EFFECTS OF CHEMICAL CUES ON THE LARVAL SETTLEMENT, METAMORPHOSIS AND MORTALITY RATE OF TWO SPECIES OF TROPICAL OYSTERS (*Crassostrea iredalei* and *Crassostrea belcheri*)

by

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µm	Micrometer
°C	Degree Celsius
‰	Part per thousand
ppt	Part per thousand
%	Percentage
mL	Milliliter
L	Liter
RM	Ringgit Malaysia
h	Hour
min	Minute
g	Gram
cm	Centimeter
M	Molarity
N	Moles
$gmol^{-1}$	Gram per mol
gmL^{-1}	Gram per milliliter
n	Number of mole
M_1	Initial molarity
M_2	Final molarity
V_1	Initial volume
V_2	Final volume
mg	Milligram
µg	Microgram

mm	Millimeter
R1	Replicate 1
R2	Replicate 2
R3	Replicate 3
SL	Shell length
±	Plus or minus
>	More than
<	Less than

KESAN BAHAN KIMIA KE ATAS PEMENDAPAN, METAMORFOSIS DAN KADAR MORTALITI LARVA DUA SPECIES TIRAM TROPIKA (*Crassostrea iredalei* DAN *Crassostrea belcheri*)

ABSTRAK

Pemendapan dan metamorfosis larva adalah penting untuk pengkulturan tiram tropika di Malaysia. Aruhan yang lebih berkesan terhadap pemendapan dan metamorfosis tiram komersial dengan menggunakan bahan-bahan kimia neuroaktif boleh meningkatkan pengeluaran benih tiram di hatcheri. Walaupun kedua-dua tiram ini telah dikulturkan di Malaysia, tetapi permintaan pasaran terhadap tiram adalah lebih tinggi berbanding dengan pengeluaran. Maka, kajian ini bertujuan untuk mengenalpastikan pengaruhan pemendapan dan metamorfosis larva dengan menggunakan empat jenis bahan kimia neuroaktif iaitu epinephrine (EPI), L-3, 4-dihydroxyphenylalnine (L-DOPA), norepinephrine (NE) and serotonin (5-HT) terhadap dua jenis tiram tropika, *Crassostrea belcheri* dan *C. iredalei*. Larva tiram didedahkan kepada lima kepekatan yang berbeza (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} M) selama 1 jam dan 24 jam. Larva tiram dari induk-induk yang berbeza menunjukkan tindakbalas terhadap bahan-bahan kimia neuroaktif yang berbeza-beza. Keputusan kajian ini menunjukkan bahawa NE pada kepekatan 10^{-7} M memberikan peratusan pemendapan yang tertinggi pada larva *C. belcheri* yang berpanjang cengkerang (SL) 290 µm (11 kali ganda pada 10^{-7} M berbanding dengan kawalan). Kepekatan 10^{-7} M 5-HT berjaya mengaruh dua kali ganda pemendapan dan metamorfosis larva *C. belcheri* bersaiz 300 µm SL. L-DOPA pada kepekatan 10^{-7} M telah mengaruhkan pemendapan dan metamorfosis yang optimum (iaitu 6 kali ganda lebih berbanding

kepekatan bahan kima yang lain) pada *C. iredalei* bersaiz 280 µm. Larva *C. iredalei* bersaiz SL 290 µm yang terdedah kepada 10^{-5} M NE manghasilkan pemendapan dan metamorfosis yang optimum dengan tiga kali ganda berbanding kawalan. Kesemua bahan kimia neuroaktif berkesan meningkatkan pemendapan dan metamorfosis larva kedua-dua tiram tropika pada kepekatan yang lebih rendah (10^{-5}, 10^{-6} atau 10^{-7} M). Tindakbalas ketoksikan tertinggi diperhatikan pada 10^{-3} dan 10^{-4} M L-DOPA, 10^{-3} M NE dan 10^{-3} M 5-HT di mana kematian larva mencapai 100%. Menurut hasil kajian, EPI adalah berkesan mengaruhkan pemendapan dan metamorfosis larva kedua-dua tiram dan pada masa yang sama mempunyai kematian larva yang lebih rendah berbanding dengan bahan kimia neuroaktif yang lain. Hasil kajian ini menunjukkan bahan-bahan kimia neuroaktif mempunyai potensi untuk meningkatkan peratusan pemendapan dan metamorfosis larva *C. belcheri* dan *C. iredalei* di hatcheri dan seterusnya peningkatan penghasilan tiram dalam perkembangan industri pengkulturan tiram di kawasan tropika.

EFFECTS OF CHEMICAL CUES ON THE LARVAL SETTLEMENT, METAMORPHOSIS AND MORTALITY RATE OF TWO SPECIES OF TROPICAL OYSTERS (*Crassostrea iredalei* AND *Crassostrea belcheri*)

ABSTRACT

Larvae settlement and metamorphosis were important for the cultivation of tropical inoysters in Malaysia. Inducing greater degree of larval settlement and metamorphosis of commercial oysters using neuroactive compounds can significantly improved the seed production in hatcheries for these valuable species. Although these oysters are cultivated in Malaysia, the market demand was higher compared to the production. For this purpose, this study was aimed at the laboratory assessment of the larval settlement and metamorphosis of two tropical oysters, Cupped oyster (*Crassostrea belcheri*) and Slipper oyster (*C. iredalei*) using four different neuroactive compounds, namely epinephrine (EPI), L-3,4-dihydroxyphenylalnine (L-DOPA), norepinephrine (NE) and serotonin (5-HT) at five different concentrations (10^{-3}, 10^{-4}, 10^{-5},10^{-6} and 10^{-7} M) for 1 h and 24 h exposure time. Larvae from different broodstock showed different efficiency toward different neuroactive compounds. This results show that NE at 10^{-7} M yielded the highest larval settlement and metamorphosis of *C. belcheri* larvae with 290 µm shell length (SL) (11 folds at 10^{-7} M compared with control). 10^{-7} M 5-HT managed to induce two folds of larval settlement and metamorphosis of *C. belcheri* 300 µm SL. The optimum yield, approximately six folds of settlement and metamorphosis was recorded at in larvae *C. iredalei* with SL 280 µm exposed to 10^{-7} M L-DOPA. *C. iredalei* larvae with 290 µm SL exposed to 10^{-5} M NE yielded the optimum larval settlement and

metamorphosis, which is three folds compared to control. All neruoactive compounds did induce larval settlement and metamorphosis of both tropical oysters at lower concentrations. The highest toxicity responses were observed at L-DOPA at 10^{-3} and 10^{-4} M; 10^{-3} M NE and 5-HT, where the larval mortality reached 100%. Overall results showed EPI had effectively induced larval settlement and had lower larval mortality. The results of this study showed the neuroactive compounds have the potential to increase the percentage of settlement and metamorphosis of *C. belcheri* and *C. iredalei* larvae in hatcheries and thus the increase in production in oyster cultivation in tropical area.

