respectively. The seawater was filtered (5 μm pore size), sterilized with chlorine (5 ppm), and neutralized with sodium thiosulfate prior to culture. The culture water (25 °C, 33 ppt) was not renewed during the test period.

The numbers of surviving *Artemia* were estimated based on three 10 mL samples taken from the tanks. Total length of 30 *Artemia* individuals was measured daily from each tank. The specific growth rate of *Artemia* was calculated as the natural logarithm of the proportion of total length at 4 dah compared with the initial total length at 0 dah because *Artemia* in some treatment groups had died by 5 dah.

4.1.3.4 Larval morphological measurements

We measured the length of the carapace (CL) and chela (CHL) in Z4 larvae using a microscope in experiment 1. In addition to the absolute length of the chela, the ratio of the chela length (relative chela length, CHLr) to the CL were calculated (as percentages) as an index of the Z4 larvae megalopal features. CL and length of the dorsal spine (DSL) retained on the MG carapace were measured, and the frequency of megalopae retaining the dorsal spine (DSf) and furcae on their telsons (TELf) were calculated as indices of immature morphology in MG in experiments 1 and 2. These measurement methods are described in Chapter 3.2 and 3.3 (Dan et al. 2013, 2014). The C1 juveniles with close-set eyes in experiments 1 and 2 were observed in some treatment

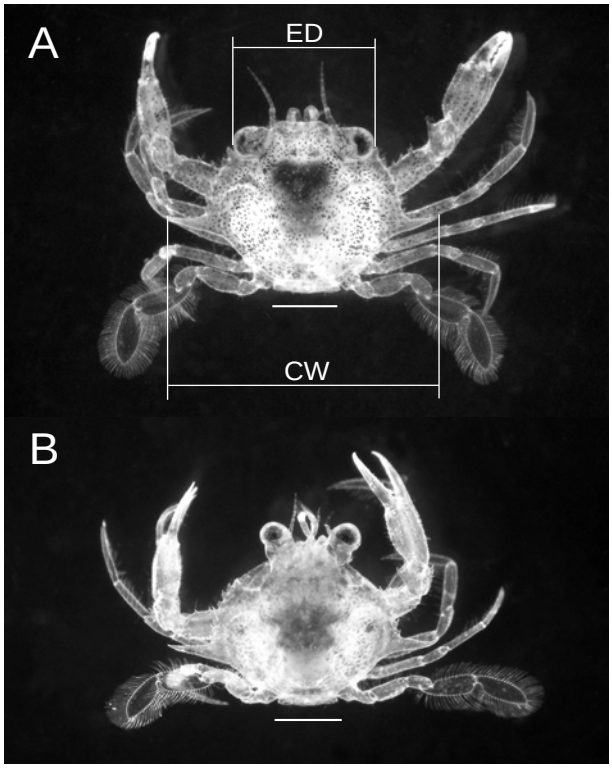

Figure 4.1.1. Photographs of first stage crabs of *Portunus trituberculatus* with schematic diagrams of morphological measurements. A, normal crab; B, morphologically abnormal crab with close-set eyes. Abbreviations: CW, carapace width; ED, eye distance. Scale bar = 1 mm

groups (Fig. 4.1.1), these C1 juveniles were unviable, and most died immediately after moulting. Therefore, we measured CW (lateral spine to lateral spine) and distance between bilateral eyes (ED) of C1, and the ratio of the ED (EDr) to the CW was calculated (as a percentage) in experiments 1 and 2 (Fig. 4.1.1).

4.1.3.5 Chemical analysis

Artemia sampled in experiment 2 were washed, concentrated with distilled water, and their density was determined by the volumetric method. Subsamples (0.5 mL including 3,110–5,020 individuals) were weighed and dried to constant weight at 110 °C.

The samples were washed with distilled water and stored at −80 °C until total lipid and fatty acid analyses. We measured total lipid and fatty acids using a method modified slightly from a method in Chapter 3.1 (Dan and Hamasaki, 2011), as follows. Helium was used as the gas-liquid chromatograph carrier gas at a pressure of 120 kPa. Column temperature was initially held at 170 °C and then increased at a rate of 2 °C min^{-1} to the final temperature of 230 °C.

4.1.3.6 Data analysis

Statistical analyses were performed using R (R 3.1.0; R Development Core Term, 2014) with a 5 % significance level. We used a generalized linear model (GLM) (McCullagh and Nelder, 1989; Everitt and Hothorn, 2009) and a generalized linear mixed-effects model (GLMM) (Everitt, 2005; Everitt and Hothorn, 2009; Zuur et al., 2009) to evaluate the effect of treatment on survival, developmental rate, and morphological measurements of larvae, as well as survival, growth, and nutritional status of *Artemia*. The GLM and GLMM procedures with methods to test the significance of explanatory variables are summarized in Table S4.1.1 in the Appendix.

4.1.4 Results

4.1.4.1 Experiment 1: Effect of starving rotifers and *Artemia* on larval survival and morphology

The changes in the densities of *Nannochloropsis*, rotifers, and *Artemia* retained in the larval rearing water every morning are shown in Fig. S4.1.1 in the Appendix. The density of *Nannochloropsis* retained was low due to consumption by rotifers in the Rotifer + Nannochloropsis group. In contrast, a large number of cells was retained in the Artemia + Nannochloropsis group, as *Artemia* could not digest the *Nannochloropsis* (Fig. S4.1.1A in the Appendix). Rotifer density was high in the Rotifer + Nannochloropsis group compared with that in the Rotifer group (Fig. S4.1.1B in the Appendix). *Artemia* were supplied the previous morning at densities of 2.0 and 4.0 individuals mL^{-1} during the zoeal period and the MG were retained at 1.4 individuals mL^{-1} and 2.6 individuals mL^{-1}, respectively, in the larval rearing water the following morning (Fig. S4.1.1C in the Appendix).

Larval survival rate to C1 moult was 21.8–26.6 %, and no significant treatment effect was observed (Table 4.1.1). The C1 juveniles in the *Artemia*-fed groups (Artemia and Artemia + Nannochloropsis groups) had close-set eyes (Fig. 4.1.1) and were inactive and inviable compared with juveniles in the rotifer-fed groups (Rotifer and Rotifer + Nannochloropsis groups). The developmental rate (LSI) was significantly faster in the *Artemia*-fed groups than that in the rotifer-fed groups (Fig. 4.1.2).

The CL values of Z4 and MG were larger in the *Artemia*-fed groups than those in the rotifer-fed groups, and the Rotifer + Nannochloropsis group had larger CL values than those in the Rotifer group (Fig. 4.1.3A, C). In contrast, none of the treatments had an effect on CW of C1 (Fig.

Table 4.1.1. Mean survival rates of swimming crab, *Portunus trituberculatus*, larvae in each treatment of experiments 1 and 2

Treatment		Survival rate (%)			
		Z3-Z4	Z4-MG	MG-C1	Z3-C1
Experiment 1					
Rotifer		100 (0.0)	52.8 (4.8)	41.5 (11.2)	21.8 (5.0)
Rotifer + Nanno.		100 (0.0)	66.7 (14.4)	41.4 (10.7)	26.6 (2.4)
Artemia		97.2 (4.8)	54.0 (11.2)	44.1 (13.4)	22.2 (1.8)
Artemia + Nanno.		97.2 (4.8)	54.8 (16.1)	46.8 (10.9)	23.8 (0.3)
Treatment effect	*F*	1.816	0.8209	0.4318	2.018
	Df	3, 8	3, 8	3, 8	3, 8
	P(>*F*)	0.2223	0.5180	0.7360	0.1900
Experiment 2					
NH Artemia		95.0 (3.3)	99.1 (1.7)[b]	3.5 (4.1)[a]	3.3 (3.8)[a]
Starved Artemia		96.7 (3.8)	88.9 (5.6)[a]	1.9 (3.8)[a]	1.7 (3.3)[a]
d-Nanno. Artemia		95.0 (4.3)	89.5 (4.9)[a]	40.7 (13.2)[b]	34.2 (9.6)[b]
Treatment effect	*F*	0.2531	8.414	21.58	22.46
	Df	2, 9	2, 9	2, 9	2, 9
	P(>*F*)	0.7817	0.0087	0.0004	0.0003

Values in parentheses represent standard deviations. Different superscript letters indicate the significant differences between treatments. Z3, third zoeal stage; Z4, fourth zoeal stage; MG, megalopal stage; C1, first crab stage. Nanno., *Nannochloropsis oculata*; d-Nanno., commercially available digestible *Nannochloropsis oculata*; NH Artemia, newly hatched *Artemia* nauplii

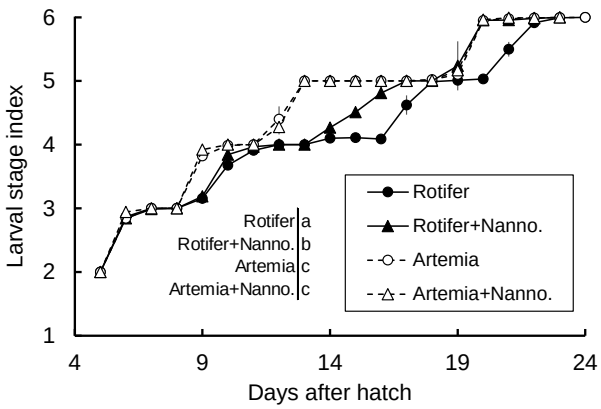

Figure 4.1.2. Changes in larval stage index (LSI) as an index of the larval development rate of *Portunus trituberculatus* cultured under different dietary conditions during the third and fourth zoeal stages in experiment 1. Vertical bars indicate standard deviations of triplicates in each treatment group. Age effect: $F = 3144$, $df = 1, 213.7$, $P < 0.0001$. Treatment effect: $F = 23.79$, $df = 3, 7.998$, $P = 0.0002$. Differences in LSI between treatments ($P < 0.05$) are indicated by a different lowercase letter in the table following the treatment

4.1.3F). The megalopal features in Z4 (CHL and CHLr) tended to be larger in the *Artemia*-fed groups than those in the rotifer-fed groups (Fig. 4.1.3B), whereas the ED and EDr values in the *Artemia*-fed groups, which produced the abnormal C1 juveniles with close-set eyes (Fig. 4.1.1), were smaller than those in the rotifer-fed groups (Fig. 4.1.3G). No significant differences in immature morphology of MG (DSL, DSf, or TELf) were observed between treatment groups (Fig. 4.1.3D, E).

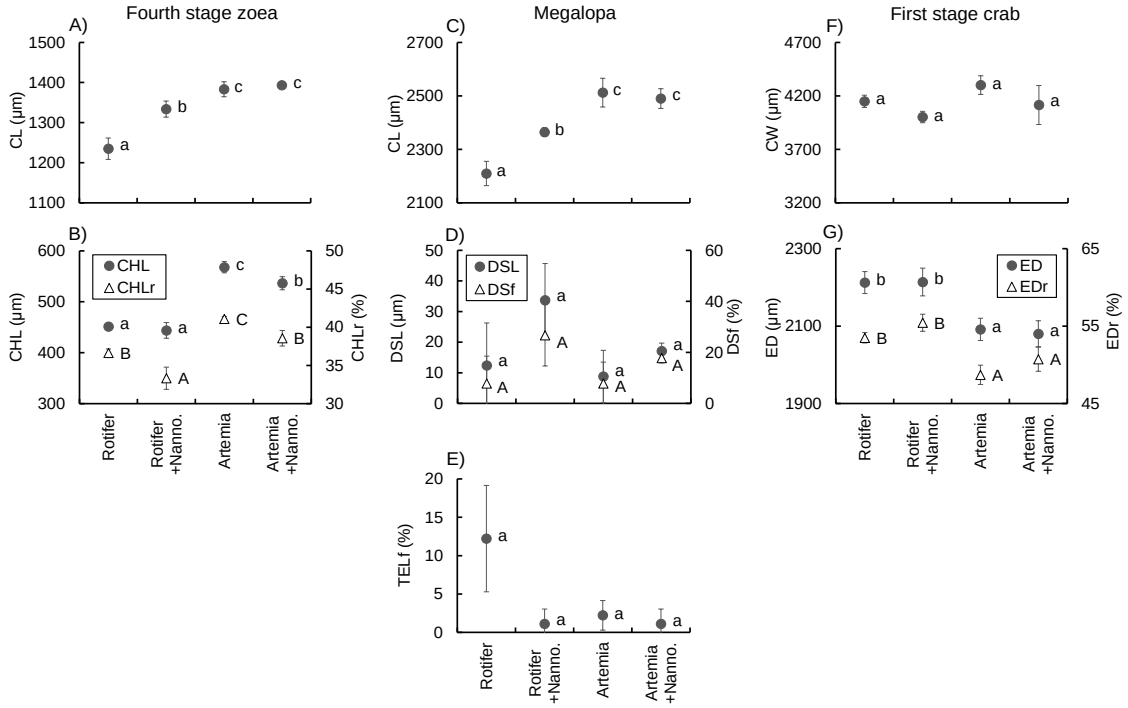

Figure 4.1.3. Differences in carapace length (CL) (A), chela length (CHL), and relative chela length (CHLr) (B) of fourth stage zoeae; CL (C), dorsal spine length (DSL) and frequency of dorsal spine retention (DSf) (D), frequency of telson furcae retention (TELf) (E) of megalopae, carapace width (CW) (F), and eye distance (ED) and relative eye distance (EDr) (G) of the first stage crab of *Portunus trituberculatus* cultured under different dietary conditions during the third and fourth zoeal stages in experiment 1. Vertical bars indicate standard deviations of triplicates in each treatment group. Treatment effect: CL of fourth stage zoeae, $F = 35.33$, $df = 3, 8$, $P = 0.0001$; CHL, $F = 82.19$, $df = 3, 8$, $P < 0.0001$; CHLr, $F = 37.01$, $df = 3, 8$, $P < 0.0001$; CL of megalopae, $F = 35.33$, $df = 3, 8$, $P = 0.0001$; DSL, $F = 3.483$, $df = 3, 8$, $P = 0.070$; DSf, $F = 2.3026$, $df = 3, 8$, $P = 0.1537$; TELf, $F = 5.742$, $df = 3, 8$, $P = 0.0215$; CW, $F = 3.867$, $df = 3, 8$, $P = 0.0560$; ED, $F = 16.09$, $df = 3, 8$, $P = 0.0009$; EDr, $F = 18.52$, $df = 3, 8$, $P = 0.0006$. Differences among treatments are indicated by a different letter ($P < 0.05$)

Overall, the *Artemia*-fed groups exhibited faster development, larger somatic growth in Z4 and MG, and accelerated zoeal morphogenesis compared with those in the rotifer-fed groups. However, the difference in somatic size disappeared in C1, and feeding *Artemia* induced unviable C1 juveniles with close-set eyes.

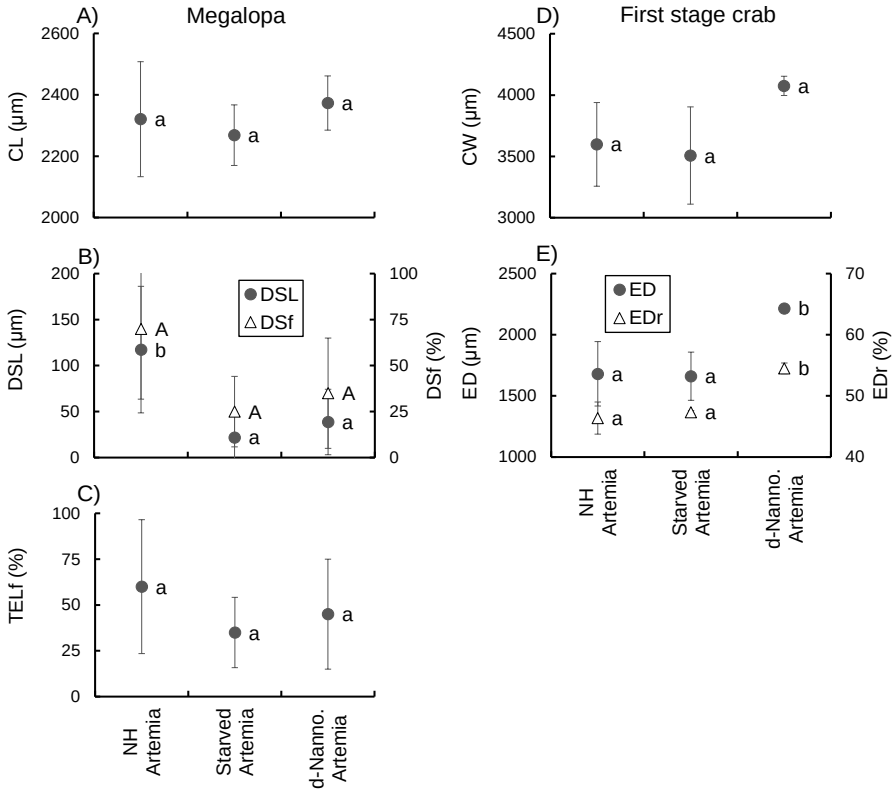

Figure 4.1.4. Differences in carapace length (CL) (A), dorsal spine length (DSL), frequency of dorsal spine retention (DSf) (B), frequency of telson furcae retention (TELf) (C) of megalopae, carapace width (CW) (D), eye distance (ED), and relative eye distance (EDr) (E) of the first stage crab of *Portunus trituberculatus* cultured with differently treated *Artemia* in experiment 2. Vertical bars indicate standard deviations of four replicates in each treatment group. Treatment effect: CL, $F = 0.6226$, $df = 2, 9$, $P = 0.5581$; DSL, $F = 4.706$, $df = 2, 9$, $P = 0.0399$; DSf, $F = 1.960$, $df = 2, 9$, $P = 0.1965$; TELf, $F = 0.7128$, $df = 2, 9$, $P = 0.5160$; CW, $F = 4.735$, $df = 2, 5$, $P = 0.0702$; ED, $F = 13.64$, $df = 2, 5$, $P = 0.0094$; EDr, $F = 31.47$, $df = 2, 5$, $P = 0.0015$. Differences among treatments are indicated by a different letter ($P < 0.05$)

4.1.4.2 Experiment 2: Effect of starved or non-starved *Artemia* on larval survival and morphogenesis

The larvae exhibited high survival (88.9–99.1 %) in all treatment groups until reaching the MG (Table 4.1.1). However, survival declined largely during the MG to C1 period in the NH Artemia (3.5 %) and starved Artemia groups (1.9 %), whereas the d-Nannochloropsis Artemia group had significantly higher survival (40.7 %). Overall survival during the Z3 to C1 period was also highest in the d-Nannochloropsis Artemia group. In contrast, no treatment had an effect on developmental rate (Fig. S4.1.2 in the Appendix).

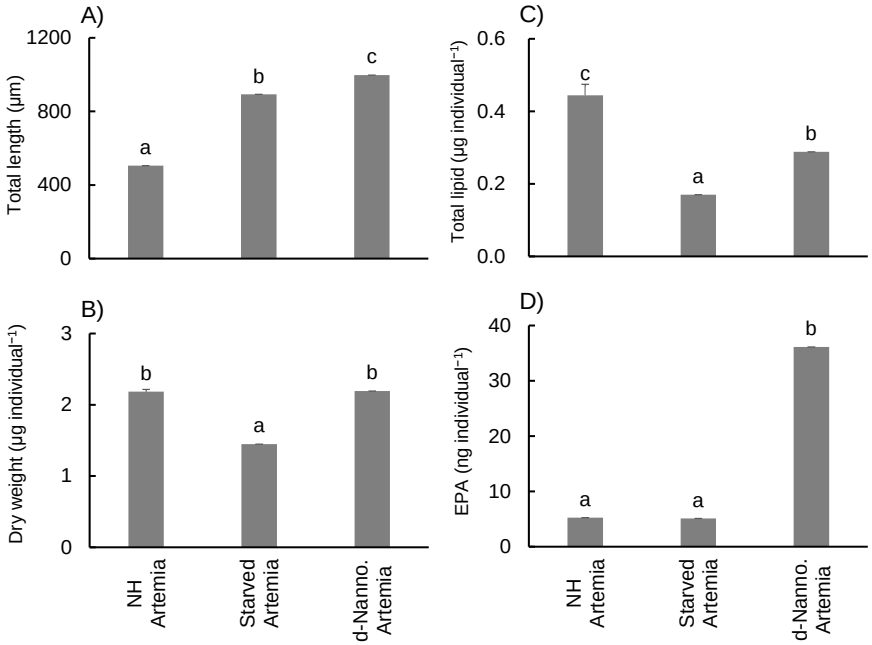

Figure 4.1.5. Differences in total length (A), dry weight (B), total lipid content (C), and eicosapentaenoic acid (EPA) content (D) in individual newly hatched *Artemia*, 48 h starved *Artemia*, and *Artemia* cultured with digestible *Nannochloropsis oculata* for 48 h used in experiment 2. Vertical bars indicate standard deviations of replicated analyses (duplicate for total length and dry weight, triplicate for total lipids and EPA) in each sample. Treatment effect: total length, $F = 1682$, $df = 2, 3$, $P < 0.0001$; dry weight, $F = 15.32$, $df = 2, 3$, $P = 0.0266$; total lipid, $F = 306.4$, $df = 2, 6$, $P < 0.0001$; EPA, $F = 271.3$, $df = 2, 6$, $P < 0.0001$. Differences among treatments are indicated by a different letter ($P < 0.05$)

No treatment had an effect on somatic size (CL) or telson morphology (TELf) of MG (Fig. 4.1.4A, C). Retention of the dorsal spine tended to be higher in the NH Artemia group than that in the other groups; the value was significantly larger in DSL but not in DSf (Fig. 4.1.4B). No significant difference was observed in CW of C1 between the treatment groups, although the CW of the d-Nannochloropsis Artemia group tended to be larger (Fig. 4.1.4D). The ED and EDr of C1 were significantly higher in the d-Nannochloropsis Artemia group than those in the other treatment groups (Fig. 4.1.4E).

