INFLUENCE OF DIET AND ABIOTIC FACTORS ON THE SURVIVAL AND REPRODUCTION OF CALANOID COPEPOD *Pseudodiaptomus annandalei*

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FACULTY OF SCIENCE
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INFLUENCE OF DIET AND ABIOTIC FACTORS ON THE SURVIVAL AND REPRODUCTION OF CALANOID COPEPOD *Pseudodiaptomus annandalei*

ABSTRACT

The calanoid copepod, *Pseudodiaptomus annandalei,* due to its good nutritional value and significance as a natural diet of many tropical fish, is a superior live feed for marine fish larvae. Fish larvae needs a sustainable supply of good quality live feed for optimal growth and survival. Aims of this study were to investigate the effects of (1) marine mircroheterotrophs grown in palm oil mill effluent (POME) as copepod diet and (2) abiotic factors i.e. salinity (ppt), temperature (˚C), pH, photoperiod and light intensity on the survival and nauplii production of *P. annandalei.* Both biotic and abiotic experiments were conducted with the initial stocking density of fifty copepod nauplii stage I in 250 ml of sterile 15 ppt brackish water and nauplii were cultured in water baths under controlled condition. Each treatment was conducted in triplicates with a completely randomized design. Survival and reproduction of *P. annandalei* were determined every 2-day intervals for a duration of 14 days. *P. annandalei* fed on a mixed diet of 75% POME-grown *Shewanella algae* (POME-SA) and 25% POME-grown *Aurantiochytrium limacinum* (POME-AL) had significantly ($p < 0.05$) higher survival (71.3±11.6%) and nauplii production on day 8 (27±1.2 nauplii) compared to other POME-based diets tested and a commercial microalgae product *Nannochloropsis oculata*. Salinities of 5, 15, 25 and 35 ppt had no effect on the survival of *P. annandalei* ($p > 0.05$) but influenced F2 nauplii production. The highest nauplii density was recorded at 15 ppt (103±48.8 nauplii) on day 14. The survival of *P. annandalei* was affected by temperature ($p < 0.05$) where 26˚C gave the highest survival (day 8: 85.3±4.7% and day 14: 58.7±9.6%) compared to those cultured at other temperatures (28˚C to 36˚C). In contrast, significant nauplii production recorded at 28˚C (42±12.4 nauplii). The survival of *P. annandalei* was

affected by pH ($p < 0.05$) where 49.3±3.7% survival was recorded at pH 8 on last day but the nauplii production was not significant among treatments. Photoperiod did not affect the survival and nauplii production of *P. annandalei* where 82.0±11.4% survival and 11±4.0 nauplii was recorded at 12h light: 12h dark. The light intensity of 4.05 µmol/m^2/sec gave the highest survival of *P. annandalei* (86.7±2.4%) compared to other treatments tested. No variations between treatments were detected for sex ratio and body growth in salinity, pH, photoperiod and light intensity experiments except for feed. At a larger scale experiment (1L tank), the survival of *P. annandalei* was high and maintained above 70% through experiment. Fatty acid analysis shows that a consortium of POME-grown SA and POME-grown AL biomass contained 0.80±0.01% of docohexanoic acid (DHA) whilst *P. annandalei* fed on this mixed diet contained high amount of arachidonic acid (ARA) (0.91±0.35%), eicosapentaenoic acid (EPA) (4.36±0.02%) and DHA (7.21±0.06%). As conclusion, a mixed diet of POME-grown SA and POME-grown AL is the most suitable diet for mass culturing *P. annandalei* in captivity, incorporating the optimal culture conditions of 15±1 ppt salinity, 26.0±1.0 ºC, pH 7-8, 12h light: 12h dark photoperiod and light intensity of 4.05 µmol/m^2/sec.

Keywords: *Pseudodiaptomus annandalei,* waste-grown microheterotrophs, abiotic, survival, reproduction, fatty acids

PENGARUH DIET DAN ABIOTIK FAKTOR KE ATAS KELANGSUNGAN DAN PENGHASILAN KALANOID KOPEPOD, *Pseudodiaptomus annandalei*

ABSTRAK

Kalanoid kopepod, *Pseudodiaptomus annandalei,* mempunyai nutrisi profil yang bagus dan penting sebagai makanan semulajadi ikan tropikal, sangat berpotensi untuk dijadikan sebagai makanan hidup untuk larva ikan marin. Larva ikan memerlukan makanan yang berkualiti untuk pembesaran optima. Tujuan kajian ini adalah untuk menyiasat kesan (1) mikroorganisma marin heterotrofik yang dibesarkan dalam POME sebagai makanan kopepod (2) faktor abiotik seperti kemasinan (ppt), suhu (˚C), fotoperiod dan keamatan cahaya ke atas kelangsungan hidup dan penghasilan naupli *P. annandalei.* Kesemua eksperimen biotik dan abiotik dijalankan dengan stok awal sebanyak 50 ekor nauplii tahap I telah diletakkan ke dalam 250 ml air masin 15 ppt dan nauplii dikultur dalam kukusan air di bawah keadaan makmal yang terkawal. Setiap rawatan mengandungi tiga replikasi dengan "completely randomized designed" (CRD). Jangka hayat dan tumbesaran *P. annandalei* diperhatikan setiap selang dua hari selama 14 hari. Keputusan menunjukkan bahawa makanan campuran berasaskan 75% POME-*Shewanella algae* (SA) dan 25% POME-*Aurantiochytrium limacinum* (AL) secara statistiknya ketara ($P<0.05$) ke atas jangka hayat (71.3±11.6%) dan penghasilan nauplii yang ketara pada hari kelapan (27±1.2 nauplii) berbanding dengan rawatan berasaskan POME yang lain dan produk mikroalga komersil *Nannochloropsis oculata*. Kemasinan 5, 15, 25 dan 35 ppt tidak memberi kesan ke atas kelangsungan hidup *P. annandalei* ($P<0.05$) tetapi mempengaruhi penghasilan nauplii generasi kedua. Kepadatan nauplii yang paling banyak telah direkodkan pada 15 ppt di hari ke 14 (103 ± 48.8). kelangsungan hidup *P. annandalei* telah dipengaruhi oleh suhu ($p < 0.05$) yang mana 26˚C mengekalkan kelangsungan hidup yang paling tinggi (hari kelapan: 85.3 ± 4.7% dan hari ke-14:

58.7±9.6%) dibandingkan dengan kultur di suhu 28˚C hingga 36˚C. Berbeza dengan pengeluaran naupli tertinggi yang telah direkodkan pada 28˚C (42.0±12.4 nauplii). Kadar kelangsungan hidup turut dipengaruhi oleh pH ($p < 0.05$) yang mana 49.3±3.7% kelangsungan hidup telah direkodkan pada pH 8 pada hari terakhir eksperimen tetapi penghasilan nauplii tidak ketara antara rawatan. Fotoperiod tidak memberi kesan ketara ke atas kelangsungan hidup *P. annandalei* serta penghasilan nauplii yang mana 82.0±11.4% kelangsungan dan 11±4.0 nauplii direkodkan pada 12 jam cerah: 12 jam gelap hari ke 14. Keamatan cahaya 4.05 μmol/m^2/sec memberi kesan paling tinggi ke atas kelangsungan hidup *P. annandalei* (86.7 ± 2.4%) dibandingkan dengan rawatan lain yang diuji. Tiada kelainan antara semua rawatan dikesan untuk nisbah jantina dan pertumbuhan badan dalam eksperimen kemasinan, pH, fotoperiod dan keamatan cahaya kecuali makanan. Pada skala besar (tangki 1 liter), kelangsungan hidup *P. annandalei* tinggi dan kekal melebihi 70% sepanjang eksperimen berlangsung. Analisa asid lemak menunjukkan biojisim POME-*Shewanella algae* (SA) dan POME-*Aurantiochytrium limacinum* (AL) mengandungi 0.30±0.02% ARA dan 0.80±0.01% DHA sementara itu *P. annandalei* yang telah diberi makan diet campuran ini mengandungi ARA (0.91±0.01%), EPA (4.36±0.02%) dan DHA (7.21±0.06%). Kesimpulannya, diet campuran berasaskan POME-SA dan POME-AL adalah yang paling sesuai digunakan untuk mengkultur massa *P. annandalei* dalam peliharaan, menggabungkan keadaan kultur optima 15±1 ppt kemasinan, 26.0 ± 1.0 ºC, pH 7-8, 12 jam cahaya: 12 jam fotoperiod dan keamatan cahaya 4.05 μmol/m^2/sec.

Kata kunci: *Pseudodiaptomus annandalei,* mikroheterotropi kultur dari sisa air kelapa sawit, abiotik, kelangsungan, pembiakan, asid lemak

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

&	:	And
>	:	Greater than
<	:	Less than
µmol	:	Micromole
%	:	Percent
±	:	Plus minus
AL	:	*Aurantiochytrium limacinum*
ANOVA	:	Analysis of variance
ARA	:	Arachidonic acid
BOD	:	Biological oxygen demand
ºC	:	Degree celcius
cells/mL	:	Cells per mililitre
CO_2	:	Carbon dioxide
COD	:	Chemical oxygen demand
CT	:	*Candida tropicalis*
dw	:	Dry weight
DHA	:	Docosahexaenoic acid
EFA	:	Essential fatty acid
EPA	:	Eicosapentaenoic acid
et al.	:	And others
FAA	:	Free amino acid
FAO	:	Food and Agriculture Organization
g	:	Grams
h	:	Hours
HCL	:	Hydrochloride acid
HUFA	:	Highly unsaturated fatty acid
i.e	:	In other words

L	:	Litre
LC-PUFA	:	Long chain polyunsaturated fatty acid
m^2/sec	:	Metre squared per second
MUFA	:	Monounsaturated fatty acid
mL	:	Mililitre
mg	:	Miligram
mg/L	:	Miligram per litre
MPOB	:	Malaysian Palm Oil Board
MPOC	:	Malaysian Palm Oil Council
NaOH	:	Sodium hydroxide
NI	:	Nauplius stage I
PDA	:	Potato dextrose agar
PDB	:	Potato dextrose broth
POME	:	Palm oil mill effluent
ppt	:	Parts per thousand
PUFA	:	Polyunsaturated fatty acid
RM	:	*Rhodotorula mucilaginosa*
RS	:	*Rhodovulum sulfidophilum*
SA	:	*Shewanella algae*
SE	:	Standard error
SPSS	:	Statistical Package for Social Science
v/v	:	Volume per volume

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Aquaculture is one of the fastest growing sectors as compared to other major food production sectors globally with high production in finfish farming (FAO, 2018). This sector could potentially provide adequate food access, reduce poverty around the world and enhance food security (Fathi *et al.,* 2018). Nonetheless, one of the major bottleneck faces by this sector is the limited production of high quality live feed that could meet the nutrition needs of marine fish larvae and fingerlings.

Malnutrition is the major factor that leads to the high mortality rates of first-feeding fish larvae (Hamre *et al.* 2013). This is because marine fish larvae are very vulnerable and fragile during the early stage of their development and they have strict requirements in order to survive, develop and grow properly (Conceicao *et al*., 2010; Barroso *et al.,* 2013; Hamre *et al.,* 2013). Feeding of nutritious larval feed during this phase is of great importance for optimal development of fish larvae (Ohs *et al.,* 2010). Marine fish larvae need essential fatty acids (EFA) i.e. docosahexaenoic acid (DHA) and eicosapenteanoic acid (EPA) that are classed under long chain polyunsaturated fatty acids (LC-PUFA) for good growth, survival and development (Conceicao *et al*. 2010; Pinto *et al.,* 2013).

Live feed and formulated diet are commonly used in larviculture. Although formulated diet is preferred choice due to its availability, ease of storage, long shelf-life and easy to use, their low digestibility and inconsistent nutritional quality are not the excellent starter feed for fish larvae even formulated diet likely to meet all the elements required for growth (Carneiro *et al.,* 2003; Das *et al.,* 2012). In contrast, live feeds have high water content and can be easily ingested by fish larvae (Das *et al.,* 2012; Wittenrich *et al.,* 2007; Conceicao *et al.,* 2010). On top of that, their nutritional value can be manipulated to cater to the nutrient requirements of fish larvae, they are constantly available for larvae and their movement can stimulate fish feeding behaviour (Kolkovski, 2001; Carter, 2015).

Live feeds that are widely used in hatcheries are rotifers and *Artemia*. However, the main disadvantage of these two zooplankton is that they are naturally deficient in EFA such as EPA and DHA that are crucial for larval development (Rajkumar & Vasagam, 2006). The said zooplankton usually undergo an additional or extra process of enrichment to meet nutrient requirement prior feeding to fish larvae (Rasdi & Qin, 2014). Some of the enrichment techniques used were oil-based emulsions and microencapsulated preparations (Barclay & Zeller, 1996; Sorgeloos *et al.,* 2001). On the other hand, copepod could be the potential candidate for larviculture due to excellent PUFA contents as compared to *Artemia* and rotifers (Drillet *et al.,* 2011; Qin, 2013).

Copepods particularly calanoid species for instance *Pseudodiaptomus pelagicus* and *Acartia tonsa* play a central role in most marine food webs and are a natural food source for many important marine larval fish species such as grouper (Li *et al.,* 2008; Gopakumar & Santhosi, 2009; Santhanam *et al.,* 2015; Rayner, *et al.,* 2017). Copepods have high protein around 44-52% (FAO, 1996) and free amino acid contents which are essential in improving protein utilization and growth performance of fish larvae. Moreover, amino acids act as impotant major sources of energy for first-feding larvae (Park *et al.,* 2006). Free amino acid content is easy to be digested by fish larvae because they have an undeveloped digestion system that is unable to process the complex protein content or inert diet (Chen *et al.,* 2006).

Copepods are the good sources of vitamin E, vitamin C, antioxidants and astaxanthin (Barroso *et al.,* 2013). Besides that, they are also rich in exogenous digestive enzymes for example proteinases and lipases that aid in the digestion of larval fish (Freese *et al.,* 2012; Zaleha *et al.,* 2012). Hence, copepods can fullfill the criteria needed for the growth and development of early marine fish larvae. Nevertheless, a reliable production protocol for copepods in captivity is still underdeveloped due to our poor understanding on their feed

preferences and environmental conditions to thrive well (Rasdi & Qin, 2014).

Besides that, growth and production of copepods are also depending on the abiotic factors such as salinity, temperature, pH, light intensity and photoperiod. There are numbers of studies relating the effect of abiotic factors on the development of calanoid copepods were reported, some of which were reported by Nagaraj (1992), Milller *et al.* (1994), Pedersen & Hansen (2003) and Peck & Holste (2006). Salinity for example had a marked impact on copepods where it could affect lifespan, survival rate and reproduction (Chen *et al.,* 2006). Besides, temperature is also one of the key factors for copepods where it influences egg hatching success (Castro-Longoria, 2003), production of nauplii (Isla & Perissinotto, 2004) and survival of copepods (Li *et al.,* 2009). Therefore, it is essential to study the effects of environmental conditions on copepod physiology in order to understand their tolerance limits. Hence, an optimized condition can be provided for their growth under captivity.

The genus and species of choice for this research is *Pseudodiaptomus annandalei* because it is a significant natural diet of many tropical fish such as grouper (Gusmão & McKinnon, 2014). Besides that, *P. annandalei* is able to survive in a wide range of salinity, heavy aeration and high level of ammonia (Hwang *et al.,* 2010). It is also an excellent source of arachidonic acid (ARA), EPA and DHA ((Rayner *et al.,* 2015; Lee *et al.,* 2010; Chen *et al.,* 2006).

Most calanoid copepods such as *Pseudodiaptomus hessi* (Siqwepu *et al.,* 2017), *Acartia erythraea* (Rajkumar & Rahman, 2016) and *Nannocalanus minor* (Santhanam *et al.,* 2015) gained HUFA and DHA through microalgae diets. This has been supported by Hernández Molejón & Alvarez-Lajonchère (2003) that the nutritional composition of copepods can be easily manipulated. However, microalgal production is not economically

feasible as high production costs remains a limitation to hatcheries (Kaparapu, 2018). This is because microalgae require specific growth conditions such as light and additional nutrients to grow well (Loo *et al*., 2012).

Marine microheterotrophs such as bacteria, yeast and protists can be an alternative to the conventional diets as copepod feed. Bacteria and yeast fungi have been widely used as mariculture feeds as microheterotrophs are much easier to be grown and cultured. Heterotrophic growth does not need the investment (in terms of labour and expensive equipment) as much as in phototrophic growth (Harel *et al.,* 2002). Besides that, marine bacteria such as *Photobacterium* species could produce PUFA thus provide the vital nutrients needed by copepods for growth. Other than that, protist species *Auranthiochytrium limacinum* is also a primary producer of PUFA in marine environment where they produce a large amount of DHA and long chain PUFA (Monroig *et al.,* 2013).

Palm oil mill effluent (POME) is a viscous and organic brownish colloidal mixture of water, oil and fine suspended solid by product produced from palm oil industry (Yahaya & Seng, 2013). Although POME is a nontoxic by product, discharging untreated POME into environment could have detrimental effect particularly to the aquatic organisms due to its residual oil content that cannot be separated using conventional gravity-based systems (Yahaya & Seng, 2013). Nonetheless, POME is still rich in carbohydrate, protein, lipids, nitrogenous compound and minerals (Bala *et al.,* 2014, Habib *et al.,* 1997).

Rich organic residue in POME makes it favourable to be used as a substrate to grow specific microorganisms (Nurul-Adela *et al*., 2016). It could be potentially used to proliferate marine microheterotrophs such as bacteria (Loo *et al.,* 2012), protists and yeasts that are able to consume the complex molecules in POME as their food source. Indirectly, it could enhance the nutrient content of the microorganisms and copepods that

consume it as a feed. Furthermore, high content of chemical oxygen demand (COD) in POME can be potentially reduced using microheterotrophs (Oswal *et al.,* 2002; Loo *et al.,* 2012). By this way, the wastewater is not only being treated cheaply, but also being transformed into aquaculture products.

Therefore, this research was to investigate the efficacy of POME-grown microheterotroph diets and optimize the environmental factors that could affect the production of *P. annandalei* in captivity.

The objectives of this study were:

1. to evaluate the effects of POME-grown fed microheterotrophs on survival, growth and reproduction of *Pseudodiaptomus annandalei.*
2. to optimize the captive environmental conditions i.e. salinity, temperature, pH, photoperiod and light intensity for *P. annandalei.*
3. to establish a stable production at the end of experiment for captive *P. annandalei* raised on POME-grown microheterotrophs.

CHAPTER 2: LITERATURE REVIEW

2.1 Global aquaculture production

Aquaculture is the farming of aquatic organisms such as fish, crustaceans and molluscs and it is the fastest growing industry compared to other food sectors. Finfish is the most highly traded seafood in international market to meet the demand for protein source. Aquaculture sector could be an alternative solution to provide good-quality seafood products to meet the consumers' demand (FAO, 2018). This led to a robust economic growth where in 2016 the first-sale value was estimated at USD 243.5 billion with 110.2 million tonnes of global aquaculture production (FAO, 2018). Currently, China is the leading country in fish production and exportation, followed by Norway, Vietnam and Thailand. This sector is expected to gradually increase for next few years.

2.2 The importance of live feeds

The critical cycle of growth in most fish species is during the larvae period (Akbary *et al.,* 2007). Altricial larvae has low digestive capacity because the digestive system is still rudimentary and lacking a stomach. In most cases, they are unable to ingest formulated diets as compared to live feeds (Conceicao *et al.,* 2010). They need suitable diets that are readily consumed, can be digested efficiently and provide the required nutrients to support good growth and health (Girri *et al.,* 2002). Live feed is the best choice for larvae because they swim in the water column thus constantly available for fish larvae (Conceicao *et al.,* 2010). This is different with formulated diets that are commonly less available to the larvae as they tend to aggregate on the water surface or sink quickly to the bottom.

Besides that, for predatory type of fish larvae, the swimming pattern of live feed in water column stimulates the feeding responses of fish larvae that naturally attack the moving prey (Conceicao *et al.,* 2010). Furthermore, live feeds are highly digestible due to high water content and thin exoskeleton which are more palatable to larvae

(Conceicao *et al.,* 2010). One of the significant characteristics is also due to the various sizes of live feeds making them suitable for many kinds of fish larvae with different mouth gapes (Conceicao *et al.,* 2010). Live feed could also provide important nutrients such as lipids particularly the essential fatty acids (EFA) which are crucial for early development and the main source of energy for larvae (Bell & Sargent, 2003; Lall & Lewis-McCrea, 2007). EFA include DHA, EPA and ARA. Marine fish larvae are incapable to produce EFA from their precursors i.e. oleic acid, linolenic acid and linoleic acid. Thus, they need to obtain EFA through diet (Bell *et al.,* 2002). Deficiency in essential nutrients could disrupt the normal development of fish as it affects the normal morphogenesis and skeletogenesis at early stages (Cahu & Zambonino Infante, 2001; Lall & Lewis-McCrea, 2007; Boglino *et al.*, 2012).