CHAPTER I

INTRODUCTION

1.1 General introduction

Oyster is a bivalve mollusc and that can be found around the world except in the Antarctica and the Arctic region (Carriker and Gaffney, 1996). It is commonly found in the low intertidal to the regions of shallow, sheltered estuaries of the subtidal area. Oyster is one of the prevalent categories of utilized mollusc across the globe, which is an essential protein resource in many countries. Oyster industry has increased 58 % in production by 2004. The top oyster producers, judging from their production estimates are China, Japan, USA and Australia (Markid, 2002). Oysters are typically sold in whole and live, fresh half shell, frozen half shell, frozen meat, canned meat and smoked meat. In Malaysia, oysters has identified as commercial and luxury food where they have the sweet flavour and usually served in half shell form in hotels and restaurants (Nair *et al.,* 1993). As a result of the increase in human population and higher level of human life style, oysters become a luxury food (Nair *et al.,* 1993). Thus, the demand of oysters undoubtedly increased. From the annual statistical report by Department Fisheries Malaysia, the production of tropical oyster in aquaculture sector was decreasing after 2009. This might be due to the insufficient of the oyster seed supplies for the oyster production.

The oyster farmers started to produce the hatchery oyster seed to solve the insufficiency of seed supplies. However, there are some constraints that were found in the process of the development of the oyster in hatchery. The main constraint for the oyster farmer was the delayed of the larval settlement and metamorphosis of the oyster larvae and caused the high mortality. Larval settlementt and larval metamorphosis were defined by different researchers as two different phenomena where but settlement was also defined as attachment and metamorphosis defined as morphogenetic development (Hadfield and Paul, 2001). The definition of settlement process of Pacific oyster (*C. virginica*) in the study by Zimmer-Faust and Tamburri (1994) was when the larvae attached on the substrate by using foot. In this study, the terms of settlement and metamorphosis were used to discuss the metamorphic induction of tropical oyster larvae where the larvae had either attached or unattached on the substrate and lost the ability as free-swimming larvae. The larvae were considered underwent settlement and metamorphosis where they had the same description (settlers, prodissoconch postlarvae, dissoconch postlarvae or juveniles) as mentioned in the study of Baker and Mann (1994).

In most of the invertebrate larvae, the settlement behaviours is reversible with the exception of oyster larvae where oyster larvae will settle permanently once a suitable substrate had been found. Larval settlement occurred where the planktonic larvae descent to the sea bottom. When the larvae developed ciliated velum, they can swim freely in the water column. The free-swimming larvae were indicated by the presence of velum and cilia for locomotion and organ to filter food from water column. When the larvae were ready, they started to search by their foot for suitable substrate and settle.

After the larvae had cemented to substrate, metamorphosis would take place where a layer of new shell would be excreted. However, the description of the larval metamorphosis was not clear. Based on the study by Baker and Mann (1994), the metamorphosis of larvae can be categorized to four different phases, which were settler, prodissoconch postlarvae, dissoconch postlarvae and juvenile (Figure 1.1). The larvae which possess any of the categories which had been described by Baker and Mann (1994) were considered undergoing metamorphosis.

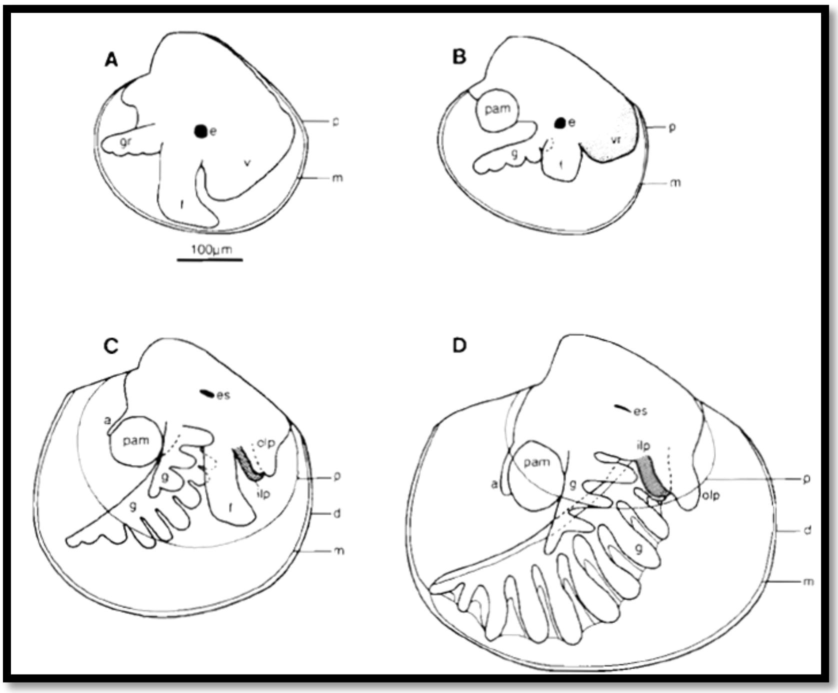

Figure 1.1: Four metamorphosis phases in *Crassostrea virginica* (A) Settler. (B) Prodissoconch postlarvae. (C) Dissoconch postlarvae. (D) Juvenile. a: anus; d: dissoconch edge; e: eyespot; es: eyestreak; f: foot; g: gills; gr: gill rudiments; ipl: inner labial palps; m: mantle edge; olp: outer labial palps; p: prodissoconch edge; pam: posterior adductor muscle; v: velum; vr: velar remnant (modified from Baker and Mann, 1994)