The total length of *Artemia* supplied to each treatment group was longest in the d-Nannochloropsis Artemia group and shortest in the NH Artemia group (Fig. 4.1.5A). Dry weight of individual *Artemia* was significantly lower in the starved Artemia group than that in the NH Artemia and d-Nannochloropsis Artemia groups (Fig. 4.1.5B). Total lipid content of individual *Artemia* was highest in the NH Artemia group and lowest in the starved Artemia group (Fig. 4.1.5C). Eicosapentaenoic acid (EPA) content in individual *Artemia* was higher in the d-

Nannochloropsis Artemia group than that in the NH Artemia and starved Artemia groups (Fig. 4.1.5D). The fatty acid composition of *Artemia* in each treatment group is detailed in Table S4.1.2 in the Appendix.

Thus, feeding NH *Artemia* and starved *Artemia*, which contained low EPA levels, resulted in larval mortality during the MG to C1 period and produced C1 juveniles with close-set eyes. These effects were prevented by feeding d-Nannochloropsis *Artemia* containing a higher EPA level.

4.1.4.3 Experiment 3: Effect of phytoplankton and rotifer co-existence on starvation of *Artemia*

The densities (mean ± sd) of *Nannochloropsis* retained in culture water in the morning were 22.3 ±1.3, 5.7 ± 0.6, 2.7 ± 0.4, 2.7 ± 1.2, 1.7 ± 0.6, and 0.7 ± 0.4 × 10^4 cells mL^{-1} in the Nannochloropsis, Nannochloropsis + LR, Nannochloropsis + HR, d-Nannochloropsis, d-Nannochloropsis + LR, and d-Nannochloropsis + HR groups, respectively. Almost all supplemented *Nannochloropsis* in the Nannochloropsis group were retained the following day.

Artemia survival was higher in the d-*Nannochloropsis*-supplemented groups (d-Nannochloropsis, d-Nannochloropsis + LR, and d-Nannochloropsis + HR) than that in the control and *Nannochloropsis*-supplemented groups (Nannochloropsis, Nannochloropsis + LR, and Nannochloropsis + HR) (Fig. 4.1.6A). The d-Nannochloropsis group showed higher survival than that in the d-Nannochloropsis + LR and d-Nannochloropsis + HR groups. Almost all *Artemia* died at 4 dah in the Nannochloropsis + LR and Nannochloropsis + HR groups, but these groups had significantly higher survival than that in the Nannochloropsis group. The Nannochloropsis group showed similar survival to the control group; their survival declined abruptly at 3 dah. The *Artemia* growth rate was faster in the d-*Nannochloropsis*-supplemented groups than that in the control and *Nannochloropsis*-supplemented groups (Fig. 4.1.6B).

Thus, *Artemia* could not digest the untreated *Nannochloropsis*, and feeding the untreated *Nannochloropsis* resulted in mortality and delayed growth due to starvation, similar to that observed in the control group without phytoplankton supplementation. Mortality that occurred during *Nannochloropsis* supplementation slightly but significantly decreased with rotifers in the culture water. However, d-*Nannochloropsis* supplementation maintained high survival and continuous growth of *Artemia*. The rotifers decreased *Artemia* survival in cultures supplemented with d-*Nannochloropsis*.

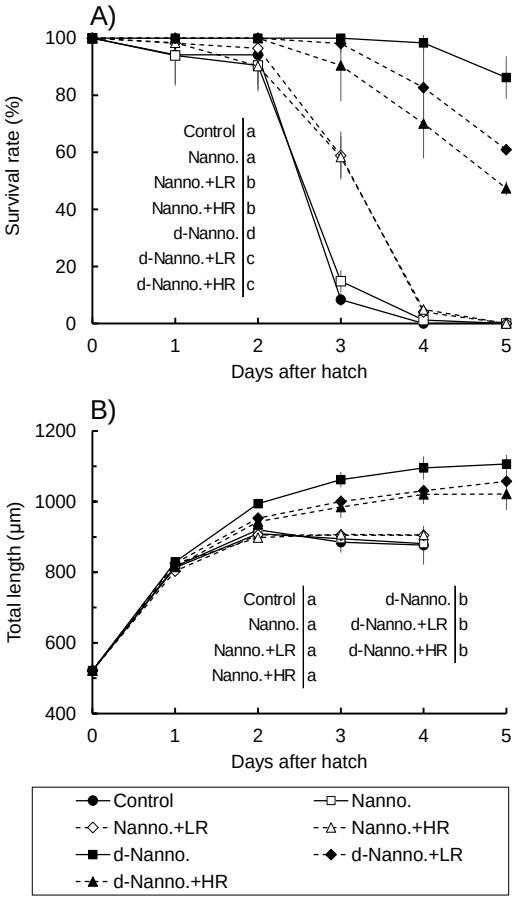

Figure 4.1.6. Changes in survival rate (A) and total length (B) of *Artemia* cultured under different feeding conditions for 5 days. Vertical bars indicate standard deviations of triplicates in each treatment group. Age effect on survival: $\chi^2 = 387779$, $df = 1$, $P < 0.0001$. Treatment effect on survival: $\chi^2 = 1059$, $df = 6$, $P < 0.0001$. Treatment effect on specific growth rate calculated by total length at 4 days after hatch; $F = 27.25$, $df = 6, 118$, $P < 0.0001$. Differences between treatments ($P < 0.05$) are indicated by a different lowercase letter following the treatments in the table

4.1.5 Discussion

P. trituberculatus larvae can be cultured to reach MG by being fed only rotifers (Hamasaki et al., 1998; Takeuchi et al., 1999a), and successfully reared with only *Artemia* after the Z3 stage (Takeuchi et al., 1999b, c). Supplementing with *Artemia* suppresses cannibalism among late zoeal larvae (Takeuchi et al., 1999c; Suprayudi et al. 2002b). In addition, feeding only rotifers makes it difficult to fulfil larval dietary intake during the late zoeal period (Hamasaki, 1997); thus, *Artemia* were supplied from the Z2 or Z3 stages during all seed production trials conducted recently by Japanese hatcheries (Dan et al. 2013; Chapter 3.2). In experiment 1, *Artemia* fed during the late zoeal period achieved larger Z4 and MG, faster developmental rate, and accelerated zoeal morphogenesis compared with feeding only rotifers. A similar positive effect of *Artemia* supplementation on zoeal development and morphogenesis has been reported in mud crab *Scylla serrata* (Suprayudi et al. 2002b). Based on these results, it appears reasonable to commence *Artemia* supplementation from the Z3 stage during *P. trituberculatus* seed production.

Z4 and MG somatic size and zoeal morphogenesis and developmental rates decreased when larvae were reared with rotifers during the late zoeal period compared with those in the *Artemia*-fed groups. However, the somatic size of C1 in the rotifer-fed groups recovered to the same size as the *Artemia*-fed groups by feeding *Artemia* after moulting to MG. In contrast to the rotifer-fed groups in which viable and morphologically normal C1 were observed, feeding *Artemia* during the late zoeal period induced unviable and abnormally close-set eyed C1, regardless of *Nannochloropsis* supplementation. The eye sockets of these close-set eyed C1 were located in the normal position (Fig. 4.1.1), but their eyestalks protruded abnormally frontward and moved very little. We speculate that the close-set eyes were caused by poor vitality of MG larvae; inactive larvae might take more time to shed their exoskeletons, and the eyestalks may have been caught.

As larvae in both the rotifer- and *Artemia*-fed groups were supplied with the same food (*Artemia*) after MG in experiment 1, the difference in dietary nutrients assimilated during the late zoeal period appeared to affect vitality and survival during the post-larval period (from MG to C1). This seemed to be a carry-over effect. Interestingly, feeding *Artemia* during the late zoeal period, which had a positive effect on growth and morphogenesis until MG, exerted a negative effect on vitality and morphology during the MG to C1 period. This distinct difference in the effect of *Artemia* supplementation between these periods (from Z3 to MG and from MG to C1) suggests that larval nutritional requirements differ between these times. As *P. trituberculatus* juveniles have been cultured successfully from C1 to the third crab stage eating only NH *Artemia* (Hamasaki and Sekiya, 1998), the larvae may require a special nutrient(s) only during the late zoeal period. Taken together, larvae may need to assimilate a special nutrient during the late zoeal period to develop successfully during the post-larval period, and this nutrient was lacking in *Artemia* but not rotifers in experiment 1.

Some decapod crustacean species have a non-feeding decapodid stage (i.e., MG) termed secondary lecithotrophy; e.g., decapodid larvae of some hermit crab, king crab, and spiny lobster species moult into successive juvenile stages without food (Anger, 2001c). Nutritional and energetic reserves that have been assimilated during the zoeal stage in these species are carried over during the period from decapodid to the next juvenile stage (Anger, 1989, 2001c). Calado et al. (2010) investigated starvation tolerance during the last zoeal, decapodid, and juvenile stages in four species of caridian shrimp and demonstrated that the decapodid stage exhibit higher starvation tolerance compared with that of the last zoeal and juvenile stages even in species that exhibit feeding behaviour during the decapodid stage (called facultative secondary lecithotrophy).

Facultative secondary lecithotrophy during the MG stage has also been reported in the brachyuran crab, *Mithraculus forceps* (Figueiredo et al., 2008). As the decapodid stage is a transition from the planktonic to the benthic stage, higher starvation tolerance may be a strategy to overcome the food deprivation encountered and explore suitable settlement (Anger, 1989, 2001c; Abrunhosa et al., 2008; Calado et al., 2010; Figueiredo et al., 2008). Although the MG of *P. trituberculatus* have the ability to eat, which contributed to increase C1 somatic size in the rotifer-fed groups in experiment 1, they also exhibit more swimming (locomotion) ability and planktonic and benthic behaviour (Hamasaki, 1997; Yatsuzuka and Sakai, 1982), indicating that MG is a period to explore for settlement. In this sense, dietary intake during the late zoeal period appeared to be as important as nutritional and energetic reserves consumed during MG; thus, larvae may require a special nutrient to transition this period.

Although late *Artemia* metanauplii at 4 dah can digest *Nannochloropsis* (Fuentes et al., 2011), we demonstrated that early *Artemia* nauplii could not digest *Nannochloropsis*, resulting in mortality and delayed growth due to starvation, similar to the non-supplemented treatment. Unenriched NH *Artemia* were supplied at a rate of 67.6 % in all *P. trituberculatus* seed production trials conducted in Japan (Dan et al., 2013; Chapter 3.2). In addition, *Artemia* are generally added at a sufficient density beyond the number consumed by larvae to obtain better growth and survival during larval rearing of brachyuran crabs (Andrés et al., 2007; Baylon, 2009; Minagawa and Murano, 1993; Suprayudi et al., 2002b). In fact, about 70 % of the *Artemia* supplied were retained in the larval rearing water the following morning in experiment 1. Furthermore, the proportion of seed production trials that supplemented larval rearing water with *Nannochloropsis* sp. and *Chlorella vulgaris* (hereafter, *Chlorella*) reached 88.2 % (*Nannochloropsis* and/or *Chlorella*, 61.2% and *Nannochloropsis* and diatom combination, 27.0 %) (Dan et al., 2013; Chapter 3.2). *Artemia* nauplii are unable to digest *Chlorella* species, as they have rigid cell walls like *Nannochloropsis* (Gerken et al., 2012; Sick, 1976). These seed production procedures adopted in Japanese hatcheries may lead to *Artemia* starvation.

As feeding starved *Artemia* to late zoeal larvae clearly reduced survival during the MG to C1 period in experiment 2, starving *Artemia* after adding them to larval rearing water may cause a low survival rate after MG during seed production. Supplementing with *Artemia* in combination with *Nannochloropsis* and *Chlorella* has been adopted during seed production of various species including crustacean and finfish species (Kogane et al., 2007; Lee and Ostrowski, 2001; Marte,

2003; Shields, 2001). Starving *Artemia* can occur and exert a negative effect on cultured animals in these cases.

We think there are three reasons why attention to *Artemia* starvation has not been reported by culturists. One is that *Artemia* can grow and survival until 2 dah (48 h) by consuming yolk reserves (Fig. 4.1.6) (Bengtson et al., 1991; Sorgeloos et al., 2001), which makes it difficult to notice the starvation. The second reason is that the negative effects of *Artemia* starvation during the late zoeal period carry over into the post-larval period, as mentioned above. This conceals whether starvation occurred or not. The third reason is that the negative effects of *Artemia* starvation are cancelled when food for *Artemia* is unintendedly supplied. The coexistence of rotifers in the *Artemia* culture water in experiment 3 improved *Artemia* survival. Because rotifers have a feeding apparatus (trophi in mastax) that crushes solid phytoplankton cell walls (Kleinow and Wratil, 1996; Yu and Cui, 1997), the *Artemia* may have fed on *Nannochloropsis* crushed by rotifers and/or fed on rotifer excrement. In addition, *Artemia* can use bacteria as food (Intriago and Jones, 1993; Toi et al., 2013), suggesting that bacteria proliferating because of rotifer excrement might be available as food for *Artemia*. *Artemia* and rotifers are supplied simultaneously during late zoeal period of *P. trituberculatus* seed production and coexist in the larval rearing water (Dan et al., 2013; Chapter 3.2). Therefore, *Artemia* may feed on these organisms even after supplementation with *Nannochloropsis* and *Chlorella*. However, if these organisms are not controlled by the culturist, their balance can be disrupted. Thus, the negative effect of *Artemia* starvation appears to be unintendedly induced/cancelled occasionally, which carries over. It appears to be difficult for culturists to perceive such a complex situation.

We demonstrated that starvation of *Artemia* could be overcome by supplementing with digestible *Nannochloropsis* in which the cell walls are physically broken. In addition, supplying *Artemia* that were fed digestible *Nannochloropsis* and contained higher EPA levels succeeded in improving survival after MG and producing morphologically normal C1. Although other phytoplankton, such as *Chaetoceros* sp., *Isochrysis* sp., and *Tetraselmis* sp., are available food for *Artemia*, *Nannochloropsis* contains higher EPA content among these phytoplankton (Liao et al., 2001; Lora-Vilchis et al., 2004; Renaud et al., 1991). The starved *Artemia* in the present study had a low dry weight and total lipid and EPA contents, indicating a drop in nutrition during starvation, as reported previously (Coutteau and Mourente, 1997; Estévez et al., 1998; Evjemo et al., 2001; Han et al., 2001). NH *Artemia* also contained lower EPA levels, similar to starved *Artemia*, and a continuous supply of NH *Artemia* resulted in low survival during the MG to C1

period in experiment 2. Takeuchi et al. (1999b) reared *P. trituberculatus* larvae after Z3 with *Artemia* enriched with various levels of EPA and DHA and reported that EPA improves survival during MG. This result agrees with the results of our study. These results suggest that MG requires EPA as a special nutrient that must be assimilated during the late zoeal period.

Our results suggest that it is necessary to supply EPA during the late zoeal period of *P. trituberculatus* for better survival to reach C1 and to prevent *Artemia* starvation. Supplementing larval rearing water with digestible *Nannochloropsis* is suggested to be effective based on our results. In contrast, supplementing larval rearing water with phytoplankton, such as *Nannochloropsis* and *Chlorella*, may cause morphologically abnormal MG resulting in mass mortality via assimilation of these phytoplankton by rotifers (Dan et al., 2013, 2014, Chapter 3.4). The critical period at which phytoplankton induce an abnormal MG is estimated to be the Z3 premoult (Dan et al., 2014, Chapter 3.4), and this period coincides with the *Artemia* feeding period during seed production (Dan et al., 2013; Chapter 3.2). Measures to prevent starvation of *Artemia* while preventing abnormal MG morphology should be developed to produce viable C1 with high survival rates. We speculate that such a complicated requirement is the reason why survival rates during seed production have been so low in Japanese hatcheries. Because larval intake of phytoplankton (digestible *Nannochloropsis*) via consumption of *Artemia* did not induce the abnormal morphology of MG in experiment 2, supplementing larval rearing water with enriched *Artemia* and adding digestible *Nannochloropsis* without rotifers may be a possible measure to overcome the low larval survival rates during seed production.

4.1.6 Conclusions

We conclude that starvation of *Artemia* in larval rearing water can be easily induced during *P. trituberculatus* seed production, and that starving *Artemia* during the late larval period exerted negative carry-over effects on survival and morphology during the post-larval period that were associated with a drop in nutrition, particularly EPA content. Supplementing with digestible *Nannochloropsis* containing high EPA content is a suggested measure to overcome *Artemia* starvation and its negative effects on larvae. These findings indicate that larval nutritional requirements during seed production are closely associated with ecological adaptation in their natural habitat (e.g., EPA requirement and facultative secondary lecithotrophy). It is important to improve our knowledge of the physiological and ecological adaptation strategies of cultured animals and their relationships to larval nutritional requirements, while considering the biological

characteristics of the live food (phytoplankton, rotifer, and *Artemia*), such as feeding, digestion, and nutritional bioconversion, to achieve further improvements in brachyuran crab larval rearing technology.

Chapter 5

LARVAL CULTURE METHOD TO CONTROL LARVAL MASS MORTALITY

5.1 Improved method of culturing the swimming crab *Portunus trituberculatus* larvae to prevent mass mortality during seed production

5.1.1 Summary

Larvae of the swimming crab *Portunus trituberculatus* are traditionally cultured with rotifers, *Artemia*, and phytoplankton. However, abnormal morphology of last stage zoeae caused by excessive nutritional intake and abnormal morphology of megalopae caused by indirect ingestion of phytoplanktons and lack of essential nutrients due to starvation of *Artemia* causes mass mortality of swimming crab larvae. To develop techniques to address these issues simultaneously, we evaluated methods to prevent megalopal abnormalities but maintain the nutritional value of the diet (experiment 1). Then, we evaluated the effects of *Artemia* feeding schedules on the occurrence of zoeal abnormalities (experiment 2). In experiment 1, megalopal abnormality was prevented by rearing larvae with enriched *Artemia* and digestible *Nannochloropsis* but without rotifers. However, the developmental rates were variable among individuals because of the large size of enriched *Artemia*, and zoeal abnormality was induced because of excessive *Artemia* feeding, resulting in low survival. In experiment 2, newly hatched *Artemia* were only supplied during the first zoeal stage, and larvae were reared with low density *Artemia* and/or low salinity. Our results suggest that *P. trituberculatus* larvae can be successfully cultured using only *Artemia*.

5.1.2 Introduction

The swimming crab, *Portunus trituberculatus*, is distributed throughout the temperate zone of the western Pacific Ocean. The species is the base of an economically important fishery that had a global harvest of 429,959 t in 2012 (FAO, 2014). To reduce pressure on wild stocks and meet consumer demands, culturists have expanded *P. trituberculatus* seed production efforts in Japan, China, and Korea (Dan et al., 2013; Hamasaki et al., 2011; Lee et al., 2013; Qui et al., 2013). The crab seeds are used for aquaculture and stock enhancement; aquaculture production exceeded 91,050 t in 2010 in China (Jin et al., 2013) and > 30 million and 10 million juveniles are released annually into the wild in Japan and Korea, respectively (Hamasaki et al., 2011; Lee et al., 2013). However, survival to the juvenile stage has been low and variable because of frequent mass larval mortality (Dan et al., 2013; Hamasaki et al., 2011; Chapter 3.2).

Mortality during seed production of the portunid crab including *P. trituberculatus* and *Scylla serrata* is associated with the occurrence of morphological abnormalities in larvae (Arai et al., 2004; Dan et al., 2013; Hamasaki et al., 2002a, 2011; Chapter 3.2). Two distinct types of

morphological abnormality have been reported. The first occurs in last stage zoeae and is characterized by development of excessively advanced morphological features, similar to those of megalopa, including large chelae and pleopods (Arai et al., 2004; Hamasaki et al., 2002a). The second type of abnormality occurs in megalopae and is characterized by retention of zoeal immature morphological features, such as small dorsal spines and telson furcae (Dan et al., 2013; Chapter 3.2, 3.4). Both of these abnormalities are related to larval moulting failure during metamorphosis into megalopae. The increase in size of the last zoeal chelae and retention of the megalopal dorsal spine increase the frequency of megalopae retaining old exuviae on their carapace and/or appendages (Dan et al., 2013; Chapter 3.2). The mortality caused by moulting failure during metamorphosis to the megalopal stage has been termed "moult death syndrome" among brachyuran crab species, including those that are intensively cultured such as *Mithrax caribbaeus*, *Portunus pelagicus*, *P. trituberculatus*, *S. serrata*, *Scylla tranquebarica*, and *Ucides cordatus* (Arai et al., 2004; Baylon, 2009; Hamasaki et al., 2002a; Holm et al., 2009; Lárez et al., 2000; Mann et al., 1999; Nurdiani and Zeng, 2007; Rodzuani et al., 2012; Silva et al., 2012; Soundarapandian et al., 2007; Suprayudi et al., 2002b). The occurrence of immature morphology in megalopae is thought to be the primary cause of moult death syndrome of *P. trituberculatus* larvae in Japanese hatcheries (Dan et al., 2013; Chapter 3.2).

The extrinsic factors influencing the occurrence of larval morphological abnormalities and their relationship to intrinsic mechanisms controlling larval morphogenesis have been widely studied in portunid crabs. Morphogenesis of body parts such as the chelae and pleopods, which are enlarged during metamorphosis into megalopae, is continuously accelerated throughout zoeal development under the control of the neurosecretory system located in the eyestalk (Dan et al., 2014; Chapter 3.3). The rate of morphogenesis is accelerated when larvae are reared under certain dietary and environmental conditions. Increased docosahexaenoic acid (DHA) content in rotifers, supplementation of *Artemia* at high density, and high (optimal) rearing water salinity are associated with enlarged zoeal chelae (Arai et al., 2007; Dan and Hamasaki, 2011; Hamasaki et al., 2002b; Suprayudi et al., 2002b; Chapter 3.1). Because the enlargement of these body parts may require a large amount of energy, it is reasonable that larvae would advance morphogenesis prior to megalopae as much as possible when nutritional and environmental conditions allow during the zoeal stage (Dan et al., 2014; Chapter 3.3). Thus, excessive intake of nutrition under non-stressful conditions may induce excessive morphogenesis of these body parts, resulting in abnormal morphology of last stage zoeae. Conversely, the morphogenesis of body parts that are

resorbed during metamorphosis into megalopae (e.g., dorsal spine and telson furcae) is controlled instantaneously at a critical period by the eyestalk neurosecretory system (Dan et al., 2014; Chapter 3.3). The critical period occurs at premoult during the penultimate zoeal stage (third zoeal stage for *P. trituberculatus*). The addition of phytoplankton such as *Chlorella vulgaris* and *Nannochloropsis oculata* into the rearing water indirectly induces the retention of these body parts in megalopae via the consumption of rotifers at this critical period, resulting in abnormal morphology of megalopae. Thus, the phytoplankton appear to have a factor that disrupts larval endocrine control of body part resorption (Chapter 3.4).