2.2.1 Conventional live feeds

The commonly used conventional live feeds for larval fish are *Artemia* spp. or also known as brine shrimp*, Moina* and rotifers. Rotifers or *Brachionus* species are widely used as the first food for larvae of many marine fish species after the resorption of vitelline reserves (Conceicao *et al.,* 2010). This is because they can be cultured in a large quantity and high density (Rajkumar & Rahman, 2016). They have short proliferation time and easy to be cultured. However, the drawback of using rotifers is their fatty acid profile which is inadequate to promote optimal larval growth (Olivotto *et al.,* 2003).

In rearing fish and crustacean larvae, *Artemia* is still the most preferred live feed (Dhont *et al*., 2013) due to its good tolerance to various culture conditions and ease of handling. Besides that, they are commercially available in the market (Conceicao *et al.*, 2010). Nevertheless, the disadvantage of *Artemia* is their PUFA deficiency as PUFA are vital to the optimal nervous system functions and essential to the maintenance of larvae functional efficiency (Harrocks & Yeo, 1999). They are also not cost-effective live prey

in aquaculture because of high cost (due to low and unreliable natural resources) and nutritional value that lack in essential n-3 highly unsaturated fatty acids (Conceicao *et al.*, 2010).

2.2.2 Alternative live feeds: Copepods for larva fish feeding

In nature, free-living copepods are the most common diet for various types of fish larvae. There are ten orders of copepods, but the general three orders used in aquaculture are Calanoida, Cyclopoida and Harpacticoida (Stottrup, 2003). Each order possesses their own advantages and disadvantages. Calanoid copepod species is the most well-known species studied because they are the main natural food for most of fish larval species. They are broadcast spawners where the eggs are freely spawned into the environment and this is practical for intensive cultures because it is easier to harvest the eggs for storage purpose. However, it is different with harpaticoids and cyclopoids that bear their egg in egg sacs until nauplii hatching (Stottrup, 2003).

The disadvantage of calanoid copepods is they cannot be kept in large densities that will result in high mortality. Harpaticoid copepod species are easier to culture, have high fecundity and short life cycles. Compared to calanoid species, their cultures can be maintained at very high densities but their nauplii are particularly difficult to be separated from debris because they are commonly benthic gazers. Similarly, cyclopoids which are quite easy to culture with short development times, can be maintained in high density but their major disadvantage is the difficulty to harvest eggs compared to calanoid species (Stottrup, 2003).

Besides that, various size of copepods in each developmental stage from first nauplii to adult is a major advantage in aquaculture (Stottrup, 2006; Santhanam *et al.,* 2012). Small sizes of early nauplii around 80 μm are ideal prey sizes for first feeding of cultured

larvae with small mouth gapes. This is supported by previous study done by Knuckey *et al.* (2005) reported that the grouper preferred newly hatched copepod nauplii as their feed over rotifers when fed with mixture of copepods and rotifers. The motion pattern of copepod nauplii stimulates fish feeding behavior (Barroso *et al.,* 2013). The jerking swimming motion of copepods acts as visual stimulus for fish larvae. This indirectly would result in an improved ingestion rate of fish larvae (Stottrup, 2000).

2.2.3 Nutritional profiles of copepods

Copepods are an ideal food for fish larvae due to its high nutrient contents (Anandan *et al.,* 2013; Fereidouni *et al.,* 2013). The biochemical profile of this crustacean shows that it is rich in essential amino acids, proteins, essential fatty acids and lipids (Aman & Altaff, 2004; van der Meeran *et al*., 2008). For instance, copepods contained high amount of EPA (8.3-24.6%), DHA (13.9-42.3%) and ARA (0-2.6%) compared to other live feeds (van der Meeran *et al*., 2008; Rasdi & Qin, 2014). DHA has significant influenced on larval stress resistance, growth promoter in fish and in immune system development. Furthermore, copepod nauplii and copepodid have high ratio DHA/EPA ratios of > 1 that are highly crucial to promote the development of fish larvae and reduce the occurrence of morphological abnormalities (Satoh *et al.,* 2009).

In general, the average ash content (% to dw) of copepods was between 9.5 and 10.5% (van der Meeran *et al*., 2008). Aditionally, van der Meeran *et al*. (2008) also reported that copepods contained moderate levels of lipids [6.9-22.5% of dry weight (dw)], with polar lipids (37.9-70.2% of the total lipids). Besides PUFA, copepods also rich in palmitic acid containing 10.8-17.1% of total lipids. Copepods generally have high amount of protein content ranging from 44% to 52% and a good amino acid profile, with exclusion of histidine and methionine (FAO, 1996). The total content of essential amino acids recorded in copepods was approximately 37.3-43.2% (van der Meeran *et al*., 2008).

Furthermore, high portion of polar lipid content in copepods is also an advantage because polar lipids are more biologically available to fish larvae compared to triacylglycerols (Schipp, 2006). Besides that, copepods contained high levels of exogenous digestive enzymes such as amylase and protease that help in improving digestion of fish larvae as larval fish have incomplete functional gut (Stottrup, 2000; Stottrup, 2006; Conceicao *et al.,* 2010; Zaleha *et al.,* 2012). Copepods are also rich in free amino acids (FAA) that act as major source of energy for first-feeding larvae (Park *et al.,* 2006; van der Meeran *et al*., 2008). Apart of that, copepods are also good sources of vitamin C, E and astaxanthin that are significant retinoid source for larvae (Barroso *et al.,* 2013).

2.2.4 Calanoid copepod *P. annandalei*

Calanoid copepods play a crucial role in environment because they cycle nutrients and energy by forming a trophodynamic link between primary (phytoplankton) and tertiary production (DeYoung *et al.,* 2004). They are predominantly pelagic with 75% living in marine waters and the remaining 25% can be found in fresh water habitat. Many of them are selective feeders that feed on small phytoplankton by filtration and herbivorous. The common way to distinguish calanoid with other copepod orders is through their long antennules that could be as long as their body or even longer (Støttrup, 2003) (Reference to Figure 1.1).

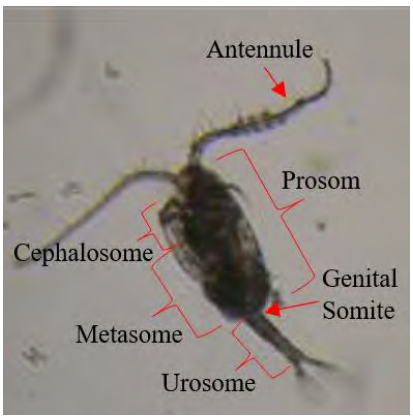

Figure 1.1 External morphology of a male calanoid

Calanoid copepods have cylindrical bodies with narrow abdomens. The region consist of thorax (metasome) and an abdomen (urosome) is known as trunk. The head (cephalosome) fused with thorax are known as the prosome region that also consists of anteriorly median naupliar eye, a set of antennae and various appendages used for feeding, swimming and mating. The abdomen region has no appendages except for the caudal rami and it is also narrower than the thorax. The first abdominal segment consists of genital opening (genital somite) that located dorsally which differs them between male and female while the anal opening (anal somite) is located at the last segment of the abdomen. The genital system consists of paired glands, ducts and genital aperture that is placed ventrally (Bengston, 2003).

The life cycle of calanoid comprises of nauplii, copepodid and adult. After the eggs released into water, the larva that hatched from the copepod eggs is called nauplius (NI) where the size is varied between species. The life cycle is divided into six naupliar stages (NI, NII, NIII, NIV, NV, NVI) and five copepodid stages (CI, CII, CIII, CIV, CV) where each stage separated by a molt process and lastly an adult stage (CVI) (Golez *et al.,* 2004). Generally, the size of the nauplii is between 80 and 250 µm; copepodid (80-350 µm) and adult (250-600 µm) (Støttrup, 2003). Normally adult females are larger than males and once adult females reach maturity, the somatic growth ceases and egg production is thus

often used as an expression of growth.

The sexual dimorphic characters are usually developed during the later stage of copepodid where the male and female copepods could be distinguished. Normally the body size of males and females is different where males are smaller than females. They reproduce sexually where a sac containing viable sperm (spermatophore) will be deposited by the male near the genital aperture of female. Most of them are broadcasters, shedding eggs individually in the water column (Dhont *et al.,* 2013). The resting eggs are primary mode of dormant state in calanoids where the spherical eggs protected by chitinised envelope can remain viable for several years and can withstand unfavourable conditions such as heat, cold or desiccation for long periods (Bengston, 2003).

P. annandalei is a commonly study species where it is an euryhaline species distributed widely in subtropical and tropical Indo-Pacific regions all over the year (Hwang *et al.,* 2010; Dhanker *et al.,* 2013). This species can be found in mangroves (Chew *et al.,* 2015), coastal waters (Beyrend-Dur *et al.,* 2011), estuarine (Beyrend-Dur *et al.,* 2013) and brackish waters (Rayner *et al.,* 2015). There were also other several species that have been studied for mass culture purposes which are commonly from the following genera such as *Acartia*, *Parvocalanus* and *Gladioferens* (O'Bryen & Lee, 2005; Støttrup, 2003). Overall, calanoid copepods appear to be well suited for culture systems due to the following characteristics: 1) pelagic life cycle, 2) natural prey for many fish larvae, 3) capacity of storing eggs, 4) most of the species freely spawn the eggs which can then be easily harvested (Støttrup, 2006) 5) diversity of copepod size range for fish larvae based on their mouth sizes (Santhanam *et al.,* 2015).

2.3 Influence of abiotic factors on copepods

2.3.1 Salinity

Each calanoid species could adapt to different range of salinity conditions for survival and reproduction due to habitat variations (Castro-Longoria, 2003). As for *P. annandalei* the appropriate salinity ranges are between 5 to 20 ppt (Chen *et al*., 2006). The optimum salinity for fecundity and the naupliar survival rate is at 15 ppt that achieved the highest total nauplii reproduction (Chen *et al*., 2006). Low salinity could slow down the development time of calanoid copepod to reach maturity compared to higher salinity (Shayegan *et al.,* 2016; Karlsson *et al*., 2018).

The clutch size of *P. annandalei* has been affected by salinity where Beyrend-Dur *et al.* (2011) recorded that salinity of 5 gave the lowest clutch size whereas salinity of 15 have the highest clutch size. Besides that, higher salinity could result in poor hatching success of *P. annandalei* eggs (Chen *et al.,* 2006). The eggs could not hatch when the salinity exceeded the range of 0-30 ppt. Most of calanoid copepods that inhabit estuarine waters could tolerate broad range of salinities conditions (Millione and Zeng, 2008).

2.3.2 Temperature

Temperature could affect the maturation time of copepod, developmental stages, mortality fecundity and percentage of ovigerous female (Li *et al.,* 2009; Anzueto-SÃnchez *et al.,* 2014). The normal temperature range of *P. annandalei* in their habitat was between 26ºC to 32ºC (Lehette *et al.,* 2016). This is coincided with the finding by Rhyne *et al*. (2009) who observed the temperature between 24-30ºC had the highest mean survival of *P. pelagicus* copepods nauplii turn to adult stage.

Temperature that exceed the normal range will have negative effect on the *P. annandalei.* Recent study of Doan *et al.* (2019) found that high temperature of 34ºC that

exceed *P. annandalei* upper thermal optimum negatively affects the population in terms of reduced size at maturity, low hatching success and decrease of naupliar production. The clutch size was also reduced at temperature of 34ºC compared to 30ºC which is the main temperature of the coastal regions water in Southest Asian (Doan *et al.,* 2019).

2.3.3 pH

Generally, copepods that live in neutral conditions can tolerate to the pH ranges between 6.5 to 8.0 for optimum survival and reproduction (Suárez-Morales, 2015). Nevertheless, low pH value could be lethal to the marine zooplankton due to pH stress (Yamada & Ikeda, 1999). The study done by Yamada & Ikeda (1999) on ten species of oceonic zooplankton found that they are significantly sensitive to acidic pH (pH 4.7 to pH 5.8) as the mortality increased with increased exposure time but the tolerance varied between each species. The effect of acidification and high CO_2 varied between species where some can tolerate low seawater pH. For example, low seawater pH does not affect the offspring production of *Eurytemora affins* (Almén *et al.* 2016). The reason for adaptation in changes of pH is because they are naturally exposed to large pH fluctuations in the environment.

Acidic pH levels (pH 6) could adversely affect nauplii survival and recruitment in some species such as *A. tonsa* where the nauplii could not survive in acidic water column (Hansen *et al.,* 2017). The response of copepod against ocean acidification is also developmental stage-dependent (Wang *et al.,* 2018). The earlier developmental stages of copepods are the most sensitive to this stress. Besides that, the fatty acid composition of DHA, ARA and EPA were also affected and reduced in acidified pH (Jayalakshmi *et al.,* 2016). These fatty acid composition are crucial for copepods as they are the key component of food web for fish larvae that enhances their somatic growth. Calcerous marine organisms and crustaceans that are exposed to acidified pH for a long term could

have profound impact through reduction in concentration of calcium carbonate that is a main composition of their skeleton (Gazeau *et al.,* 2007 and Vehmaa *et al.,* 2016).

There is no specific study has been done on the effect of pH on *Pseudodiaptomus annandalei* and not much is known about their tolerance to pH. But it has been reported that estuarine copepods are more tolerant to elevated pH (Hansen *et al*., 2017). Generally, acidic pH has more negatie effect to the calanoid copepods where it will reducing the brood size and reduce egg production rates (Aguilera *et al*., 2013).

2.3.4 Photoperiod

Photoperiod also can affect the productivity and growth of copepods (Peck & Holste, 2006; Farhadian *et al*., 2014). The most favourable photoperiod range for calanoid copepods is at least 12 hours of light (Støttrup, 2003). Egg production, hatching success and naupliar development are significantly influenced by photoperiod. As reported by Peck & Holst (2006), longer illumination period could increase the egg production and hatching success percentage of calanoid copepod *Acartia sinjiensis.* The long hour of light period gave the positive effect to hatching success of copepod and nauplii development (Peck, *et al.,* 2008).

Similar finding was made by Camus & Zeng (2008), increasing in illumination period of photoperiod would accelerate the development of naupliar and copepodite. The mean development time from egg to adult was shortest at constant light 24L: 0D followed by 18L: 6D respectively. In terms of survival, photoperiod could affect adult life expectancy with shortest life span recorded under constant light (24 h L: 0 h D) compared to another treatments (Camus & Zeng, 2008). Similar founding was recorded by Nogueira *et al.* (2017) where lowest population number were found at constant light conditions. Therefore, manipulating the culture condition could help to maximize its productivity for

aquaculture hatcheries.

2.3.5 Light intensity

Studies on the light level are scarce and rarely reported in literature. Low level of light intensity was applied in general and there is no exact intensity of light is provided and recorded (Støttrup, 2003). Davis (1983) had reported that the intensity used on rearing calanoid copepod *Pseudocalanus* sp. was only at the level of 18 uE m^2/sec and the light intensity used in the system by Stottrup *et al* (1986) was 25 uE m^2 /sec. Person-Le Ruyet (1975) found that density fluctuations of some calanoid species did not depend on light regimes. As for cyclopod species *A. dengizicus*, their production was significantly highest under low light intensity and highest survival rate was achieved under continuous light (Farhadian *et al.,* 2014). This can be summarised that the effect of light intensity varied between species and population.

2.4 Microalgae and heterotrophic microorganisms as copepod diet

Microalgae is the conventional feed used as a diet for copepod in aquaculture. There are various types of microalgae used as feed such as *Isochrysis galbana, Nannochloropsis oculata, Chlorella marina, Chaetoceros affinis* and *Chaetoceros muelleri.* The nutritional content between the species are varied depending on their biochemical composition, digestibility and size of cells (Das *et al.,* 2012). Basically, the key nutrients they provide are fatty acids, protein, pigments, energy and vitamins. The effects of different dietary microalgae have on culture parameters have been evaluated with numbers of copepods including *Acartia sinjiensis* (Millione & Zeng, 2009), *Acartia erythraea* (Rajkumar & Rahman, 2016), *Pseudodiaptomus pelagicus* (Ohs *et al.,* 2010) and *Nannocalanus minor* (Santhanam *et al.,* 2015).

Dietary microalgae can support the survival and production of copepods as shown by *I. galbana.* It increases the population density of *Paracalanus parvus* and their egg production (Jeyaraj & Santhanam, 2012). The drawback of dietary microalgae usage is on their production cost where it is quite expensive due to space, skilled labour requirement, and energy (FAO, 1996). The culture needs specific growth condition such as constant illumination for growth (Loo *et al*., 2012).

Heterotrophic microorganisms such as marine yeasts and bacteria may be an alternative feed to replace the conventional dietary microalgae as copepod feed. These organisms are rich in essential nutrients and may provide the requirement for growth of copepods. Marine yeasts such as the red coloured colony *R. mucilaginosa* is a good source of long chain poly-unsaturated fatty acid that can meet the nutrient requirement of copepods (Perrier *et al.,* 1995). Other than that, the marine heterotrophic protist, *Aurantiochytrium limacinum* is a source of diet rich with DHA (Abad & Turon, 2015). Copepods need to obtain that essential fatty acid from their diet because they could not produce it on their own (Bell *et al.,* 2007).

Furthermore, these versatile microorganisms are easier to maintain, and their growth requirement is not as fastidious as microalgae production. They can be grown in POME and utilize the organic contents of the waste that are high in lipids, protein, carbohydrates, minerals and nitrogenous compounds (Phang, 1990). They are nontoxic wastewater with light brown colloidal suspension but are pollutents to the environment if discharged without treatment. This is due to high biological oxygen demand (BOD) content, organic and inorganic solids content. The application of POME as a medium for growing heterotrophic microorganism could be one of the ways to help in reducing the environmental impact of the waste (Salihu & Alam, 2012). The multiplication of microorganisms was contributed by high organic matter in POME because they could

obtain the nutrients from conversion process of the complex molecules into simpler substances.

2.5 Malaysia's palm oil industry

Malaysia is the second largest of world crude palm oil (CPO) producers and exporters after Indonesia that accounts for 12% of the world's oil and fat production and 27% of world export (MPOC, 2013). The production of CPO in 2016 was 17.3 million tonnes (MPOB, 2016). It is one of the largest and important industries in Malaysia where this commodity significantly contributes the most to national revenue.

POME is a nontoxic wastewater generated from the palm oil extraction process which required a large amount of water around 5-7.5 tonnes for each tonne of CPO (Wang *et al.,* 2015; Idris *et al.,* 2018). Previous study by Yacob *et al.* (2006) reported that POME is the major waste produced from the extraction process where 372 mills in Malaysia were generating more than 40 million tonnes of POME in year of 2004. The characteristics of raw POME is unfavourable to the environment. This thick brownish liquid with unpleasant odour is acidic (pH between 4 to 5), contained mostly 95-96% of water, 0.6-0.7% of oil and 4–5% of suspended solid (Ahmad *et al.,* 2009; Ma, 2000; Ahmad *et al.,* 2003).

Dissolved constituents of POME also contain high levels of carbohydrate, nitrogenous compounds, lipids and mineral with vital nutrients contents which are nitrogen, phosphorus, potassium, magnesium and calcium (Rupani *et al.,* 2010; Muhrizal *et al.,* 2006). Besides that, POME possess excessive BOD and chemical oxygen demand (COD) that could result in serious environmental pollution if discharged on lands or into the rivers that may deteriote the surrounding (Bala *et al.,* 2015; Rupani *et al.,* 2010). Thus, effective treatment of POME is essential before being discharged in order to avoid any

complications and help to conserve the environment.

In order to overcome this problem, there were numbers of studies have been done to convert POME into various useful materials such as cheap organic fertilizers (Wu *et al.,* 2009; Guo *et al.,* 2007), fermentation media to produce various products or metabolites ranging from antibiotics (Lin *et al.,* 2005), organic acids (Din *et al.,* 2012), enzymes (Masngut *et al.,* 2007) and cellulase (Hii *et al.,* 2012); and as live foods for animals (Wu *et al.* 2009). Considering POME that is rich in carbon supply and nutrient content, Idris *et al.* (2018) has used POME as an alga (*Chlorella* sp.) cultivation medium for biodiesel production. The cultivation of *Chlorella* sp. in POME shows good growth where high content of lipid and biomass were produced. It has good potential as a non-food type biodiesel feedstock.

Other than that, POME can be used as a substrate for growing microheterotrophic organisms as feed for aquaculture organism. The POME was cultured with phototrophic bacteria *Rhodovulum sulfidophilum* which then were successfully fed to rotifers (Loo *et al.,* 2015). Those microheterotrophic organisms can grow directly in high-oganic load wastewater. They can exploit the nutrients and organic materials in POME for its growth and at the same time reducing COD and BOD of wastewater. Hence, it is a low-cost alternative to culture microbes and POME can also be transformed into useful products thus potentially reduced environmental problems.