Larval settlement and metamorphosis are the foundation yet critical stage in the whole life cycle in most of the marine invertebrates including oyster. Free-swimming larvae were developed to pediveliger stage where they are actually preparing their transformation to a new mode of life. They are preferentially settled and metamorphosed at the suitable habitat where the habitat has matched the requirement of the adult life. This transformation was essential and dangerous because the larvae will need to adopt a new lifestyle in their life cycle as this might lead to lethal if they failed to undergo settlement and metamorphosed to a new lifestyle. Larval settlement was the most important stage where the larvae search for the suitable substrate or habitat to continue their life in another form. The settlement stage is often regulated by chemical cues of various biological origins and these cues usually bind to an assortment of receptors in the neural tissues of the larvae (Hay, 2008; Mackie and Mitchell, 1981). These stimulations will lead to metamorphosis or transformation in the larvae in terms of behaviours and morphologies. In the span of over forty years, plenty of studies reported that the larval settlement and metamorphosis of marine invertebrate can be affected by several factors for example physical cues such as substrate (Hartati and Suryono, 2000), temperature (Tan and Wong, 1996), salinity and larvae densities (Helm and Bourne, 2004) and food availability (Laing, 1995; Rico-Villa *et al.*, 2009), environmental cues (bottom current or turbulence) and chemical cues (epinephrine (EPI), L-3, 4-dihydroxyphenylalanine (L-DOPA), norepinephrine (NE) and serotonin (5-HT)).

Chemical inducers studies on the oyster settlement began in the mid-century when researchers reported gregarious settlement in several marine sessile invertebrates (Knight-Jones, 1953; Knight-Jones and Crisp, 1953; Knight-Jones and Stevenson, 1950). These findings inspired more researchers to determine how the larvae recognize the presence of the marine surface films after the discovery melanin; this precursor was L-3, 4-dihydroxyphenylaline (L-DOPA) (Weiner *et al.*, 1985).

Subsequently, L-DOPA was evaluated for the effects on the settlement of the Pacific oyster, *C. gigas* (Coon *et al.,* 1986). Of all the chemicals studied, L-DOPA, epinephrine (EPI), norepeniphrine (NE) and serotonin (5-HT) constantly produced an inductive effect on marine invertebrate organisms (Fang *et al.,* 2001; Feng *et al.,* 2006; Gao and Liu, 2006; Liu *et al.,* 2006; Mesías-Gansbiller *et al.,* 2008; Voronezhskaya *et al.,* 2008).

However, some larvae would not undergo settlement and metamorphosis in some test experiment which reflects a lacking of some positive cues or factors that prohibited the induction of larval settlement and metamorphosis. This may be due to the failure of the larvae to detect the positive cues or the cues might be toxic towards the larvae (Hadfield and Paul, 2001). In addition, current constraint on oyster culture in Malaysia is the insufficient and inconsistent seeds supply, so improvement of larval settlement and metamorphosis is an important aim. Although numerous studies of the effectiveness of chemicals on larvae settlement been conducted, the studies of chemicals that are capable to induce the larval settlement of tropical oysters in Malaysia are very limited.

Therefore, this thesis aims to increase the understanding of the larval settlement process induced by various chemical stimuli in difference concentrations with different exposure timing. By doing so, valuable insights into the biochemistry of the cultured oysters in Malaysia may be gained. Based on the results, comprehensive discussions on the effectiveness of the chemical stimuli based on exposure timing and concentrations on the settlement induction of oyster larvae will provide platform for future study and help hatchery increases the chances of settlement on the provided suitable substrate. Small scale experiments of the larval settlement and metamorphosis were conducted under controlled laboratory conditions to determine the relative importance and effects of the neuroactive compounds. The chemical cues affecting larval settlement and metamorphosis were studied in this research and it might bring some light to the induction of larval settlement and metamorphosis in tropical oyster larvae *C. belcheri* and *C. iredalei*.

1.2 Thesis aims and objectives

This dissertation is designed to improve the knowledge of the larval settlement and metamorphosis in two tropical oysters, *Crassostrea belcheri* (Slipper oyster) and *Crassostrea iredalei* (Cupped oyster) by discovering chemical compounds which have the capability to induce settlement.

The tropical oysters, *C. belcheri* and *C. iredalei* were selected as the organisms in this dissertation to examine the larval settlement process due to limited information on the larval settlement and metamorphosis process of these two species by chemical compounds. In addition, these species provide a foundation of profits for Malaysia via aquaculture, and also the prospects for establishing new technologies to enhance oyster production.

The main objectives of this dissertation were:

1. To identify the effects of the four different neuroactive compounds namely Epinephrine (EPI), L-3, 4-dihydroxylalanine (L-DOPA), Norepinephrine (NE), serotonin (5-HT) on larval settlement and metamorphosis of both tropical oysters, *C. belcheri* and *C. iredalei* in Malaysia.

2. To determine the best concentrations and exposure times to induce the optimum settlement and metamorphosis.

3. To investigate the best neuroactive compound to induce the optimum larval settlement and metamorphosis with low larval mortality and low cost.

CHAPTER II:

LITERATURE REVIEW

2.1 The various oyster species in Malaysia

Oysters, mussels, clams and scallops are referred as bivalves. All oysters belong to the Family Ostreaidea. The oysters are normally described based on the shell. For oyster, the two valves are inadequate where the left and right shells are not of equal shape. Oysters can be classified in two different categories which are edible and not edible oysters (Galtsoff, 1964). Edible or commercial oysters are those oysters that can be served as food in either fresh or in dry form such as *Crassostrea* and *Saccostrea*.

Oyster is one of the seafood which is essential and important part of our daily diet. Most of the bivalves have less fat, high protein and easy to digest (Appukuttan, 1998; Helm and Bourne, 2004). Oysters that were collected from the wild were consumed by the coastal inhabitant in the past and now oysters are enjoyed throughout the world in the city or outside the city. In Malaysia, there were several species of oysters identified such as *Crassostrea iredalei* (slipper oyster), *Crassostrea belcheri* (mangrove oyster), *Saccostrea echinata*, *Saccostrea* spp. (rock oyster), *Ostrea folium* and *Hyotissa hyotis* (Figure 2.1) (Devakie and Ali, 2000a, b; Mohamad Yatim, 1993). Among the species found in Malaysia, the most commercially important species are *C. iredalei* and *C. belcheri* that are normally eaten half shell or raw. The slipper oyster (*C. iredalei*) is a commercially potential species, which is preferred for its creamy coloured meat and its sweet flavour (Najiah, *et al.*, 2008). According to Devakie and Ali (2000a, b), *C. iredalei* is the oyster species that can only be found in the Asian regions. In the Philippines, *C. iredalai* is an economically important bivalve and native oyster which is predominantly farmed.