In addition to morphological abnormalities in the last stage zoeae and megalopae, low nutritional value of *Artemia* has also been associated with mortality during the period from megalopal to first crab stages (Chapter 4.1). Newly hatched and starved *Artemia* have a low eicosapentaenoic acid (EPA) content. Furthermore, *Artemia* are unable to digest phytoplankton such as *Chlorella* and *Nannochloropsis* because of their rigid cell walls (Chapter 4.1). These phytoplankton species are commonly used during seed production as food for live feed in Japanese hatcheries (Dan et al., 2013; Chapter 3.2), but their use may result in starvation of *Artemia*. Because, EPA is an essential fatty acid for *P. trituberculatus* larvae (Takeuchi et al., 1999a, b), larvae that feed on *Artemia* with low EPA content during late zoeal development (third and fourth stage zoeae) die during the megalopal period or moult to an unviable first stage crab.

Thus, the causes and/or phenomena associated with larval mass mortality during seed production of portunid crabs are relatively well understood. To prevent excessive morphogenesis of last stage zoeae, culturists can control the dietary nutritional level, feeding density, and environment to suppress morphogenesis of chelae (Dan and Hamasaki, 2011; Dan et al., 2014; Hamasaki et al., 2002b; Chapter 3.1, 3.3). To prevent immature morphology of megalopae, culturists can prevent larval intake of phytoplankton via consumption of rotifers during the critical period (Chapter 3.4). Last, to prevent nutritional deficiency of *Artemia*, culturists can enrich *Artemia* with EPA and supplement digestible *Nannochloropsis*, which contain EPA and in which the cell walls are ruptured, into the larval rearing water (Chapter 4.1).

To minimize larval mass mortality during seed production, all of these measures must be implemented concurrently. However, this poses some practical problems. For example, rotifers and *Artemia* typically coexist in the late zoeal rearing water during seed production (Dan et al., 2013; Chapter 3.2). Thus, there is potential that supplementation of the rearing water with digestible *Nannochloropsis* would prevent *Artemia* starvation but indirectly induce megalopal

immature morphology via consumption of rotifers. Our objective was to develop a rearing technique that would address these conflicting requirements. In experiment 1, we evaluated the interactive effect of 1) the timing of feeding enriched or non-enriched *Artemia* feeding, 2) the rotifer feeding schedule, and 3) supplementation with digestible or non-digestible *Nannochloropsis* on the incidence of megalopal abnormal morphology and nutritional deficiency of *Artemia*. In experiment 2, we evaluated methods to control last zoeal abnormal morphology using enriched *Artemia* as a primary food source for larvae reared at different feeding densities and salinities. Based on these results, we propose a new rearing technique for *P. trituberculatus* to prevent larval mass mortality.

5.1.3 Materials and Methods

5.1.3.1 Experiment 1: Effect of feeding schedule on larval survival and morphology

Recently hatched larvae (first stage zoeae, Z1) were obtained from two females (173 and 181 mm carapace width (CW)) on July 30, 2014 following the method described in Chapter 3.3 (Dan et al., 2014). The larvae from the two females were pooled and stocked into 18 polyethylene tanks (500 L seawater; temperature, 26.1 ºC; salinity, 33 ppt) at a mean density of 11,250 individuals 500 L^{-1} (range: 9,833 to 12,500). We evaluated the effect of timing of supplementation with enriched or newly hatched non-enriched *Artemia* (EAr or NHAr), enriched rotifers, and digestible or non-digestible *Nannochloropsis oculata* (hereafter, *Nannochloropsis*). The tanks were divided into six treatment groups (3 tanks/treatment): 1) EAr supplied from the first zoeal stage (EAr-Z1), 2) EAr supplied from the second zoeal stage (EAr-Z2), 3) EAr supplied from the third zoeal stage (EAr-Z3), 4) EAr supplied from the fourth zoeal stage (EAr-Z4), 5) NHAr supplied from the Z1 stage (NHAr-Z1), and 6) NHAr supplied from the Z3 stage (NHAr- Z3). The NHAr-groups were supplemented with *Artemia* (Utah Strain, Pacific Trading Co., Ltd., Fukuoka, Japan) that had hatched within the past 24 h (at 25 °C) and non-digestible *Nannochloropsis* (Yanmarine K-1, Chlorella Industry Co., Ltd, Tokyo, Japan). The EAr-groups, were supplemented with *Artemia* (200 individuals mL^{-1}) that were enriched with 10,000,000 cells mL^{-1} of commercially available digestible *Nannochloropsis* (Marine Alfa, Marinetech Co. Ltd, Aichi, Japan) for 24 h after hatching. Additionally, the rearing water of these groups was supplemented with digestible *Nannochloropsis*. Rotifers enriched with Baker's yeast fortified with DHA (Asuka Pharmaceutical Co., Ltd, Tokyo, Japan) were provided to all tanks until the initiation of *Artemia*

Table 5.1.1. Protocols for rearing larvae of the swimming crab *Portunus trituberculatus* in experiments 1 and 2

Treatment	*Artemia* addition (ind. mL^{-1})						Rotifer addition	Phytoplankton supplementation (10^4 cell mL^{-1})			Salinity
	Type	Z1	Z2	Z3	Z4	MG	Duration	Type	Density	Duration	(ppt)
Experiment 1											
EAr-Z1	Enriched[1]	3.0	3.0	3.0	3.0	3.0	–	d-Nanno.[3]	20	Z1 - MG	33
EAr-Z2	Enriched[1]	–	3.0	3.0	3.0	3.0	Z1[2]	d-Nanno.[3]	20	Z2 - MG	
EAr-Z3	Enriched[1]	–	–	3.0	3.0	3.0	Z1 - Z2[2]	d-Nanno.[3]	20	Z3 - MG	
EAr-Z4	Enriched[1]	–	–	–	3.0	3.0	Z1 - Z3[2]	d-Nanno.[3]	20	Z4 - MG	
NHAr-Z1	Newly hatched	3.0	3.0	3.0	3.0	3.0	–	Nanno.	20	Z1 - MG	
NHAr-Z3	Newly hatched	–	–	3.0	3.0	3.0	Z1 - Z2[2]	Nanno.	20	Z3 - MG	
Experiment 2											
EAr	Newly hatched	–	–	–	–	–	–	d-Nanno.[3]	20	Z1 - MG	32
	Enriched[1]	3.0	3.0	3.0	3.0	3.0					
NH-EAr	Newly hatched	3.0	–	–	–	–			20		32
	Enriched[1]	–	3.0	3.0	3.0	3.0					
NH-EAr (LD)	Newly hatched	1.5	–	–	–	–			10		32
	Enriched[1]	–	1.5	1.5	1.5	3.0					
NH-EAr (HD-LD)	Newly hatched	3.0	–	–	–	–			10		32
	Enriched[1]	–	1.5	1.5	1.5	3.0					
NH-EAr (LS)	Newly hatched	3.0	–	–	–	–			20		25[4]
	Enriched[1]	–	3.0	3.0	3.0	3.0					
NH-EAr (LS+LD)	Newly hatched	1.5	–	–	–	–			10		25[4]
	Enriched[1]	–	1.5	1.5	1.5	3.0					

Moulting stage: Z1, first zoeal stage; Z2, second zoeal stage; Z3, third zoeal stage; Z4, fourth zoeal stage; MG, megalopal stage
[1] Enriched with commercially available digestible *Nannochloropsis oculata* (Marine Alfa, Marinetech Co. Ltd, Aichi, Japan)
[2] Rotifers enriched with commercially available frozen baker's yeast were added at densities of 10, 15, and 20 ind. mL^{-1} at the Z1, Z2, and Z3 stages, respectively
[3] Commercially available digestible *Nannochloropsis oculata* (Marine Alfa)
[4] Salinity was reduced gradually over 2 days (32 ppt at hatching, 28 ppt on the first day after hatching, and 25 ppt 2 days after hatching)

supplementation, except the EAr-Z1 and NHAr-Z1 groups. In these groups (EAr-Z2, EAr-Z3, EAr-Z4, and NHAr-Z3), the rotifer supplementation was terminated after supplying *Artemia* commenced, but the rotifers remained in the larval rearing water thereafter. The supplementation schedules for each treatment group are shown in Table 5.1.1. The larval rearing procedures were the same as those described in Chapter 3.3 (Dan et al., 2014).

Twenty larvae were sampled daily from each tank and their larval moulting stage was determined by observation under a stereomicroscope. A larval stage index (LSI) was calculated using the formula: LSI = (absolute value × number of larvae)/20 (Villegas and Kanazawa, 1980; Millamena and Quinitio, 2000). Each individual was classified (as absolute value) using scale of 1, 2, 3, 4, or 5 corresponding to Z1, Z2, Z3, Z4, and megalopae (MG), respectively. The numbers of surviving larvae were estimated by the volumetric method for each stage from Z2 to MG, and all surviving individuals were counted at the first crab stage (C1). Thirty recently moulted Z4, MG, and C1 stage individuals were sampled from each tank for morphological measurements. In each tank of the EAr-Z1, EAr-Z3, NHAr-Z1, and NHAr-Z3 groups, thirty *Artemia* were sampled each morning (08:00) prior to additional supplementation, and their total length was measured during the zoeal period.

5.1.3.2 Experiment 2: Effect of *Artemia* feeding method on larval survival and morphology

Recently hatched Z1 were obtained from a female (194 mm CW) on September 3, 2014 and stocked into 18 tanks (500 L seawater; temperature, 25.4 ºC) at a mean density of 12,008 individuals 500 L^{-1} (range: 10,167 to 13,167). We evaluated the effect of *Artemia* size (small newly hatched or large enriched *Artemia*) on Z1 and the effect of feeding density of *Artemia* on the incidence of abnormal morphology in the Z4 stage. Additionally, we evaluated the effect of low salinity (LS) on the morphology of Z4 individuals in the LS-groups, following methods used in *S. serrata* (Dan et al., 2011; Chapter 3.1). The tanks were divided into six treatment groups (3 tanks/treatment): 1) EAr supplemented throughout the zoeal stage, 2) a combination of NHAr during the Z1 stage followed by EAr from the Z2 stage (NH-EAr), 3) NH-EAr and low density *Artemia* (LD) supplementation, 4) NH-EAr and high density *Artemia* supplementation at the Z1 stage followed by low density supplementation after the Z2 stage (HD-LD), 5) NH-EAr and low salinity larval rearing water (LS), and 6) NH-EAr with LS and LD (LS+LD). The hatching and methods for enriching *Artemia* were the same as in experiment 1. The rearing water was supplemented with digestible *Nannochloropsis* in all groups, but rotifers were not supplied to any groups. The supplementation schedule for each treatment group is shown in Table 5.1.1. In the low

salinity (LS)-groups, the salinity was gradually reduced from 32 ppt at hatching to 25 ppt 2 days after hatching (dah) by adding sterilized fresh water. Other rearing procedures, including the methods for estimating larval survival, rate of development, and larval sampling were the same as in experiment 1.

5.1.3.3 Morphological measurements of larvae

We measured the length of the carapace (CL) and chela (CHL) of Z4 stage individuals using a microscope. Additionally, we calculated the ratio of CHL to CL (relative chela length, CHLr, %) as an index of abnormal morphology in Z4 stage individuals. We measured CL and the length of the dorsal spine (DSL) retained on the MG carapace. The frequency of MG stage individuals retaining the dorsal spine (DSf) and furcae on their telsons (TELf) were used as indices of abnormal immature morphology of MG stage individuals. The occurrence of C1 juveniles with close-set eyes is related to nutritional deficiency in larvae (Chapter 4.1). Therefore, in addition to the CW, we measured the distance between the bilateral eyes (ED) of C1 stage individuals, and the ratio (%) of the ED (EDr) to the CW. These measurement methods are described in Chapter 3.2, 3.3, and 4.1 (Dan et al., 2013, 2014). In both experiments, the larvae that failed to moult to the MG stage were counted. As an index of serious moulting failure, we calculated the frequency of larvae that were trapped by the exuviae (OEf) (Hamasaki et al., 2002a). Additionally, a total moulting failure index (MFf) was calculated by summing OEf and the frequency of larvae that had scars on their chelae and walking legs because of moulting failure (but without old exuviae).

5.1.3.4 Data analysis

Statistical analyses were performed using R (R 3.1.0; R Development Core Term, 2014) with a 5 % significance level. We used the generalized linear model (GLM) with the *glm* function to evaluate the effect of the treatments on larval survival (McClullagh and Neilder, 1989; Everitt and Hothorn, 2009). The numbers of moulting and not moulting (died before reaching successive moulting stages) individuals from Z1 to Z4, from Z1 to MG, and from Z1 to C1 were used as the two-vector response variable with the quasi-binomial family (logit link) to account for over dispersion of the error distribution. The categorical fixed-factor “treatment” was used as the explanatory variable in these analyses.

A generalized linear mixed-effects model (GLMM) using the *lmer* function in the lme4 package (Everitt, 2005; Everitt and Hothorn, 2009; Zuur et al., 2009) was used to evaluate the effect of treatment on larval development rate (lme4: http://github.com/lme4/lme4/ “Accessed 1

November 2014”). In this analysis, the LSI value was used as the response variable with the Gaussian family (identical link), and the explanatory variables were larval age (dah) and treatment (as a categorical fixed factor). LSI data were collected from the same tank every day. Therefore, the identity of each rearing tank was included in the GLMM as a random intercept effect to account for a potential correlation among tank observations (Everitt, 2005; Everitt and Hothorn, 2009; Zuur et al., 2009).

The effects of the treatments on larval morphology were analyzed using a GLM. The morphological measurements were used as response variables with a Gaussian distribution and an identical link (CL, CHL, CHLr, DSL, CW, ED, and EDr) or quasi-binomial distribution with a logit link (DSf, TELf, OEf, and MFf) to account for over dispersion of the error distribution. The explanatory variables were the treatments in these analyses.

To evaluate the statistical significance of the explanatory variables, the *F* test (both for the quasi-binomial and Gaussian families in the GLM analyses), or the Wald *F* test with Kenward–Roger degrees of freedom (Gaussian family in the GLMM analyses) was performed using the *Anova* function (type II) implemented in the car package (pbkrtest: http://people.math.aau.dk/~sorenh/software/pbkrtest/ “Accessed 1 November 2014”) (Kenward and Roger, 1997; Fox and Weisberg, 2011). Differences between treatments were evaluated with Tukey’s method using the *glht* function implemented in the multcomp package (Hothorn et al., 2008).

Moulting failure, represented by the OEf and MFf indices, corresponded to the occurrence of excessively advanced Z4 morphology and/or immature MG morphology. To evaluate the relationship between moulting failure and the incidence of larval morphological abnormalities, we used a GLM with CHLr and DSL as explanatory variables and OEf and MFf as the response variables (binomial family, logit link). In this analysis, the independent effects of CHLr and DSL, the additive effect (CHLr + DSL), and the interaction (CHLr × DSL) were analyzed to determine which factor had the largest effect. Model selection was conducted based on Akaike’s information criterion (AIC), where the best model had the lowest AIC value (Akaike, 1973; Burnham and Anderson, 2002).

5.1.4 Results

5.1.4.1 Experiment 1: Effect of feeding schedule on larval survival and morphology

The total length (mean ± standard deviation) of *Artemia* in the larval rearing water during the zoeal period was 1008 ± 5, 939 ± 13, 848 ± 4, and 814 ± 12 μm in the EAr-Z1, EAr-Z3, NHAr-Z1, and NHAr-Z3 groups, respectively. Thus, the *Artemia* were larger in the larval rearing water of groups that were supplemented with enriched *Artemia* and digestible *Nannochloropsis* than in the groups that were supplemented with newly hatched *Artemia* and untreated non-digestible *Nannochloropsis*.

There was no significant difference in larval survival to the Z4 stage between groups, though survival was lowest in the EAr-Z4 group (Table 5.1.2). However, the majority of larvae in the EAr-Z2, EAr-Z3, NHAr-Z1, and NHAr-3 groups died during metamorphosis into MG, and larval survival to the MG stage was very low. In the EAr-Z1 and EAr-Z4 groups, the survival rates were also low, but they were higher than those in other groups. Survival to the C1 stage was low in the EAr-Z1 (8.2 %), EAr-Z2 (5.7 %), and EAr-Z4 (7.6 %) groups but was still higher than in the remaining groups (0.2–0.7 %).

The developmental rates during the early zoeal period (until Z3, 6 dah) were retarded in the EAr-Z1 group (Fig. 5.1.1A). Conversely, there was no delay in the development of the NHAr-Z1 group. During the later zoeal period after Z3, the rate of development was retarded in the EAr-Z4 group and we observed cannibalism. Overall, the time to reach the MG stage was significantly delayed in EAr-Z4 group relative to the other groups, except for the EAr-Z1 group.

The treatments significantly affected the morphology of Z4 and MG (Table 5.1.3). The CL of Z4 stage individuals tended to decrease as *Artemia* feeding was delayed, except in the EAr-Z1 group (Fig. 5.1.2A). The chelae size of Z4 stage individuals (CHL and CHLr) was largest in the NHAr-Z1 group followed by the EAr-Z1 group, and smallest in the EAr-Z4 group (Fig. 5.1.2B). The degree of dorsal spine retention in MG (DSL and DSf) was highest in the NHAr-Z1 and NHAr-Z3 groups, followed by the EAr-Z3 group (Fig. 5.1.2D). Additionally, these values were higher in the EAr-Z2 group than in the EAr-Z1 and EAr-Z4 groups. There was a similar pattern with the retention of the telson furcae in MG (Fig. 5.1.2E). The CL of MG individuals was low in groups exhibiting immature megalopal morphology (NHAr-Z1, NHAr-Z3, and EAr-Z3 groups), because their rostrum was bent (Fig. 5.1.2C). The MG CL was largest in the EAr-Z1 group.

Table 5.1.2. Mean survival of swimming crab *Portunus trituberculatus* larvae in each treatment group in experiments 1 and 2

Treatment		Survival rate (%)		
		Z1-Z4	Z1-MG	Z1-C1
Experiment 1				
EAr-Z1		81.3 (6.9)	23.4 (4.9)^{b}	8.2 (0.6)^{b}
EAr-Z2		97.0 (5.2)	5.8 (1.2)^{a}	5.7 (1.0)^{b}
EAr-Z3		90.3 (10.7)	3.4 (2.0)^{a}	0.7 (0.2)^{a}
EAr-Z4		77.1 (10.2)	25.3 (13.3)^{b}	7.6 (4.1)^{b}
NHAr-Z1		91.3 (15.0)	2.9 (1.5)^{a}	0.2 (0.1)^{a}
NHAr-Z3		85.9 (9.8)	1.1 (1.2)^{a}	0.3 (0.3)^{a}
	F	1.313	15.85	27.93
Treatment effect	*df*	5, 12	5, 12	5, 12
	P(>*F*)	0.3223	0.0001	< 0.0001
Experiment 2				
EAr		95.0 (4.9)	20.2 (6.5)^{a}	6.7 (1.6)^{a}
NH-EAr		97.3 (4.7)	23.9 (7.2)^{a}	5.9 (1.3)^{a}
NH-EAr (LD)		94.6 (4.8)	47.3 (5.1)^{b}	16.5 (3.2)^{b}
NH-EAr (HD-LD)		95.0 (8.6)	58.2 (5.4)^{bc}	19.1 (1.4)^{bc}
NH-EAr (LS)		100.0 (0.0)	66.9 (11.3)^{c}	22.3 (1.7)^{c}
NH-EAr (LS+LD)		94.2 (10.0)	48.2 (2.2)^{b}	22.0 (1.5)^{c}
	F	0.6158	21.41	47.18
Treatment effect	*df*	6, 14	6, 14	6, 14
	P(>*F*)	0.7147	< 0.0001	< 0.0001

Different superscripts in the same column represent a significant difference between treatments (comparison of generalized linear model with Tukey method, $P < 0.05$). Z1, first zoeal stage; Z4, fourth zoeal stage; MG, megalopal stage; C1, first crab stage

At the C1 stage, the CW and ED were smallest in the NHAr-Z1 group (Fig. 5.1.2F, G), but there was no difference in EDr between groups (Table 5.1.3, Fig. 5.1.2G).

Moulting failure during metamorphosis into MG was observed in all groups (Fig. 5.1.3A). The results of the GLM analysis of the relationships between moulting failure and the morphology of Z4 and MG individuals are summarized in Table 5.1.4 and Fig. 5.1.4. Because the relationship between MG morphology and moulting failure can be observed individually, the frequency distribution for moulting failure against DSL classes are also shown as bar graphs using the pooled data from individual MG from all tanks (Fig. 5.1.4B). In the GLM analysis, the MFf and OEf were significantly affected by both CHLr and DSL. The OEf and MFf increased as the CHLr and DSL increased (Table 5.1.4, Fig. 5.1.4A, B). The AIC was lower when DSL was used as an explanatory variable than when CHLr was used, but the lowest AIC score was obtained when using the additive effect (CHLr + DSL) as the explanatory variable for both OEf and MFf.