2.6 Conventional and unconventional feed for copepod culture

Feed is a crucial key factor that contributes the essential nutrients needed for growth and reproduction of copepod. It is essential to identify which food is suitable for continuous copepod production because optimising the copepod diet for batch culture production could improve the growth performance of copepod, nutrient content in their

body, fecundity, egg production in female copepod and success of egg hatching rate (Payne & Rippingale, 2000; Rajkumar & Rahman, 2016; Santhanam & Perumal, 2012 and Santhanam *et al.,* 2015).

2.6.1 Microalgae as a conventional diet for copepod culture

Microalgae can be defined as unicellular photosynthetic micro-organisms that can be found in freshwaters or saline environments which convert sunlight, water and carbon dioxide to alga biomass (Ruane, *et al.,* 2010). They are the basic and nutritious diet used for zooplankton culture specifically copepods in the aquaculture hatcheries (Kassim, *et al.,* 2014). Algae diets have been reported to have significant effects on copepod growth and development (Knuckey, *et al.,* 2005), egg production (Camus, *et al.,* 2009) and success of egg hatching (Milione & Zeng, 2007).

Some of the main commercial genera grown are *Isochrysis, Tetraselmis, Nannochloropsis* and *Chlorella.* Each species of microalgae has different level of PUFAs where most of them show moderate to high percents of EPA, while some species also has poor nutritional value which make them not suitable for use as a single-species diet (Guedes & Malcata, 2012). The use of mixed diets was suggested in order to meet the nutritional requirement of the target copepods. This has been proved by Milione & Zheng (2007) that found combination of two microalgae diets given to *Acartia sinjiensis* was significantly better than with monospecific diets for their development rate.

Copepods development, survival and reproduction can be significantly improved when appropriate microalgae diets were provided at each stage of the life cycles. This was confirmed by Xiaoxa *et al.* (2019), who found that calanoid copepods *P. dubia* has different nutritional needs and food preferences at distinct life stages. This has been observed that suitable feed for developing larvae was *Platymonas subcordiformis* while

Cyclotella meneghiniana was a better feed for breeding adult. The best feed for *P. dubia* throughout its whole life cycle was both *Chaetoceros muelleri* and *I. zhanjiangensis.*

However due to photoautotroph's characteristic, major requirements of microalgae growth are light, water and inorganic nutrients (Xu *et al.,* 2009). This makes their mass production costly due to huge manpower, space requirements and operations (Kassim *et al.,* 2014). A cost effective and nutritionally adequate alternative should be considered.

2.7 Unconventional diets for copepod culture

2.7.1 Marine yeasts

Marine yeasts are the yeasts isolated from the marine environment such as seawater, plants of marine habitat and sediments (Chi *et al.,* 2010; Kutty & Philip, 2008). They can grow well in the media prepared from seawater compared to the fresh water media. It was reported that marine yeasts have unique properties for instance higher special enzyme productivity, unique industrial enzyme production and high osmotic tolerance (Zaky *et al.,* 2014). Besides that, marine yeasts are also rich with bioactive substances such as enzymes, amino acids, glucan, vitamins and glutathione. The potential applications of marine yeast include marine culture, industries of food, pharmaceutical and chemical (Sarkar *et al.,* 2010).

The general genera of marine yeasts are *Candida, Saccharomyces, Rhodotorula* and *Guehomyces* (Chen *et al.,* 2009). Sarlin & Philip (2016) stated that yeasts have been used as feed for aquaculture organisms such as marine shrimps because of high nutrients content where it contains protein (29-63%), carbohydrates (21-39%) and lipids (1-23%). *Candida, Saccharomyces* and *Torula* sp are known for high protein content up until 60%. The efficiency of yeast-based diets in aquaculture organisms were tested in many previous studies. A study has been done by Payne & Rippingale (2000) on baker's yeast

(*Saccharomyces cerevisae*) as the diet for *Gladioferens imparipes* reported that the yeast was less efficacious diet to copepod. In another recent study, it was reported that the population density of harpaticoid copepods *Tisbe furcata* increase and reached greater mass when fed with a diet supplemented with yeast and corn flour compared to other feed treatments (Wang *et al.,* 2017). Therefore, the extensive research of marine yeast as feed for aquaculture organism such as copepods has gained attention of researchers.

2.7.2 Marine bacteria

Marine bacteria are unicellular prokaryotic organism with size generally less than 0.5-1.0 μm (Ducklow, 2001). Some of the marine bacteria species do have metabolic capacity to produce high levels of PUFAs and most of them are mostly from Gram-negative bacteria. To date, it was reported that there are two bacteria phyla producing PUFAs which are the Gammaproteobacteria (such as *Shewanella, Photobacterium* and *Colwellia*) and the *Bacteroidetes* (such as *Psychroserpens* and *Flexibacter*) (Nichols & McMeekin, 2002). Due to this advantage, there are growing of interest among researchers to utilize bacteria as one of food sources especially for zooplankton groups used as live feed in aquaculture hatcheries. Besides that, they are also easy to culture especially heterotrophic bacteria that can utilize carbon as their nutrients.

There was previous feeding experiment from 1978 done by Rieper on harpaticoids fed with bacteria as food. It was reported that the development of harpaticoids were positive and had no difference with the development of copepods fed on fish food diet. Other study on harpaticoids feeding on bacteria also has been done by De Troch *et al.* (2012) where *Microarthridion littorale* were fed with ^{13}C labelled bacteria and fatty acid bioconversion have been observed. Bacteria has potential to be a provider of essential nutrients for the copepods and substituting their conventional feed.

2.7.3 Marine protist

Protists are marine single-celled eukaryotes that provide essential links in the marine food web (Fuhrman & Caron, 2016). They are a significant component in the diet of copepod that are rich in nutrient content which can support higher growth in copepod (Levinsen *et al.,* 2000 & Broglio *et al.,* 2003). A group of marine protists that have continuously gained attention is thraustochyrids because of their biotechnological potential (Aasen *et al.,* 2016).

Thraustochyrids are unicellular heterotrophic protists that have high lipid contents and are abundantly distributed in the marine ecosystem (Caamaño *et al.,* 2017). Thraustochyrids have gained attention of researchers due to their simpler PUFA profiles and cost-effective culture conditions (Singh *et al.,* 2014). They can synthesize lipids with a high percentage of PUFAs such as DHA and EPA and some bioactive compounds (Gupta *et al.,* 2012). Their ability to produce high levels of DHA is significant to aquaculture industry where PUFAs are essential dietary component for marine finfish and crustacean larval.

One of the marine protists that act as an excellent DHA producer is *Aurantiochytrium* strain (Manikan *et al.,* 2015). The most well known highly oleaginous and excellent DHA producer strain is *Aurantiochytrium limacinum* strain or previously known as *Schizochytrium limacinum* (Leong *et al.,* 2019). This is supported by a study done by Visudtiphole *et al.* (2018) on shrimp *Penaeus vannamei* post-larval (PL). It has been reported that HUFA supplementation via enrichment of *Artemia* with *Aurantiochytrium limacinum* has positive effects on the PL shrimps in terms of growth performance, increasing hypo-salinity tolerance and swimming strength. Application of DHA-rich thraustochyrids can potentially be an alternative to improve the diets of copepod cultures.

CHAPTER 3: MATERIALS AND METHODS

3.1 Microorganism cultures

The stock cultures of marine heterotrophic microorganisms i.e. (1) bacteria: *Shewanella algae* (SA) and *Rhodovulum sulfidophilum* (RS), (2) yeasts: *Candida tropicalis* (CT) and *Rhodotorula mucilaginosa* (RM) and (3) a protist: *Aurantiochytrium limacinum* (AL) were obtained from the Feed-to-Fish Laboratory, University of Malaya. These microorganisms were isolated from local coastal waters.

They were either maintained on potato dextrose agar (PDA) or 112 synthetic agar plates (Table 3.1) and these cultures were stored in a chiller at temperature of 4±2˚C. Prior to scaling up, the microbial cultures were streaked onto fresh agar plates and incubated under temperature of 30±2°C for 24-48h.

Table 3.1: Preparation of synthetic media for culturing specific marine microbes

Microorganism	Type of Synthetic Medium	Amount of Sodium Chloride (g/L)	pH	Incubation Periods (hours)
Shewanella algae (SA)	112 medium	30	7.0	24
Rhodovulum sulfidophilum (RS)	112 medium	30	7.0	24
Rhodotorula mucilaginosa (RM)	Potato dextrose broth	15	7.0	24-48
Aurantiochytrium limacinum (AL)	Potato dextrose broth + 3% Glucose	15	7.0	24-48
Candida tropicalis (CT)	Potato dextrose broth	15	7.0	24

After inoculation in a sterile condition, the bottles containing 10% inoculum (v/v) were incubated in the culture room at 30±2˚C with light intensity of 32.5 μmol/m^2/sec.

3.2 Synthetic media and POME preparation

3.2.1 Preparation of synthetic media

The culture media used to culture microheterotrophs are shown in Tables 3.2-3.4. The pH medium was adjusted to 7.0 by adding hydrochloric acid (HCL) prior to autoclaving the culture medium at 121˚C for 15 minutes.

Table 3.2: Composition of 112 medium

Composition	Amount	
Dipotassium hydrogen phosphate	1.0 g	0.25 g
Magnesium sulphate	0.5 g	0.125 g
Yeast Extract	10.0 g	2.5 g
Sodium chloride	30.0 g	7.5 g
[1]Bacto agar	20.0 g	5.0 g
pH	7.0–7.2	
Distilled water	1 L	250 mL

[1]For 112 medium agar preparation, bacto agar was added

Table 3.3: Composition of potato dextrose broth (PDB)

Composition	Amount
Potato Dextrose Broth	24.0 g
Sodium chloride	15.0–30.0 g[2]
pH	7.0
Distilled water	1L

[2]Amount of salt used depends on the microorganism

Table 3.4: Composition of potato dextrose agar (PDA)

Composition	Amount
Potato Dextrose Agar	39.0 g 9.8
Sodium chloride	15.0–30.0 g[3]
Agar	30.0 g 7.5
pH	7.0–7.2
Distilled water	1L
Glucose (only for protist culture)	3.0 g

[3]Amount of salt used depends on the microorganism

3.2.2. POME preparation

Raw POME was collected from the sludge separator at West mill of Sime Darby Plantation, Pulau Carey, Selangor, Malaysia. The raw POME was kept in 25 litre jerry cans for two days to allow the settlement of solid particles then filtrated by using a 40 µm filter net. Subsequently, the liquid was kept in plastic containers and stored in a 20˚C freezer.

For the preparation of culture medium, diluted POME medium was prepared by mixing 25% of filtered POME (filtered with a 40 µm scoop net) with 75% of distilled water (v/v). Ratio of 25% filtered POME was used because it is the optimized concentration for culture medium, developed by Loo *et al.* (2012). The salinity of POME medium was adjusted according to the specific requirement of each microorganism (Table 3.1). Then, 900 ml of POME medium was then filled up into 1 litre Schott bottle and autoclaved at 121˚C for 15 minutes.

3.3 Mass culture of microorganisms for copepod feeding trials

3.3.1 Bacterial culture

The agar plate of *Shewanella algae* from the chiller was taken out and left in the room temperature. Under sterile condition of the laminar flow, cut the solid agar in the plate into small pieces by using a sterile knife. Then, the cutted small pieces of agar from the plate was transferred into 1 litre of synthetic media (v/v) by using a sterile tweezer. The bottles of inoculum were then incubated in the culture room for 24 h at 30±2˚C with light intensity of 32.5 µmol/m^2/sec.

After 24 h, 10%- or 100-ml inoculum containing microorganism that has been cultured in synthetic medium previously was inoculated into 900 ml of 25% autoclaved POME medium (v/v) (Subsection 3.2.2) in the sterile condition. The bottles were than incubated

in the culture room for a specific duration at 30±2˚C with light intensity of 32.5 µmol/m^2/sec before used for copepod feeding trials.

3.3.2 Yeast culture

Each agar cultures of *Candida tropicalis*, *Rhodotorula mucilaginosa* and *Auranthiochytrium limacinum* were transferred separately into each 1 litre of sterile potato dextrose broth (PDB) in a sterile condition. The media containing yeasts were incubated in culture room at 30±2˚C with light intensity of 32.5 µmol/m^2/sec for 48 hours except CT which required only 24 h cultivation.

After 48 hours, 10% of inoculum containing microorganism in PDB synthetic medium was inoculated into 900 ml of 25% autoclaved POME medium (v/v) (Subsection 3.2.2) in the sterile condition. The bottles were than incubated in the culture room for a specific duration at 30±2˚C with light intensity of 32.5 µmol/m^2/sec before used for copepod feeding trials.

3.3.3 Microalgae

The instant microalgae *Nannochloropsis oculata* (Nanno 3600) paste was procured from the Reed Mariculture, United States. The paste was diluted with 15 ppt saline solution to get a final density of $2x10^5$ cells/ml from the original concentration of $6.8x10^{13}$ cells/ml (stated on the pack). Saline solution was prepared by mixing synthetic sea salt (Instant Ocean Sea Salt) with freshwater until the desired salinity was obtained and then, it was sterilized prior to use.

3.4 *Pseudodiaptomus annandalei* culture and rearing conditions

The stock culture of copepod nauplii *Pseudodiaptomus annandalei* was obtained from the Marine Culture Unit (hatchery) in Rimba Ilmu, University of Malaya. The copepods

were isolated from the local coastal waters. The copepod culture was adapted to laboratory conditions at room temperature of 28˚C prior to starting the copepod feeding trials. They were fed with *Nannochloropsis oculata* and POME-grown diets consisting bacteria and yeast. The copepods *Pseudodiaptomus annandalei* were cultured in 15 ppt salinity water which they were isolated from.

3.5 Experimental design

For all feeding experiments, fifty copepods (naupliar I stage) per replicate were stocked in a 300 ml beaker containing 250 ml of sterile 15 ppt salinity water (except for the salinity experiment) for a single replicate and each treatment was carried out in three replicates. This stocking density was chosen in order to avoid overcrowded effect. Assignment to treatments were completely randomized in Memmert WB14 electric water bath during the culture period (Figure 3.1). The schematic representation of completely randomized design (CRD) were shown in Figure 3.2.

Figure 3.1: Beakers were labelled and placed in completely randomized design (CRD)

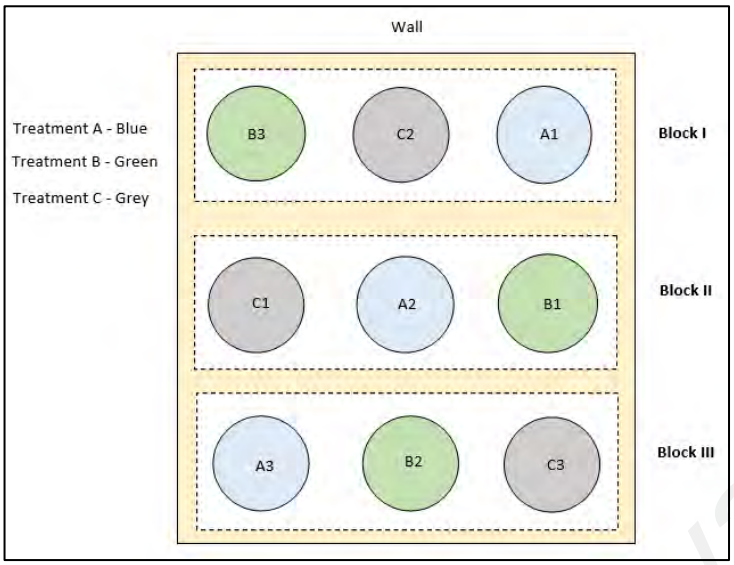

Figure 3.2: Schematic representation of CRD treatments in water bath.

The survival (%) and reproduction of *P. annandalei* were observed every 2-day intervals for a duration of 14 days. Three subsamples of 10ml from each beaker were counted to determine the number of surviving copepod and their developmental stages (nauplii, copepodid, adult male, adult female and gravid female) (Golez *et al*., 2004) under a stereomicroscope (Leica EZ4). The samples were then returned into the culture beakers. At every two days intervals, 50% of culture water was replaced with new sterile brackish water of 15 ppt (unless stated otherwise) to maintain water quality of the culture treatments. Total count of copepod population was performed on day 8 (all nauplii reached the adult stages) and day 14 (final day of experimental period). The sex ratio of male over female also was counted on day 8 in every experiment.

The environmental parameters i.e. pH, salinity (ppt), temperature (˚C) and concentration of dissolved oxygen (mg/l) were taken daily at 10 am using a multi-parameter (Eutech Instruments PCD650) and a portable dissolved oxygen meter (YSI 550A) was used to measure oxygen concentration. The culture conditions for feeding trials were maintained based on normal condition in natural environment of *P. annandalei* reported by previous literature or on other related calanoid species. The culture conditions

were setted at 15 ppt salinity (Chen *et al.,* 2006), pH 7-8, 28˚C (Rhyne *et al.,* 2009), light intensity of 4.05 µmol/m^2/sec and photoperiod of 12 h light and 12 h dark (Støttrup, 2003) that mimics the natural environment. Each beaker were supplied with medium aeration from the tube that was connected with portable aerator. Light intensity source was from white fluorescent bulbs which were placed directly above the water bath until required intensity level achieved. The intensity of light were measured by using a portable lux meter. These conditions were maintained unless otherwise stated.

3.5.1 Calculation for survival, nauplii production and sex ratio of copepods

In experiments 1-7, the initial density of nauplii was 50 numbers and 200 numbers were stocked in the 1 litre scale-up experiment. Survival of the initial nauplii (stage I) population in the duration of 14 days was measured as the copepod survival. The calculation of survival was as follows:

Day 4

$$\textit{Average of copepods between replicates (Av)} = \frac{[(Rep_1N+C)+(Rep_2N+C)+(Rep_3N+C)}{3}$$

$$\textit{Survival of Copepods (\%)} = \frac{(Av)}{N_i} \times 100 \qquad (3.1)$$

Day 6

$$\textit{Average of copepods between replicates (Av)} = \frac{[(Rep_1N+C)+(Rep_2N+C)+(Rep_3N+C)}{3}$$

$$\textit{Survival of Copepods (\%)} = \frac{(Av)}{N_i} \times 100 \qquad (3.2)$$

Day 8, 10, 12 and 14

$$\textit{Average of copepods between replicates (Av)} = \frac{[(Rep_1N+C)+(Rep_2N+C)+(Rep_3N+C)}{3}$$

$$\textit{Survival of copepods (\%)} = \frac{(Av)}{N_i} \times 100 \qquad (3.3)$$

N represent number of nauplii, *C* equal to number of copepodites, *A* was total number of male and female adults and N_i was initial number of nauplii (stage I). Then, the survival from a total of three replicates per treatment were calculated and averaged. As for

reproduction, total number of F2 nauplii were calculated from day 8 onwards where average number of nauplii individuals were calculated from three replicates per treatment.

Sex ratio of male adults to female adults in population were calculated on day 8 because highest number of adults were observed throughout all experiments. The sex ratio was presented as a percentage of mean value from three replicates per treatment.

3.5.2 Experiment 1 – Effect of feed on survival and reproduction of *P. annandalei*

This experiment was conducted to evaluate the efficiency of POME-based diets on the survival (%), growth in length (μm) and reproduction (nauplii individuals) of *P. annandalei* in order to determine their food preference which was then be used for the subsequent experiments unless otherwise stated. The feeding trial was carried out according to culture protocols described by Vidhya *et al.* (2014). List of diets tested were: microalgae *Nannochloropsis oculata,* POME-grown: *Aurantiochytrium limacinum*, *Candida tropicalis*, *Rhodotorula mucilaginosa, Rhovulum sulfidophilum*, *Shewanella algae* and a mixed diet of POME-grown SA:AL (Refer Table 3.1 and Subsection 3.3 for the preparation steps).

Prior to feeding, the concentrations of marine yeast and bacteria grown in POME were calculated using a Neubauer hemocytometer. The feed concentration was $2x10^5$ cells/ml where copepods were fed twice a day (1 ml at 8 am and 5 pm). The control conditions setted were 15 ppt of salinity, temperature of 28˚C, pH 7±1, 12 hours light: 12 hours dark and 4.05 μmol/m^2/sec of light intensity.

Table 3.5: Summary of treatments used in feed experiment

Treatments
1a. Frozen instant Nanno 3600 (*Nannochloropsis oculata*) (Control)
1b. POME-grown *Aurantiochytrium limacinum* (POME-grown AL)
1c. POME-grown *Candida tropicalis* (POME-grown CT)
1d. POME-grown *Rhodotorula mucilaginosa* (POME-grown RM)
1e. POME-grown *Rhovulum sulfidophilum* (POME-grown RS)
1f. POME-grown *Shewanella algae* (POME-grown SA)
1g. A mixed diet of POME-grown SA : POME-grown AL (1:1 ratio)

3.5.3 Experiment 2 – Effect of feed ratio on survival and reproduction of *P. annandalei*

In order to study the effect of POME-based feed ratio on the survival, growth in length and reproduction of *P. annandalei,* the feeding trial was carried out based on the best feed obtained from experiment 1 (Treatment 1g: Mixed diets of POME-grown SA : POME-grown AL). The feeding ratio tested in the experiment were POME only (as control), POME-grown AL: POME-grown SA in ratio of 3:1 and also ratio 1:3 (Table 3.6). The control conditions setted were 15 ppt of salinity, temperature of 28˚C, pH 7±1, 12 hours light: 12 hours dark and 4.05 µmol/m^2/sec of light intensity.