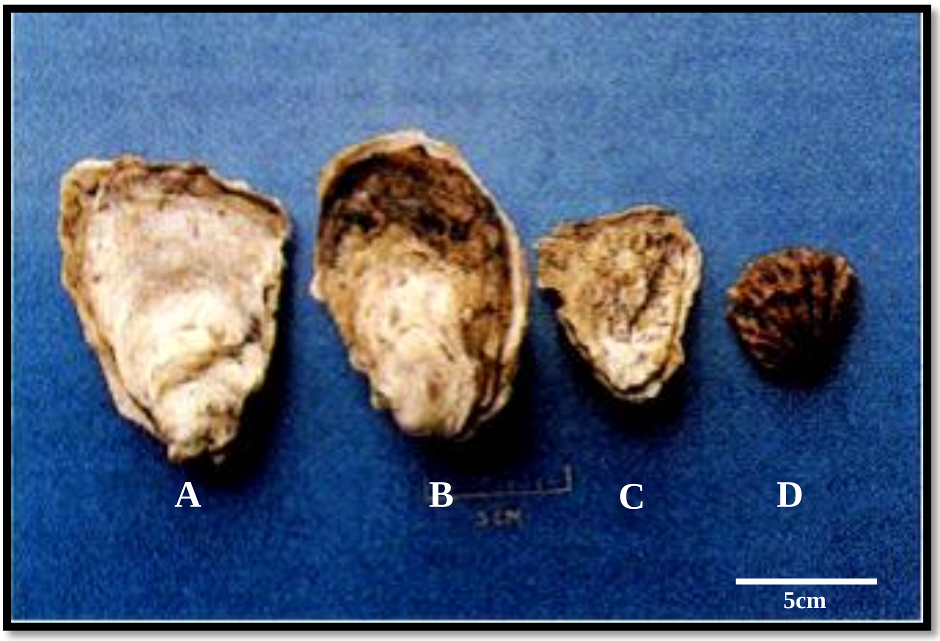

Figure 2.1: The various oysters found in Malaysia, A: *Crassostrea iredalei*; B: *Crassostrea belcheri*; C: *Saccostrea* sp.; and D: *Ostrea folium* (modified from Mohamad Yatim, 1993).

The importance of oysters can be divided into several criteria such as ecological and economical. Oyster reefs provided habitat for other marine life such as barnacles, sea anemones and mussels. Besides providing nursery grounds for the small marine animals, oysters also served as a food source to fishes and other organisms. Since oysters are filtered feeders, they played an important role in the natural habitat where they have the ability to filter the excess nutrient and particles in the water. As an example, oysters in Chesapeake Bay helped to clean up the estuary. According to Sun (2006), depletion of oysters and destruction of oyster reefs in Chesapeake Bay results the ecosystem to become unhealthy due to excess nutrient and turbid. The depletion of oysters was caused by the high demand of oyster for the local market and the extra pollutant deposited to the Chesapeake Bay.

Oysters have economical value since ancient times. It becomes a popular food because of its good taste and rich nutrient such as vitamins, omega'3, protein, carbohydrate and mineral especially zinc. The good taste of the oysters made it a luxury food among the Malaysian especially the Chinese community. In Malaysia, oysters (normally *C. iredalei* and *C. belcheri*) that served in the hotels and restaurants are raw and half-shell (Marked, 2002). In contrast, rock oysters (*S. cucullata*) are served as oyster omelette by hawkers.

2.2 Oyster culture in Malaysia

Oysters were not popular and were less consumed formerly. People started to consume oysters after they realized the value of the oyster and it became the preferred shellfish and staple food in the annual food cycle in Malaysia. Oysters had created a new wealth to the community and brought the economic prospect in Malaysia. High demand of oysters in the market had caused more farmers to engage in collecting oysters and oyster cultivations. Trials of oyster cultivation in Malaysia were started in 1960's with the small scale family business. However, at year 1987, a project entitled 'Oyster culture in Malaysia' has been established through the Fisheries Research Institute in Penang. Figure 2.2 shows that the oyster production in Malaysia had decreased rapidly after 2007 and there was a sharp increase of oyster production in year 2009 and decreased sharply again after year 2009 (Department of Fisheries Malaysia, 2012). The fluctuation of the oyster production might due to the inconsistent and insufficient in seed supply. From the previous study, oyster can be collected from the mangrove roots (Mangrove area), rocks at the mud area and sandy beaches. In Malaysia, the oyster that is collected from the wild is *S. cucullata*, while the oysters used for mariculture are *C. iredalei* and *C. belcheri*.

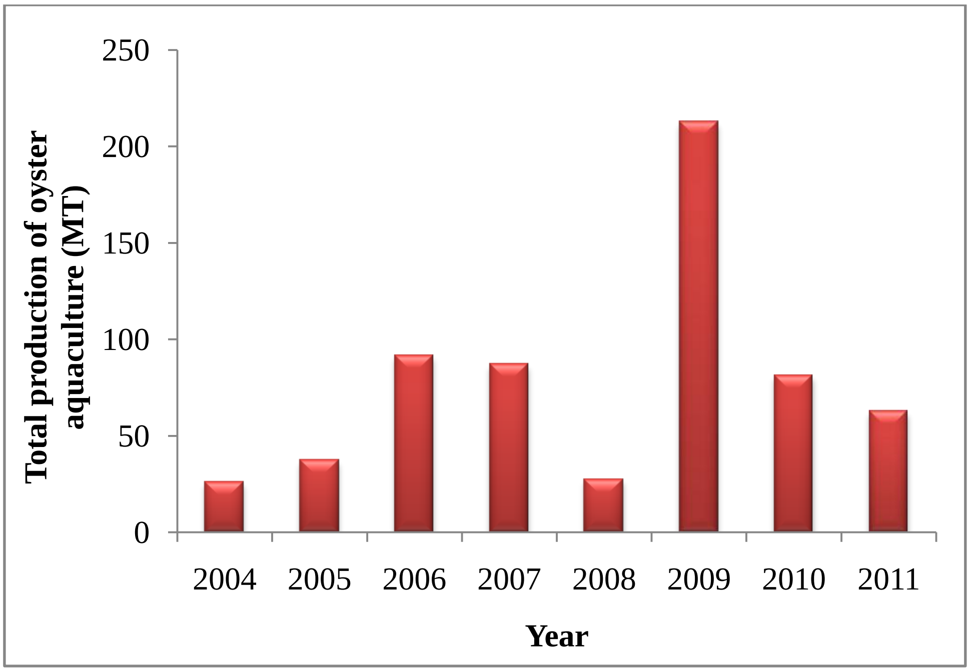

Figure 2.2: Total production of local oysters in Malaysia from year 2004 to year 2011 (modified from Department of Fisheries Malaysia, 2012).