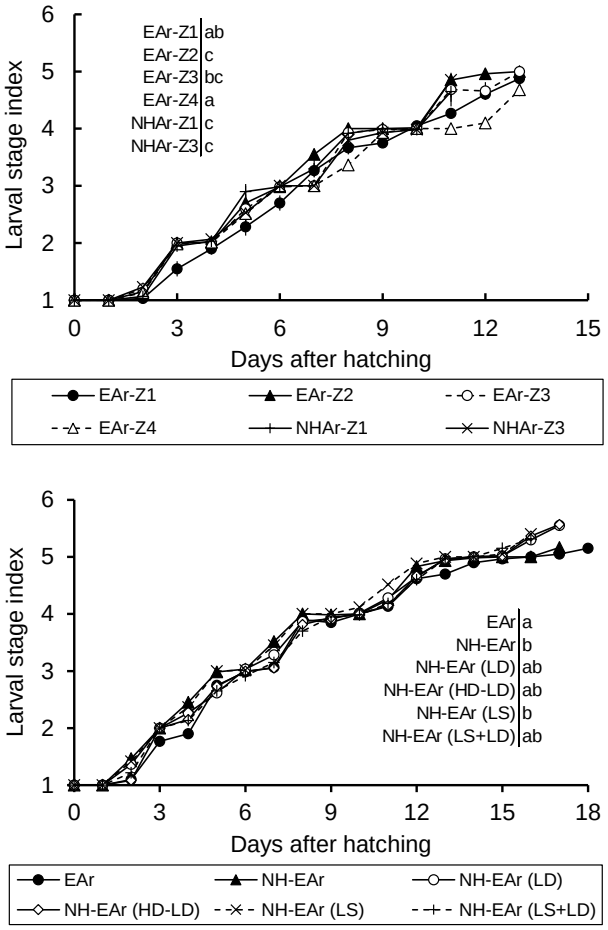

Figure 5.1.1. Changes in larval stage index (LSI) as an index of the larval developmental rate of *Portunus trituberculatus* cultured under different dietary conditions in experiment 1 (A) and 2 (B). Vertical bars represent the standard deviation of triplicate treatment groups. Age effect: experiment 1, F = 7172, df = 1, 221.1, P < 0.0001; experiment 2, F = 6351, df = 1, 302.0, P < 0.0001. Treatment effect: experiment 1, F = 8.405, df = 5, 12.11, P = 0.0012; experiment 2, F = 4.723, df = 5, 12.04, P = 0.0128. Differences in LSI between treatments (P < 0.05) are indicated by a different lowercase letter in the table following the treatment

Thus, our data suggest that both excessive morphogenesis in Z4 individuals and immature morphology in MG individuals lead to moulting failure, but their effects are independent. Our results suggest that immature morphology of MG individuals contributes more to moulting failure than does excessive morphogenesis in Z4 individuals. OEf and MFf were significantly affected by the treatment (Table 5.1.3), and were higher in groups that had higher incidences of immature morphology of MG stage individuals (NHAr-Z1 > NHAr-Z3 > EAr-Z3 > EAr-Z2) (Fig. 5.1.2D, E, 5.1.3A). Although immature morphology of MG individuals was not observed in the EAr-Z1 group, moulting failure did occur because of the occurrence of relatively large chelae in Z4 individuals (Fig. 5.1.2B, 5.1.3A). The larvae of the EAr-Z4 group that exhibited smaller chelae during the Z4 stage and slightly immature morphology at the MG stage did not fail to moult.

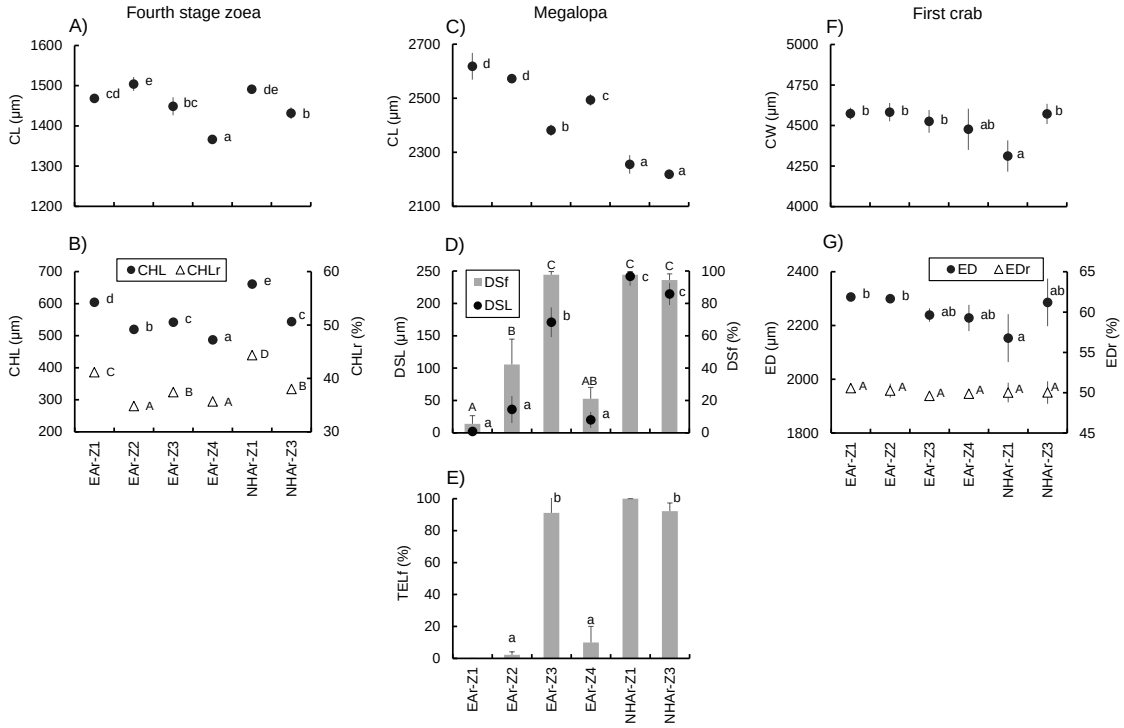

Figure 5.1.2. Differences in carapace length (CL) (A), chela length (CHL), and relative chela length (CHLr) (B) of fourth stage zoeae; CL (C), dorsal spine length (DSL) and frequency of dorsal spine retention (DSf) (D), frequency of telson furcae retention (TELf) (E) of megalopae, carapace width (CW) (F), and eye distance (ED) and relative eye distance (EDr) (G) of the first stage crab of *Portunus trituberculatus* cultured under different feeding schedules in experiment 1. Vertical bars represent the standard deviation of triplicate treatment groups. Differences among treatments are indicated by a different letter ($P < 0.05$)

Taken together, our results suggest that larvae in the EAr-Z2, EAr-Z3, NHAr-Z1, and NHAr-Z3 groups died because of abnormal immature morphology at the MG stage. In the EAr-Z1 group, survival to the C1 stage was suppressed because of excessively advanced morphology (chelae) in Z4 individuals. In EAr-Z4, Z4 and MG larvae were morphologically normal, but the delay in *Artemia* feeding resulted in retardation of development and cannibalism. Thus, high survival was not achieved in any of the groups.

Table 5.1.3. Analysis of deviance table for an *F* test (type II) evaluating the effects of treatment (explanatory variable) on morphological measurements (response variables) in the swimming crab *Portunus trituberculatus*

Morphological measurement		SS	*df*	*F*	*P*(>*F*)
Experiment 1					
Z4	CL	36633	5, 12	42.05	< 0.0001
	CHL	58934	5, 12	143.5	< 0.0001
	CHLr	192.7	5, 12	87.93	< 0.0001
MG	CL	411998	5, 12	102.2	< 0.0001
	DSL	171398	5, 12	125.4	< 0.0001
	DSf	396.7	5, 12	65.47	< 0.0001
	TELf*	318	3, 8	50.73	< 0.0001
	OEf*	180.9	4, 10	38.85	< 0.0001
	MFf*	170.6	4, 10	42.97	< 0.0001
C1	CW	156171	5, 11	4.664	0.0157
	ED	49668	5, 11	3.575	0.0365
	EDr	1.655	5, 11	0.4663	0.7938
Experiment 2					
Z4	CL	15082	5, 12	9.559	0.0007
	CHL	16298	5, 12	17.85	< 0.0001
	CHLr	38.09	5, 12	12.53	0.0002
MG	CL	42097	5, 12	3.019	0.0542
	DSL	669.5	5, 12	14.93	0.0001
	DSf	33.86	5, 12	4.976	0.0107
	OEf*	118.5	3, 8	19.82	0.0005
	MFf	125.2	5, 12	14.76	0.0001
C1	CW	219482	5, 12	7.056	0.0027
	ED	10953	5, 12	2.622	0.0796
	EDr	14.44	5, 12	6.887	0.0030

* To avoid the error attributed to zero data in the analyses using quasi-binomial family, the groups with 0 % or 100 % for TELf, OEf, and MFf were excluded from the analysis

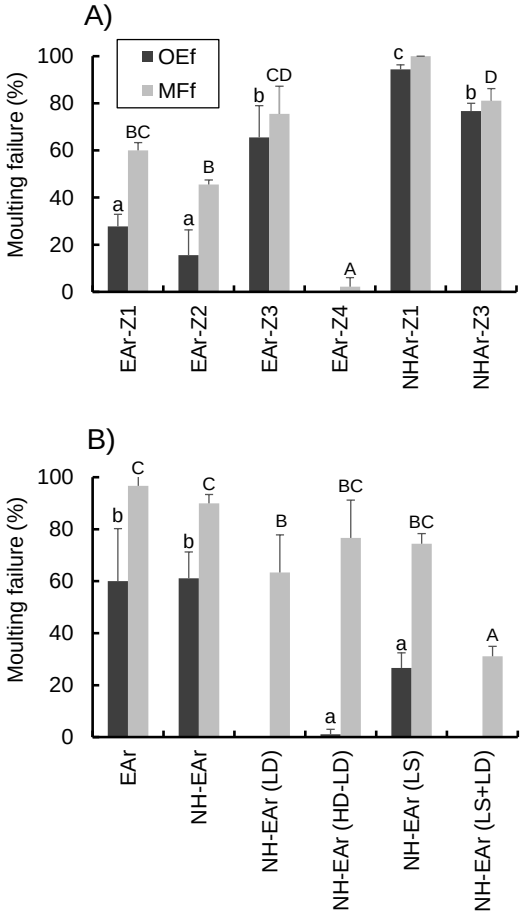

Figure 5.1.3. Differences in the frequency of severe moulting failure (OEf) and total moulting failure (MFf) (see text for description of these terms) of cultured *Portunus trituberculatus* during metamorphosis into megalopae under different feeding schedules in experiment 1 (A) and 2 (B). Vertical bars represent the standard deviation of triplicate treatment groups. Differences among treatments are indicated by a different letter ($P < 0.05$)

5.1.4.2 Experiment 2: Effect of *Artemia* feeding method on larval survival and morphology

Survival to the Z4 stage was high in all groups (94.2–100.0 %, Table 5.1.2). Survival to the MG stage decreased to 20.2 and 23.9 % in the EAr and NH-EAr groups, but was higher in the NH-EAr (LD), NH-EAr (HD-LD), and NH-EAr (LS+LD) groups (range: 47.8 to 58.2 %), and was highest in the NH-EAr (LS) group (66.9 %). Survival to the C1 stage was highest in the NH-EAr (LS) and NH-EAr (LS+LD) groups (22.3 and 22.0 %, respectively), followed by the NH-EAr (HD-LD) and NH-EAr (LD) groups (19.1 and 16.5 %, respectively). Survival was lowest in the EAr and NH-Ar groups (6.7 and 5.9 %, respectively). The rate of development was significantly retarded in the EAr group, which was supplemented with enriched *Artemia* throughout zoeal development, relative to the groups that were fed newly hatched *Artemia* during the Z1 stage (Fig. 5.1.1B).

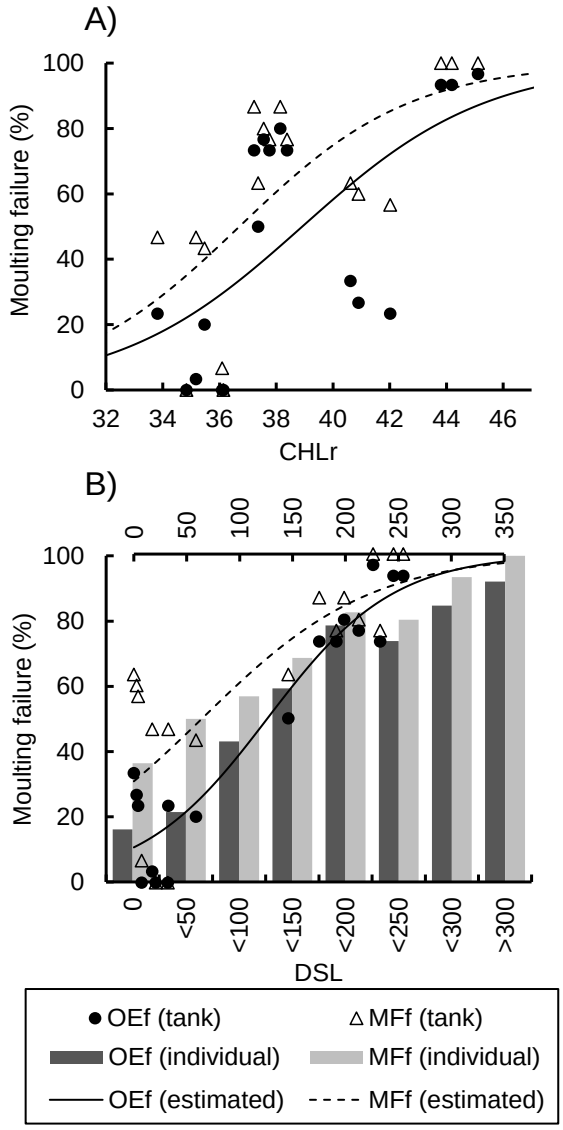

Figure 5.1.4. Relationships between the frequency of moulting failure during metamorphosis into megalopae (severe failure; OEf, total failure; MFf) and relative chela length (CHLr) of fourth stage zoeae (A) and dorsal spine length (DSL) of megalopae (B) of *Portunus trituberculatus* cultured under different feeding schedules in experiment 1. Scatter plots indicate the mean of each rearing tank, and the bar graph shows the frequency distributions of individual data from all tanks. Solid and broken lines show the logistic curves estimated using generalized linear models. The upper scale is shown for the scatter plot in (B)

The treatments significantly affected the morphology of Z4, MG, and C1 stage individuals, except for the CL of MG individuals and ED of C1 individuals (Table 5.1.3). The CL of Z4 individuals was smaller in the NH-EAr (HD-LD) and NH-EAr (LS+LD) groups than in other groups (Fig. 5.1.5A). The chelae size (CHL and CHLr) of Z4 individuals was largest in the EAr group and smallest in the NH-EAr (HD-LD) and NH-EAr (LS+LD) groups (Fig. 5.1.5B). There was no difference in the CL of MG individuals between groups (Fig. 5.1.5C). We rarely observed immature morphology in MG individuals (DSL, DSf, and TELf); the DSL was larger in the NH-EAr groups than in other groups, but was still small (Fig. 5.1.5D, E). The CL of C1 individuals was largest in the NH-EAr (LS) and NH-EAr (LS+LD) groups and smallest in the NH-EAr (HD-LD) group (Fig. 5.1.5F). There was no significant difference in ED between groups, but EDr was

Table 5.1.4. Estimated intercept, coefficients, and Akaike's information criteria (AICs) for the generalized linear models used to examine the effects of larval morphology (explanatory variables) on the frequency of moulting failure (response variables) of the swimming crab *Portunus trituberculatus* larvae reared in experiments 1 and 2. *Δi* represents the difference in the AIC between a given model and the model with the lowest AIC, for each response variable

Response variables	Explanatory variables		Intercept	Coefficient			AIC	*Δi*
				Estimated	SE	*P*		
Experiment 1								
OEf	CHLr		−12.00	0.308	0.0323	< 0.0001	258	168
	DSL		−2.121	0.0168	0.00125	< 0.0001	117	27
	CHLr+DSL	(CHLr)	−10.76	0.232	0.0461	< 0.0001	90	0
		(DSL)		0.0149	0.00129	< 0.0001		
	CHLr×DSL		−2.061	0.000418	0.0000315	< 0.0001	110	20
MFf	CHLr		−12.15	0.331	0.0366	< 0.0001	219	84
	DSL		−0.810	0.012495	0.00115	< 0.0001	181	45
	CHLr+DSL	(CHLr)	−10.38	0.257	0.0400	< 0.0001	136	0
		(DSL)		0.010854	0.00126	< 0.0001		
	CHLr×DSL		−0.799	0.000319	0.0000295	< 0.0001	172	37
Experiment 2								
OEf	CHLr		−31.15	0.726	0.0784	< 0.0001	194	93
	DSL		−2.12	0.166	0.0172	< 0.0001	185	84
	CHLr+DSL	(CHLr)	−33.37	0.751	0.0909	< 0.0001	101	0
		(DSL)		0.160	0.0180	< 0.0001		
	CHLr×DSL		−2.163	0.00413	0.000420	< 0.0001	180	79
MFf	CHLr		−25.36	0.647	0.0827	< 0.0001	124	12
	DSL		0.422	0.144	0.0259	< 0.0001	155	43
	CHLr+DSL	(CHLr)	−20.50	0.520	0.0859	< 0.0001	112	0
		(DSL)		0.0751	0.0225	0.00082		
	CHLr×DSL		0.411	0.00355	0.000626	< 0.0001	153	41

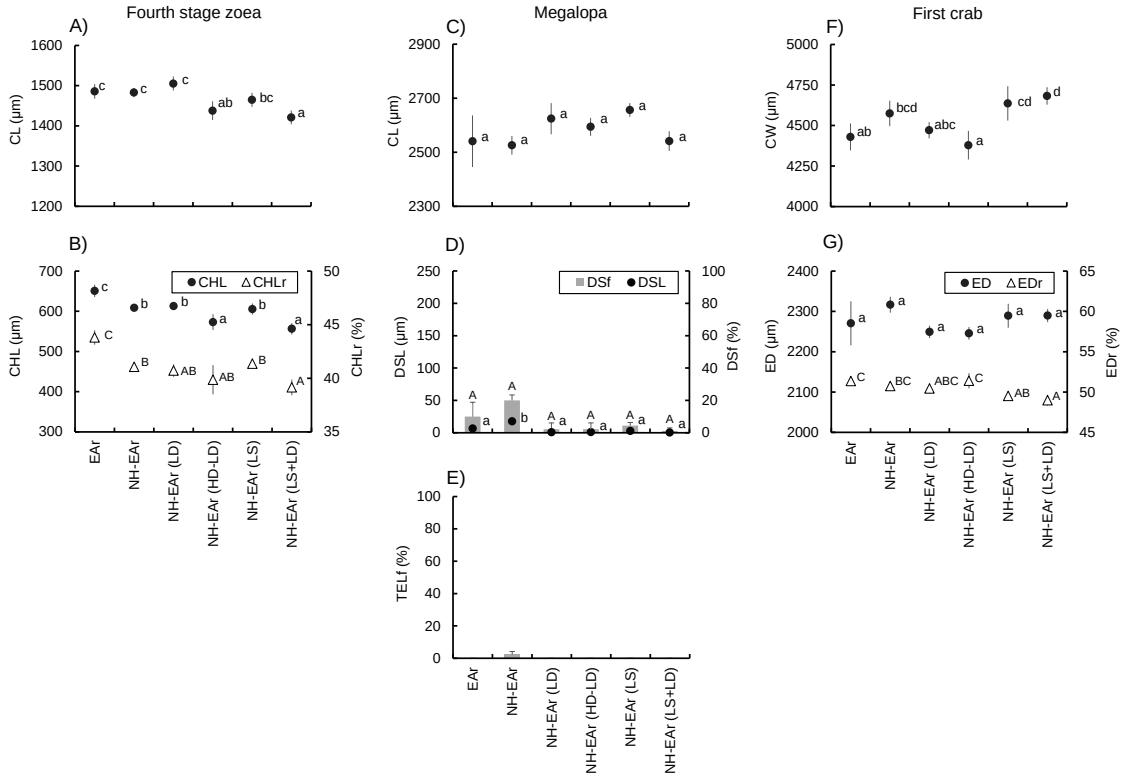

Figure 5.1.5. Differences in carapace length (CL) (A), chela length (CHL), and relative chela length (CHLr) (B) of fourth stage zoeae; CL (C), dorsal spine length (DSL) and frequency of dorsal spine retention (DSf) (D), frequency of telson furcae retention (TELf) (E) of megalopae, carapace width (CW) (F), and eye distance (ED) and relative eye distance (EDr) (G) of the first stage crab of *Portunus trituberculatus* cultured under different feeding schedules and salinity levels in experiment 2. Vertical bars represent the standard deviation of triplicate treatment groups. Differences among treatments are indicated by a different letter ($P < 0.05$)

lower in the NH-EAr (LS) and NH-EAr (LS+LD) groups than in other groups (Fig. 5.1.5G).