Table 3.6: Summary of treatments used in the ratio of feed experiment

Treatments
2a. POME (Control)
2b. POME-grown SA (50%) + POME-grown AL (50%)
2c. POME-grown SA (75%) + POME-grown AL (25%)
2d. POME-grown SA (25%) + POME-grown AL (75%)

3.5.4 Experiment 3 – Effect of salinity on survival and reproduction of *P. annandalei*

Four salinities i.e. 5ppt, 15ppt, 25ppt and 35ppt were used in this experiment (Table 3.7). The saline water was prepared by mixing artificial salt (Aquarium Systems Instant Ocean Reef Salt) with aged fresh water to achieve the respective salinity (See Table 3.7).

Prior to the experiment, the copepods were acclimated slowly for one week to the corresponding salinity. During the culture period, they were fed with a mixed diet of POME-grown SA and POME-grown AL in a ratio of 3:1 twice a day (10 am and 5 pm) and the temperature was set at 28^{o}C, pH 7±1 and 12 h of L: 12 h of D and 4.05 µmol/m^2/sec of light intensity.

Table 3.7: Summary of treatments used in salinity experiment

Treatments
3a. Salinity of 5 ppt (Mixture of 5 g of salt + 1L of water)
3b. Salinity of 15 ppt (Mixture of 15 g of salt + 1L of water)
3c. Salinity of 25 ppt (Mixture of 25 g of salt + 1L of water)
3d. Salinity of 35 ppt (Mixture of 35 g of salt + 1L of water)

3.5.5 Experiment 4 – Effect of temperature on survival and reproduction of *P. annandalei*

Experiment was carried out to test the effect of temperature on the survival and reproduction of *P. annandalei.* Temperature tested were 26˚C, 28˚C, 30˚C, 32˚C, 34˚C and 36˚C where 28˚C was set as a control temperature based on literature (Rhyne *et al.,* 2009) (Table 3.8). The respective temperatures were maintained using water bath. Before starting the experiment, the copepod was cultured in the respective temperatures for two days. Then, 50 nauplii that have been produced in the respective temperatures were collected and introduced into each treatment. The feed, ratio of feed and salinity were determined in Subsection 3.5.1, 3.5.2 and 3.5.3. Other control conditions were pH 7±1, 12 hours light: 12 hours dark and 4.05 µmol/m^2/sec of light intensity.

Table 3.8: Summary of treatments used in the temperature experiment

Treatments
4a. 26˚C
4b. 28˚C (Control)
4c. 30˚C
4d. 32˚C
4e. 34˚C
4f. 36˚C

3.5.6 Experiment 5 – Effect of pH on survival and reproduction of *P. annandalei*

The pH experiments were carried out to test the effect of different level of pH on survival and growth of *P. annandalei.* pH tested were pH 5, pH 6, pH 7, pH 8 and pH 9 and control which is between pH 7 to 8 (Table 3.9). Small amount of 0.1 mole of sodium hydroxide (NaOH) and hydrochloric acid (HCL) solutions were used to adjust the water pH until required pH were obtained. The nauplii produced from copepod cultured grown in respective pH were collected and introduced into each treatments in order to observe their survival and reproduction in every two days interval. The culture condition used were determined in previous Subsection 3.5.1 to 3.5.4. Other control conditions setted were 12 hours light: 12 hours dark and 4.05 μmol/m^2/sec of light intensity.

Table 3.9: Summary of treatments used in pH experiment

Treatments
5a. pH 5
5b. pH 6
5c. pH 7
5d. pH 8
5e. pH 9
5f. Control (pH 7-8)

3.5.7 Experiment 6 – Effect of photoperiod on survival and reproduction of *P. annandalei*

The method described by Peck & Holste (2006) was adopted to study the effect of photoperiod on the survival and reproduction of *P. annandalei*. Three conditions of photoperiods (hours of Light: Dark) used were 24 h L: 0 h D, 0 h L: 24 h D and 12 h L: 12 h D (Table 3.10). To obtain complete darkness, an opaque cover material was used to cover the opening of waterbath. Other culture conditions used were determined in previous Subsection 3.5.1-3.5.5 and light intensity was setted at 4.05 μmol/m^2/sec.

Table 3.10: Summary of treatments used in photoperiod experiment

Treatments
6a. 24 hour Light: 0 hour Dark
6b. 0 hour Light: 24 hour Dark
6c. 12 hour Light: 12 hour Dark

3.5.8 Experiment 7 – Effect of light intensity on survival and reproduction of *P. annandalei*

This experiment was carried out to test the effect of light intensity on survival, reproduction and growth in length of *P. annandalei* were adopted from Farhadian *et al*. (2014). Three levels of light intensity namely low (4.05 μmol/m^2/sec), medium (20.2 μmol/m^2/sec) and high (42.9 μmol/m^2/sec). The low light intensity (4.05 μmol/m^2/sec) was served as control where the lighting source was from cool white fluorescenct bulb (Table 3.11). The culture conditions used were determined in previous Subsection 3.5.1-3.5.6.

Table 3.11: Summary of treatments used in light intensity experiment

Treatments
7a. 4.05 µmol/m^2/sec (Control)
7b. 20.2 µmol/m^2/sec
7c.42.9 µmol/m^2/sec

3.6 Performances of *Pseudodiaptomus annandalei* fed with different type diets, cultured in an optimized conditions

Four different type of diets [POME-grown SA (3): POME-grown AL (1), biomass, POME (positive control) and no feed given (negative control) were tested on *P. annandalei* in the optimal culture parameters (Table 3.12). The aim of this experiment was to study and observe the effect of diet types on *P. annandalei* based on optimize parameters resulted from previous experiments (from subsection 3.5.1 to 3.5.7). Fifty numbers of nauplii were stocked in the 250 ml of 15 ppt saline water with triplicates for each treatment.

Table 3.12: Summary of treatments used in feed experiment with optimized conditions

Treatments
Diet 0: No feed given
Diet 1: POME-grown SA (3): POME-grown AL (1) liquid
Diet 2: POME-grown SA (3): POME-grown AL (1) biomass
Diet 3: POME

3.7 Scale up 1L culture of *Pseudodiaptomus annandalei*

P. annandalei was cultured in large volume in order to observed their survival based on the best optimize parameter decided in previous experiment. In 1000 ml volume of 15 ppt saline water, 200 numbers of *P. annandalei* nauplii were stocked in the culture. The sampling method was done as described in Subsection 3.5. The feed given was POME-grown SA (75%): POME-grown AL (25%) liquid as determined in subsection 3.6.

Feeding were given two times (10 am and 5 pm). The other optimize parameters used were salinity of 15 ppt (Subsection 3.5.3), temperature of 26˚C (Subsection 3.5.4), pH of 7-8 (Subsection 3.5.5), 12 h L: 12 h D photoperiod (Subsection 3.5.6) and light intensity of 20.2 µmol/m^2/sec (Subsection 3.5.7).

3.8 Nutritional profiles of feed and *P. annandalei*

3.8.1 Nutritional profile of feed uses in feeding experiment

POME-grown feed of 50 grams were prepared for proximate analysis, fatty acid analysis and amino acid analysis. The samples were prepared by centrifuged the stated amount using a Beckman J2-MI refrigerated centrifuge machine (7000 rpm speed for 5 minutes at 4ºC). The samples then were kept in freezer at -20ºC before being sent to UKM Unipeq Sdn Bhd, Selangor, Malaysia. (Full list of standard method of analysis can be referred to Appendix B).

3.8.2 Nutritional analysis of *P. annandalei*

As preparation for analysis of *P. annadalei*, biomasses of *P. annadalei* fed with diet composed POME-grown SA (3): POME-grown AL (1) and only with POME were collected by mass culturing the copepods (in the 200L tanks) in Marine Culture Unit, Rimba Ilmu of University Malaya. The copepods were then harvested every 7 days using scoop net with the size of 200 µm and rinsed off with distilled water. Then, the harvested adult copepods were kept in freezer at -20ºC before undergoing the freeze-drying process by using ALPHA 1-4 LCS basic.

Standard IUPAC method 2.301 (IUPAC, 1987) was referred for fatty acid methyl esters (FAMEs) preparation. In Addition, Waters Accq-Tag methods were used to analysed the amino acid profiles. Meanwhile, the proximate analysis was done for protein (No. 981.10), moisture (No. 950.46), ash (No. 923.03) and total fat (No. 991.36) based

on the AOAC 16th edition (1995) methods for POME, POME-grown AL and POME-grown SA feed. Carbohydrates were analysed based on Pomeranz & Meloan (1987) with in-house methods and for energy (by calculation) based on Pearson (1970) (Full list of standard method of analysis can be referred in Appendix B).

Next step of analytical techniques, sufficient amount of freeze dried copepod samples fed with SA (3) : AL (1) and POME were gained and then the fat extraction were done before outsourced the samples to UKM Unipeq Sdn Bhd, Selangor, Malaysia for analyses. After going through freeze-dried process, two mililitres of hexane was added into the test tube sample containing 15 mg of copepods. The sample was then vortexed vigorously for one minute and were left to deposit at room temperature for two hours. Next, the sample was centrifuged for five minutes at 3000 rpm speed.

The glass beaker was pre-weighed, and the supernatant were transferred into the beaker after centrifuge process took place. Then in order to remove any water residue, sodium sulphate was added, and the mixture was filtered. This procedure was repeated for three times. The glass beaker containing solution were left to dry and the concentrated oil extract was weighed to determine the copepod lipids that have been extracted. The oil extract samples were kept in -20ºC freezer before sent to external laboratory for fatty acid analysis. These procedure methods of copepod lipid extraction process were adapted from Zhang *et al.* (2013).

3.9 Measurement of the length (as growth in length) of *P. annandalei*

Figure 3.3: The measurement of body length (A) and width (B) of a male copepod

The body length of *P. annandalei* was measured from rostrum to caudal ramus (labelled as 'A' in the Figure 3.1) and the width of prosome which is the widest part of the body (labelled as 'B' in Figure 3.1). Copepod body sizes were measured by using Leica DMIL LED inverted microscope with an attached camera. The images were measured in µm using the software of Leica Application Suite X (LAS X).

3.10 Statistical analysis

The mean and standard error of *P. annandalei* survival, production for nauplii, male to female sex ratio and body growth of copepods in length were calculated from the triplicate of each treatments. Differences in treatment means of each experiment in terms of survival, production of naplii, male to female sex ratio and body growth were compared against days and analyzed by one-way analysis of variance (ANOVA) using analytical software IBM SPSS Statistic version 24. The statistical significance for all parameters was at 95% confidence interval ($p < 0.05$). Tukey HSD test was then performed in order to determine the differences between treatment if ANOVA results were significant ($p < 0.05$).

CHAPTER 4: RESULTS

4.1 Effects of POME-grown diets on the survival, growth and reproduction of *P. annandalei*

It has been observed that the survival of *P. annandalei* was supported by POME grown heterotropic microorganism diets as compared to copepods fed with the instant microalgae *Nannochloropsis oculata*, except for POME-grown RM and POME-grown CT which gave low survivals (Figure 4.1). Generally, POME-grown RS gave the stable survival from day 4 (67±0%) to day 12 (61±11.1%) with highest value on day 10 (72±11.1%) but the survival of *P. annandalei* decreased on the day 14 (40.7±4.7%).

Observation made on the day 8 showed that the survival rates of the four POME-grown diets i.e. POME-grown AL (56±16.4%), followed by POME-grown RS (55.3±12.7%), a mixed diet of POME-grown SA: POME-grown AL (54.7±10.9%) and POME-grown SA (51.3±11.7%) were about the same. The other three POME-grown diets presented low survival of *P. annandalei* were POME-grown CT (31.0±8.1%), POME-grown RM (26.7±4.4%) and microalgae diet (15.3±7.7) (Figure 4.1).

By day 14, a mixed diet of POME-grown SA: POME-grown AL had the highest survival rate (73.3±3.7%) followed by POME-grown AL (55.3±21.6%), POME-grown RS (40.7±4.7%), POME-grown SA (37.0±10.4%), POME-grown CT (22.0±7.0%), POME-grown RM (21.3±3.7%) and the lowest survival rate recorded was instant microalgae (18.0±8.7%) ($p < 0.05$) (Figure 4.1).

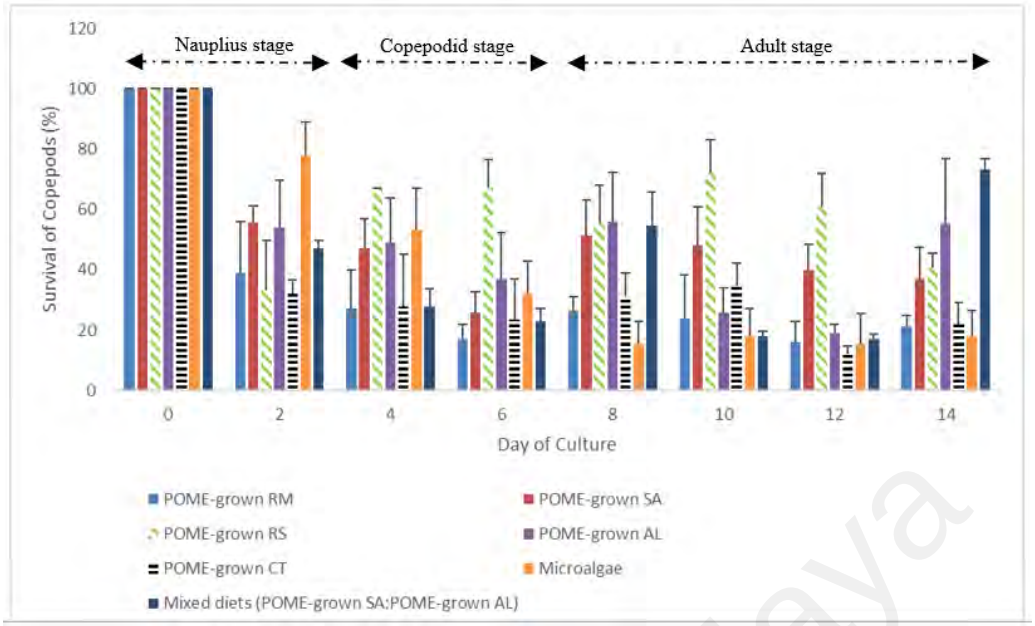

Figure 4.1: Survival of *P. annandalei* (mean±SE) fed with seven types of feed for the duration of two weeks

Overall, nauplii F2 production after 14 day of culture was not statistically affected by feeds tested ($p > 0.05$). The highest number of F2 nauplii on day 8 was observed in the mixed diet of POME-grown SA: POME-grown AL (66.0±32.6) followed by POME-grown AL (26.0±16.6) recorded on day 14, although not statistically different. No nauplii detected in the other feeds for both day 8 and day 14 (days of total count) (Table 4.1). Summary data of statistical analysis can be referred in Appendix E.

Table 4.1: F2 nauplii production (mean±SE) from copepods given POME-based diets and instant microalgae on day 8 and day 14. Different letters denote significant differences among treatments

	Type of Feed						
	POME-grown RM	**POME-grown SA**	**POME-grown RS**	**POME-grown AL**	**POME-grown CT**	**Micro-algae**	**Mixed POME-grown SA:POME-grown AL**
F2 Nauplii Production							
Day 8	0±0^{a}	0±0^{a}	0±0^{a}	15.0±2.8^{a}	0±0^{a}	0±0^{a}	66.0±32.6^{a}
Day 14	0±0^{a}	0±0^{a}	0±0^{a}	26.0±16.6^{a}	0±0^{a}	0±0^{a}	11.0±9.1^{a}

The mean ratio of adult males to adult females fed with seven diets on day 8 is shown in Figure 4.2. Sex ratio was significantly influenced by the type of feed given to *P. annandalei* ($p < 0.05$). The sex ratio of copepods fed on POME-grown RM, POME-grown AL and mixed diets of SA:AL had the closest equal ratio of male to female as compared to other feed treatments.

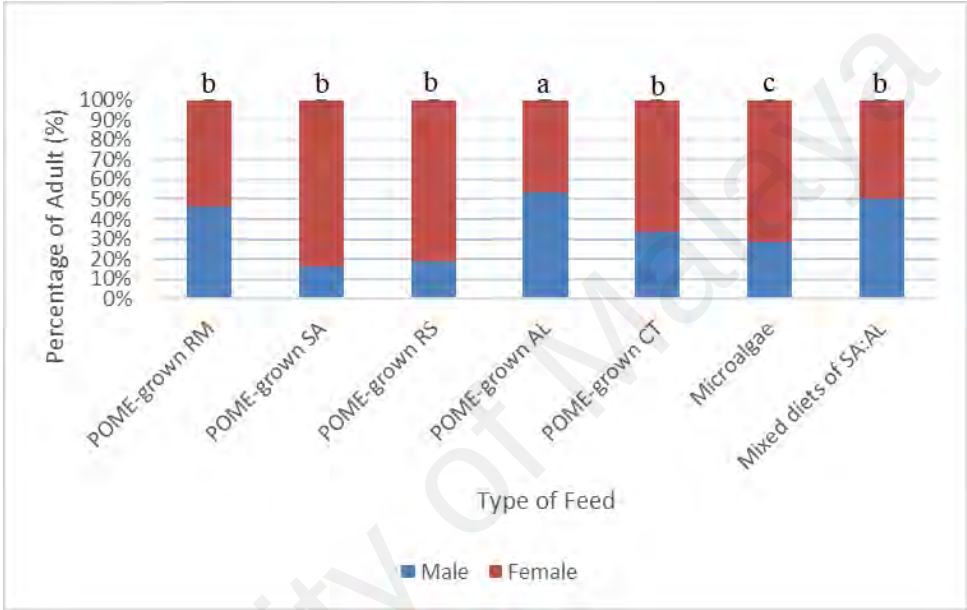

Figure 4.2: The percentage of adult males and females given various feeds on day 8

The average body size of *P. annandalei* raised on different feed types shows that feed have a significant effect on the growth (in total length and width) of copepods. The longest adult male of *P. annandalei* was on a diet of POME-grown RM with length of 1051.1±10.0 µm which it was also the largest in size (Table 4.2). While, no variations detected between all feed diets of adult females. Summary data of statistical analysis can be referred in Appendix D.

Table 4.2: Growth (in total length and width) of *P. annandalei* fed with seven types of waste grown microbial feed on day 14. Different letters denote significant differences among treatments

Body size (µm)	Micro-algae	AL	SA	RM	RS	CT	Mix
Male							
Length	946.5± 31.1[c]	925.3± 20.1[c]	878.0± 34.8[c]	1051.1 ±10.0[a]	887.4± 37.5[c]	1027.7 ±36.7[b]	945.6± 40.7[c]
Width	243.9± 12.6[a]	242.0± 4.2[a]	230.8± 9.9[a]	240.0± 10.0[a]	218.1± 3.4[a]	232.7± 11.1[a]	249.2± 4.7[a]
Female							
Length	1058.0 ±6.7[a]	972.6± 55.9[a]	949.5± 71.7[a]	1188.4 ±5.1[a]	1020.5 ±56.2[a]	1182.7 ±47.9[a]	1056.5 ±18.5[a]
Width	269.6± 1.4[a]	295.8± 12.1[a]	281.0± 8.5[a]	267.8± 3.6[a]	263.0± 29.1[a]	309.1± 12.8[a]	275.5± 6.9[a]

Table 4.3: The environmental parameters recorded for all feed treatments tested (see Appendix C for details)

	pH	Salinity (ppt)	Temperature (°C)	Dissolved oxygen (mg/L)
Range of values	7.3-8.13	14.8-16.2	27.5-8.4	3.3-6.2

Based on the results obtained, the suitable feed for culturing *P. annandalei* is mixed diet of POME-grown SA: POME-grown AL because the survival of *P. annandalei* significantly higher by end of experiment compared to other feeds tested. It is crucial to find feed that could maintaining life sustainability of copepods. This feed also showed the highest amount of nauplii numbers were produced, although the F2 nauplii production was not significant between the treatments. Furthermore, one to one male and female adults sex ratio was observed in this feed treatment.

4.2 Effect of feed ratios on the survival, growth in length and reproduction of *P. annandalei*

The survival of *P. annandalei* fed with different ratios of a mixed diet of POME-grown AL and POME-grown SA were statistically different among treatments ($p < 0.05$). The

highest survival between the treatments was contributed by POME-grown SA (75%) : POME-grown AL (25%) feed recorded on the day 2 with the value of 89±5.6%, followed by POME feed with value of 88.9±5.6% and POME-grown AL (50%): POME-grown SA (50%) (83.3±0%) (Figure 4.3).