2.2.1 Problems of oyster culturing in natural habitat

Most of the oyster cultivation in the Asian countries such as Thailand, Indonesia, Vietnam and Malaysia depend on natural seeds collected from the intertidal area such as mangrove areas and rocky shores. In Malaysia and most of the Asian countries, oysters were collected from the rocks and mangrove areas and sold to local restaurants (Markid, 2002; Szuster *et al.*, 2008). The number of oysters in natural environment began to decrease due to over-fishing and degradation of natural habitat. Another reason for the depletion of the oysters sources was due to the destruction of mangrove areas for development. Over-harvesting and human had caused the bottom environment of the oyster habitat less suitable for the newly larvae to attach and grow (Devakie and Ali, 2000a, b; Fusetani, 2004; Sudradjat, 1990).

The spawning and spatfall period of oysters were hard to determine and predict in the natural condition (Hartati and Suryono, 2000). Inconsistency of the weather, lack of husbandry, declined of water quality, illegal disposal of sewage and chemical into the river or coastal area were the factors that affect the natural oyster production. For oyster cultivation, the most important factors that affect the taste and the quality of the oysters are the quality of the water and nutrients. In the natural condition, salinity, temperature and sedimentation change constantly with other factors are difficult to monitor. Strong wind and storms will definitely damage the natural oyster farm. Nevertheless, even simple tidal changes will also cause the serious effect to the farm. All these variables in the natural environment are causing the quality and quantity of oysters inconsistent.

Biofouling is also one of the constraints of the oyster farming. The larvae of the barnacles, mussels, sponges and tubeworm would normally attach faster than the oyster larvae (Fusetani, 2004). In addition, some of the larvae of biofoulers such as barnacles, mussels, tubeworms attached on the oyster shells and compete for space and food (Figure 2.3). When there is lack of food and space, the growth of the oyster will be retarded and may cause death to oysters. Besides competing the food and space, some organism laid eggs on the oyster shell and used the shell as a nursery and protection for their juvenile (Goldsborough and Meritt, 2001). This biofouling problem can be solved, but it requires vigorous cleaning process which will increase the labour cost on the investor (Nair *et al.*, 1993). Predators also play an important role in oyster farming. The predators are crabs, whelks, starfish and worms (Beal, 1993; Pauly *et al.*, 1998).

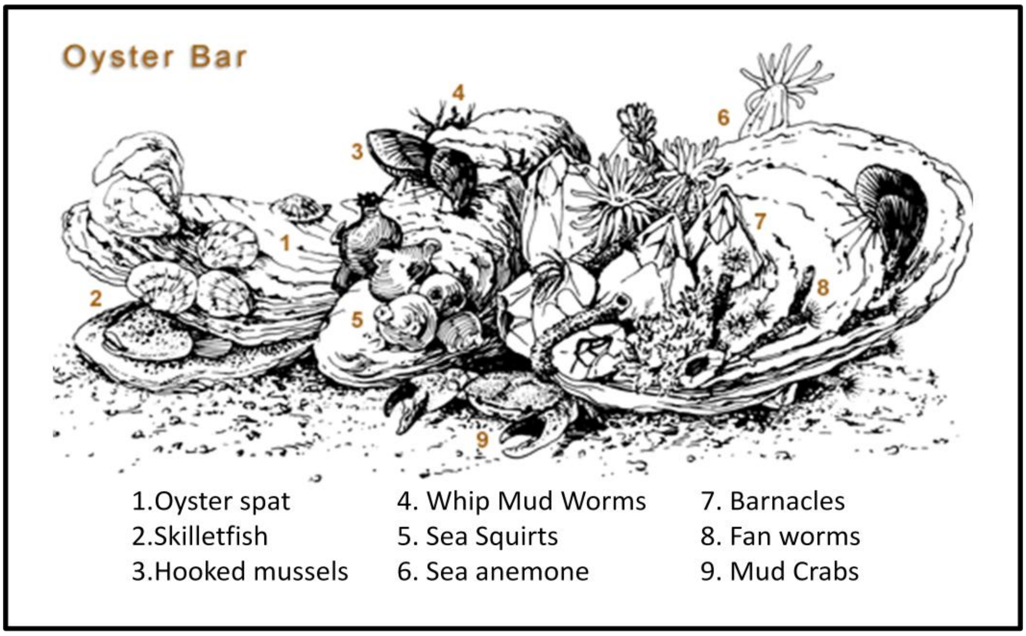

Figure 2.3: Biofoulers found on the adult oyster in natural habitat (modified from Goldsborough and Meritt, 2001).

Natural oysters seem to suffer higher mortality compared to the hatchery produced oyster. This may due to the poor water quality in the natural habitat. Oysters are able to act as bioaccumulator where oysters would take up the heavy metals in the natural environment. Hence, oysters are always exposed to several diseases and pathogens due to illegal dumping and pollution and while filtering the food from the water column, the oyster take up the parasite spores and transmit them to their tissue (Lim *et al.*, 1998; Goldborough and Meritt, 2001). The heavy metal and diseases not only have negative effect on the organism itself but human do not dare to consume because the low quality of the wild oysters. The bacteria pathogen (such as *Vibrio parahaemolyticus*), parasites and biofouling that on or inside of oyster would caused illness to the human who consumed it.

As described above with all the factors affecting the oysters, intensive labour is needed in order to collect the seed from wild. In Malaysia, hatchery is needed to increase the production to support the oyster demand of the local market. However, most of the oyster cultivation still depends on the wild seed to support the oyster industry in Malaysia.