Total moulting failure (MFf) was observed frequently in all groups, whereas serious moulting failure (OEf) was observed only in the EAr, NH-EAr, and NH-EAr (LS) groups. This suggests that the moulting failure occurring in other groups (NH-EAr (LD), NH-EAr (HD-LD), and NH-EAr (LS+LD)) were only mild (Fig. 5.1.3B). The relationships between moulting failure and the morphology of Z4 and MG are shown as a mean value for each tank in Fig. 5.1.6, but individual DSL data are not shown because the majority of individual DSL values were zero. In the GLM analysis, the lowest AIC was obtained for the model with the additive effect (CHLr+DSL) as the explanatory variable for both OEf and MFf (Table 5.1.4). The CHLr and DSL were positively related to the OEf and MFf (Fig. 5.1.6), but the AIC values were lowest in the models that used

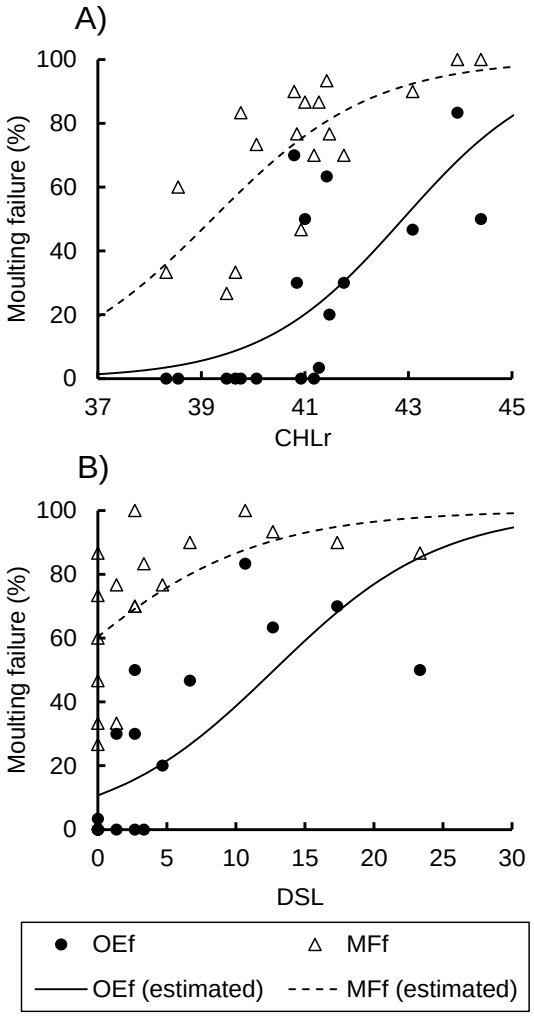

Figure 5.1.6. Relationships between the frequency of moulting failure during metamorphosis into megalopae (severe failure; OEf, total failure; MFf) and relative chela length (CHLr) of fourth stage zoeae (A) and dorsal spine length (DSL) of megalopae (B) of *Portunus trituberculatus* cultured under different feeding schedules and salinity levels in experiment 2. Each plot indicates the mean of each rearing tank. Solid and broken lines represent the logistic curves estimated using generalized linear models. The bar graph for individual DSL data is not shown because the majority of individual DSL values were zero

DSL to explain OEf and CHLr to explain MFf. These results indicate that both excessive morphogenesis of Z4 individuals and immature morphology of MG individuals is associated with moulting failure, and that excessive morphogenesis of Z4 individuals is the primary cause of the mild moulting failure observed in this experiment. Consequently, the groups exhibiting advanced morphogenesis at the Z4 stage (enlarged CHL and CHLr) also had the higher MFf values (Fig. 5.1.3B, 5.1.5B).

Thus, the rate of development was improved by feeding of newly hatched *Artemia* during the Z1 stage. Abnormal morphology was rare in all groups at the MG stage, but excessive morphogenesis (size of chelae) of Z4 individuals affected larval survival via by increasing the frequency of moulting failure into MG. The excessive morphogenesis of the Z4 stage (size of chelae) were suppressed by lowering the feeding density of *Artemia* and the salinity of the rearing water. These measures resulted in higher survival to the C1 stage.

5.1.5 Discussion

The larval morphology of Z4 and MG individuals varied among the treatment groups in experiment 1. The chelae size of Z4 individuals increased as *Artemia* were supplied earlier in the zoeal development period. The relationship between *Artemia* feeding schedule and morphogenesis of zoeal chelae was similar to that observed in *P. trituberculatus* and *S. serrata*. Earlier supply of *Artemia* and higher density *Artemia* in the rearing water were associated with enlarged chelae in the last zoeal stage (Suprayudi et al., 2002b; Chapter 3.4). Previously, we demonstrated that morphogenesis of body parts that become enlarged during metamorphosis into MG (e.g., chelae and pleopods) is accelerated when zoeae experience good nutritional and environmental conditions (e.g., dietary n-3 highly unsaturated fatty acid content and salinity) throughout the zoeal period, a phenomenon that is regulated by the eyestalk neurosecretory system (Arai et al., 2007; Dan and Hamasaki, 2011, Dan et al., 2014; Hamasaki et al., 2002b; Chapter 3.1, 3.3). Taken together, supplementation of *Artemia* appears to accelerate morphogenesis of these body parts, and the effect can be observed early in zoeal development. Thus, an inappropriate *Artemia* feeding schedule can cause abnormal morphology in Z4 individuals.

We observed abnormal immature morphology of MG individuals (retention of dorsal spine and telson furcae) in the groups that were supplemented with enriched *Artemia* and digestible *Nannochloropsis* from the Z2 and Z3 stages (EAr-Z2 and EAr-Z3) or supplemented with newly hatched *Artemia* (NHAr-Z1 and NHAr-Z3). Previously, we demonstrated that immature megalopal morphology was induced when larvae indirectly ingested phytoplankton such as *Chlorella* and *Nannochloropsis* via consumption of rotifers. The critical period at which ingestion of phytoplankton led to immaturity occurred during the premoult of Z3 individuals (Chapter 3.4). This critical period coincided with the critical period at which resorption of body parts (e.g., dorsal spine and telson furcae) is regulated by the eyestalk neurosecretory system (Dan et al., 2014; Chapter 3.3, 3.4). This suggests that phytoplankton have a factor (chemical) that disrupts the endocrine control of the eyestalk neurosecretory system. In the EAr-Z2, EAr-Z3, and NHAr-Z3 groups, rotifers that were fed untreated non-digestible or digestible *Nannochloropsis* were supplied until the Z2 and Z3 stages so were present (remained) in the rearing water during the critical period (premoult of Z3). Thus, larvae likely ingested these rotifers, resulting in immature megalopal morphology. In contrast, the group that was supplemented with digestible *Nannochloropsis* from the Z4 stage (EAr-Z4) exhibited little immature morphology of MG individuals. These observations are consistent with our previous results (Chapter 3.4). We observed a high

incidence of immature morphology of MG individuals in the NHAr-Z1 group that was supplied with newly hatched *Artemia* and untreated *Nannochloropsis*, but not with rotifers. Because newly hatched *Artemia* are not able to digest the untreated *Nannochloropsis* (Chapter 4.1), this suggests that newly hatched *Artemia* also have a factor that induces immature morphology of MG. Because *Artemia* are a crustacean, we speculate that increased hormone(s) levels in newly hatched *Artemia* affect(s) larval endocrine control. On the other hand, the EAr-Z1 group that was not supplemented with rotifers and newly hatched *Artemia* but with enriched *Artemia* and digestible *Nannochloropsis* did not exhibit immature morphology of MG individuals. This indicates that larval indirect ingestion of digestible *Nannochloropsis* via consumption of enriched *Artemia* does not induce abnormal immature morphology of MG individuals, unlike via consumption of rotifers and newly hatched *Artemia*.

Both the excessive morphogenesis of Z4 individuals and immature morphology of MG individuals appear to cause moulting failure during metamorphosis into MG. The frequency of moulting failure increased with an increase in the chelae size of Z4 individuals and frequency of dorsal spine retention in MG individuals, and the effects were independent. The same phenomenon has been reported in mass seed production trials of *P. trituberculatus* (Dan et al., 2013; Chapter 3.2). These results clearly indicate that factors causing morphological abnormalities of Z4 and MG individuals must be addressed concurrently to prevent moulting failure (moult death syndrome).

The chelae size of Z4 individuals in the EAr-Z4 group was small, we observed a low incidence of immature morphology in MG individuals, and the frequency of moulting failure was low, but survival to the C1 stage was low. In this group, the rate of development was retarded after the Z3 stage and cannibalism was observed among larvae. A similar negative effect associated with a delay in *Artemia* feeding has been reported in other brachyuran crabs (Baylon et al., 2009; Davis et al., 2005; Harms and Seeger, 1989; Suprayudi et al., 2002b). Additionally, late zoeal stage larvae (Z3 and Z4) must ingest sufficient EPA to develop successfully from the MG to C1 stages; EPA deficiencies result in mortality after the MG stage (Chapter 4.1). EPA is an essential fatty acid for *P. trituberculatus* larvae (Hamasaki, 1997; Takeuchi et al., 199a, b, c). Because the larvae in EAr-Z4 group were reared to the Z4 stage with rotifers but not phytoplankton or *Artemia*, they may not have accumulated sufficient energy and nutrition including EPA, resulting in low survival after MG.

Rotifers are an essential part of the diet of early *P. trituberculatus* zoeae so are used in all seed production trials conducted in Japan (Dan et al., 2013; Hamasaki et al., 2011; Chapter 3.2). It is generally thought to be impossible to successfully rear larvae from hatching to the C1 stage using only *Artemia* (i.e., without rotifers) (Hamasaki, 1997). Indeed, almost all larvae that were reared with newly hatched *Artemia* and untreated (non-digestible) *Nannochloropsis* from the Z1 stage (NHAr-Z1) died prior to the C1 stage because of morphological abnormalities at the Z4 and MG stages. However, 23.4 and 8.2 % of larvae that were reared with enriched *Artemia* and digestible *Nannochloropsis* (EAr-Z1) survived to the MG and C1 stages, respectively. Thus, our feeding schedule can be used to prevent the occurrence of immature morphology in MG individuals and provide sufficient nutrition during the zoeal period, thereby increasing survival. This is the first report of successfully rearing *P. trituberculatus* larvae from hatch to the C1 stage using only *Artemia* as a food source.

We identified two problems with the use of enriched *Artemia* as main food for larvae in experiment 1. First, the enriched *Artemia* (total length, 786 ± 97 μm) were too large and fast swimming for Z1 stage larvae (565 ± 23 μm CL), making them difficult for larvae to capture and ingest. This led to delayed and variable development rates. Increased variation in development rates can increase the incidence of cannibalism between small and large larvae in later developmental stages. Second, a longer *Artemia* feeding period accelerated the morphogenesis of last zoeal chelae excessively and resulted in moulting failure. Because of these reasons, survival to the C1 stage was negatively affected in the EAr-Z1 group.

In experiment 2, we facilitated larval feeding by supplementing with newly hatched *Artemia* (497 ± 24 μm) during the Z1 stage and enriched *Artemia* (837 ± 11 μm) beginning at the Z2 stage. Additionally, the density of *Artemia* was held low to prevent excessive morphogenesis in the Z4 stage and/or the larvae were reared at low salinity. These changes improved the developmental rates and had a positive effect on zoeal morphogenesis, consistent with previous observations of the effects of *Artemia* density in *P. trituberculatus* and *S. serrata* and salinity in *S. serrata* (Dan et al., 2011; Suprayudi et al., 2002b; Chapter 3.1, 3.4). As a result, the treatment groups in experiment 2 had higher survival to the MG (47.8–66.9 %) and C1 (16.5–22.3 %) stages. Because moulting failure during metamorphosis into MG was correlated with the chelae size of Z4 individuals, even in these groups, we speculate that survival could be further improved by additional suppression of zoeal morphogenesis (size of chelae). A reduction in the level of

Artemia enrichment (e.g., enrichment time and materials) and optimization of *Artemia* feeding density at each moulting stage may improve control of zoeal morphogenesis.

We demonstrated for the first time that newly hatched *P. trituberculatus* larvae could be cultured successfully to the juvenile stage in stagnant water using only *Artemia* and digestible *Nannochloropsis*. This method does not result in abnormal morphology of MG or nutritional deficiency during the late zoeal period, the two of the major causes of mass mortality during seed production (Dan et al., 2013; Chapter 3.2, 3.4, 4.1). The excessive morphogenesis of Z4 individuals can also be controlled, and further improvements may result in reliable seed production with high and stable survival while preventing moulting failure. Moreover, the labour, facility, and energy costs can be reduced because rotifer culture and water exchange are unnecessary. The absence of rotifers may also reduce the opportunity to transfect various virulent bacteria and fungi that are pathogenic to portunid crab larvae (Dan and Hamasaki, 2014; Hamasaki and Hatai, 1993a, b; Muroga et al., 1989; Nakamura and Hatai, 1994; Olafsen, 2001; Verdonck et al., 1997).

The most important factors to consider in the use of *Artemia* during larval culture of aquatic organisms include: size (newly hatched or enriched), enrichment level corresponding to the nutritional requirements of the cultured animals (enrichment materials and level), supplementation density, and maintenance of nutritional condition after addition to the rearing water (supplementation of digestible food). An understanding of the optimal levels or requirements for each of these factors can improve larval rearing techniques for many cultured species that rely on *Artemia* as food.

Chapter 6

CONCLUDING REMARKS

6.1. Causes of larval mass mortality during the seed production of portunid crabs

We defined four major causes of mass mortality occurring during seed production of the portunid crabs *Portunus trituberculatus* and *Scylla serrata*: bacterial necrosis disease, two types of abnormal morphology in last stage zoeae and megalopae (MG), and nutritional deficiency (Table 6.1). Bacterial necrosis disease was caused by infection of larvae with the bacterium family Flavobacteriaceae. Infection resulted in mass mortality during the zoeal period. The abnormal morphology of last stage zoeae was characterised by excessively advanced (enlarged) morphogenesis of chelae and pleopods. The abnormal morphology of MG stage individuals was characterised by retention of the immature dorsal spines and telson furcae. The occurrence of both abnormal morphologies induced moulting failure during metamorphosis into MG, a phenomenon known as moult death syndrome (MDS). Nutritional deficiency occurred when late zoeal stage larvae fed on newly hatched or starved *Artemia* that had low EPA levels. This deficiency resulted in mortality during the period from MG to first crab stage (C1). The frequency with which each of these causes leads to mass mortality differs among species (e.g., bacterial necrosis disease is most common in *S. serrata* whereas abnormal MG morphology is most common in *P. trituberculatus*). Regardless, there is an urgent need for countermeasures to prevent mass mortalities in Japanese hatcheries.

We demonstrated that each of these causes can independently induce larval mass mortality in small scale (1−30 L) and pilot-scale (500 L tank) larval rearing experiments. However, it is difficult to separate the underlying factors in a mass seed production setting. For example, supplementation of the culture tanks with phytoplankton (e.g., n-3 HUFA fortified *Chlorella* or *Nannochloropsis*) indirectly induces abnormal morphology of MG via consumption of rotifers. This effect was mediated by disruption of the endocrine control of tissue resorption (Chapter 3.4) and excess accumulation of n-3 HUFA, which results in abnormal morphology of last stage zoeae (Arai et al., 2007; Hamasaki et al., 2002b; Chapter 3.1). Furthermore, supplementation with these phytoplankton species led to nutritional deficiency during the late zoeal period because *Artemia* nauplii were unable to digest *Chlorella* and *Nannochloropsis*, resulting in their starvation (Chapter 4.1). Thus, a single factor (supplementation of phytoplankton) resulted in mass mortality via three mechanisms. I speculate that such complex interactions explain why survival is still low in Japanese hatcheries, despite attempts to improve culture techniques during the past 50 years.

Table 6.1. Causes of larval mass mortality during seed production of the portunid crab species *Portunus trituberculatus* and *Scylla serrata*, and the measures developed for their control

Mortality pattern	Morphological trait	Cause	Causal pathway	Critical period	Control measure
Death during the zoeal period	Tissue necrosis of zoeal spines and appendages	Infection with the causative bacterium (Flavobacteriaceae family)	Contamination of larvae, live-foods, and water with causative bacteria.	Throughout culture (primarily during the zoeal period)	Probiotics (not yet practical), NFS-Na bath treatment, Reduction in use of live-foods, Stagnant water culture
Moulting failure and death after metamorphosis into megalopae	Excessive morphogenesis of last stage zoea (large chelae and pleopod)	Excessive nutritional accumulation in a low-stress environment	High n-3 HUFA (effect: DHA>EPA) content in rotifers, Prolonged feeding of *Artemia* at high density High salinity	Throughout the zoeal period	Reduction in nutritional level in rotifers, Reduction in *Artemia* feeding density, Reduction in culture water salinity
	Immature morphology of megalopae (retention of dorsal spines and telson furcae)	Disruption of endocrine control of tissue resorption by the eyestalk neurosecretory system	Indirect ingestion of phytoplankton via rotifers, Ingestion of newly hatched *Artemia*	Premoult of the penultimate zoeal stage (premoult of Z3 for *P. trituberculatus*)	Elimination of rotifers, Nutritional enrichment of *Artemia*
Death during the megalopal stage or moulting into a non-viable first stage crab	Normal	Nutritional deficiency	Low EPA content in newly hatched and starved *Artemia*	Late zoeal period (Z3−Z4 for *P. trituberculatus*)	Nutritional enrichment of *Artemia* with EPA, Supplementation of digestible *Nannochloropsis* into larval culture water

6.2. Bacterial necrosis disease and control with probiotics

Bacterial necrosis disease, caused by the bacterium family Flavobacteriaceae, results in significant mortality during seed production of *S. serrata*. Almost all larvae died prior to the last zoeal stage in trials without an NFS-Na bath treatment (Chapter 2.1). During seed production of *P. trituberculatus*, *Portunus pelagicus*, and *Scylla paramamosain*, bacterial disease accounted for 26.4% of the larval mass mortality incidences (Hamasaki et al., 2011) and symptoms of necrosis have been reported in these species (Hamasaki, 1997). However, in contrast to *S. serrata*, bacterial necrosis disease was not always lethal during seed production of *P. trituberculatus*, *P. pelagicus*, and *S. paramamosain*. *S. serrata* larvae appear to be particularly sensitive to the causal bacterium of necrosis disease. In the wild, *S. serrata* spawn offshore (Hill, 1994; Hyland et al., 1984; Ogawa et al., 2012) whereas *P. trituberculatus* and *S. paramamosain* spawn in the nearshore and/or estuary regions (Hamasaki, 1996; Ogawa et al., 2012). Thus, *S. serrata* larvae rear in clean offshore water whereas the larvae of *P. trituberculatus* and *S. paramamosain* rear in nearshore water that is rich in organic compounds and has a range of bacteria (Azam et al., 1983). However, during seed production, all larvae are reared in microbial- and organic-rich water because of the intensive feeding and supplementation procedures. I speculate that the difference in sensitivity to pathogenic bacteria is because of species differences in the immune systems of larvae that have adapted to different natural habitats.

There is significant interest in the potential for probiotics to provide an alternative method of disease control to antibiotics. Supplementation with probiotic bacteria did suppress larval necrosis and improve larval survival even during commercial-scale mass seed production of *S. serrata* (Chapter 2.1). However, the beneficial effect of the probiotics decreased over time and the majority of larvae died prior to reaching the C1 stage. I speculate that this reduction occurred because the bacteria were unable to proliferate and produce extracellular substances in the larval culture tanks. The success of probiotics as a disease control measure during seed production will be contingent on being able to ensure continuous proliferation of probiotic bacteria and production of inhibitory factors in the larval culture water. A number of studies suggest that selection of probiotic strains that have a high affinity for live foods can improve their effectiveness (D'Alvise et al., 2012; Gatesoupe, 1991, 2002; Pintado et al., 2010; Verschuere et al., 2000b; Villamil et al., 2010). The biological characteristics of the pathogenic bacteria (e.g., adhesion, invasion route, and extracellular production) should also be taken into consideration when selecting probiotic bacteria. Additionally, the inflow of nutrients, bacteria, and organic matter can be reduced by the

use of stagnant water larval culture and disinfection of the live foods (Sorgeloos et al., 2001; Tomoda et al., 2011). The larval culture methodology I propose (Chapter 5.1) represents a starting point for using probiotics. The method does not require renewal of culture water and the larvae are supplied only with *Artemia*, which can be disinfected (Sorgeloos et al., 2001).

6.3 Mechanism of larval morphogenesis and to the induction of abnormal morphology

I defined two patterns of larval morphogenesis based on the results of zoeal eyestalk ablation experiments in *P. trituberculatus* (Chapter 3.3, and 3.4). The first pattern was characterized by the morphogenesis of body parts (e.g., chelae and pleopod) that were enlarged during development prior to metamorphosis into MG. This morphogenesis (enlargement) was continuously accelerated throughout zoeal development under the control of the neurosecretory system located in the eyestalk (X-organ sinus grand complex). The second pattern was characterized by morphogenesis of body parts (e.g., dorsal spines and telson furcae) that were resorbed during metamorphosis into MG. This morphogenesis (resorption) was controlled instantaneously at a critical period by the eyestalk neurosecretory system. The critical period was at the premoult of the penultimate zoeal stage (Z3 for *P. trituberculatus*). The absence (ablation) of eyestalks before this critical period resulted in the occurrence of a supernumerary zoeal stage (Z5 for *P. trituberculatus*) in which some body parts (chelae and pleopods) were not enlarged sufficiently and other body parts (dorsal spines and telson furcae) were not resorbed. Taken together, these observations suggest that larval metamorphosis, which has been treated as a single event to date, consists of two patterns of morphogenesis. Furthermore, both patterns are controlled by the eyestalk neurosecretory system, though using two different pathways. Although the morphogenesis of body parts that exhibit complex morphological changes (e.g., maxillipeds and gills) were not examined in this study, I infer that they are also organized by enlargement and resorption of the tissue.

I evaluated the extrinsic factors that may affect these two patterns of morphogenesis. The morphogenesis of enlarged body parts was accelerated when larvae were reared under high dietary nutrition levels and under certain environmental conditions. Higher DHA content in rotifers, feeding *Artemia* for a longer duration and at higher density, and high salinity were associated with enlarged zoeal chelae. Because enlargement of these body parts requires a large amount of energy, it is reasonable that larvae would promote morphogenesis prior to the megalopal stage as much

as possible when nutritional and environmental conditions allow during the zoeal stage, even though these body parts remain non-functional (rudimentary) during the zoeal stage. Conversely, the resorption of body parts such as the dorsal spines and telson furcae does not place energy demands on the individual. These structures also play important roles in preventing predation and facilitating swimming during zoeal stages (Morgan 1989). Thus, the larvae require these body parts during the zoeal stages, but they must then be quickly resorbed during metamorphosis. To achieve this, the cue for resorption is given before metamorphosis at a critical period (premoult during the penultimate zoeal stage). The resorption is then triggered in the internal tissue during the last zoeal stage and appears as an external shape after moulting (metamorphosis) into MG. This tissue resorption was indirectly inhibited by larval intake of phytoplanktons via consumption of rotifers at this critical period, suggesting that the phytoplankton have a factor/s that disrupts/inhibits regulation by the eyestalk neurosecretory system (Chapter 3.4). Conversely, indirect ingestion of phytoplankton (digestible *Nannochloropsis*) via consumption of *Artemia* did not induce abnormal morphology of MG (Chapter 4.1 and 5.1). The supplementation of newly hatched *Artemia* also appears to disrupt/inhibit this regulation (Chapter 5.1). However, supplementation of newly hatched *Artemia* did not always induce abnormalities (see Chapter 3.4 and 4.1). I speculate that the causal factor is only present in *Artemia* nauplii during the period immediately after hatch. Because of this, I observed variability in the occurrence of abnormalities associated with differences in exposure of larvae during this critical period caused by differences in *Artemia* hatching time, time of feeding, and feeding concentration.

