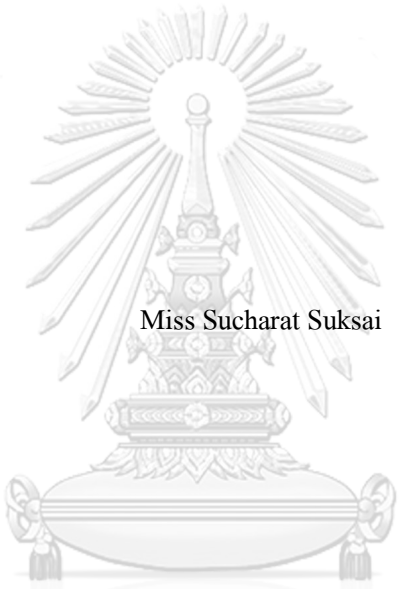
EFFECT OF TEMPERATURE ON ANTIOXIDANT ENZYME ACTIVITIES AND GENE EXPRESSION IN SAND WORM *Perinereis quatrefagesi* (Grube, 1878)

Miss Sucharat Suksai



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ผลของอุณหภูมิต่อแอกทิวิตีของเอนไซม์ต้านอนุมูลอิสระและการแสดงออกของยีนในเพรียงทราย *Perinereis quatrefagesi* (Grube, 1878)

น.ส.สุชารัตน์ สุขใส

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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By	Miss Sucharat Suksai
Field of Study	Marine Science
Thesis Advisor	Supanut Pairohakul, Ph.D.
Thesis Co Advisor	Professor Chanpen Chanchao, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the Faculty of Science

(Professor POLKIT SANGVANICH, Ph.D.)

THESIS COMMITTEE

..... Chairman

(Associate Professor VORANOP VIYAKARN, Ph.D.)

..... Thesis Advisor

(Supanut Pairohakul, Ph.D.)

..... Thesis Co-Advisor

(Professor Chanpen Chanchao, Ph.D.)

..... Examiner

(Jes Kettratad, Ph.D.)

..... External Examiner

(Sumaitt Putchakarn, Ph.D.)

สุชารัตน์ สุขใส : ผลของอุณหภูมิต่อแอกทิวิตีของเอนไซม์ต้านอนุมูลอิสระและการแสดงออกของยีนในเพรียงทราย *Perinereis quatrefagesi* (Grube, 1878). (EFFECT OF TEMPERATURE ON ANTIOXIDANT ENZYME ACTIVITIES AND GENE EXPRESSION IN SAND WORM *Perinereis quatrefagesi* (Grube, 1878)) อ.ที่ปรึกษาหลัก : ดร.ศุภณัฐ ไพโรหกุล, อ.ที่ปรึกษาร่วม : ศ. ดร.จันทร์เพ็ญ จันทร์เจ้า