2.2.2 Constraints of oyster hatchery

The main constraint of oyster cultivation in most of the countries including Malaysia is lack of good quality seed. The problems of the oyster in natural habitat can be solved by using the technology in hatchery industry. With the hatchery cultivation system, water quality can be determined and controlled. Sewage and pollution that contributed diseases and bioaccumulation processes can be avoided. In the hatchery, oyster larvae are more secured compared to natural habitat, where the larvae are not easily washed off by the current, less temperature and salinity shock during wet season or dry season. Predators such as crabs and starfish can be prevented in the hatchery. Oyster cultivation need large amount of hatchery produced seeds, therefore the seed production become the most important stage in oyster industry. High technology is needed in order to build the oyster cultured system which local farmers cannot afford the high cost of the hatchery cultivation. Local farmers have limited knowledge in maintaining and managing systems in a hatchery. Those problems can be solved technically but the seeds productions remains a big constraint although oyster seeds can be produced from the hatchery.

The most critical and important problem in the hatchery seed production is the larval settlement and metamorphosis. This stage is the most significant and is the foundation phase in the life cycle of oysters. Delay of the larval settlement and metamorphosis will cause mortality to the larvae and affect the production of oysters in hatchery (Devakie and Ali, 2000a, b). Larval settlement and metamorphosis are the key factor which affects the survival and quality of the oyster seeds

2.3 The life history of oyster larvae

Oysters are a highly prolific organism, where a mature oyster would probably produce approximately 50 to 100 millions of eggs during spawning (Quayle and Newkirk, 1989). The sex of oyster cannot be determined according to their external morphology. Oyster has different genera where all the genera have different spawning mode. There are two categories of sexual habit specifically incubatory (larviparous) and nonincubatory (oviparous). Larviparous oysters such as *Ostrea* have larger eggs and the eggs were fertilized inside the bodies which are in the gill cavity. After fertilization, the larvae are incubated and discharged into the water after they have developed into larvae (Coe, 1938). Whereas, oviparous oysters have smaller eggs and the eggs and sperms are released to the water column and fertilization will occur outside of the oyster body. The oysters belonging to this category are *Crassostrea* and *Saccostrea*. According to Norman (1985), larviparous species can adapt themselves to wider range of environmental parameters compared to oviparous species. Both types of oysters are hermaphrodites where they alternatively change their sexes. For the oviparous species, higher number of the gametes produced and released to the water column to ensure the possibility of fertilization occurred. Once the sperms and eggs are released to the water column, the external fertilization will take place. The eggs that released are usually isolecithal where the amount of nutrient in the egg used as food for developing and grow was low. After the eggs being fertilized, it developed to larvae and later finds a suitable place to settle. Once the oyster larvae settled on the substrate, the larvae would start the adult life (Figure 2.4).

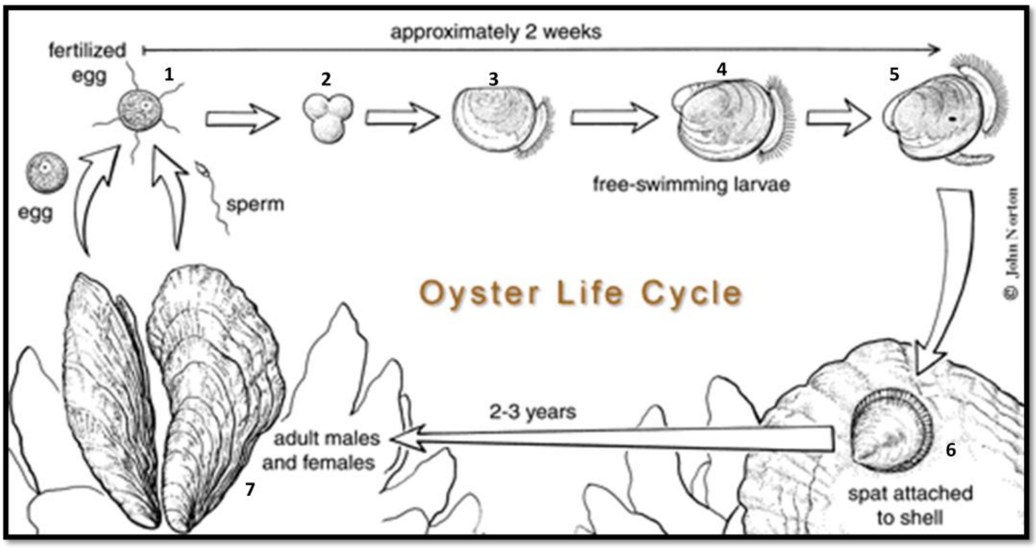

Figure 2.4: The life cycle of oyster. 1: Embryo; 2: Embryo division; 3: Free-swimming veliger; 4: Umbo-veliger; 5: Pediveliger; 6: Spat (Juvenile) and 7: Adult (modified from Goldsborough and Meritt, 2001).

2.4 Larval settlement and metamorphosis

Fertilized egg will undergo a few stages before reaching pediveliger stage. The time consumed from the fertilization stage to the advance pediveliger stage is about 18 days (Wong *et al.,* 1989), depending on species and environment. Oyster, like many marine invertebrates, have a complex life cycle and diverse behaviours which often dictated by environment and biological stimulation (Briffa and Williams, 2006; Engel *et al.,* 2002; Harder and Qian, 2000; Harder *et al.,* 2002; Wikstrom and Pavia, 2004). Adult oysters are sedentary and reef-forming organisms that produced microscopic larvae in planktonic forms (Kennedy, 1996; Thorson, 1964). The morphologies of these larvae are completely different from those of the adults and they may remain in this stage ranging from minutes to months depending on various stimulations like water temperature, food availability and suitable substratum (Coon *et al.*, 1990a; Pechenik, 1984; Kennedy, 1996; Hadfield, 1986; McClintock and Baker, 2001). During this stage, the larvae may drift a great distance inside the water column or being swept by ocean current before finding the suitable substratum and metamorphosing into adulthood (Pawlik, 1992). The benefits for having a dispersed larval stage are less competition for resources with adults, reducing benthic mortality while in planktonic form, decreases in breeding, and decreases the chance of a local wipe-out (Pechenik, 1999). However, prolong of the pelagic stages due to insufficient of the variables, increases the chances of predation and exposure to diseases (Nestlerode *et al.*, 2007; Underwood and Fairweather, 1989).