The relationships between intrinsic and extrinsic factors affecting larval morphogenesis and causing abnormal morphology are summarised in Table 6.2. The abnormal morphology of last stage zoeae is caused by excessive larval dietary intake and accumulation of nutrients (n-3 HUFA, particularly DHA) associated with high feeding density and high nutritional value of live foods (rotifers and *Artemia*). Such conditions are unlikely to occur in natural habitats. Furthermore, low stress culture conditions appear to reduce energy consumption (e.g., low osmoregulatory effort under optimal salinity) and promote energy accumulation in larvae (Anger, 2001e), resulting in further acceleration of morphogenesis. Morphological abnormality of MG also occurs as a result of disruption of the endocrine control of tissue resorption by the eyestalk neurosecretory system. My results show that this is associated with indirect larval intake of phytoplanktons via consumption of rotifers or direct consumption of newly hatched *Artemia*. These two types of abnormal

Table 6.2. Relationship between intrinsic and extrinsic factors affecting larval morphogenesis and abnormal morphologies of the swimming crab *Portunus trituberculatus*

Morphological change during metamorphosis into megalopa	Body parts	Intrinsic control by eyestalk neurosecretory system	Intrinsic control period	Extrinsic factors that increase the incidence of abnormal morphology	Outcome of abnormal morphology
Enlargement	Chelae, Pleopods	Acceleration of enlargement	Throughout zoeal development (Z1−Z4)	High n-3 HUFA (particularly DHA) content in rotifer (acceleration), Prolonged (earlier) duration of feeding *Artemia* (acceleration), High *Artemia* feeding density (acceleration), High culture water salinity (acceleration)	Excess enlargement resulting moulting failure during meta-morphosis
Resorption	Dorsal spines, Telson furcae	Cue for resorption	Premoult during the penultimate zoeal stage (premoult of Z3)	Indirect ingestion of phyto-plankton via rotifers during premoult of the penultimate zoeal stage (inhibition/disruption), Intake of newly hatched *Artemia* (the critical period is unidenti-fied) (inhibition/disruption)	Retention of immature traits resulting in moulting failure during metamorphosis and/or mortality immediately after metamorphosis

morphologies induced moulting failure during metamorphosis into MG and resulted in MDS. The exact cause of MDS occurring during seed production has not been well understood (Baylon, 2009). My results suggest that the two types of abnormal morphologies are the major causes of MDS. To determine which type of abnormal morphology causes MDS during seed production trials, culturists should carefully observe the morphology of last stage zoeae and MG under a microscope. As larvae suffer MDS, they settle to the bottom of large culture tanks. These dead or moribund larvae should be sampled and checked for abnormal morphology.

6.4 Characteristics in nutritional requirement of portunid crab larvae

Portunid crab larvae require n-3 HUFA's such as DHA and EPA. These nutrients improve larval survival, developmental rate, and body size to the C1 stage in a dose-dependent manner (Hamasaki et al., 1998; Kobayashi et al., 2000a, b; Suprayudi et al., 2002a, 2004 Takeuchi et al., 1999a, b, c). Interestingly though, larvae that are fed non n-3 HUFA enriched rotifer and *Artemia* have high survival (~70%) to the last zoeal stage (Hamasaki et al., 1998; Kobayashi et al., 2000a, b; Suprayudi et al., 2004; Takeuchi et al., 1999a, b, c). Additionally, the survival of *P. trituberculatus* juveniles from C1 to third crab stages is optimized by feeding non-enriched newly hatched *Artemia* containing low EPA and no DHA (Hamasaki and Sekiya, 1998). In Chapter 4.1, I showed that supplying late zoeal larvae (Z3 and Z4) with *Artemia* that had low EPA content had no effect on larval survival and development to the MG stage, but did have an effect during the period between MG and C1, and led to mass mortality. Taken together, these results suggest that *P. trituberculatus* larvae only require dietary EPA during the late zoeal period and it is necessary for successful development from MG to C1.

The MG stage represents the end of the planktonic phase and is associated with settlement behaviour. The MG and C1 of *P. trituberculatus* have been found on drifting seaweeds and *S. serrata* juveniles have been found on seagrass (Shiota, 1993; Tanigawa, 2001; Webley et al., 2009). Because the migration (dispersion) is largely passive during the planktonic zoeal period, late zoeal larvae must accumulate sufficient energy reserves prior to the MG stage to avoid starvation during the exploratory (swimming) phase of the dispersion, thereby increasing the likelihood of finding suitable habitat for settlement (drifting seaweeds or seagrass). The larval requirement for EPA during the late zoeal period is most likely related to this characteristic migratory behaviour. However, portunid crab larvae can utilize an alternative developmental

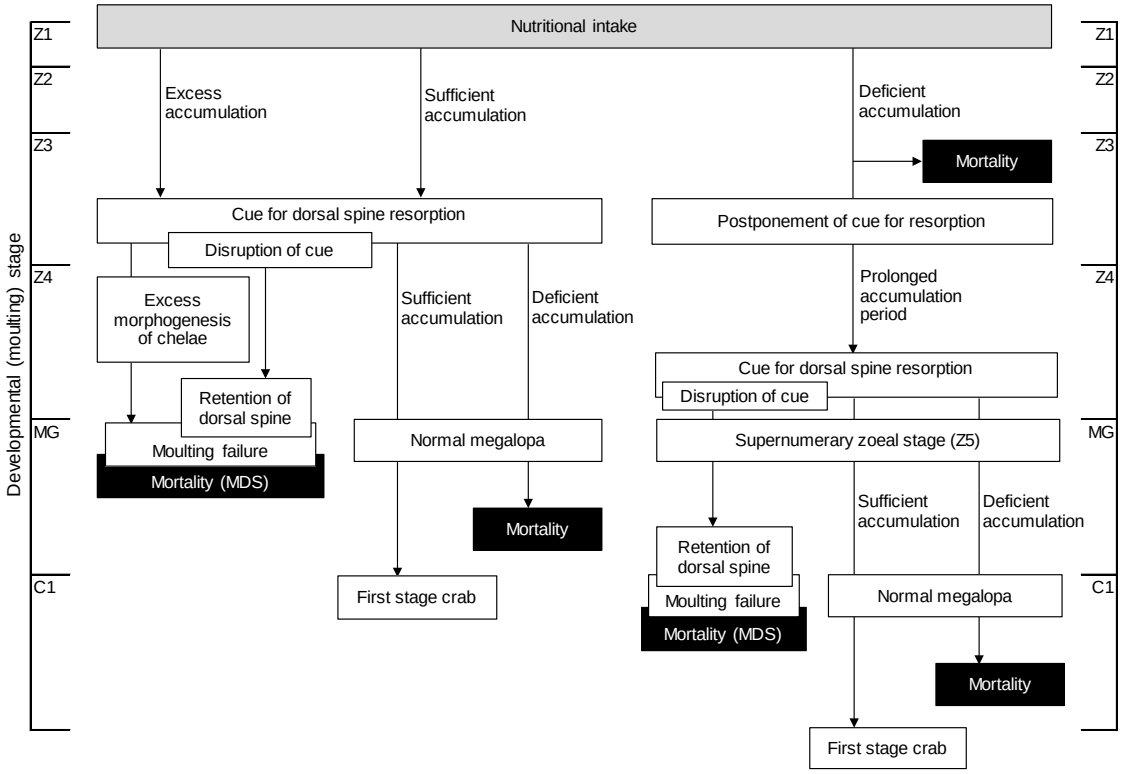

Figure 6.1. Schematic diagram of the occurrence of larval mass mortality and the alternate developmental pathways. The choice of pathway is dependent on larval nutritional intake and energy accumulation in the swimming crab *Portunus trituberculatus*. Morphogenesis of chelae is accelerated by uptake of excess dietary n-3 HUFA (effect, DHA > EPA). Disruption of cues that trigger tissue (dorsal spine) resorption is induced by indirect ingestion of phytoplankton via consumption of rotifers or ingestion of newly hatched *Artemia*. The negative effect of nutritional deficiency is most evident when intake of dietary EPA is insufficient

pathway involving an additional (supernumerary) zoeal stage (Z5 for *P. trituberculatus* and sixth zoeal stage for *S. paramamosain*) when larvae experience unfavourable dietary conditions and cannot accumulate sufficient energy, a phenomenon known as terminal additive staging (Gore, 1985; Hamasaki, 1997; Zeng et al., 2004). These supernumerary stage zoeae can develop into normal viable MG. The co-existence of flexible developmental pathways that are utilized based on body condition and the occurrence of mass mortality due to low body condition seems contradictory. However, this contradiction can be explained by taking into account the mechanism of larval morphogenesis, as shown in Fig. 6.1. When the larvae accumulate sufficient energy the cue for tissue (dorsal spines and telson furcae) resorption is triggered at premoult during the penultimate zoeal stage and the chelae and pleopods are enlarged, resulting in normal metamorphosis via the normal developmental pathway. When the larval accumulation of energy is

below a threshold, the cue for tissue resorption is postponed to the next moulting stage and enlargement of chelae is also retarded, resulting in occurrence of the supernumerary zoeal stage. When the body condition of larvae declines after the cue for tissue resorption is given (e.g., because of feeding with starved *Artemia*), the larvae moult into normal MG via the normal developmental pathway, but are unable to survive from the MG to C1 stages. Excessive zoeal energy accumulation induces excessive morphogenesis in last stage zoeae and eventually results in MDS. Thus, culturists should carefully control larval nutritional conditions at each larval moulting stage, and pay particular attention to the levels of EPA and DHA.

6.5 Starvation of *Artemia* and its relation to larval nutritional deficiency

Artemia are typically the main food source for late stage zoeae during seed production of portunid crabs (Baylon, 2009; Dan et al., 2013; Hamasaki, 1997; Kobayashi et al., 2002a; Roscoe et al., 2004; Suprayudi et al., 2002b; Takeuchi et al., 1999b). The nutritional state of larvae during the late zoeal period is thought to be linked to the nutritional state of *Artemia*. Because brachyuran crab zoeae are passive feeders their feeding rate is a function of encounter rates with prey (Anger, 2001c; Baylon et al., 2004; Minagawa and Murano, 1993). Thus, the density of *Artemia* is generally maintained at a level that is higher than larval consumption to increase encounter rates and improve survival and growth during seed production. After each feeding event, a large number of *Artemia* remain in the larval culture water until the tanks are supplemented the following day. I evaluated the nutritional status of *Artemia* after addition to the rearing tanks and demonstrated that early *Artemia* nauplii could not digest the phytoplankton (*Chlorella* and *Nannochloropsis*) because of their rigid cell walls (Chapter 4.1). Thus, supplementation with *Nannochloropsis* did not prevent starvation of *Artemia*, and instead promoted starvation. The survival of *P. trituberculatus* larvae that were fed starved *Artemia* during the late zoeal period decreased after the MG stage because of their low nutrient status, and particularly their low levels of EPA. *Chlorella* and *Nannochloropsis* are generally supplemented into larval culture water during seed production (Dan et al., 2013; Chapter 3.2). Thus, my results suggest that in many instances *Artemia* may starve after being supplied into larval culture water and this causes mass mortality during seed production. To prevent *Artemia* starvation while supplementing essential EPA, I recommend supplementation of the larval culture water with digestible *Nannochloropsis* that have physically broken cell walls. The condensed digestible *Nannochloropsis* (Marine Alfa, Marinetech Co. Ltd, Aichi, Japan) is commercially available in Japan.

Table 6.3. Comparison of the procedures used during typical seed production and in the larval culture method developed for *Portunus trituberculatus* in this study

Culture method	Food type/task	Treatment/species	Larval stage (density/rate)					
			Z1	Z2	Z3	Z4	MG	(unit)
Standard (former)	Rotifer	Enriched with n-3 HUFA	10	10	20	20	–	(individuals mL^{-1})
	Artemia	Newly hatched	–	–	0.5	1.0	3.0	(individuals mL^{-1})
	Phytoplankton	*Chlorella vulgaris* or *Nannochloropsis oculata*	10	10	10	10	–	(10^4 cell mL^{-1})
	Other food	–	–	–	–	–	Minced mysids	(–)
	Water exchange	Flow through or intermittent exchange	–	–	50	50	100	(% volume daily)
New	Rotifer	–	–	–	–	–	–	
	Artemia	Newly hatched	3.0	–	–	–	–	(individuals mL^{-1})
		Enriched with digestible *Nannochloropsis*	–	1.5	1.5	1.5	3.0	(individuals mL^{-1})
	Phytoplankton	Digestible *Nannochloropsis*	10	10	10	10	20	(10^4 cell mL^{-1})
	Other food	–	–	–	–	–	–	
	Water exchange	Stagnant	–	–	–	–	–	

6.6 Culture method to prevent larval mass mortality

A comparison of the standard culture method and a new method for larval culture of *P. trituberculatus* that prevents mass mortality is shown in Table 6.3. In the new culture method, the occurrence of abnormal MG morphology is reduced by feeding larvae only with *Artemia* (i.e., no rotifers). To simplify larval feeding, small newly hatched *Artemia* nauplii are supplied at high density (3.0 individuals mL^{-1}) during the Z1 stage. Then, *Artemia* that are enriched with digestible *Nannochloropsis* are supplied from the Z2 stage to provide EPA for larvae and to avoid larval ingestion of newly hatched *Artemia* during the critical period (premoult of Z3) at which they

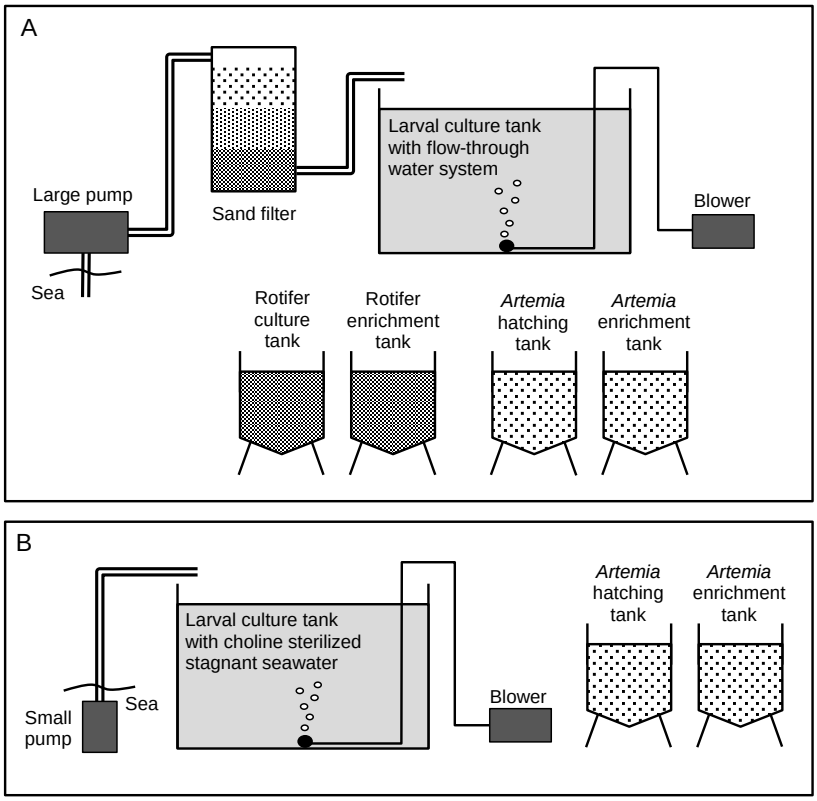

Figure 6.2. Schematic comparison of the equipment required for (A) standard larval culture and (B) newly developed method for culturing the swimming crab *Portunus trituberculatus*

might induce abnormal morphology of MG. To control the occurrence of excessive morphogenesis of last stage zoeae, the *Artemia* feeding density is held at a low level (1.5 individuals mL^{-1}) from the Z2 to last zoeal stage and the salinity of the culture water is lowered throughout the culture period. Digestible *Nannochloropsis* were supplemented into the culture tank to prevent starvation of *Artemia* to maintain their nutritional value. The larval culture water was not exchanged to reduce the potential for contamination with pathogens. These measures resulted in high survival to the C1 stage, demonstrating for the first time that *P. trituberculatus* larvae can be cultured using only *Artemia*.

Because the new culture method does not require rotifers or water exchange, the cost and energy, labour, and facility (e.g., rotifer culture tanks and water pumps) needs are reduced (Fig. 6.2). These changes will allow small hatcheries that do not have large water pumps and rotifer culture systems to conduct mass seed production of *P. trituberculatus* using only larval culture tanks, an aeration system, and *Artemia* hatching and enrichment tanks.

6.7 Future directions

I succeeded in developing a new method for larval culture that prevents multiple causes of mortality during seed production of *P. trituberculatus*. However, this methodology has not yet been trialled in other portunid species such as *P. pelagicus*, *S. serrata*, and *S. paramamosain*. These species have five zoeal instar stages, one more than *P. trituberculatus*, and their newly hatched larvae are smaller. There is a possibility that newly hatched *Artemia* are too large for these Z1 (Baylon et al., 2004; Heasman and Fielder, 1983; Roscoe et al., 2004). However, Kobayashi et al. (2000a) and Suprayudi et al. (2002b) demonstrated that *S. paramamosain* and *S. serrata* larvae could be cultured to final stage zoeae using only newly hatched *Artemia* with high survival (100 % and 81.7 %, respectively). The necessity of rotifer supply at the Z1 stage of these species should be examined prior to adopting the new culture method. If Z1 larvae of the other species require rotifers, they should be removed from the larval culture water prior to the premoult of the Z4 stage to prevent abnormal morphology of MG individuals. If the larvae can be cultured using only *Artemia*, culturists must then pay more attention to the occurrence of abnormal morphology of last stage zoeae, because they will be fed *Artemia* for a longer duration than are *P. trituberculatus*. The *Artemia* feeding density, nutritional enrichment level, and culture conditions (e.g., salinity, water temperature, and light intensity) should be carefully controlled to suppress excessive morphogenesis of last stage zoeae.

In Chapters 4 and 5, improvements in the use of *Artemia* resulted in optimization of a larval culture method for *P. trituberculatus*. The most important factor was maintenance of nutritional condition by supplementing digestible *Nannochloropsis* into the larval culture water. This prevented starvation of *Artemia*, an issue that has been largely overlooked to date. I expect that this technique will improve seed production technologies for many species, because *Artemia* are one of the most important live-foods in the seed production of many species.

In this study, I revealed the relationships between larval morphogenesis and extrinsic environmental and dietary factors, and improved our knowledge of mechanisms controlling larval metamorphosis phenotypes. The larval eyestalk ablation experiments provided insight into endocrine control mechanisms by revealing the coexistence of two distinct pathways of regulation. However, the endocrine pathways controlling these phenomena remain unknown. To address this, there is a need for research into the relationship between the occurrence of morphological abnormalities and the dynamics of hormones such as ecdysone, moult-inhibiting hormone, and juvenile hormone. Portunid crabs are an ideal model for further study as ovigerous females are

easy to obtain, larvae can be easily cultured and exhibit both enlarged and resorbed traits during larval metamorphosis, and larval morphogenesis can be experimentally manipulated.

Many species of portunid crabs (e.g. *P. trituberculatus*, *P. pelagicus*, *S. serrata*, *S. paramamosain*, and *C. sapidus*) exhibit fast growth and reach breeding size (fishery size) within a year (Davis et al., 2005; Hamasaki, 1996; Hamasaki et al., 2011; Ogawa et al., 2012; Xiao and Kumar, 2004). Their annual harvest varies considerably (Hamasaki et al., 2011; Lipcius and Stockhausen, 2002; Obata et al., 2006) and there is a need to understand the factors contributing to these fluctuations. A recent report suggested there was a significant positive correlation between the abundance of *P. trituberculatus* MG on drifting seaweeds in summer and the abundance of fishery recruits in the following autumn, whereas the relationship between spawning stock abundance and recruitment was weak (Shiota, 1993). Thus, the author concluded that larval survival from hatch to MG has a significant effect on the abundance of fishery recruits. However, the larval ecology and factors affecting larval survival in the natural habitat remain poorly understood. In this study, I demonstrated that nutritional accumulation during zoeal period affects post-larval (megalopal) survival and development. The same phenomenon is likely to occur in natural habitats. The larval culture method developed in this study has a very simple feeding procedure, and can eliminate the noise of abnormal morphology. The density of *Artemia* and digestible *Nannochloropsis* supplied under artificial culture conditions can be used as a proxy for the abundance of prey zooplankton and phytoplankton in natural habitats. By doing so, it may be possible to simulate the conditions of a natural food web. Further research into the effect of varying dietary conditions on larval survival, development, and starvation tolerance may contribute to improvement in our understanding of larval ecology, recruitment dynamics, and fluctuation in the abundance of wild stocks.