By day 14, the survival of *P. annandalei* in all treatments decrease over the time but the copepods fed on POME-grown SA (75%): POME-grown AL (25%) feed was not really affected with survival of 71.3±11.6% on the day 14. For the POME-grown AL (50%): POME-grown SA (50%) almost half population survived was (40.7±6.8%). Then, followed by POME-grown AL (75%): POME-grown SA (25%) treatment with 25.3±5.3% survival. Meanwhile, *P. annandalei* fed on POME diet could not survived and had massive mortality (7.3±4.7%) (Figure 4.3).

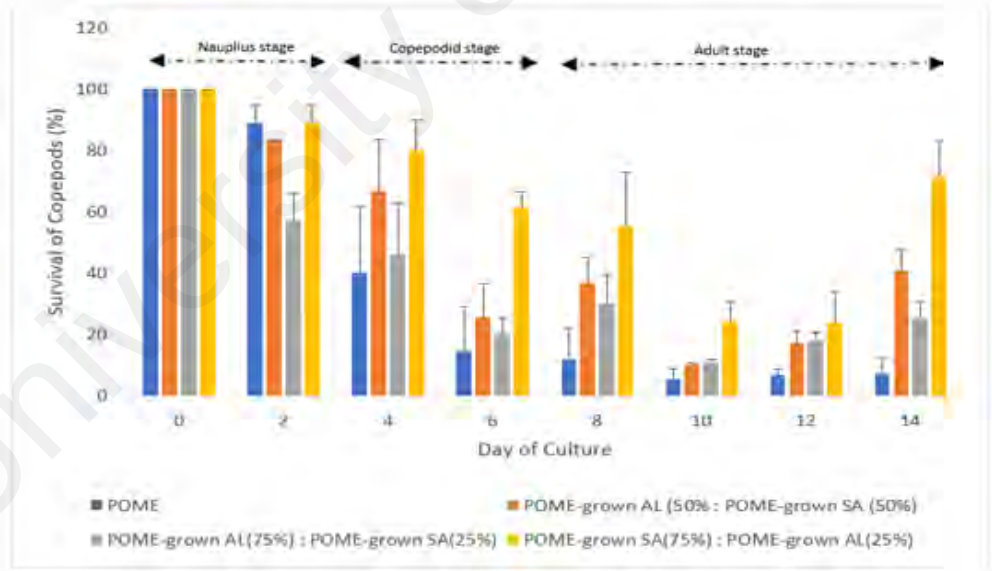

Figure 4.3: Survival of *P. annandalei* (mean±SE) given four different ratios of a mixed diet of POME-grown AL and POME-grown SA for a period of two weeks

The F2 nauplii production was significantly affected by feed ratios tested ($p < 0.05$). On the day 8, highest nauplii production was recorded in cultures given diet of POME-grown SA (75%): POME-grown AL (25%) (27.0±1.2), followed by POME-grown AL (50%): POME-grown SA (50%) (5.0±2.7), POME-grown AL (75%): POME-grown SA

(25%) (2.0±0.7) while no nauplii detected at all in cultures fed with POME. The F2 nauplii production between each feed ratio on day 14 were not significant ($p > 0.05$) (Table 4.4). Summary data of statistical analysis can be referred in Appendix G.

Table 4.4: Mean (±SE) of *P. annadalei* nauplii produced on day 8 and day 14. Copepods were fed with different ratios of a mixed diet of POME-grown SA and POME-grown AL. Different letters denote significant differences among treatments

	Ratio of Feed			
	POME	**POME-grown AL : POME-grown SA**	**POME-grown AL (75%) : POME-grown SA (25%)**	**POME-grown SA (75%) : POME-grown AL (25%)**
F2 Nauplii Production				
Day 8	$0±0^{c}$	$5.0±2.7^{b}$	$2.0±0.7^{b}$	$27.0±1.2^{a}$
Day 14	$1.0±0.3^{a}$	$2.0±1.2^{a}$	$6.0±4.1^{a}$	$9.0±4.6^{a}$

On day 8, male to female population was not signficantly influenced by different ratios of a mixed diet of POME-grown SA and POME-grown AL ($p > 0.05$) and the population recorded was balance 1:1 ratio except for POME-grown AL: POME-grown SA tratment which a bit skewed to male-biased (Figure 4.4).

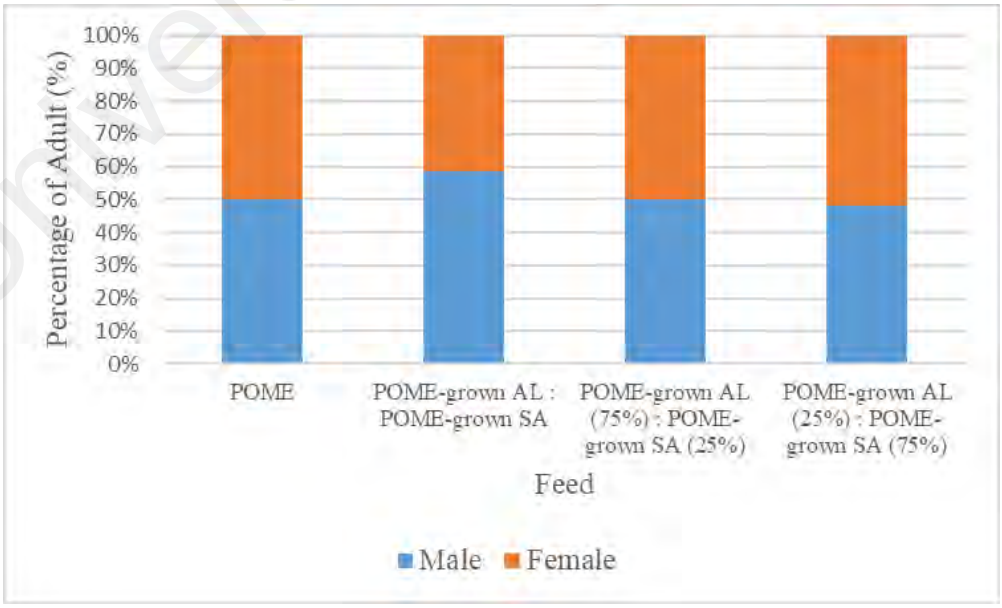

Figure 4.4: The percentage of adult males to adult females given various ratios of a concoction diet on day 8

The average body size of *P. annandalei* raised on different feed ratio shows that feed does not have a significant effect on the growth (in total length and width) of copepods ($p > 0.05$). No variations were detected between all treatments of adult males and females (Table 4.5). Average body length of adult males was ranged from 876.4-1001.3 µm and 218.6-250.8 µm for width. While for adult females, average body length was ranged from 932.0-1049 µm and 234.7-270.9 µm.

Table 4.5: Growth (in length and width) of *P. annandalei* fed with different ratio of a mixed diet as compared to only POME as control on day 14. Different letters denote significant differences among treatments

Body size (µm)	POME	AL:SA	AL (3):SA (1)	SA (3):AL (1)
Male				
Length	1001.3±26.7[a]	943.9±32.5[a]	876.4±54.0[a]	939.6±41.2[a]
Width	231.8±10.7[a]	250.8±6.6[a]	218.6±11.0[a]	229.2±3.2[a]
Female				
Length	1032.2±25.0[a]	932.0±48.7[a]	1049.4±58.4[a]	995.8±16.3[a]
Width	267.5 ± 6.5[a]	270.9±8.3[a]	261.3±15.4[a]	234.7±8.8[a]

Table 4.6: The environmental parameters recorded for all feed ratio treatments tested (see Appendix C for more details)

	pH	Salinity (ppt)	Temperature (°C)	Dissolved oxygen (mg/L)
Range of values	7.3-8.0	15.1-16.7	27.5-28.1	3.5-5.6

Thus, the appropriate ratio of feed for *P. annandalei* is POME-grown SA (75%): POME-grown AL (25%) due to high survival and reproduction *P. annandalei* were observed from the results obtained.

4.3 Effect of salinity on the survival, growth and reproduction of *P. anandalei*

Salinity did not have significant effect on survival of *P. annandalei* (P>0.05). Figure 4.7 showed that the survivals of *P. annandalei* cultured in each different salinities were fluctuated, although not statistically different. The survival in each cultures were

declining by day 6 where survivals in 25 ppt cultures were (38.0±24.8%), followed by 35 ppt (21.6±12.5%), 5 ppt (21.3±18.3%) and 15 ppt (16.4±4.4%).

On the day 8, the survivals of *P. annandalei* were increased where 35 ppt (53.3±12.3%), followed by 25 ppt (48.7±11.8%), 15 ppt (34.7±6.4%) and 5 ppt (24.7±10.4%), although not statistically different ($p > 0.05$). By day 14, the survival between 25 ppt (33.3±8.4%), followed by 35 ppt (32.7±4.8%) and 15 ppt (27.3±4.1%) were comparable (Figure 4.5).

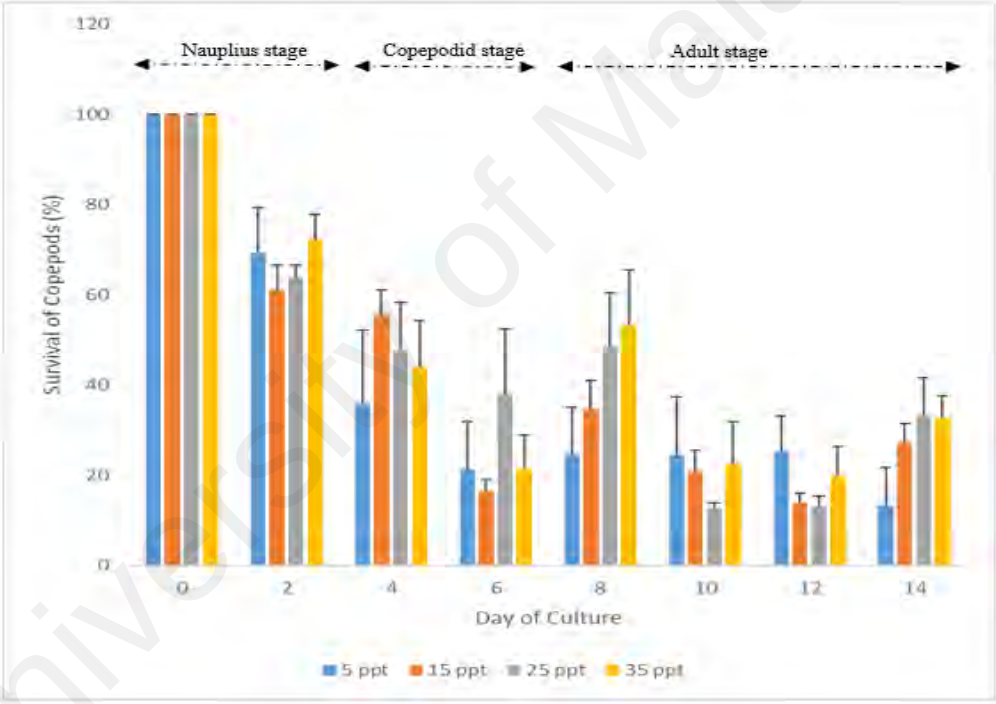

Figure 4.5: Survival of *P.* annandalei (mean±SE) cultured in four salinities for duration of two weeks

The F2 nauplii production of *P. annandalei* was significantly affected by salinity ($p < 0.05$). On day 14, 15 ppt gave the highest mean of F2 nauplii (103.0±48.8) followed by 5 ppt (10.0±8.3), 25 ppt (6.0±1.2) and 35 ppt gave the lowest mean of F2 nauplii production (1.0±0.3) (Table 4.7). No variations in F2 nauplii production on day 8 was detected ($p >$

0.05). Overall, F2 nauplii cultured at 15 ppt had the highest population as compared to other salinities tested. Summary data of statistical analysis can be referred in Appendix I.

Table 4.7: Mean (±SE) of *P. annadalei* nauplii produced on day 8 and day 14. Copepods were cultured in four salinities. Different letters denote significant differences among treatments

	Salinity (ppt)			
	5	**15**	**25**	**35**
F2 Nauplii Production				
Day 8	4.0 ± 4.2^{a}	9.0 ± 3.2^{a}	0 ± 0^{a}	0 ± 0^{a}
Day 14	10.0 ± 8.3^{b}	103.0 ± 48.8^{a}	6.0 ± 1.2^{b}	1.0 ± 0.3^{c}

Adult males to adult females population was not significantly influenced by salinity ($p < 0.05$). The mean number of male and female adults on day 8 is shown in Figure 4.6. Most of the population recorded on male-biased with more than 50% of male population except for 25 ppt treatment with 30% of male population.

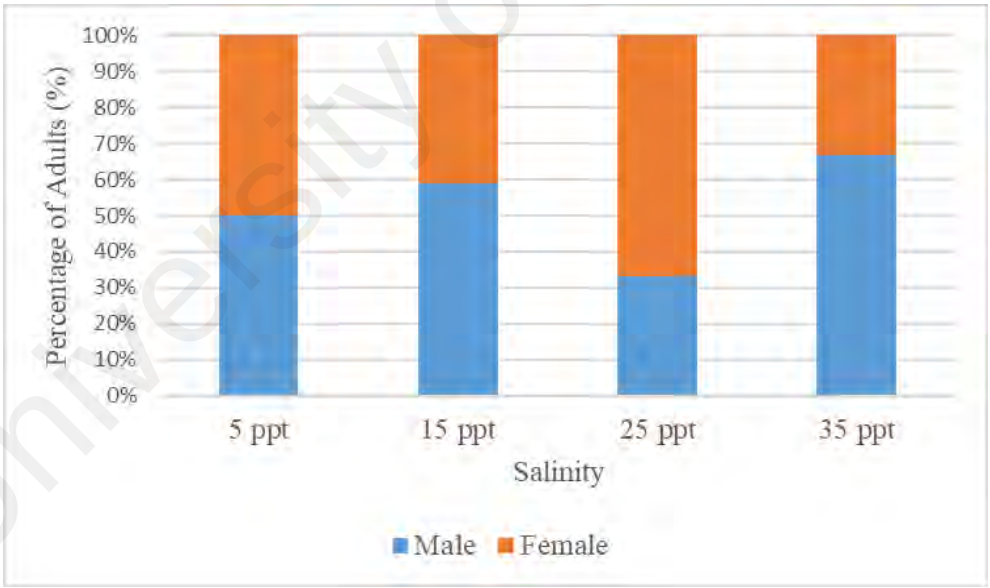

Figure 4.6: The percentage of the ratio of adult males to adult females cultured in four salinities on day 8

The average body size of *P. annandalei* raised on different salinity shows that it does not have a significant effect on the growth (in total length and width) of copepods ($p > 0.05$). There were no variations detected between all treatments of adult males and females (Figure 4.3). Average body length of adult males recorded was ranged from

946.4-971.5 µm and 251.0-261.6 µm for width. While for adult females, average body length was ranged from 1127.5-994.4 µm and 259.9-285.5 µm (Table 4.8).

Table 4.8: Growth (in length and width) of *P. annandalei* cultured in different salinity on day 14. Different letters denote significant differences among treatments

Body size (µm)	5 ppt	15 ppt	25 ppt	35 ppt
Male				
Length	946.4±3.9[a]	971.5±21.4[a]	958.5±26.6[a]	969.9±25.9[a]
Width	243.0±17.9[a]	261.4±11.5[a]	251.0±4.9[a]	251.6±2.4[a]
Female				
Length	1072.5±26.8[a]	994.4±18.9[a]	1109.2±32.9[a]	1127.5±57.5[a]
Width	285.8±22.0[a]	259.9±5.5[a]	285.5±3.0[a]	265.0±2.6[a]

Table 4.9: The environmental parameters recorded for all salinity treatments tested (Details can be referred to in Appendix C)

	pH	Salinity (ppt)	Temperature (ºC)	Dissolved oxygen (mg/L)
Range of values	7.3-8.0	6.1-36.8	27.5-28.2	3.9-5.3

Overall, even the survival of *P. annandalei* was not affected by salinity where survival can be maintained in salinity between 15 ppt to 35 ppt throughout the experiment, 15 ppt has been chosen as suitable salinity condition that will be set for subsequent series of experiments. This is because even 15 ppt salinity does not give excellent survival result, result of nauplii production was taken into consideration which the highest F2 nauplii production was observed in 15 ppt treatment compared to other treatments tested. High F2 nauplii production is one of the favourable characteristics desired in culturing copepods.

4.4 Effect of temperature on the survival, growth and reproduction of *P. annandalei*

The survival of copepods was significantly affected by temperature ($p < 0.05$). The survival of *P. annandalei* cultured at 26˚C on the day 8 was significantly the highest

(85.3±4.7%) compared to other temperatures tested ($p < 0.05$) (Figure 4.7). The survival of the cultures at 26˚C maintained the highest for the next subsequent day 10 (67.1±16.5) and on the day 14 (58.7±9.6). It was also recorded that by day 14, the copepods survival at temperature of 36˚C decreased dramatically with survival rate less than 20%.

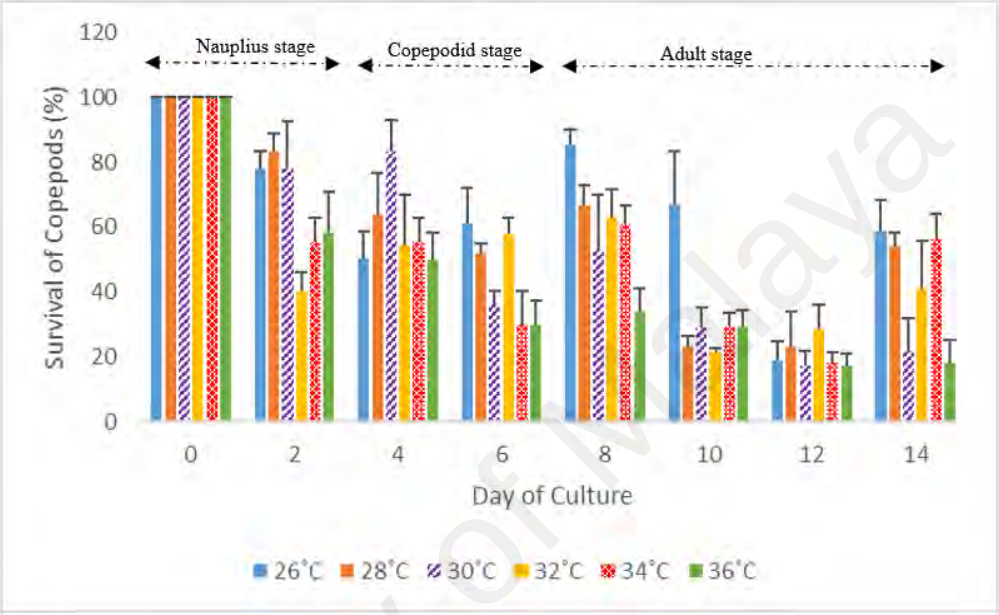

Figure 4.7: Survival of *P. annandalei* (mean±SE) cultured at six different temperatures for a duration of two weeks

The F2 nauplii production of *P. annandalei* was not affected by temperature on day 8 ($p > 0.05$) but the production was significantly affected by temperature on day 14 ($p < 0.05$). On day 14, the temperature of 28˚C gave the highest mean value of nauplii production (42.0±12.4), followed by 34˚C (27.0±4.8) and 30˚C (26.0±8.8). No nauplii was produced at 36˚C (0±0) (Table 4.10). Summary data of statistical analysis can be referred in Appendix K.

Table 4.10: Mean (±SE) *P. annandalei* nauplii produced on day 8 and day 14. Copepods were cultured at different temperatures. Different letters denote significant differences among treatments

	Temperature (°C)					
	26	**28**	**30**	**32**	**34**	**36**
F2 Nauplii Production						
Day 8	0±0^{a}	1.0±0.3^{a}	13.0±13.2^{a}	9.0±6.2^{a}	10.0±5.9^{a}	0±0^{a}
Day 14	7.0±4.4^{b}	42.0±12.4^{a}	26.0±8.8^{a}	9.0±3.4^{b}	27.0±4.8^{a}	0±0^{c}

Adult males to adult females ratio was significantly affected by temperature ($p < 0.05$) where the population recorded was male-biased in most treatments. The population cultured in temperature of 28˚C, 30˚C, 32˚C and 36˚C was male-biased. While cultured in temperature of 26˚C and 34˚C had the equal ratio of male to female populations (Figure 4.8).