The cycle of marine larvae which are between a planktonic larval to a sessile condition are critical for the survival of that species (Sukumar and Joseph, 1988). In the natural habitat, free swimming larvae would be dispersed by the wave and current. The planktonic larvae would spend about two weeks in the water column. Larvae were consisted two transparent valves which joined together with a hinge called umbo. Planktonic larvae normally use the velum and cilia to swim. The larvae in the pediveliger stage are also defined as eye-spotted larvae or pediveliger consisting mouth, cilia, eye, foot and velum (Figure 2.5). Towards the end of the pelagic stage, the swimming larvae will eventually settle out of the water column onto the suitable substrate and metamorphose into adult form. This stage of the life cycle of the oyster is defined as the movement from a pelagic environment to a benthic and subsequent attachment to a particular substratum often being species-specific (Pawlik, 1992;

Rodriquez *et al.,* 1993). This process begins when the larvae search for a suitable substrate to set on and this phase is known as settlement. Pediveliger larvae swim through the water in a spiral movement till making contact with a solid object. Upon contact, they will crawl on the substrate surface and locating specific stimuli with their foot (Prytherch, 1934). This process can be reversed if the larvae deem the surface unsuitable and resume their search for the suitable substrate (Scheltema, 1974). The evaluation of the substrate surface by using the foot is termed as settlement behaviour (Bonar *et al.,* 1990; Coon *et al.,* 1986; Coon *et al.,* 1990a; Coon *et al.,* 1990b; Fitt and Coon, 1992; Walch *et al.,* 1999). Once the appropriate substrate has been found, the larvae will cement itself to the substrate using the crystalline cement from its foot and the permanent attachment will be its left valve (Harper, 1992). The newly cemented larvae are now called “spat” and commence metamorphosis eventually losing their larval feeding organ; velum, develop gills, resort the foot and produce adult shell (Kennedy, 1996). Once the larvae attached to the substrate, they would take up the calcium carbonate ($CaCO_3$) in the surrounding to build the shells. If the condition is not being disturbed, the larvae will permanently settle at that substrate and metamorphosis and remain there for its whole life.

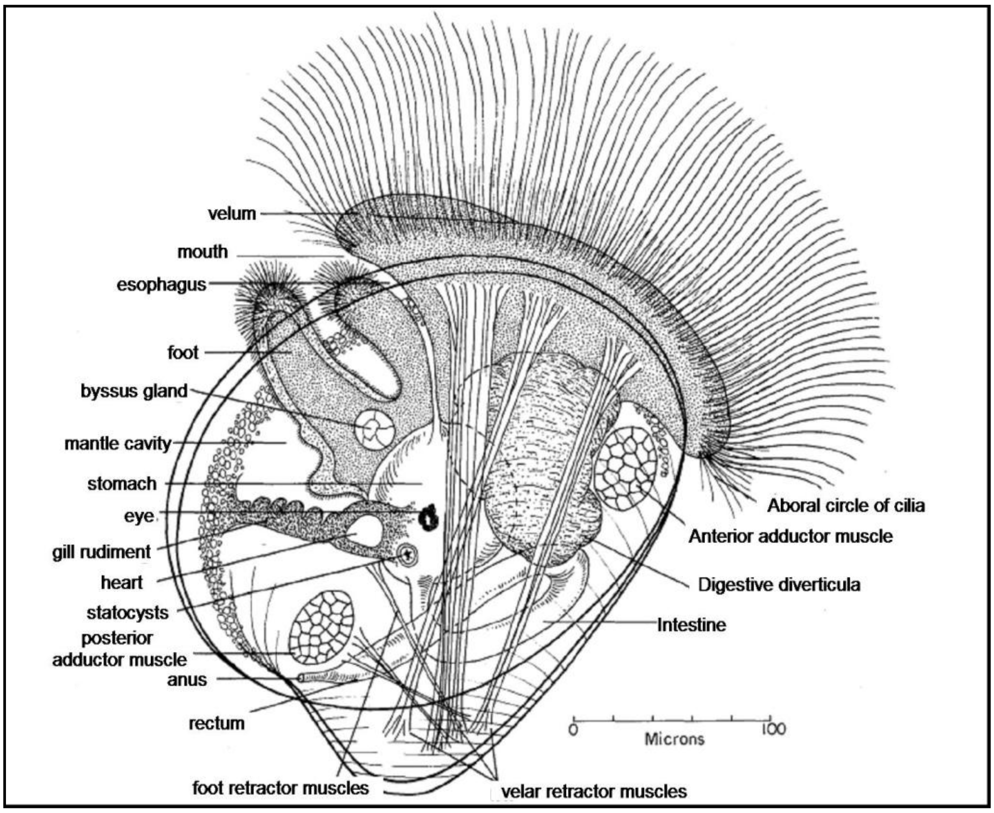

Figure 2.5: Anatomy of the Prodissoconch II (veliconcha) larvae of oyster (*Crassostrea*) viewed from the left side, in the swimming position (modified from Galtsoff, 1964).

The setting process can be initiated after the organism had completed the process of larval development. Figure 2.6 showed the setting process and the path of the larvae from the planktonic larvae until cemented on the substrate. Process of setting usually occurred in continuous steps namely, swimming, searching, crawling and cementation. Based on the study of Prytherch (1934), most of the larvae skipped the searching process and straight sank down to the bottom and started the crawling stage. Crawling larvae secreted the byssus or sticky thread when they contacted and crawling on the substrate and ready for cementation. After cementation, the larvae were sessile and started to metamorphose and development of gills, internal organs and shell. The larvae cemented on the substrate were not easily slough off by current or wave in the natural habitat.