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Appendix

Supplementary data for Chapter 2.1

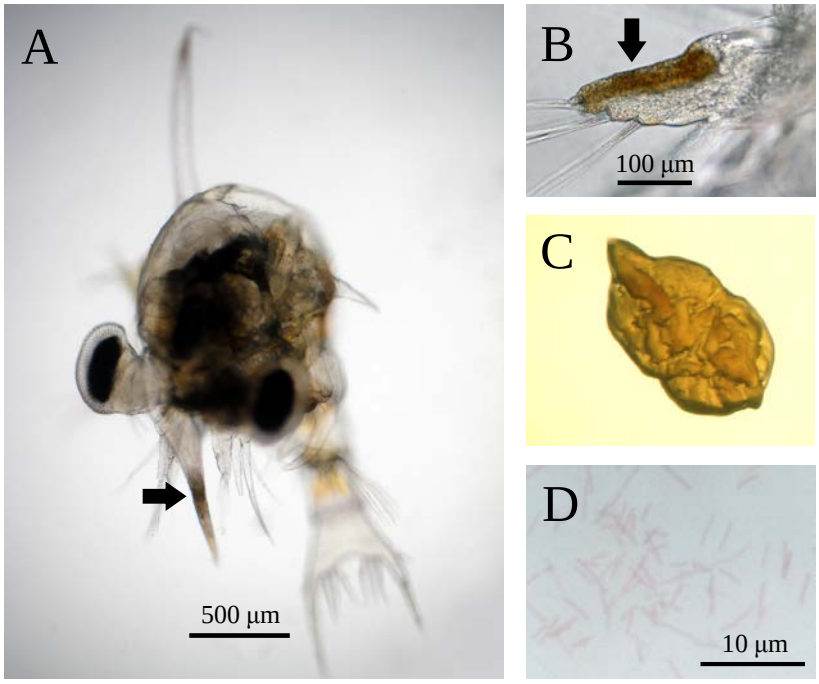

Figure S2.1.1. (A) Fourth stage zoea of *Scylla serrata* showing symptoms of bacterial necrosis disease. (B) Tissue necrosis in an antennule. (C) Colony of the pathogenic bacterium isolated from a zoea showing necrosis disease. (D) Gram-stained pathogenic bacteria. Arrows in A and B indicate the necrotic tissues

Table S2.1.1. Colony counts for the dominant bacteria and their percentages of total bacterial counts in zoeal larvae of *Scylla serrata* showing symptoms of tissue necrosis

Tank	Number of larvae	Total bacteria (log cfu larva^{-1})	Dominant bacteria (log cfu larva^{-1})	Dominance rate (%)
1	8	4.98 ± 0.83	4.74 ± 0.85	69.5 ± 32.6
2	6	5.61 ± 0.35	5.29 ± 0.29	57.5 ± 35.5
3	6	5.29 ± 0.73	4.56 ± 0.87	34.1 ± 33.8
4	11	5.66 ± 0.38	5.32 ± 0.46	60.0 ± 30.0

Mean ± sd
cfu, colony-forming units

Table S2.1.2. Phenotypic characteristics of pathogenic bacteria (NY strain) isolated from *Scylla serrata* larvae showing symptoms of tissue necrosis, and probiotic candidate bacteria screened from larval rearing water (strain 7) and surrounding coastal water (strains 12, 16, 18, 25 and 26)

Characteristic	Bacterial strain						
	NY	7	12	16	18	25	26
Date of collection	2000/7/7	2000/7/18	2000/8/1	2000/8/1	2000/8/1	2000/8/1	2000/8/1
Collection site	Larvae	Larval rearing water	Coastal seawater	Coastal seawater	Coastal seawater	Coastal seawater	Coastal seawater
Colony color	Yellow	Black	Orange	Dark yellow	Pale yellow	Yellow	Brown
Endospore	−	−	−	−	−	−	−
Gram stain	−	−	−	−	−	−	−
Motility	−	−	+	−	+	+	−
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Catalase production	+	−	+	+	+	−	−
Oxidase production	+	+	+	+	+	+	+
Oxidation/Fermentation (D-glucose)	−/−	−/−	−/−	−/−	+/+	−/−	−/−
Acid/Gas production (D-glucose)	−/−	−/−	−/−	−/−	+/−	−/−	−/−
Aerobic growth	+	+	+	+	+	+	+
Anaerobic growth		−	−			−	−
Growth at temperature:							
4°C		−	−			−	−
30°C		+	+			+	+
37°C	−			+	−		
45°C	−			+ weak	−		
API 20NE*							
Arginine dihydrolase	−						
β-glucosidase (aesculin hydrolysis)	−						
β-galactosidase (p-nitrophenyl-alpha-D-galactopyranoside hydrolysis)	−						
Cytochrome oxidase	+						
D-glucose fermentation	−						
Indole production	−						
Nitrate reduction	+						
Protease (gelatin hydrolysis)	+						
Urease	−						
Assimilation of:							
Adipic acid	−						
Arabinose	−						
Capric acid	−						
Citrate	−						
Gluconate	−						
Glucose	−						
Malate	−						
Maltose	−						
Mannitol	−						
N-acetyl-glucosamine	−						
Phenylacetic acid	−						
Family	Flavobacteriaceae	Alteromonadaceae	Alteromonadaceae	Flavobacteriaceae	Aeromonadaceae	Alteromonadaceae	Alteromonadaceae

*API 20NE rapid identification system (bioMérieux, Marcy L'Etoile, France)

Table S2.1.3. Mortality rates of first stage zoeae of *Scylla serrata* inoculated with candidate probiotic strains and incubated for 24 h

Strain	Family	Collection sites	Inoculation (log cfu mL^{-1})	Mortality (%)
Control	–	–	–	0
7	Alteromonadaceae	Larval rearing water	5.74	5
12	Alteromonadaceae	Coastal seawater	5.85	5
16	Flavobacteriaceae	Coastal seawater	5.36	5
18	Aeromonadaceae	Coastal seawater	5.11	10
25	Alteromonadaceae	Coastal seawater	5.81	5
26	Alteromonadaceae	Coastal seawater	5.30	100

Newly hatched Z1 were placed in six-well culture plates with 10 mL of sterile seawater (34 ppt salinity) in each well (20 individuals $well^{-1}$), which was inoculated with each candidate strain from MA plates at 5.11–5.85 log cfu mL^{-1}. The larvae in the control group were not inoculated with any bacteria. After 24 h at 29 ± 1 °C, surviving larvae were counted and the survival rate was determined

Supplementary data for Chapter 3.3

Table S3.3.1. Morphological measurements (CL, CHL, PL, DSL and FL) of fourth stage zoeae of *Portunus trituberculatus* treated during the postmoult and premoult of the second (Z2) or third (Z3) zoeal stages. Values are mean ± sd. See Table 3.3.1 and Fig. 3.3.1 in the main text for definitions of measurement abbreviations and further details

Developmental stage at treatment	Treatment	Morphological measurement				
		CL (μm)	CHL (μm)	PL (μm)	DSL (μm)	FL (μm)
Z2-postmoult	Control	1488 ± 8	541 ± 16	345 ± 7	1182 ± 58	971 ± 36
	Darkness	1399 ± 36	500 ± 5	318 ± 8	1116 ± 46	992 ± 7
	Dorsal spine ablation	1510 ± 22	551 ± 15	345 ± 7	587 ± 133	968 ± 8
	Eyestalk ablation	1388 ± 15	300 ± 18	148 ± 13	1318 ± 74	993 ± 37
Z2-premoult	Eyestalk ablation	1385 ± 24	354 ± 40	217 ± 16	1269 ± 71	947 ± 58
Z3-postmoult	Control	1469 ± 38	524 ± 14	338 ± 9	1253 ± 2	1031 ± 26
	Darkness	1443 ± 30	515 ± 21	333 ± 12	1243 ± 55	1017 ± 48
	Dorsal spine ablation	1494 ± 23	538 ± 17	348 ± 10	386 ± 48	998 ± 40
	Eyestalk ablation	1473 ± 24	458 ± 14	293 ± 14	1457 ± 33	1000 ± 34
Z3-premoult	Eyestalk ablation	1471 ± 10	505 ± 9	322 ± 9	1320 ± 42	1014 ± 21

Table S3.3.2. Morphological measurements (CHLr, PLr, DSLr and FLr) of fourth stage zoeae of *Portunus trituberculatus* treated during the postmoult and premoult of the second (Z2) or third (Z3) zoeal stages. Values are mean ± sd. See Table 3.3.1 and Fig. 3.3.1 in the main text for definitions of measurement abbreviations and further details

Developmental stage at treatment	Treatment	Morphological measurement			
		CHLr (%)	PLr (%)	DSLr (%)	FLr (%)
Z2-postmoult	Control	36.4 ± 0.9	23.2 ± 0.4	88.2 ± 4.0	72.4 ± 1.3
	Darkness	35.8 ± 1.3	22.7 ± 0.8	83.1 ± 3.9	73.8 ± 1.3
	Dorsal spine ablation	36.5 ± 0.7	22.8 ± 0.4	41.9 ± 9.6	69.2 ± 2.0
	Eyestalk ablation	21.6 ± 1.2	10.7 ± 0.9	99.5 ± 2.5	75.0 ± 3.1
Z2-premoult	Eyestalk ablation	25.5 ± 2.5	15.6 ± 1.0	96.6 ± 3.3	72.3 ± 3.6
Z3-postmoult	Control	35.7 ± 1.0	23.0 ± 0.3	86.1 ± 2.8	70.9 ± 2.4
	Darkness	35.7 ± 0.9	23.0 ± 0.5	87.3 ± 3.9	71.5 ± 2.8
	Dorsal spine ablation	36.0 ± 1.2	23.2 ± 0.8	26.4 ± 2.9	68.6 ± 4.1
	Eyestalk ablation	31.1 ± 0.7	19.9 ± 0.7	98.3 ± 11.7	70.8 ± 1.5
Z3-premoult	Eyestalk ablation	34.3 ± 0.6	21.9 ± 0.6	91.9 ± 3.6	70.6 ± 2.0

Table S3.3.3. Morphological measurements (CL, CHL, DSL and FL) of fifth instar larvae of *Portunus trituberculatus* treated during the postmoult of the second (Z2), third (Z3) or fourth (Z4) zoeal stages, premoult of Z2 or Z3, or intermoult or premoult of Z4. Z5 = supernumerary fifth zoeal stage occurring after eyestalk ablation in Z2 or Z3; MG = megalopae. Values are mean ± sd. See Table 3.3.1 and Fig. 3.3.1 in the main text for definitions of measurement abbreviations and further details

Developmental stage at treatment	Treatment	Morphological measurement				
		CL-Z5 (μm)	CL-MG (μm)	CHL (μm)	DSL (μm)	FL (μm)
Z2-postmoult	Control	–	2550 ± 54	1401 ± 30	0 ± 0.0	25 ± 14.7
	Darkness	–	2416 ± 27	1353 ± 8	1 ± 2.0	49 ± 35.7
	Dorsal spine ablation	–	2535 ± 53	1394 ± 28	4 ± 3.0	30 ± 23.9
	Eyestalk ablation	1594 ± 14	–	480 ± 13	1425 ± 77.7	1044 ± 81.3
Z2-premoult	Eyestalk ablation	1670 ± 33	–	596 ± 18	1368 ± 74.1	1026 ± 149.1
Z3-postmoult	Control	–	2468 ± 33	1377 ± 15	17 ± 3.1	20 ± 5.8
	Darkness	–	2424 ± 36	1370 ± 11	2 ± 2.6	36 ± 10.7
	Dorsal spine ablation	–	2464 ± 28	1359 ± 19	15 ± 9.4	24 ± 12.1
	Eyestalk ablation	1756 ± 57	–	655 ± 20	1595 ± 57.1	867 ± 205.2
Z3-premoult	Eyestalk ablation	1873 ± 52	2440 ± 132	1048 ± 86	569 ± 233.5	757 ± 103.7
Z4-postmoult	Control	–	2484 ± 12	1412 ± 21	15 ± 6.2	2 ± 2.5
	Darkness	–	2451 ± 28	1376 ± 17	2 ± 1.1	0 ± 0.0
	Dorsal spine ablation	–	2528 ± 30	1403 ± 12	21 ± 9.6	3 ± 4.8
	Eyestalk ablation	–	2501 ± 48	1364 ± 16	3 ± 0.7	21 ± 22.0
Z4-intermoult	Eyestalk ablation	–	2637 ± 68	1396 ± 25	4 ± 6.3	1 ± 2.2
Z4-premoult	Eyestalk ablation	–	2649 ± 43	1394 ± 11	69 ± 45.5	39 ± 26.5

Table S3.3.4. Morphological measurements (CHLr, DSLr and FLr) of fifth instar larvae of *Portunus trituberculatus* treated during the postmoult of the second (Z2), third (Z3) or fourth (Z4) zoeal stages, premoult of Z2 or Z3, or intermoult or premoult of Z4. Z5 = supernumerary fifth zoeal stage occurring after eyestalk ablation in Z2 or Z3; MG = megalopae. Values are mean ± sd. See Table 3.3.1 and Fig. 3.3.1 in the main text for definitions of measurement abbreviations and further details

Developmental stage at treatment	Treatment	Morphological measurement					
		CHLr-Z5 (%)	CHLr-MG (%)	DSLr-Z5 (%)	DSLr-MG (%)	FLr-Z5 (%)	FLr-MG (%)
Z2-postmoult	Control	–	56.0 ± 1.5	–	0.0 ± 0.0	–	1.0 ± 0.6
	Darkness	–	56.1 ± 0.7	–	0.0 ± 0.1	–	2.0 ± 1.5
	Dorsal spine ablation	–	56.1 ± 1.1	–	0.2 ± 0.1	–	1.2 ± 0.9
	Eyestalk ablation	29.4 ± 0.8	–	89.9 ± 5.1	–	64.3 ± 3.8	–
Z2-premoult	Eyestalk ablation	35.4 ± 0.8	–	81.9 ± 2.6	–	61.3 ± 8.7	–
Z3-postmoult	Control	–	55.8 ± 0.9	–	0.7 ± 0.1	–	0.8 ± 0.2
	Darkness	–	56.3 ± 0.8	–	0.1 ± 0.1	–	1.5 ± 0.5
	Dorsal spine ablation	–	55.0 ± 0.9	–	0.6 ± 0.4	–	1.0 ± 0.5
	Eyestalk ablation	38.4 ± 0.8	–	91.3 ± 5.9	–	48.5 ± 12.8	–
Z3-premoult	Eyestalk ablation	37.1 ± 3.5	46.8 ± 2.4	86.6 ± 4.8	17.6 ± 13.5	61.9 ± 8.2	29.0 ± 6.1
Z4-postmoult	Control	–	56.8 ± 0.8	–	0.6 ± 0.2	–	0.1 ± 0.1
	Darkness	–	57.1 ± 0.8	–	0.1 ± 0.0	–	0.0 ± 0.0
	Dorsal spine ablation	–	56.7 ± 0.8	–	0.8 ± 0.4	–	0.1 ± 0.2
	Eyestalk ablation	–	54.4 ± 0.8	–	0.1 ± 0.0	–	0.8 ± 0.9
Z4-intermoult	Eyestalk ablation	–	54.2 ± 2.0	–	0.2 ± 0.2	–	0.0 ± 0.1
Z4-premoult	Eyestalk ablation	–	54.6 ± 1.1	–	2.6 ± 1.7	–	1.5 ± 1.0

Table S3.3.5. Morphological measurements (Z5f, DSf and TELf) of fifth instar larvae of *Portunus trituberculatus* treated during the postmoult of the second (Z2), third (Z3) or fourth (Z4) zoeal stages, premoult of Z2 or Z3, or intermoult or premoult of Z4. Values are mean ± sd. See Table 3.3.1 and Fig. 3.3.1 in the main text for definitions of measurement abbreviations and further details

Developmental stage at treatment	Treatment	Morphological measurement		
		Z5f (%)	DSf (%)	TELf (%)
Z2-postmoult	Control	0	0.0 ± 0.0	19.6 ± 6.8
	Darkness	0	2.5 ± 5.0	24.1 ± 11.0
	Dorsal spine ablation	0	7.1 ± 4.8	23.3 ± 20.5
	Eyestalk ablation	100 ± 0.0	100 ± 0.0	100 ± 0.0
Z2-premoult	Eyestalk ablation	100 ± 0.0	100 ± 0.0	100 ± 0.0
Z3-postmoult	Control	0	21.6 ± 6.7	26.9 ± 10.1
	Darkness	0	2.8 ± 3.2	22.1 ± 12.6
	Dorsal spine ablation	0	18.9 ± 5.7	27.5 ± 12.1
	Eyestalk ablation	100 ± 0.0	100 ± 0.0	100 ± 0.0
Z3-premoult	Eyestalk ablation	14.1 ± 4.6	77.6 ± 10.1	95 ± 3.6
Z4-postmoult	Control	0	26.3 ± 3.9	2.1 ± 2.4
	Darkness	0	3.8 ± 2.6	0.0 ± 0.0
	Dorsal spine ablation	0	35.9 ± 17.0	3.6 ± 4.8
	Eyestalk ablation	0	6.5 ± 1.8	12.3 ± 11.3
Z4-intermoult	Eyestalk ablation	0	6.4 ± 8.1	2.8 ± 5.6
Z4-premoult	Eyestalk ablation	0	57.3 ± 27.0	21.0 ± 10.0

Supplementary data for Chapter 3.4

Table S3.4.1.Statistical analysis methods to evaluate the effects of treatment on survival, rate of development, and morphological measurements of swimming crab *Portunus trituberculatus* larvae reared in experiments 1–3

Analysis object	Experiment	Method	Error distribution	Link function	Response variable	Explanatory variable	Test of significance of explanatory variables
Larval survival	1 and 3	GLM[1]	Quasi-binomial	Logit	Numbers of live and dead animals	Treatment[3]	*F* test[5]
	2	GLMM[2]	Binomial			Treatment[3, 4], Larval age (days)	Wald χ^2 test[5]
Rate of development	1, 2 and 3	GLMM[2]	Gaussian	Identical	Larval stage index (LSI)	Treatment[3, 4], Larval age (days)	Wald *F* test with Kenward–Roger degrees of freedom[5, 6]
Morphological measurements	1 and 3	GLM[1]	Gaussian	Identical	CL, CHL, CHLr, DSL, and FL	Treatment[3]	*F* test[5]
			Quasi-binomial	Logit	DSf, TELf, and Z5f		
	2	GLM[1]	Gaussian	Identical	CL, CHL, CHLr, DSL, and FL	Treatment periods/timing[3]	*F* test[5]
			Quasi-binomial	Logit	DSf, TELf, and Z5f		

[1] Generalised linear model was employed using the *glm* function (McCullagh and Nelder, 1989; Everitt and Hothorn, 2009)

[2] Generalised linear mixed-effects model was employed using the *glmer* or *lmer* function in the lme4 package (Bates et al., 2012; Everitt, 2005; Everitt and Hothorn, 2009; Zuur et al., 2009). Data on larval survival in experiment 2 and LSI in all experiments were collected in the same tank every day. Therefore, to account for a potential correlation between the observations, the identity of each rearing tank was included in the GLMM as a random intercept effect

[3] Differences between treatments were evaluated with the Tukey method using the *glht* function implemented in the multcomp package (Hothorn et al., 2008)

[4] Because the treatment timing varied among treatment groups (control, Chlorella, and eyestalk ablation) in experiment 2, the data on survival and rate of development of larvae were compared when the treatment timing (days since transfer from the 500 L tank to 1 L beakers) was the same

[5] The significance of the explanatory variables was evaluated using the *Anova* function (type II) implemented in the car package (Fox and Weisberg, 2011)

[6] The values were calculated according to Kenward and Roger (1997) and Halekoh and Højsgaard (2013)

Table S3.4.2. Analysis of deviance table for an *F* test (type II) evaluating the effects of treatment periods/timing (explanatory variable) on morphological measurements (response variables) in swimming crab *Portunus trituberculatus* larvae reared in experiment 2

Treatment	Response variable	SS	*df*	*F*	*P*(>*F*)
Control	CL	20689	3	3.397	0.0526
	CHL	17360	3	7.704	0.0039
	CHLr	45.53	3	3.963	0.0355
	DSL	76116	4	11.16	0.0002
	FL	174008	4	19.44	< 0.00001
	DSf	59.14	4	8.239	0.0010
	TELf	68.45	4	16.29	< 0.00001
Chlorella	CL	6977	3	2.609	0.1042
	CHL	29650	3	28.286	< 0.00001
	CHLr	102.2	3	20.88	< 0.00001
	DSL	162863	4	12.62	0.0001
	FL	492813	4	28.39	< 0.00001
	DSf	53.49	4	12.78	0.0001
	TELf	182.8	4	25.4	< 0.00001
Eyestalk ablation	CL	28768	3	6.042	0.0095
	CHL	61531	3	28.19	< 0.00001
	CHLr	183.6	3	24.71	< 0.00001
	DSL	12479287	5	100.7	< 0.00001
	FL	6893079	5	77.72	< 0.00001
	DSf*	1.929	2	0.3325	0.7256
	TELf*	22.72	2	6.567	0.0174

* To avoid the error attributed to zero data in the analyses using quasi-binomial family, the groups showing 0 % or 100 % in DSf and TELf were excluded from the analysis

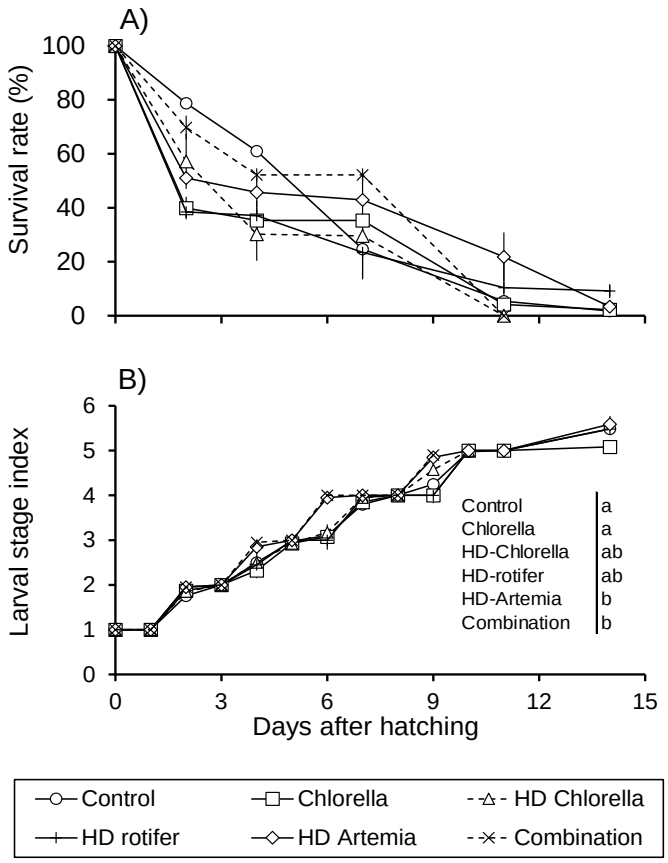

Figure S3.4.1. Changes in survival rate (A) and larval stage index (LSI, B) as an index of the rate of development of *Portunus trituberculatus* larvae cultured with different dietary conditions in experiment 1. Vertical bars indicate the standard deviations of duplicates in each treatment group. Age effect on LSI: $F = 4604$, $df = 1, 10.7$, $P < 0.0001$. Treatment effect on LSI: $F = 5.286$, $df = 5, 6.04$, $P = 0.0328$. Differences in LSI between treatments ($P < 0.05$) are indicated by a different lowercase letter following the treatment in the table