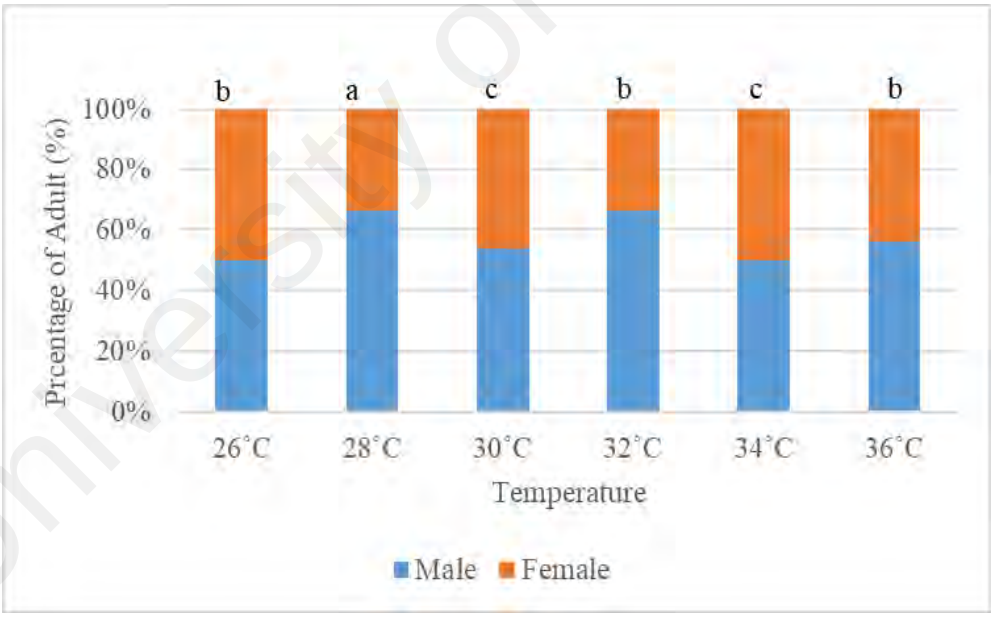

Figure 4.8: The percentage of adult males over adult females reared at various temperature on day 8

Besides that, the average body sizes of *P. annandalei* were not statistically significant between different temperatures ($p > 0.05$). There were no variations detected between all treatments of adult males and females (Figure 4.4). Average body length of adult males recorded ranged from 872.3-950.0 µm and 223.7-247.6 µm for width. While for adult

females, average body length ranged from 964.4-1016.2 µm and 244.4-262.4 µm (Table 4.11).

Table 4.11: Growth (in length and width) of *P. annandalei* cultured at different temperatures on day 14. Different letters denote significant differences among treatments

Body size (µm)	26ºC	28ºC	30ºC	32ºC	34ºC	36ºC
Male						
Length	949.4± 28.1[a]	913.6± 42.3[a]	892.0± 41.3[a]	950.9± 37.6[a]	872.3± 33.8[a]	882.3± 31.6[a]
Width	247.6± 7.9[a]	240.0± 11.2[a]	243.9± 15.0[a]	247.2± 4.5[a]	229.0± 10.9[a]	223.7± 14.9[a]
Female						
Length	983.9± 42.0[a]	1016.2 ±24.0[a]	995.6± 16.5[a]	1015.8 ±27.3[a]	964.4± 14.8[a]	979.0± 5.3[a]
Width	262.4± 4.4[a]	254.2± 15.6[a]	254.6± 7.3[a]	259.1± 1.6[a]	250.7± 6.5[a]	244.4 3.7[a]

Table 4.12: The environmental parameters recorded for all temperature treatments tested (Details can be referred to in Appendix C)

	pH	Salinity (ppt)	Temperature (ºC)	Dissolved oxygen (mg/L)
Range of values	7.1-7.9	15.4-18.4	25.6-35.4	2.0-5.7

Overall, 26˚C has been chosen as the suitable temperature condition for maintaining *P. annandalei* culture. This is because it can maintain high survival number of *P. annandalei* at the end of experiment and equal ratio male to female population was observed though the nauplii production is not high.

4.5 Effect of pH on the survival, growth and reproduction of *P. annandalei*

The survival of *P. annandalei* was significantly affected by pH ($p < 0.05$). Highly acidic pH has negative effect on *P. annandalei* where the survival of copepods cultured in pH 5 and pH 6 treatment declined over the time and total mortality occurred by day 6 for pH 5 and by day 14 for pH 6. Same effect was observed in high alkaline treatment

(pH 9) where the survival also dropped and critically low by day 14 (6±8.7%) compared to other treatments of pH 8 (33.3±12.8%), pH 7 (28.0±8.1%) and control (26.0±13.1%) (Figure 4.9).

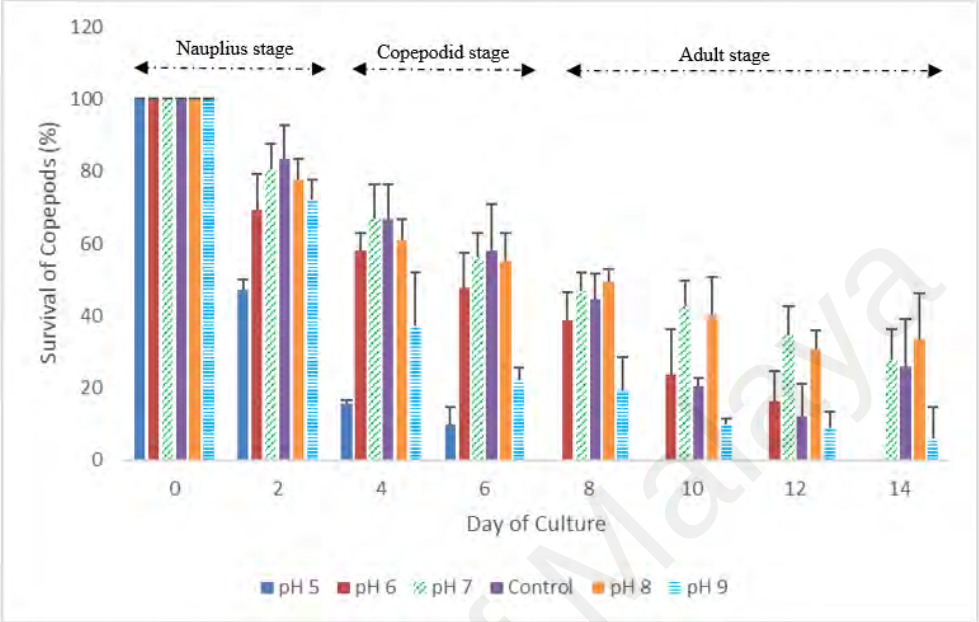

Figure 4.9: Survival of *P. annandalei* (mean±SE) cultured at six different pH for two weeks

The F2 nauplii production of *P. annandalei* was not affected by pH ($p < 0.05$). Even not the result is not statistically significant, control treatment had the highest number of nauplii (15.0±8.1 nauplii) compared to other treatments tested on last day of experiment (Table 4.13). Summary data of statistical analysis can be referred in Appendix M.

Table 4.13: Mean (±SE) *P. annandalei* nauplii produced on day 8 and day 14. Copepods were cultured at different pH. Different letters denote significant differences among treatments

	pH					
	Control	**5**	**6**	**7**	**8**	**9**
F2 Nauplii Production						
Day 8	4.0±4.0^{a}	0±0^{d}	1.0±1.0^{a}	0±0^{a}	4.0±2.0^{a}	0±0^{a}
Day 14	15.0±8.1^{a}	0±0^{a}	0±0^{a}	0±0^{a}	1.0±0.6^{a}	2.0±2.0^{a}

The ratio of adult males to adult females was not significantly affected by pH (P>0.05). The sex ratio in all treatments was relatively equal between males and females except for pH 6 and pH 9 that was male-biased (Figure 4.10).

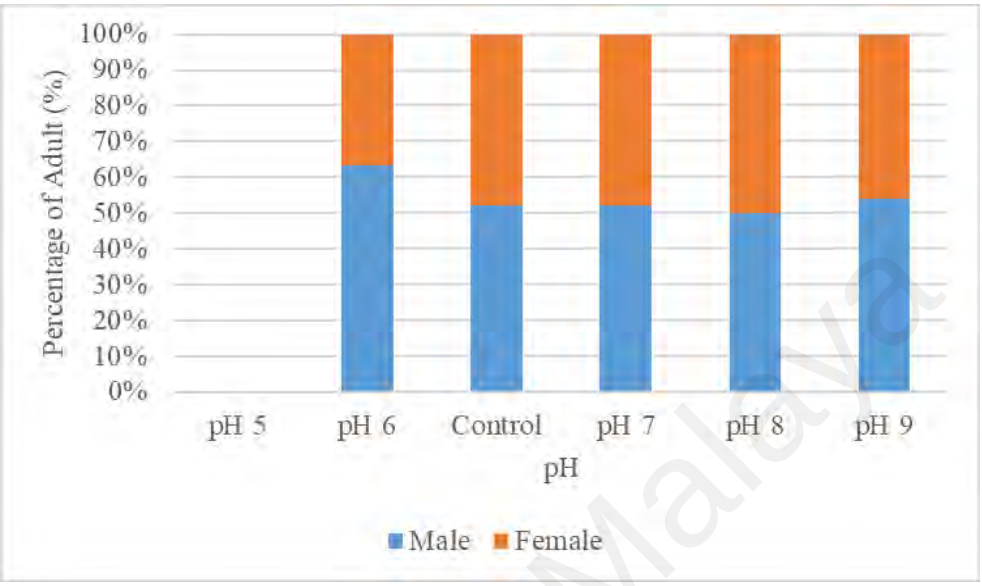

Figure 4.10: The percentage of adult males to adult females cultured in different pH on day 8

The average body sizes of *P. annandalei* were not statistically significant between different pH ($p > 0.05$). There were no variations detected between all treatments of adult males and females (Figure 4.5). Average body length of adult males recorded was ranged from 992.0-1047.8 µm and 251.8-264.3 µm for width. While for adult females, average body length was ranged from 1007.2-1163.2 µm and 272.6-288.5 µm (Table 4.14).

Table 4.14: Growth (in length and width) of *P. annandalei* cultured at different pH on day 14. Different letter denote significant differences among treatments

Body size (µm)	pH 7	pH 8	pH 9
Male			
Length	1047.8±76.7[a]	1038.3±26.1[a]	992.0±22.2[a]
Width	264.3±14.2[a]	253.7±6.1[a]	251.8±8.8[a]
Female			
Length	1163.2±11.0[a]	1152.1±52.8[a]	1007.2±0[a]
Width	279.7±1.6[a]	272.6±13.0[a]	288.5±0[a]

Table 4.15: The environmental parameters recorded for all pH treatments tested (Details can be referred to in Appendix C)

	pH	Salinity (ppt)	Temperature (°C)	Dissolved oxygen (mg/L)
Range of values	5.0-9.7	14.9-16.5	25.8-26.7	3.5-5.6

Generally, based on the results gained, pH 8 has been chosen as a suitable condition to culture *P. annandalei* since the percentage of survival recorded was the highest compared to other pH treatments. Besides that, the survival was not drastically drop throughout the experiment.

4.6 Effect of photoperiod on the survival, growth and reproduction of *P. annandalei*

The survival of *P. annandalei* was not affected by photoperiod ($p > 0.05$). Although not statistically different, the highest survival was observed in photoperiod of 24 h L:0 h D from day 2 (94.4±5.6%) to day 4 (83.3±9.6%). However, from day 6 onwards till day 14, the illumination of 12 h L : 12 h D gave the highest survival of copepods (day 6: 72.2±5.6% and day 14: 82±11.4%) (Figure 4.11).

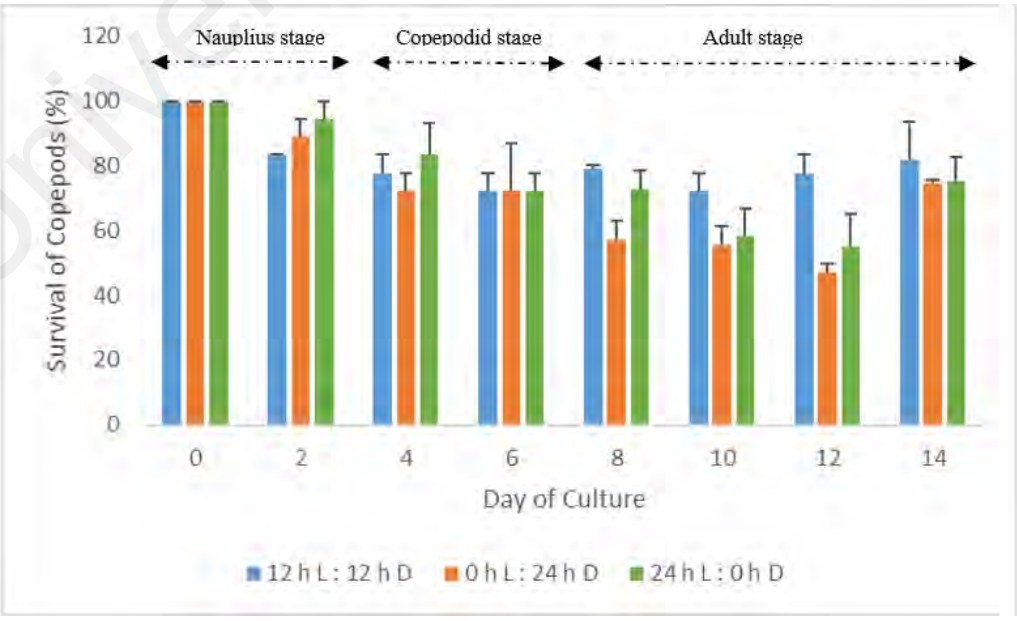

Figure 4.11: Survival of *P. annandalei* (mean±SE) cultured at three different photoperiods for a course of two weeks

The production of F2 nauplii was not affected by photoperiod ($p > 0.05$). There were no variations in nauplii production between the treatments tested. Although not statistically different, nauplii production was the highest in 12 h L: 12 h D treatment on day 8 (15.0±5.4%) and still maintained the highest on day 14 (11.0±4.0%) (Table 4.16). Summary data of statistical analysis can be referred in Appendix O.

Table 4.16: Mean (±SE) *P. annadalei* nauplii produced on day 8 and day 14. Copepods were cultured at different photoperiods. Different letters denote significant differences among treatments

	Photoperiod (Light : Dark hours)		
	12 : 12	**0 : 24**	**24 : 0**
F2 Nauplii Production			
Day 8	15.0±5.4[a]	5.0±2.7[a]	13.0±5.2[a]
Day 14	11.0±4.0[a]	3.0±2.5[a]	5.0±4.3[a]

Sex ratio was not statistically affected by the photoperiods ($p > 0.05$). The adult males to adult females population of *P. annadalei* were evenly distributed amongst the treatment tested except 0 h L : 24 h D treatment where male-skewed ratio was recorded with almost 60% of male (Figure 4.12).

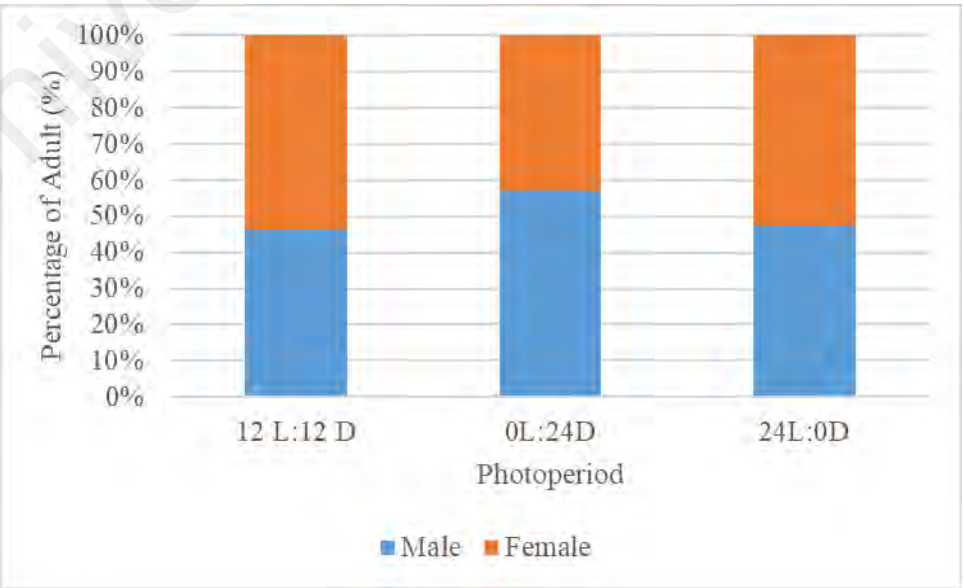

Figure 4.12: The percentage of adult males to adult females cultured in different photoperiods on day 8

Besides that, the average body size of *P. annandalei* in different photoperiod treatments was not statistically significant ($p > 0.05$). There were no variations detected between all treatments of adult males and females (Figure 4.5). Average body length of adult males recorded was ranged from 762.4-814.1 µm and 209.1-226.9 µm for width. While for adult females, average body length was ranged from 879.5-996.9 µm and 226.7-266.8 µm (Table 4.17).

Table 4.17: Growth (in length and width) of *P. annandalei* cultured at different photoperiods on day 14. Different letters denote significant differences among treatments

Body size (µm)	12L:12D	0:24D	24L:0
Male			
Length	762.4±41.4[a]	814.1±107.9[a]	904.6±48.9[a]
Width	209.1±12.1[a]	221.6±18.6[a]	226.9±14.7[a]
Female			
Length	879.5±49.9[a]	982.2±25.6[a]	996.9±18.0[a]
Width	226.7±8.5[b]	266.8±3.8[a]	254.7±4.8[a]

Table 4.18: The environmental parameters recorded for all photoperiod treatments tested (Details can be referred to in Appendix C)

	pH	Salinity (ppt)	Temperature (°C)	Dissolved oxygen (mg/L)
Range of values	7.2-8.0	14.9-16.0	25.8-26.7	3.5-5.6

Overall, 12 h light: 12 h dark photoperiod treatment was considered the best treatment based on the observation in terms of high survival and nauplii production even the reuslts were not statistically different. Besides that, it mimics natural lighting cycle that is more favourable compared to other photoperiod treatments tested.

4.7 Effect of light intensity on the survival, growth and reproduction of *P. annadalei*

The survival of *P. ananndalei* was statistically significant affected by light intensity ($p < 0.05$). The highest survival were recorded at treatment of 4.05 µmol/m^2/sec on day 8

(86.7±2.4%), although not statistically significant. By day 14, survival of *P. ananndalei* at 4.05 µmol/m^2/sec treatment (68.0±3.3%) still maintained the highest compared to 20.2 µmol/m^2/sec treatment (57.3±4.4%) and 42.9 µmol/m^2/s treatment (46.7±2.9%) (Figure 4.13).

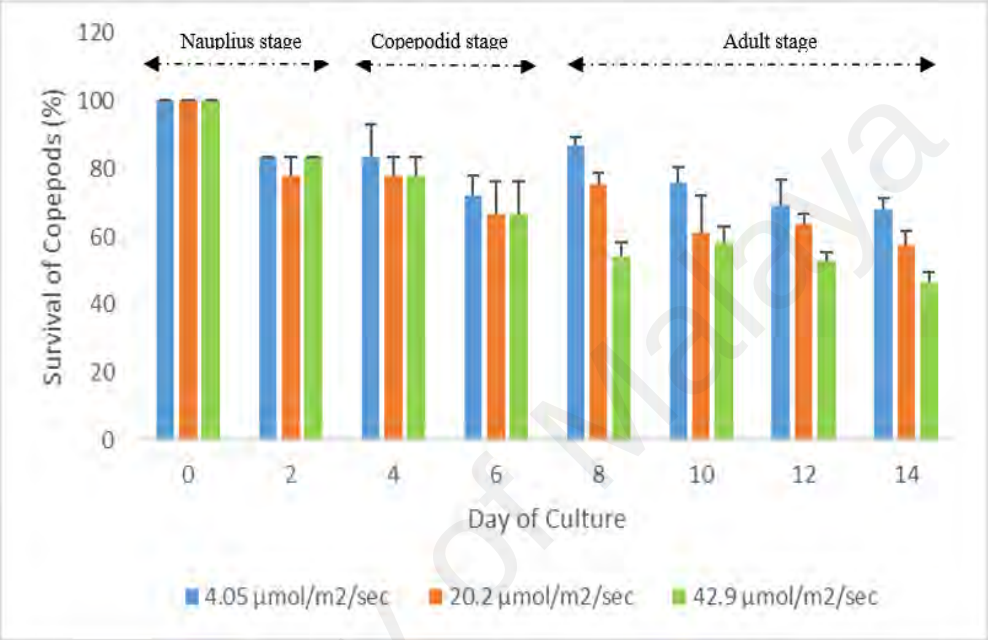

Figure 4.13: Survival of *P. annandalei* (mean±SE) cultured at three different light intensities for a course of two weeks

The F2 nauplii production was not statistically affected by light intensity ($p > 0.05$). There were no variation of nauplii production between the treatments tested. The production of F2 nauplii were comparable between the treatments (Table 4.19). Summary data of statistical analysis can be referred in Appendix Q.