ไส้เดือนทะเลหรือเพรียงทราย เป็นหนึ่งในสัตว์ที่อาศัยในเขตน้ำขึ้นน้ำลงที่เผชิญกับการแปรผันของสิ่งแวดล้อมบริเวณชายฝั่งตลอดเวลา หนึ่งในปัจจัยสิ่งแวดล้อมที่สำคัญคืออุณหภูมิเนื่องด้วยมีความแปรผันของอุณหภูมิสูงในเขตน้ำขึ้นน้ำลง นอกจากนี้ กิจกรรมมนุษย์ยังส่งผลให้เกิดการเปลี่ยนแปลงของอุณหภูมิน้ำจากการปล่อยน้ำเสียที่มีอุณหภูมิสูงจากบ้านเรือนและโรงงานอุตสาหกรรม อีกทั้ง ด้วยสถานการณ์ภาวะโลกร้อนที่ยังเป็นประเด็นสำคัญ การเพิ่มขึ้นของอุณหภูมิน้ำทะลประมาณ 3°C จึงมีความเป็นไปได้ที่จะเกิดตามการคาดการณ์ การศึกษานี้มีเป้าหมายเพื่อศึกษาผลของอุณหภูมิต่อเพรียงทราย *Perinereis quatrefagesi* ในระดับเซลล์โดยใช้การตอบสนองของ heat shock protein และ oxidative stress เป็นตัวชี้วัดเนื่องจากเป็นการตอบสนองต่อความเครียดขั้นพื้นฐาน กระบวนการทางสรีรวิทยาถูกนำมาใช้ในการแสดงผลกระทบของอุณหภูมิต่อ ในระดับเซลล์โดยใช้การแสดงของยีนด้วยวิธี real-time PCR และแอกทิวิตีของเอนไซม์ด้วย enzyme assay การศึกษานี้ประกอบด้วยสี่ชุดการทดลองของอุณหภูมิ (27 29 31 และ 33°C) สามซ้ำที่ช่วงเวลาต่างกัน (0 วัน 12 ชั่วโมง 1 วัน 3 วัน 7 วัน 14 วัน และ 28 วัน) ผลการศึกษาในส่วนของปัจจัยการเติบโต พบ การเปลี่ยนแปลงอย่างมีนัยสำคัญทางสถิติในน้ำหนักเปียกสุดท้ายที่อุณหภูมิต่างกัน ในขณะที่อัตราการรอดชีวิตและการเติบโตจำเพาะมีรูปแบบเดียวกันที่อุณหภูมิสูงสุดอย่างมีนัยสำคัญทางสถิติ ซึ่งสามารถแสดงให้เห็นอุณหภูมิที่เหมาะสมในการเพาะเลี้ยงเพรียงทรายชนิดนี้ คือ ที่ 29°C ในขณะที่อุณหภูมิ 27°C และ 31°C ยังคงเป็นช่วงอุณหภูมิที่สามารถเพาะเลี้ยงได้เนื่องจากยังคงมีอัตราการรอดสูง ในส่วนของการแสดงออกของยีน การแสดงออกมากที่สุดของยีน *hsp70 hsp90 sod* และ *catalase* ที่อุณหภูมิ 33°C พบการแสดงออกของยีนมากในช่วงเวลา 12 ชั่วโมงแรกของทุกยีนยกเว้น *sod* ที่ไม่สามารถตรวจสอบได้ การเพิ่มขึ้นของ *hsp70* ต่ออุณหภูมิอย่างมีนัยสำคัญทางสถิติในช่วงเวลา 12 ชั่วโมง สำหรับแอกทิวิตีของเอนไซม์ แอกทิวิตีของ SOD แสดงการเพิ่มขึ้นต่ออุณหภูมิในช่วง 12 ชั่วโมงแรกและการลดลงต่ออุณหภูมิใน 12 ชั่วโมงถัดมา พบแอกทิตีสูงของ SOD ในช่วงท้ายของการทดลอง แอกทิวิติของ CAT แสดงการเพิ่มขึ้นอย่างมีนัยสำคัญในช่วง 12 ชั่วโมง โดยภาพรวมการศึกษานี้ แสดงให้เห็นถึงผลกระทบทางด้านลบของอุณหภูมิสูงต่อสัตว์น้ำในแง่ของการลดลงการเติบโตอย่างมีนัยสำคัญและกระตุ้นการตอบสนองต่อความเครียดในระดับเซลล์ผ่าน heat shock protein และเอนไซม์ต้านอนุมูลอิสระ โดยเฉพาะอย่างยิ่งในช่วง 12-24 ชั่วโมงแรก

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Sucharat Suksai : EFFECT OF TEMPERATURE ON ANTIOXIDANT ENZYME ACTIVITIES AND GENE EXPRESSION IN SAND WORM *Perinereis quatrefagesi* (Grube, 1878). Advisor: Supanut Pairohakul, Ph.D. Co-advisor: Prof. Chanpen Chanchao, Ph.D.