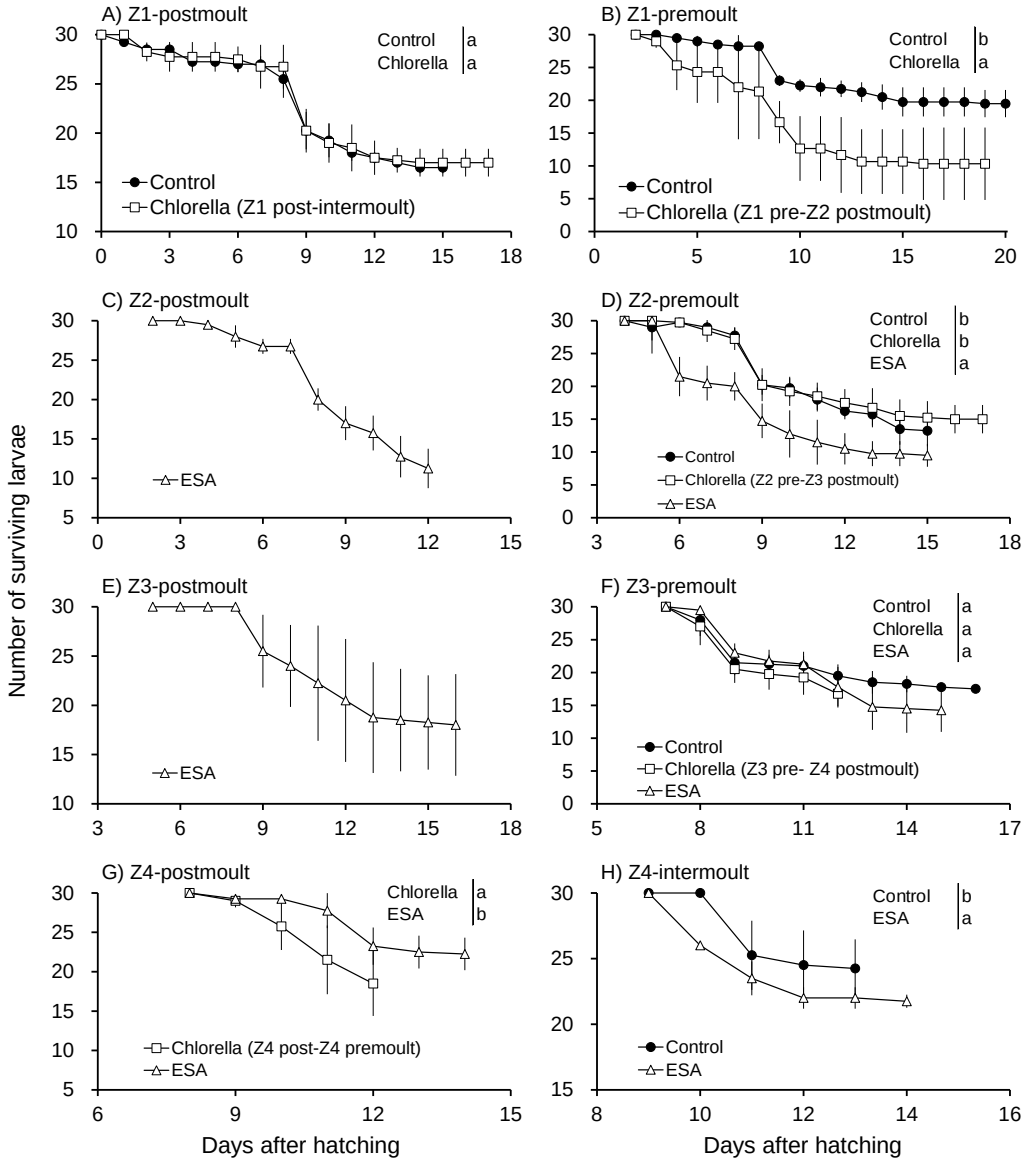

Figure S3.4.2. Changes in the number of surviving *Portunus trituberculatus* larvae treated during the postmoult of the first (A), second (C), third (E), or fourth (G) zoeal stages, premoult of the first (B), second (D), third (F) zoeal stages, and intermoult of the fourth (H) zoeal stage in experiment 2. Vertical bars represent the standard deviations of the four replicate groups in each treatment. Age effect: Z1-postmoult, $\chi^2 = 482.7$, $df = 1$, $P < 0.0001$; Z1-premoult, $\chi^2 = 427.3$, $df = 1$, $P < 0.0001$; Z2-premoult, $\chi^2 = 692.1$, $df = 1$, $P < 0.0001$; Z3-premoult, $\chi^2 = 251.0$, $df = 1$, $P < 0.0001$; Z4-postmoult, $\chi^2 = 116.6$, $df = 1$, $P < 0.0001$; Z4-intermoult, $\chi^2 = 55.87$, df = 1, $P < 0.0001$. Treatment effect: Z1-postmoult, $\chi^2 = 0.5667$, $df = 1$, $P = 0.4516$; Z1-premoult, $\chi^2 = 15.79$, $df = 1$, $P = 0.0001$; Z2-premoult, $\chi^2 = 49.50$, $df = 2$, $P < 0.0001$; Z3-premoult, $\chi^2 = 5.308$, $df = 2$, $P = 0.0704$; Z4-postmoult, $\chi^2 = 14.73$, $df = 1$, $P = 0.0001$; Z4-intermoult, $\chi^2 = 5.741$, $df = 1$, $P = 0.0017$. Differences between treatments ($P < 0.05$) are represented by a different uppercase letter following the treatment in the table: control (Control), *Chlorella vulgaris* supplementation (Chlorella), and eyestalk ablation (ESA)

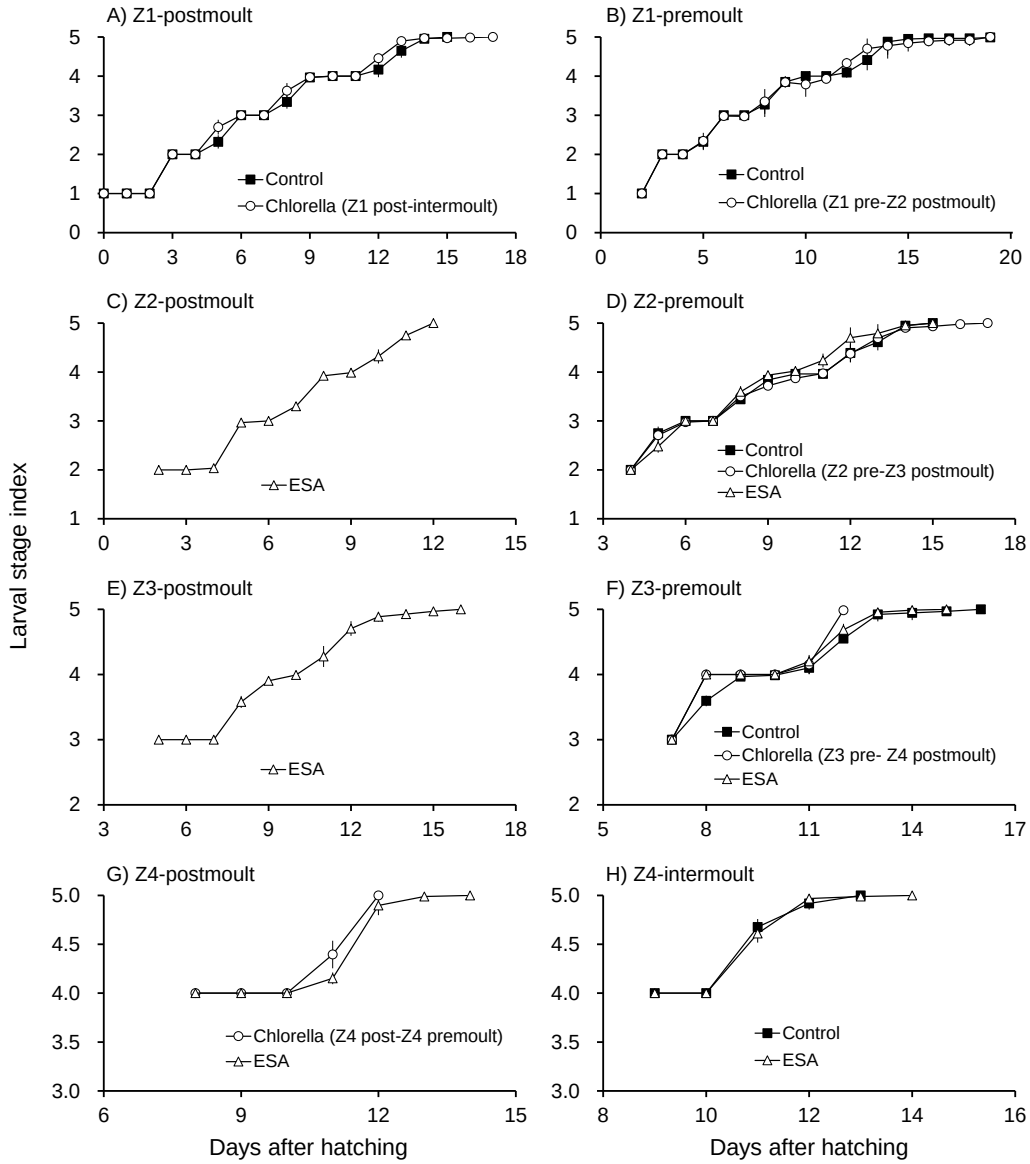

Figure S3.4.3. Changes in larval stage index (LSI) as an index of the rate of development of *Portunus trituberculatus* larvae treated during the postmoult of the first (A), second (C), third (E), or fourth (G) zoeal stages, premoult of the first (B), second (D), third (F) zoeal stages, and intermoult of the fourth (H) zoeal stage in experiment 2. Vertical bars represent the standard deviations of the four replicate groups in each treatment. Age effect: Z1-postmoult, $F = 2917$, $df = 1, 121.1$, $P < 0.0001$; Z1-premoult, $F = 1280$, $df = 1, 102.3$, $P < 0.0001$; Z2-premoult, $F = 2805$, $df = 1, 131.8$, $P < 0.0001$; Z3-premoult, $F = 444.1$, $df = 1, 80.49$, $P < 0.0001$; Z4-postmoult, $F = 122.7$, $df = 1, 36.92$, $P < 0.0001$; Z4-intermoult, $F = 192.4$, $df = 1, 31.8$, $P < 0.0001$. Treatment effect: Z1-postmoult, $F = 0.9596$, $df = 1, 5.92$, $P = 0.3656$; Z1-premoult, $F = 0.0217$, $df = 1, 4.95$, $P = 0.8886$; Z2-premoult, $F = 3.997$, $df = 2, 9.07$, $P = 0.0569$; Z3-premoult, $F = 3.485$, $df = 2, 9.22$, $P = 0.0746$; Z4-postmoult, $F = 0.8481$, $df = 1, 6.39$, $P = 0.3905$; Z4-intermoult, $F = 0.0846$, $df = 1, 5.58$, $P = 0.7817$

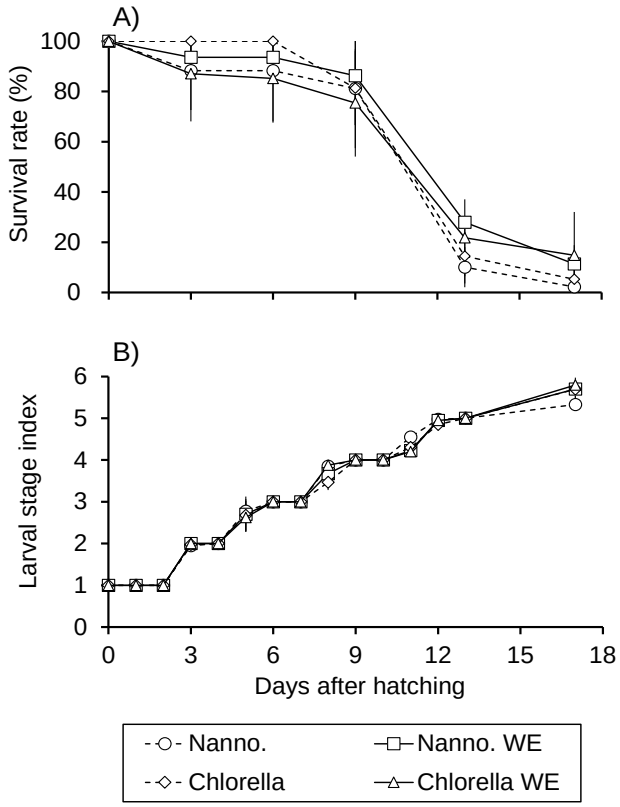

Figure S3.4.4. Changes in survival (A) and larval stage index (LSI, B) as an index of the rate of development of *Portunus trituberculatus* larvae cultured with different water exchange and phytoplankton supplementation conditions in experiment 3. Vertical bars represent the standard deviations of duplicates in each treatment group. Age effect on LSI: $F = 5715$, $df = 1, 155.7$, $P < 0.0001$. Treatment effect on LSI: $F = 0.2652$, $df = 3, 7.98$, $P = 0.8487$

Supplementary data for Chapter 4.1

Table S4.1.1. Statistical analysis methods to evaluate the effects of treatment on performance of swimming crab *Portunus trituberculatus* larvae and *Artemia* in experiments 1–3

Analysis object	Experiment	Method	Error distribution	Link function	Response variable	Explanatory variable	Test of significance of explanatory variables
Larval survival	1 and 2	GLM[1]	Quasi-binomial	Logit	Numbers of live and dead animals	Treatment[3]	*F* test[4]
Rate of development	1 and 2	GLMM[2]	Gaussian	Identical	Larval stage index (LSI)	Treatment[3], Larval age (DAH)	Wald *F* test with Kenward–Roger degrees of freedom[4,5]
Morphological measurements	1 and 2	GLM[1]	Gaussian	Identical	CL, CHL, CHLr, DSL, CW, and ED	Treatment[3]	*F* test[4]
			Quasi-binomial	Logit	DSf, and TELf		
Status of *Artemia*	2	GLM[1]	Gaussian	Identical	Total length, dry weight, total lipid, and EPA content	Treatment[3]	*F* test[4]
Artemia survival	3	GLMM[2]	Binomial	Logit	Numbers of live and dead animals	Treatment[3], *Artemia* age (DAH)	Wald *F* test[4]
Artemia growth	3	GLM[1]	Gaussian	Identical	Specific growth rate	Treatment[3]	*F* test[4]

[1] Generalised linear model was employed using the *glm* function (McCullagh and Nelder, 1989; Everitt and Hothorn, 2009)

[2] Generalised linear mixed-effects model was employed using the *glmer* or *lmer* function in the lme4 package (Bates et al., 2012; Everitt, 2005; Everitt and Hothorn, 2009; Zuur et al., 2009). Data on LSI and *Artemia* survival were collected in the same tank every day. Therefore, to account for a potential correlation between the observations, the identity of each rearing tank was included in the GLMM as a random intercept effect

[3] Differences between treatments were evaluated with the Tukey method using the *glht* function implemented in the multcomp package (Hothorn et al., 2008)

[4] The significance of the explanatory variables was evaluated using the *Anova* function (type II) implemented in the car package (Fox and Weisberg, 2011)

[5] The values were calculated according to Kenward and Roger (1997) and Halekoh and Højsgaard (2013)

Table S4.1.2. Mean total lipid content and fatty acid composition values in *Artemia* supplied to *Portunus trituberculatus* larvae in each treatment group during experiment 2 (n = 2)

Analyzed items	NH Artemia	Starved Artemia	d-Nanno. Artemia
Total lipid (% dry basis)			
	20.34 (0.72)	11.75 (0.74)	13.14 (0.87)
Fatty acid (area %)			
12:0	n.d.	3.71 (0.44)	1.85 (1.67)
14:0	0.17 (0.29)	0.80 (0.06)	n.d.
16:0	9.33 (0.34)	9.39 (0.32)	11.63 (0.29)
16:1	2.22 (0.40)	2.67 (0.11)	5.27 (0.63)
16:3n-6	0.98 (0.03)	1.00 (0.03)	n.d.
16:3n-4	0.96 (0.03)	0.21 (0.37)	n.d.
18:0	3.70 (0.15)	4.40 (0.21)	6.37 (0.67)
18:1	20.66 (0.46)	22.23 (0.54)	23.99 (1.52)
18:2n-6	5.39 (0.10)	4.12 (0.06)	4.29 (0.11)
18:3n-3	42.78 (0.54)	34.71 (0.73)	25.00 (1.09)
18:4n-3	10.17 (0.29)	9.91 (0.28)	4.53 (0.61)
20:4n-6	n.d.	0.51 (0.45)	2.48 (0.25)
20:3n-3	1.23 (0.06)	1.00 (0.04)	0.83 (0.74)
20:4n-3	1.23 (0.05)	0.20 (0.35)	n.d.
20:5n-3	1.18 (0.06)	2.99 (0.23)	12.54 (0.64)
22:0	n.d.	2.16 (0.28)	1.22 (1.15)
22:6n-3	n.d.	n.d.	n.d.
Σ n-3 HUFA	3.64 (0.16)	4.19 (0.50)	13.37 (1.34)

Values are mean ± standard deviations. NH Artemia, newly hatched *Artemia* nauplii; Nanno., *Nannochloropsis oculata*; d-Nanno., commercially available digestible *Nannochloropsis oculata*; n.d., not detected; n-3 HUFA, n-3 highly unsaturated fatty acids

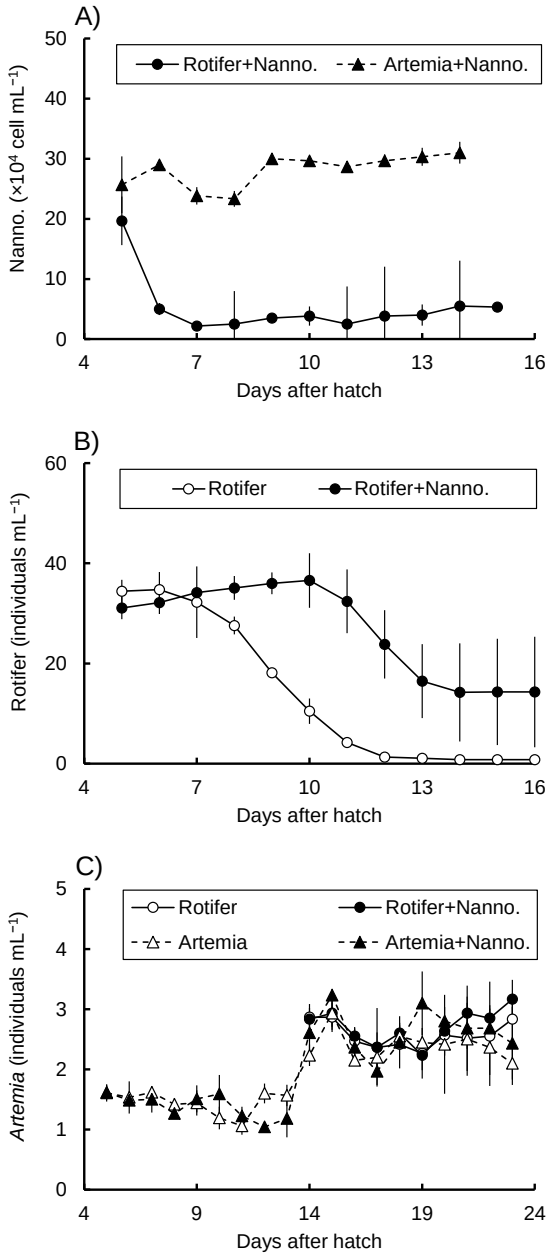

Figure S4.1.1. Changes in *Nannochloropsis oculata* (A), rotifer (B), and *Artemia* (C) densities the morning before supplementing the rearing water of *Portunus trituberculatus* larvae in experiment 1. Vertical bars indicate standard deviations of triplicates in each treatment group

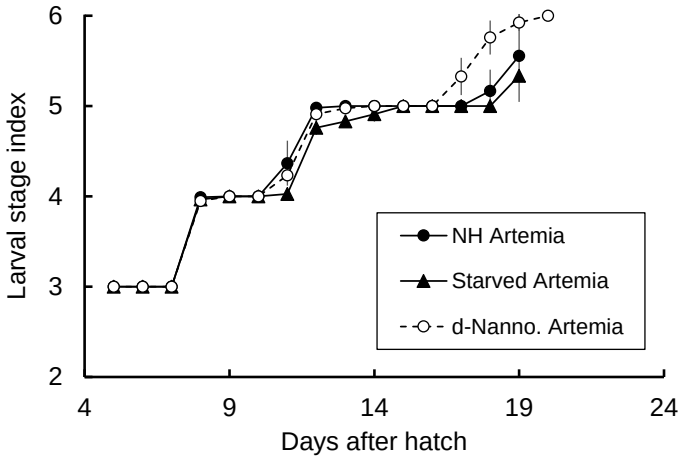

Figure S4.1.2. Changes in larval stage index (LSI) as an index of the developmental rate of *Portunus trituberculatus* larvae cultured with differently treated *Artemia* in experiment 2. Vertical bars indicate standard deviations of four replicates in each treatment group. Age effect: $F = 1442$, $df = 1, 173$, $P < 0.0001$. Treatment effect: $F = 2.779$, $df = 2, 173$, $P = 0.0649$