Table 4.19: Mean (±SE) of *P. annandalei* nauplii produced on day 8 and day 14. Copepods were cultured at three different light intensities. Different letters denote significant differences among treatments

	Light Intensity (µmol/m^2/sec)		
	4.05	**20.2**	**42.9**
F2 Nauplii Production			
Day 8	1.0±0^a	1.0±0.7^a	1.0±0.5^a
Day 14	2.0±2.3^a	1.0±3.0^a	1.0±1.0^a

The number of male and female populations recorded on day 8 was not influenced by light intensity ($p > 0.05$). The result showed that sex ratio in all treatments were male biased with 60% of male population (Figure 4.14).

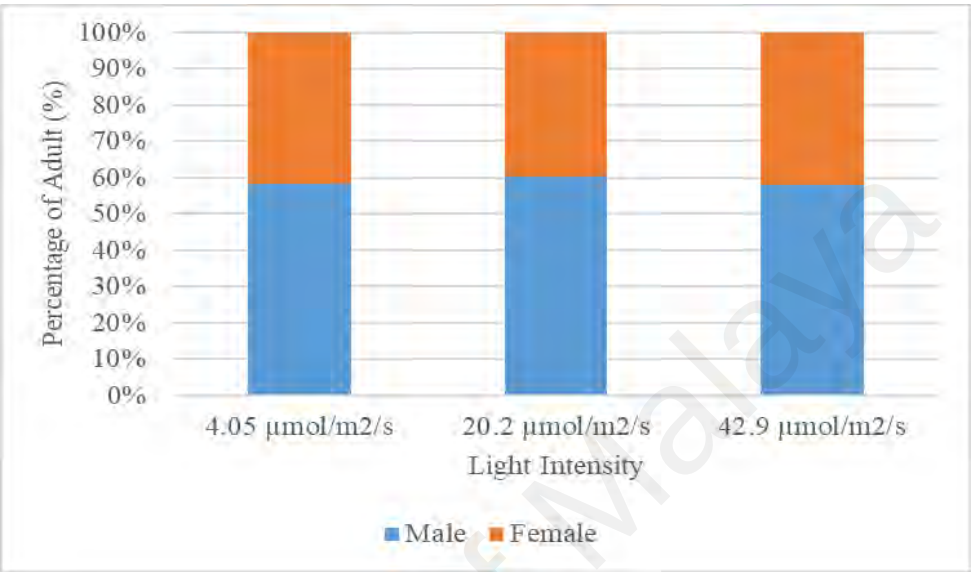

Figure 4.14: The percentage of adult males to adult females cultured in different light intensities on day 8

The average body size of *P. annandalei* in different light intentsity treatments were not statistically significant ($p > 0.05$). There were no variations detected between all treatments of adult males and females (Table 4.20). Average body length of adult males recorded was ranged from 778.8-894.0 µm and 217.8-240.7 µm for width. While for adult females, average body length was ranged from 939.2-973.5 µm and 241.2-304.5 µm.

Table 4.20: Growth (in length and width) of *P. annandalei* cultured at different light intensity treatments on day 14. Different letters denote significant differences among treatments

Body size (µm)	Control	Medium	High
Male			
Length	894.0±22.3[a]	778.8±81.4[a]	837.7±207.1[a]
Width	240.7±45.3[a]	217.8±17.1[a]	228.4±31[a]
Female			
Length	973.5±7.3[a]	963.4±29.8[a]	939.2±31.0[a]
Width	250.1 ± 4.1[a]	304.5±70.4[a]	241.2±14.3[a]

Table 4.21: The environmental parameters recorded for all light intensity treatments tested (Details can be referred to in Appendix C)

	pH	Salinity (ppt)	Temperature (ºC)	Dissolved oxygen (mg/L)
Range of values	7.3-8.1	15.1-16.6	25.7-26.1	3.0-5.2

Overall, the treatment of 4.05 µmol/m^2/sec has been chosen as suitable light intensity parameter for cultutring *P. annandalei* because of high survival has been observed compared to other light intensity treatments. The survival of *P. annandalei* cultured at this treatment maintain the highest from the start till the last day of experiment period.

4.8 Performance of *Pseudodiaptomus annandalei* in optimize parameters fed with different type diets

The survival of *P. ananndalei* was significantly affected by the types of diet ($p < 0.05$). All the diet treatments tested can maintain a good survival of the copepods, but the form of diets affected the survivality of *P. annandalei*. Unfed copepods cannot survive more than 10 days. The result shows that on day 8, POME-grown SA (75%): POME-grown AL (25%) treatment gave the highest mean survival (89.3±1.8%) (Figure 4.15). Throughout the experiment, POME-grown SA (75%): POME-grown AL (25%) treatment maintained the highest survival of *P. ananndalei* compared to other treatments tested (Figure 4.15).

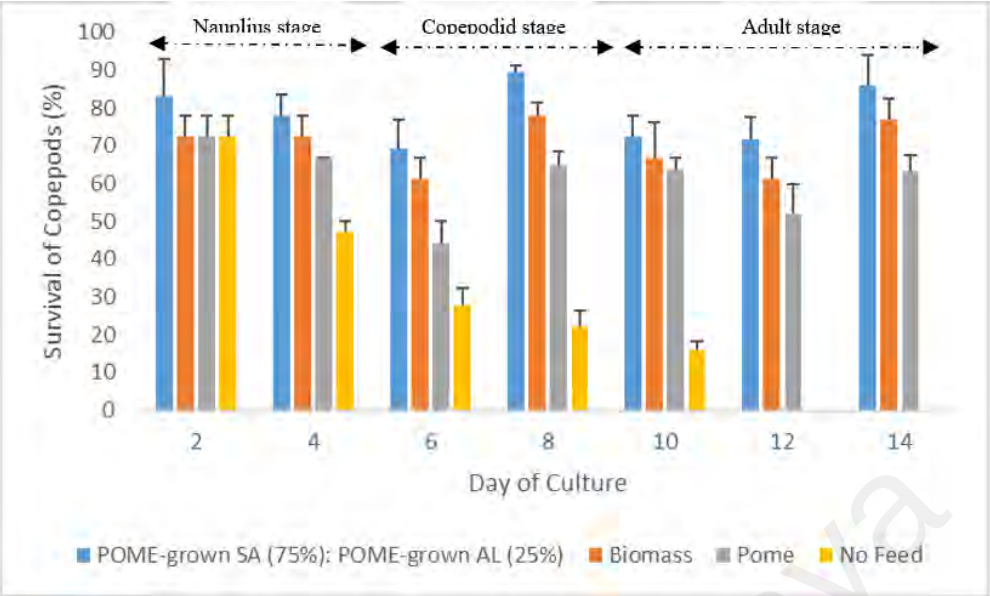

Figure 4.15: Survival of *P. annandalei* (mean±SE) cultured at four different types of feed for a course of two weeks

Table 4.22: Summarized optimal culture parameters from previous section

	Salinity (ppt)	**Temperature (°C)**	**pH**	**Photoperiod**	**Light intensity (µmol/m²/sec)**
Values	15	26-28	7.0-8.0	12 h light: 12 h dark	4.05

The number of male and female population on day 8 was not statistically affected by the type of diets ($p < 0.05$). Percentage of sex ratio in all treatments was female biased except for biomass feed with 30% of female population (Figure 4.16).

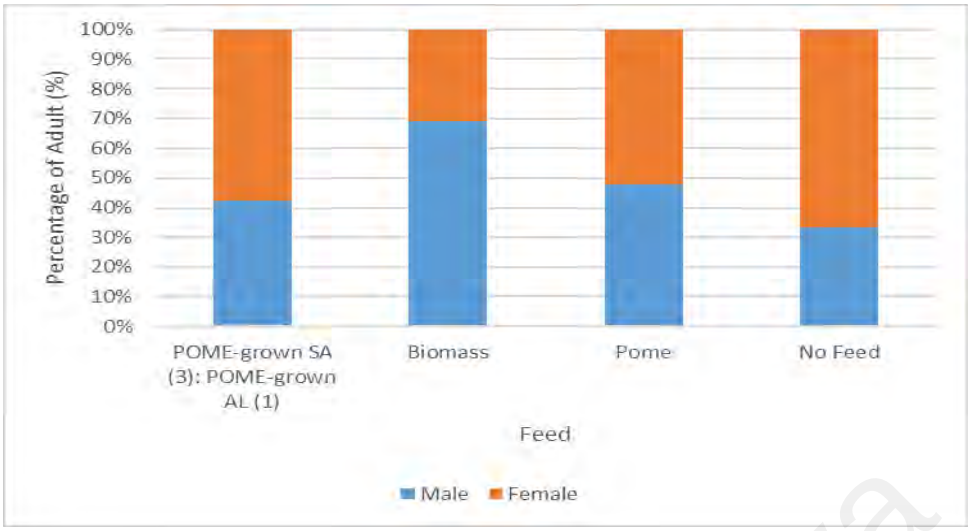

Figure 4.16: The percentage of adult males and adult females population cultured in different types of feed on day 8

Table 4.23: The environmental parameters recorded for the treatments tested (Details can be referred to in Appendix C)

	pH	Salinity (ppt)	Temperature (°C)	Dissolved oxygen (mg/L)
Range of values	7.3-7.9	15.0-16.3	25.6-26.3	3.5-5.7

Overall, the performance of *P. annandalei* in optimized parameters was relatively the best with POME-grown SA (75%): POME-grown AL (25%) diet compared to other types of diet tested. *P. annandalei* fed on POME-grown SA (75%): POME-grown AL (25%) maintained the highest survival throughout the experiment period.

4.9 Scale-up culture of *P. annandalei* in 1L culture with optimal parameters

The survival of copepods fed with POME-grown SA (75%): POME-grown AL (25%) was sustained above 70% during the experimental period. The survival rate on the day 8 was 83.8±3.9% and the survival was 81.3±16.5% on the day 14 (Table 4.23). The optimized culture parameters can be referred to table 4.21 in previous section.

Table 4.24: Survival of *P. annandalei* (mean±SE) given POME-grown SA (75%): POME grown AL (25%) for two weeks

Feed	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
POME-grown SA (75%): POME grown AL (25%)	83.3± 9.6%	77.8± 5.6%	72.2± 5.6%	83.8± 3.9%	77.8±5. 6%	77.8±5. 6%	81.3±1 6.5%

Sex ratio in this experiment was female biased where the adult males to adult females ratio was 0.76 (Table 4.24).

Table 4.25: Sex ratio of *P. annandalei* on day 8 and day 14 fed with POME-grown SA (75%): POME-grown AL (25%) diet

	POME-grown SA (75%): POME-grown AL (25%) Diet
	Sex Ratio (M/F)
Day 8	0.76±11.5
Day 14	0.88±5.0

Table 4.26: The environmental parameters recorded for the treatments tested (Details can be referred to in Appendix C)

	pH	**Salinity (ppt)**	**Temperature (°C)**	**Dissolved oxygen (mg/L)**
Range of values	7.3-8.0	15.0-15.1	25.8-26.3	3.9-5.5

4.10 Nutritional profiles of POME based feed

The proximate composition was done on POME-grown AL and POME-grown SA as they gave the highest survivorship of copepods and also POME alone as a comparison (Table 4.27). The lipid content in POME (27.1%) was higher than that of in POME-grown AL (21.3%) and POME-grown SA (13.9%) (Table 4.27). Besides that, percentage of ash and energy contents in POME alone was also greater than those in POME-grown AL and POME-grown SA. In terms of protein content, POME-grown SA had higher percentage of protein (47.3%) compared to POME alone (38.8%) and POME-grown AL (34.4%).

Table 4.27: Proximate composition (% in dry weight) of nutrients in three types of POME grown feed. Different letters denote significant differences among treatments

Proximate composition	POME	AL	SA
%	**Average**	**Average**	**Average**
Protein	38.8±2.75^{a}	34.4±0.18^{a}	47.3±5.86^{a}
Lipid	27.1±1.50^{a}	21.3±0.06^{b}	13.9±1.17^{c}
Carbohydrate	6.1±0.77^{b}	22.4±0.12^{a}	15.4±3.79^{a}
Ash	28.0±1.98^{a}	21.8±0.12^{b}	23.4±0.89^{b}
Energy (kcal/100g)	422.1±2.18	419.4±1.28	377.2±0.84

Generally, fatty acid compositions of the POME-based biomasses were mainly palmitic acid, lauric acid and oleic acid. POME contained 40.7% palmitic acid, 9.3% lauric acid and 29.5% oleic acid, POME-grown SA (40.6%, 7.17% and 28.1%, respectively) and POME-grown AL (31.7%, 24.2% and 12.3%, respectively). Some minor compositions detected in POME-based biomasses were linoleic acid, stearic and myristic (Table 4.28). DHA of 0.8% was only detected in POME-grown AL. Full list of fatty acids can be referred in appendix B.

Table 4.28: Fatty acid compositions (% of total fatty acids) of the biomasses of POME alone and microheterotrophs grown in POME. Different letters denote significant differences among treatments

% of Total Fatty Acid	POME	SA	RS	CT	RM	AL
Myristic acid	3.6±0.021^{d}	5.1±0.011^{b}	2.8±0.008^{f}	4.0±0.001^{c}	3.4±0.008^{e}	11.3±0.05^{a}
Myristoleic acid	0.1±0.005^{c}	1.0±0.015^{a}	0.1±0.001^{d}	0.2±0.011^{b}	0.1±0.002^{c}	0.1±0.004^{d}
Pentadecanoic acid	0.1±0.004^{c}	0.4±0.012^{b}	0.1±0.001^{c}	0.2±0^{c}	0.1±0.027^{c}	3.1±0.004^{a}
Palmitic acid	40.7±0.016^{c}	40.6±0.002^{d}	25.0±0.014^{f}	42.0±0.021^{b}	42.5±0.015^{a}	31.7±0.021^{e}
Palmitoleic acid	0.4±0.006^{a}	1.4±0.007^{e}	0.6±0.016^{c}	0.5±0.003^{d}	0.6±0.013^{c}	0.8±0^{b}
Heptadecanoic acid	0.3±0.008^{c}	0.5±0.011^{b}	0.3±0.006^{c}	0.2±0.015^{d}	-	1.1±0.002^{a}
Stearic acid	5.0±0.025^{c}	6.4±0.001^{b}	19.1±0.039^{a}	4.7±0.318^{c}	4.8±0.007^{c}	3.8±0.001^{d}
Oleic acid	29.5±0.063^{d}	28.1±0.031^{e}	37.8±0.07^{a}	30.0±0.028^{c}	33.6±0.021^{b}	12.3±0.018^{f}
Linoleic (Cis) acid	8.3±0.008^{a}	6.7±0^{c}	6.6±0.005^{d}	7.3±0.001^{b}	8.3±0.03^{a}	2.3±0.006^{e}
a-Linolenic acid	0.9±0.006^{a}	-	0.1±0.001^{b}	-	-	-
ARA	-	-	-	-	-	-
EPA	-	-	-	-	-	-
DHA	-	-	-	-	-	0.8±0.012^{a}
SFA	60.7	62.1	52.5	60.8	57.4	82.9
MUFA	30.0	31.1	38.5	31.8	34.3	13.4
PUFA	9.2	6.7	6.7	7.3	8.3	3.7
n-3 PUFA	0.9	-	0.1	-	-	0.8
n-6 PUFA	8.3	6.7	6.6	7.3	8.3	2.9

All biochemical analysis results were analyzed by one-way ANOVA and Tukey HSD test to determine the differences between treatment if the result was significant. Table 4.29 shows that the proportion of essential and non-essential content of amino acid in all three types of POME grown feed have no great difference. The sample size of total average content of amino acid was in duplicate. Percentage of non-essential amino acids was higher than that of in the essential amino acids in the three POME grown feeds.

Some essential amino acids were found significant between the three feeds tested where POME feed was significant with methionine (5.39±0.003%), threonine (5.41±0.01%) and tryptophan (1.77±0.001%). SA POME grown feed was significant in leusine (9.73±0.06%), isoleusine (6.27±0.01%), phenyalanine (4.62±0.02%) and valine (7.21±0.02%). Then, lysine (8.35±0.09%), methionine (5.03±0.15%) and threonine (5.82±0.30%) were found significant in AL POME grown feed. For non-essential amino acids content, only arginine (5.70±0.24%) was found significant in SA POME grown feed and cysteine (1.39±0.02%) in POME feed.

Table 4.29: Amino acid contents (% of total amino acids) of POME and *S. algae* and *A. lumacinum* grown in POME (POME-SA and POME-AL). Different letters denote significant differences among treatments

	Amino Acid	POME (%)	POME-SA (%)	POME-AL (%)
Essential	Histidine	1.66±0.38^{a}	1.56±0.20^{a}	1.15±0.25^{a}
	Lysine	5.87±0.03^{c}	6.95±0.38^{b}	8.35±0.09^{a}
	Leusine	9.41±0.09^{b}	9.73±0.06^{a}	8.57±0.37^{b}
	Isoleusine	6.01±0.06^{b}	6.27±0.01^{a}	5.78±0.15^{b}
	Methionine	5.39±0.003^{a}	3.80±0.08^{b}	5.03±0.15^{a}
	Phenylalanine	4.21±0.01^{b}	4.62±0.02^{a}	4.03±0.07^{c}
	Threonine	5.41±0.01^{a}	4.02±0.03^{b}	5.82±0.30^{a}
	Tryptophan	1.77±0.001^{a}	1.21±0.02^{b}	1.26±0.01^{b}
	Valine	6.90±0.04^{b}	7.21±0.02^{a}	6.66±0.14^{b}
	TOTAL	**46.63**	**45.37**	**46.65**
Non-Essential	Proline	3.61±0.22^{a}	3.71±0.05^{a}	3.45±0.02^{a}
	Thyrosine	2.58±0.02^{a}	2.84±0.13^{a}	2.52±0.004^{a}
	Aspartic Acid	9.34±0.10^{a}	9.89±0.43^{a}	10.34±0.24^{a}
	Serine	5.69±0.21^{a}	5.85±0.35^{a}	5.10±0.35^{a}
	Glycine	5.06±0.34^{a}	5.11±0.06^{a}	4.70±0.17^{a}
	Glutamic Acid	13.21±0.10^{a}	12.83±0.17^{a}	13.4±0.46^{a}
	Arginine	4.83±0.06^{c}	5.70±0.24^{a}	5.00±0.03^{b}
	Alanine	7.67±0.20^{a}	7.54±0.16^{a}	7.50±0.02^{a}
	Cysteine	1.39±0.02^{a}	1.17±0.06^{b}	1.34±0.03^{b}
	TOTAL	**53.38**	**54.64**	**53.35**

4.11 Nutritional profile of *P. annandalei* fed with POME based diets

Three important fatty acids i.e. ARA, EPA and DHA were detected in both *P. annandalei* fed with AL (3): AL (1) and *P. annandalei* fed with POME. The percentage of EPA (4.4%) and DHA (7.2%) content was higher in *P. annandalei* fed on POME-grown AL (3): POME-grown AL (1) compared to *P. annandalei* fed on POME alone. ARA was detected in amount of 1.3% in *P. annandalei* fed on POME alone while only 0.9% in *P. annandalei* fed with POME-grown AL (3): POME-grown AL (1) (Table 4.29). A high amount of palmitic acid was also detected in both types of *P. annandalei* with *P. annandalei* fed on POME-grown AL (3): POME-grown AL (1) (19.4%) had higher percentage content than that of in *P. annandalei* fed on POME (17.6%). Linoleic acid in *P. annandalei* fed on POME-grown AL (3): POME-grown AL (1) was 7.8% while in *P. annandalei* fed on POME alone was 8.2%.

Table 4.30: Percentage of fatty acid contents in *P. annandalei* fed with POME-grown SA (3): POME-grown AL (1) and only POME. Different letters denote significant differences among treatments

Fatty Acid	Pseudo fed with POME-grown SA (3): POME-grown AL (1)	Pseudo fed with only POME
Palmitic acid	19.38±0.03[a]	17.63±0.29[b]
Stearic acid	3.66±0.01[a]	3.29±0[b]
Linoleic acid	7.84±0.21[a]	8.21±0.09[a]
α Linolenic acid	8.82±0.12[b]	10.5±0.09[a]
Arachidonic acid (ARA)	0.91±0.35[a]	1.26±0.20[a]
Eicosapentanoic acid (EPA)	4.36±0.02[a]	3.90±0.01[b]
Docosahexaenoic acid (DHA)	7.21±0.06[a]	7.00±0.01[b]

CHAPTER 5: DISCUSSION AND CONCLUSIONS

5.1 Effect of POME-grown diets on the survival, growth and reproduction of *P. annandalei*

Dietary nutrition is one of the crucial factors that affect the survival, growth and reproduction of copepods. Among the feeds tested, the mixed feed of POME-grown SA and POME-grown AL gave the highest survival and number of nauplii of *P. annandalei*. This could be attributed to the good nutritional quality of the food which play a vital role in the development of copepods (Peterson, 2001; Daase *et al.,* 2011). Hence, the concoction diet could fulfill and meet the nutritional requirements for copepods development. The nutritional profile of *P. annandalei* fed with such mixed diets contained significant amount of PUFA i.e. 0.9% ARA, 4.36% EPA and 7.21% DHA which are crucial for copepods growth (Table 4.11).