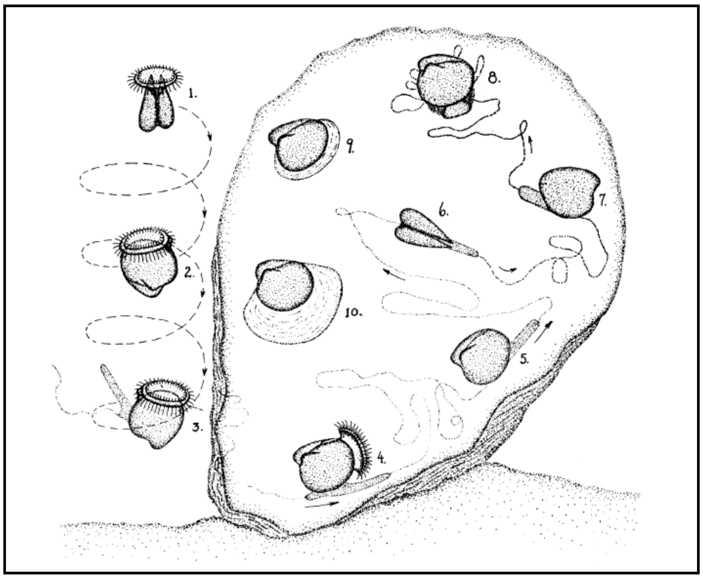

Figure 2.6: The setting process of the oyster larvae on the oyster shell. 1 and 2: swimming larvae; 3 and 4: searching stage; 5, 6 and 7: crawling stage; 8: cemented larvae; 9 and 10: spat (modified from Prytherch, 1934).

Settlement is the initial stage of metamorphosis. Several morphology and physiology changes can be observed when the larvae undergo metamorphosis. For most of the marine larvae, metamorphosis is considered as an important and indispensable process before the larvae reach the juvenile stage (Helm and Bourne, 2004). During the metamorphosis process, the foot and the velum (swim and food intake apparatus for the swimming larvae) would start to disappear; because the foot and velum are no longer needed once the larvae settled on the suitable substratum (Galtsoff, 1964). The changes in the morphology of larvae mark the transition from the swimming mode to a sedentary mode in their life. During the metamorphosis, the internal organ and the shells of the larvae are still growing rapidly. The juvenile oyster which underwent metamorphosis is now known as spat.

Settlement and metamorphosis of bivalve larvae at natural environment are inconsistent. Larvae phase in most of the bivalves will be prolonged if the larvae lack some stimuli or failed to find a suitable substrate to settle. Some of the species will face mortality if the settlement cannot occurred when the larvae was ready. This is because the larvae in pediveliger stage will lose the ability to swim and eat if they miss the chance to undergo settlement and metamorphosis process.

2.5 Factors affecting larval settlement

The settlement of the marine larvae such as oysters, barnacles, clams and scallop larvae were induced by several abiotic and biotic factors. Metamorphosis and settlement process of the bivalve larvae were affected by the intrinsic and extrinsic factors such as the genetic, age of larvae, the food availability, physical and chemical substratum, for example the types of substrates (Devakie and Ali, 2002), texture of the substrates (*C. iredalei*, Devakie and Ali, 2002; pearl oyster, Saucedo *et al*., 2005; *Tetraclita stalactifera*, Skinner and Coutinho, 2005), environmental cues, chemical cues such as epinephrine (EPI), norepinephrenine (NE), serotonin (5-HT), L-3, 4-dihydroxyphenylalanine (L-DOPA) (*C. iredalei*, Devakie and Ali, 2002; *Balanus amphitrite*, Faimali, *et al.,* 2004).

Effects of some of the physical factors on larval settlement of *C. belcheri* and *C. iredalei* were investigated in this experiment. Salinity had been reported to affect larval settlement of many invertebrates, for example: oyster (Dove and O’connor, 2007; Nell and Holliday, 1988; Devakie and Ali, 2000a; Tan and Wong, 1996), barnacles (Dineen and Hines, 1994; Nasrolahi, 2007; Thiyagarajan *et al*., 2003), polychaeta (Ushakova, 2003), clams (Gireesh and Gopinathan, 2004) and, echinoderms (Li *et al.,* 2010; Kashenko, 2006; Roller and Stickle, 1993). Filtered seawater with different salinities was used in this study on both *C. belcheri* and *C. iredalei*. Salinity used to induce the settlement of for larvae *C. belcheri* and *C. iredalei* was different where *C. belcheri* larvae needed a lower salinity compared to *C. iredalei*. The best salinity for *C. belcheri* larvae to undergo larval settlement and metamorphosis was 18 ppt (Tan and Wong, 1996).

Whereas, the salinity used to culture *C. iredalei* larvae was 22 ppt (Devakie and Ali, 2000a; 2002).

2.5.1 Chemical cues

According to Brandt (1999), EPI, L-DOPA and NE are synthesized by a sequence of enzymatic techniques in postganglionic neurons and adrenal medulla of the nervous system from Tyrosine (Figure 2.7). Serotonin is a neurotransmitter which can be synthesized from tryptophan (an amino acid). Some of the neuroactive compounds such as norepinephrine and serotonin have been found in appreciable quantities in the marine invertebrate larvae and the amount of the chemicals in the animal are changing according to the level of the development (Coon and Bonar, 1986; Masseau *et al.,* 2002).

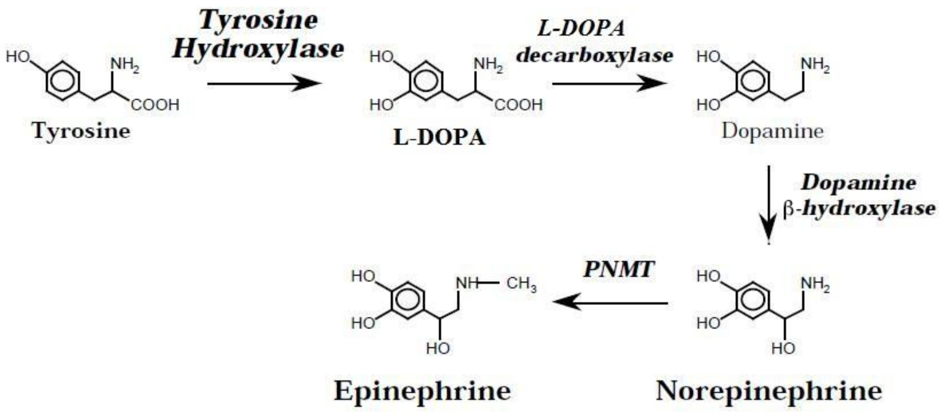

Figure 2.7 Synthesis of EPI, L-DOPA and NE from an amino acid, Tyrosine (modified from Brandt, 1999).