Shewanella algae (SA), a Gram-negative marine bacterium is an omega-3 fatty acid producer (Jiang *et al.,* 2013). *Aurantiochytrium limacinum* (AL) is a marine heterotrophic protist that could synthesize long chain fatty acids such as DHA (Abad & Turon, 2015). The results of fatty acid analysis depict that POME-grown AL contained 0.8% of DHA. DHA plays a crucial role in growth, reproduction and hatching success of copepod eggs (Lacoste *et al.,* 2001; Koski *et al.,* 2006). Selection of suitable feed based on their nutritional quality such as PUFA is vital to copepod fitness because food chemical compositions do influence the production of eggs and somatic growth of copepods (Koski *et al.,* 1998; Augustin & Boersma, 2006; Daase *et al*., 2011).

Protein is an important macronutrient for copepods as protein serves as a metabolic reserve in copepods (Perumal *et al.,* 2009). The composition of amino acids in feed may also affect the development and production of copepods (Kleppel *et al.,* 1998). For instance, they found the egg production of copepods depends on the amino acid

compostion of diet (Guisande *et al.,* 2002). Another factor that may be the reason affecting the survival of copepods is the physiological characteristics of the feed. That could be the reason why microalgae *Nannochloropsis oculata* was not a choice for *P. annandalei* may due to their physiological characteristics of cell wall containing cellulose, making them difficult to be digested (Puello-Cruz *et al*., 2009). This was also reported in previous study by Payne & Rippingale (2000) that *Nannochloropsis* resulted in low survival of calanoid copepod *Gladioferens imparipes* for similar reasons. The hard cell wall structure does not allow copepods to ingest and digest efficiently.

The result of sex ratio was significantly influenced by feed where POME-grown AL, mixed diets of POME-grown SA: POME-grown AL and POME-grown RM had the closest equal 1:1 ratio of male to female adults compared to other feeds that were female biased. It is a common occurrence for a skewed-sex ratio in favour of adult female both in the wild populations and in cultures (Gusmao & McKinnon, 2009). Interestingly, determination of copepods sex ratio could be influenced by food (Gusmao & McKinnon, 2009). Subramoniam (2017) reported that good quality of diet could influence sex change in copepods. This result is in accordance with previous report by Carotenuto *et al.* (2011) where in laboratory conditions calanoid copepod, *Temora stylifera* showed remarkable variations in sex ratios when cultivated with different microalgae species as feed. Meanwhile, feed also affects the body size of *P. annandalei.* This is probably because copepods gain essential nutrients from the feed that could meet their requirements thus facilitates the growth.

5.2 Effect of feed ratio

Combination two or more diets are much better than mono-specific diet to fulfill the requirements for growth, survival, and production of copepods (Puello-Cruz *et al.,* 2009). A mixed feed of POME-grown SA and POME-grown AL achieved higher survival value

and higher nauplii production due to their good nutrient contents i.e. DHA and ARA. The correct feed ratio is another factor that could influence the fecundity of copepods, total number of nauplii production and ovigerous female (Ohs *et al.,* 2010). The feed ratio of POME-grown SA (3): POME-grown AL (1) gave the highest survival of nauplii which was significantly higher ($p < 0.05$) compared to other feed ratios tested.

This is supported by Santhanam & Perumal (2012) who stated that zooplankton fed with a mixture of good quality feed rich in nutritive value would result in high biomass. This is because food is one of the factors that could support the good growth and density of copepod (Santhanam & Perumal, 2012). It be concluded that dietary variety is a key to the achieving of a nutritionally complete ration (Kleppel, 1993).

Feed ratio did not affect the sex ratio of *P. annandalei*. Similarly, there were also no variations detected for body length of *P. annandalei* among the feed ratio treatments tested.

5.3 Effect of abiotic factors on survival and reproduction of *P. annandalei*

5.3.1 Salinity

Salinity is one of the key factors that influences the spawning, survival rate, growth and reproduction of copepod (Chen *et al.,* 2006). Low salinity could affect the growth of copepods by delaying their development times. This suppression of growth rate observed in lower salinity (15-20% salinity) is caused by a physiological stress due to additional osmoregulation and respiration (Santhanam & Perumal, 2012).

The study done by Chen *et al.* (2006) reported that *P. annandalei* could not tolerate high salinity ranges such as 30 and 35 ppt where it would reduce the survival rate and reproduction of copepods due to osmotic stress on long temporal scale. Karlsson *et al.,*

(2018) explained that copepods need more energy for osmoregulation thus less energy allocated for development and egg production. Based on the literature, *P. annandalei* is an estuarine copepod that may adapt to low salinity conditions and could live in salinity ranges between 5 and 20 ppt (Chen *et al.,* 2006). Despite it is contradicted with the recent study where *P. annandalei* population still survived in 35 ppt salinity and not significantly different with other treatments tested. This is probably because it could adapt to a wide salinity range.

Salinity does influence the fecundity and naupliar survival rate where the result showed that the production of nauplii were significantly different ($p < 0.05$) between the treatments. The highest number of nauplii production recorded at 15 ppt was consistent with the result obtained by Chen *et al.* (2006) who investigated the effect of salinity on *P. annandalei*. Salinity ranges between 5 ppt to 30 ppt were reported tolerable for eggs hatching and the survival of nauplii but eggs could not hatch when it was more than 30 ppt. This explains why 35 ppt treatment in this study have the lowest nauplii production during the experimental period. Even though the survival result of salinity in this study is not statistically significant between all the treatments but the highest number of nauplii produced was at 15 ppt indicating it was the suitable salinity for culturing.

The sex ratio of *P. annandalei* was not influenced by the different ranges of salinities (Figure 4.6). In contrast to our result, sex ratio has been found to be influenced by salinity (Shayegan *et al.,* 2016) where accelerated female adults were obtained with decreasing salinity levels and vice verca. Nevertheless, further information to explain this effect of salinity on sex ratio were limited.

There were no variations detected between the salinity treatments of *P. annandalei* body sizes (Table 4.8). Difference result were reported by Shayegan *et al*. (2016) where

mean lengths in females of *A. tonsa* were affected by salinity. This shows that salinity can be a critical factor in the determination of copepods length in environment characterized by salinity variations (Gaudy & Verriopoulus, 2004). As a conclusion, it appears that structural response of aquatic invertebrates to salinity variations are based ultimately upon differences in metabolism which affect relative growth of body parts (Kinne, 1964, 1971).

5.3.2 Temperature

Temperature is another factor that affect the survival, mortality, maturation time, molting rate, fecundity and nauplii production of copepod (Rhyne *et al.,* 2009; Li *et al.,* 2009). It affects metabolism level and oxygen consumption of an animal where increase in consumption of metabolism and oxygen during high temperature would leading to excessive use of protein and a low survival rate (Li *et al.,* 2009). This may explain the reason for decreasing survival rate at temperature above 30˚C on Day 14.

Temperature is a crucial component that influence the survival of copepod from early nauplii to adult under optimal range of 24 - 30˚C (Rhyne *et al.,* (2009). Results indicated that temperature did significantly affect the mean survival of *P. annandalei* ($p < 0.05$) with the highest survival rate at 26˚C. These are in accordance with previous study done by Rhyne *et al.* (2009) on other *Pseudodiaptomus* species namely *P. pelagicus* where temperature between 26^{o}C to 30^{o}C is the optimal range to achieve high survival and high nauplii production which is 80 to 90 number of nauplii.

The production of nauplii also declined after the temperature exceed 30˚C due to thermal stress effects. This may be due to energy being allocated toward survival processes and not to the reproduction process (Rhyne *et al.,* 2009). The production of nauplii on the last day of experiment was statistically significant where 28˚C gave the

highest nauplii production. This is consistent with the reasons mentioned above where the optimal temperature to produce highest nauplii production is between 26 to 30˚C. Therefore, the population generation time can be adjusted by manipulating the culture temperature within a suitable temperature range. It has been observed in this study that lower temperature could slowing the maturation rate and growth while higher temperature would shorten maturation rate and the reproduction cycle.

Temperature affected sex ratio of *P. annandalei* (Figure 4.7). It showed that the population ratio of male to female cultured in treatments of 28˚C and 32˚C were male skewed whereas almost 1:1 ratio was observed in the rest of other treatments which consistent with the finding by Rhyne *et al.* (2009). The possible reason for high male ratio is copepods tend to produce extra males during the favourable temperature environments in order to maximize reproduction (Katona, 1970).

There were no variations detected between the salinity treatments of *P. annandalei* body sizes in length and width (Table 4.9). This result was contrast with the finding by Lee *et al.* (2003) where temperature had effect on the total body length of adult stage copepods. Total body length decreased with increasing temperature. The reason is because high amount of assimilated energy was used on metabolic activities and reproduction (Zakaria *et al*., 2018). Thus, producing early mature individuals at a smaller size. It can be summarized that, the differences between the result obtained may due to the difference in environmental parameter of the experiment.

5.3.3 pH

Changes of pH could give an impact to the survival of copepods and other type of zooplankton. Thus understanding the effect of pH on their survival would help to select the robust species able to resist large fluctuations in environmental parameters (Hansen

et al., 2017). The tolerance towards low and high pH by marine copepods may differ within species to some extent. For example, as reported by Hansen *et al.* (2017), *Eurytemora affinis* could tolerate high pH up to 9.51 whereby the egg hatching and nauplii survival were not affected at all.

Li *et al.* (2008) suggested that high pH value may cause the aquatic environment to become toxic due to release of some chemical compounds that in turn negatively affect the survival, health and feeding of marine copepods. Pedersen and Hansen (2003) reported that marine copepods incubated in pH 9 and 9.5 could not survive in that condition for more than five and one day, respectively, thus resulted in decrease of biomass compared to pH range from 8 to 9 where the biomass showed increment. This was consistent with finding by Li *et al.* (2008) where copepods were affected to the high pH and stopped feeding when the pH value reaches 9.5.

Other than high level of pH, low pH value also could cause various effects to biochemical and physiological processes of aquatic organism including copepods. One of the factors that lead to water acidification either in nature environment or estuaries is dissolved greenhouse gasses, specifically carbon dioxide (CO_2) (Hemraj *et al.,* 2017; Vehmaa *et al.,* 2016). High amount of CO_2 concentration in the atmosphere will dissolves in surface water, changing water carbonate chemistry and increasing dissolved inorganic carbon concentration, thus lowering the pH of water (Caldeira & Weickett, 2003). Some of the impacts that indirectly affecting copepods are difficulty in maintaining body fluid's acid-base equilibrium and negative effects on growth and reproduction of copepods due to increasing in energy reallocation into defence or repair process (Vehmaa *et al.,* 2016; Wang *et al.,* 2018; Almén *et al.,* 2016).

Based on the result obtained, *P. annandalei* could not survive in pH 9 which is consistent with the report of previous literature studies. They were also sensitive to low pH where none of the copepods survived in pH 5 incubation after day six. According to Yamada & Ikeda, (1999), the critical pH level of oceanic zooplankton was 5.0 to 6.7. Critical pH level means the pH value below 5 which mortality increases significantly compared to the control. pH of the culture significantly affects the survival of *P. annandalei* where the highest survival recorded on both day 8 and 14 was at pH 8 treatments.

However, our result on reproduction shows that pH significantly affect the production of nauplii. The control treatment gave the highest nauplii production among all. Overall, it can be concluded that pH range between 7 to 8 can be the best preference for *P. annandalei*'s culture because it is closest to natural pH in the oceanic environment, consistent with the stable pH range of oceanic waters which is between 7.5 and 8.4 (Yamada & Ikeda, 1999). This is supported by Hinga (2002) that pH of marine environment was nearly constant which around (8.0±0.5). Thus, *P. annandalei* could survive and produce better in the pH ranges that is nearest to their neutral environment because they were sensitive to acidic and high alkaline pH water.

Adult sex ratio of *P. annandalei* were not significantly affected by pH (Figure 4.7). All treatments showed 1:1 male to female ratio except for pH 6 treatment that showed more male skewed. For the body sizes of *P. annandalei,* there were no variations detected between the pH treatments (Table 4.9). The effect of pH on sex ratio and body sizes may also be species-specific that could give different result.

5.3.4 Photoperiod

The spatial and seasonal distribution of marine copepods in the environment are influenced by environmental factors such as photoperiod (Noguira *et al.,* 2017). It affects the productivity of the calanoid copepods in terms of increase nauplii growth and production under illumination period of 18L:6D (Camus & Zeng, 2008), increase egg production with 16 to 20 h of photoperiod (Peck & Holste, 2006), egg development times and egg hatching rates (16.5 h of light exposure) (Peck *et al.* 2008).

Light-dark cycles could influence the biological process of copepod where longer illumination period would increase the metabolic rate of copepod and accelerate their population development time (Noguira *et al.,* 2017). However, 24 hour constant light condition have negative effects on copepods due to higher metabolic rate needed to sustain their biological process that lead to depletion of energy reserves (Noguira *et al.* 2017).

Our result shows the survival and reproduction of *P. annadalei* were not affected by photoperiod. It did not have a significant effect on *P. annadalei*'s population growth ($p > 0.05$). Although not statistically different, the highest mean survival was found in treatment 12L:12D (day 8; 78.7±1.3% and day 14; 82±11.4%) followed by treatment 0D:24L and the lowest mean survival was at treatment 24D:0L. Treatment 12L:12D is considered the best treatment for *P. annadalei,* similar to findings agreed by Støttrup (2003) where the most favourable photoperiod range for calanoid copepods is at least 12 hr of lights which mimics natural lighting of environment. Manipulating photoperiod in aquaculture hatcheries could optimize the productivity of copepods.

5.3.5 Light Intensity

Light intentsity have significant effect on survival of *P. annadalei* with ($p < 0.05$) where the highest mean survival found in 4.05 µmol/m^2/sec and treatment followed by 20.2 µmol/m^2/sec and 42.9 µmol/m^2/sec treatment. One of the reasons control was significant than others could be due to adaptation to the low level of light. Stottrup (2003) mentioned that most researchers applied low levels of light and no particular intensities were setted.

However, reproduction of *P. annadalei* between the treatments are not statistically different. This suggest that egg production and egg hatching were not dependent on the intensity of light. A study done by Hagemann *et al.* (2017) on *A. tonsa* also found that the hatching rate of the eggs produced in intensive culture does not depend on light intensity. Meanwhile other study also observed that the most favourable conditions for population growth, reproduction and copepods development was obtain in lowest light intensity at range of 25 µmol/m^2/sec compared to high intensity of light 130 µmol/m^2/sec (Matias-Peralta *et al.,* 2005). This finding was also supported by Farhadian *et al*. (2014). It can be concluded that light intensity may not be a key factor among all environmental parameters.

5.3.6 The best feed for *P. annandalei* under optimal culture parameters

Under optimal culture parameters, *P. annandalei* best grown with the whole broth of POME-grown SA (3): POME-grown AL (1) compared to other feed types tested i.e. only POME, biomass and no feed. This shows that a mixed diet of POME-grown SA and POME-grown AL could support the growth of copepod better than other feeds such as POME feed. The reason could be due to their nutrient contents of PUFA that fullfil the requirements needed for copepod growth. Besides that, combination of two diets compared to mono-diet is better for copepods development throughout their life cycles.

Xiaoxia *et al.* (2019) suggested that different life stages of copepods may have different nutritional needs and food preferences. As for microbial biomass, high concentration of fine particles in the feed might be affecting the water quality in cultures which could be the reason for low performance of *P. annandalei* survival.

Then, optimal culture parameters also play a vital role for optimum survival and growth of copepods. The observation shows that *P. annandalei* can maintain their growth and survive along the experimental period. Water salinity, temperature and pH conditions are the most important factors affecting their life cycles. Copepods are sensitive to the fluctuation in their environment and this could give negative consequences. Thus, maintaining the optimum abiotic factors is needed to continuously reproduce populations of targeted copepods.

5.4 Nutritional content of feed biomass

5.4.1 Fatty acid and amino acid composition of feed biomass

The main composition of fatty acids detected in POME, POME grown SA and POME grown AL feeds were palmitic and oleic acids. This is because POME is mainly composed of 50% saturated and 40% unsaturated fat which is made up of palmitic acid and oleic acid (Bala *et al.,* 2014). This is also in accordance to the result of proximate composition where lipid content is significantly abundant in POME (Table 4.8). The result of POME proximate composition mostly doubles the value from the composition reported by Habib *et al*. (1997). POME is a good raw material for growing microorganism because it is rich with lipid, protein, ash and carbohydrate (Salihu & Alam, 2012). Microheterotrophs AL and SA could utilize the nutrients supplied from the POME bases for growth.

No PUFA was present in all feed biomass tested except minor DHA content present in POME grown AL feed. It has been reported that AL does naturally produce significant

amount of DHA (Abad & Turon, 2015). The reason of PUFA absence especially DHA, ARA and EPA may be due to small amount of fatty acid content in the feed that make it inadequate for the analysis to detect (Rayner *et al.,* 2015). Besides that, the preparation and duration of storage before sending to analysis may also affect the result.

The composition of non-essential amino acid was slightly higher than essential amino acid in all feeds tested. Feed rich in essential amino acids could be an added value for copepod. Amino acid is essential for early fish larvae feeding because some particular amino acids could not be brokedown from the protein (Rønnestad *et al.,* 1999).

5.5 Nutritional content of *P. annandalei*

Some small amount of PUFA were detected in copepods fed with POME feed and SA (3): AL (1). The amount DHA and EPA in the copepod fed with SA (3): AL (1) were slightly higher than the one fed with POME feed. While as for ARA, the amount in the copepod fed with POME feed was slightly higher than copepod SA (3): AL (1) feed. The presence of fatty acid in the copepod could be influenced by the fatty acid content in feed where copepod may retain the PUFA in their body (Persson & Vrede, 2006). Favourable DHA/EPA ratio value more than 1.4 in *P. annandalei* indicates that it is a good candidate for trophic transfer of these HUFAs (Rayner *et al.,* 2015). The function of DHA is as a growth promoter in fish and for immune system development and also have influence on larval stress resistance (Izquierdo & Koven, 2011).

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Limitations and recommendations of study

Some limitations of the study are the POME grown feed may contain fine particles that could deposited at the bottom of the beaker over time. It may badly ffect water quality of the cultures especially in a small-scale experiment. Thus, frequent changes of water are needed at least every two days to maintain the quality. Other limitation encountered in this study is limited laboratory equipment and apparatus. Sharing of equipment and apparatus during the experiments with other researcher could lead to risk contamination between different species. Thus, careful observation and precaution is needed to maintain axenic cultures.

Then, recommendation suggested for future study is to further optimize the *S. algae* and *A. limacinum* biomass production in POME substrate in order to achieve maximum potential of the feed. Next is to study the effect of *P. annandalei* fed with POME-grown SA and POME-grown AL as a diet for fish larvae. The efficacy of waste-grown copepods to the targeted fish larvae can be evaluated in terms of survival, growth and reproduction.

6.2 Conclusions

It is concluded that biotic and abiotic factors affected the survival, growth, sex ratio and nauplii production of *P. annandalei.* The mixed diet of marine bacteria *Shewanella algae* and marine protist *Auranthichytrium limacinum* grown in POME in a ratio of 75% to 25% [POME-grown SA (3): AL (1)] supported good survival, growth and production of *P. annandalei* compared to other feeds tested. Overall during fourteen days of culture, survival recorded was 71.3±11.6% with 27±1.2 F2 nauplii production. As for abiotic factors, salinity had no effect on the survival of *P. annandalei* but influenced the F2 nauplii production where 103±48.8 nauplii individuals was recorded in 15 ppt culture. Temperature and pH had affected the survival and nauplii production of *P. annandalei*.

However, photoperiod did not affect the survival and F2 nauplii production of *P. annandalei.* The survival of *P. annandalei* was influenced by light intentsity but it did not influence F2 nauplii production. Sex ratio of adult male and female and body growth were not influenced by feed ratio, salinity, pH, photoperiod and light intentsity factors. Basically, palmitic acid was the major component for POME (40.7%) and all POME-grown microheterotroph feeds (25-42.5%). Only POME-grown AL contained DHA with the amount of 0.8%. POME-grown SA and POME-grown AL had protein of 47.3% and 34.4, respectively, lipid (13.9% and 21.3%) and carbohydrate (15.4% and 22.4%). PUFA were detected in *P. annandalei* fed with POME-grown SA (3): POME-grown AL (1) where it contained 0.91% of ARA, 4.36% of EPA and 7.21% of DHA. Therefore, the optimum indoor culture conditions for *P. annandalei* are 15±1 ppt salinity, 26-28^{o}C, pH 7-8, 12h light: 12h dark and light intensity of 4.05 μmol/m^{2}/sec. This suggests that microheterotrophs that are grown in POME can be an alternative source of feed to copepods. The provided data could be a useful reference to understand the effects of *P. annandalei* in different abiotic conditions. Further research can be done to maximize population growth and nauplii production for commercial production of copepods.

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