Polychaetes is one of intertidal animals that face the fluctuations of coastal environment through time. One of important environmental factors to animals is temperature regarding to the fluctuation of temperature in intertidal environment. Human activities could also cause high changes in water temperature by high temperature discharges from urban and industries. Since global warming is still a main issue, an increase of seawater temperature about 3°C could be possible as a prediction. This study aimed to investigate effect of temperature in polychaetes *Perinereis quatrefagesi* at cellular level, which could be indicated by heat shock response and oxidative stress due to function of basic stress. Responses in the worms at the cellular level were measured using real-time PCR for oxidative stress gene expression and using enzyme assay for protein activities. Different temperatures (27, 29, 31, 33°C) with three replication and different exposure times (0 d, 12 hrs, 1 d, 3 d, 7 d, 14 d, and 28 d) were designed. Results of growth parameters showed a significant change in the final wet weight regarding the different temperature levels. While survival rates and specific growth rates gave a similar pattern with a significant difference at the highest temperature. This growth parameters, therefore, revealed the optimal temperature for culture this species at 29°C, while 27°C and 31°C were also acceptable with regard to the high survival rate. For gene expression, the highest upregulation of *hsp70, hsp90, sod,* and *catalase* expressed at 33°C. High rate of upregulation was found at the first 12 hours on all gene, except *sod* which was non-detectable. An increasing trend of *hsp70* with temperatures was a significant difference at the first 12 hours exposure. For enzyme activity, SOD activity showed an increasing trend at the first 12 hours exposure and a decreasing trend at 12 hours later, while the highest peak of SOD activity was found at the end of the experiment for all treatment. The CAT activity demonstrated a significant rise in the first 12 hours. Overall, this study supports the negative effect of high temperatures on aquatic organism in term of significantly low growth parameter and active stress responses; heat shock proteins and antioxidant enzymes, especially at the first 12-24 hours exposure.

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CHAPTER 1

Introductions

1.1 Rationales, theories, assumption

Marine environments have been influenced from various physical factors, especially temperature. Temperature could be applied to categorize marine ecosystem into thermally stable ecosystem, e.g. Open Ocean; and thermally unstable ecosystem, e.g. intertidal ecosystem and estuary. Thermally unstable environments are environments that have a wide range of temperature fluctuation. Changing in temperature throughout time could affect to ecosystem, for example, species diversity, species distribution, growth, survival, thermal tolerance and physiological state of organisms (Przeslawski et al., 2008). Furthermore, organisms in intertidal ecosystem seems to be vulnerable to changing in temperature because they have lived near their thermal limits already and also have a limit acclimation capacity (Madeira et al., 2012).

Coastal environments could also be influenced from various environmental changes and pollutions from human activities, especially industrial and urban discharges. Since a boiler water treatment has been used in industrial water treatment, high temperature of water has been released to the environment (Roberts et al., 2010). Moreover, global warming scenario had a prediction of increasing water temperature (+3˚C) (Parry et al., 2007). Those temperature influencing factors could impact organisms in thermally unstable environment and organisms that have been applied for wastewater treatment.

Polychaetes are some of macrofauna organisms in intertidal ecosystem that can be utilized in bioremediation for waste water especially in aquaculture system and polluted marine environment regarding to their ability to remove particulate and organic matter from water bodies (Palmer, 2010). Meanwhile, polychaetes are widely cultured due to their commercial values as a live feed and a fish bait (Olive, 1999; Olive and Cowin, 1994). The polychaetes culture are

mostly in an open-farming system which temperatures frequently change regarding to weather and season.

As an intertidal ecosystem, discharge area and open-farming system would undergo the greater changes of temperature, polychaetes, however, be able to survive in this fluctuating environment. The survival of polychaetes under these challenging temperature conditions may indicate the evidence for the thermal tolerant ability of polychaetes; however, the underlying physiological mechanisms of the ability are still limited.

In order to develop a better understanding of polychaete physiology under thermal stress, cellular mechanism investigations could be assessed through biochemical and molecular approaches as a practically convenient tool. Heat shock system and antioxidant enzyme production, which are the fundamentally cellular defense mechanisms of the animals, would be chosen to investigate considering with their early response in cellular mechanism. Changes in both mechanisms via different enzymes or genes could reveal more information in thermally physiological study and may improve the development and application of organism and protein biomarker as a bioindicator.

1.2 Objectives

1.2.1 To determine antioxidant enzyme activities of *P. quatrefagesi* in response to various temperatures

1.2.2 To investigate expression levels of some selected genes in *P. quatrefagesi* in response to various temperatures

1.3 Research Hypotheses

1.3.1 If environmental temperature rises, polychaetes will increase antioxidant enzymes activity.

1.3.2 If temperature changes, an expression of heat shock genes and antioxidant defense gene will be high upregulation in the high temperature.

1.4 Applications

The outcomes and benefits from this recent study provide fundamental knowledges of polychaetes, *P. quatrefagesi* on physiological responses especially in thermal tolerance. In term of aquaculture, the outcomes may be practically be applied to determine thermal ranges for culture this species. This works could be continually developed to represent proteins and genes as a biomarkers for environmental monitoring in the future.

CHAPTER 2

Literature Reviews

2.1 Marine ecosystem

2.1.1 Intertidal zone

Intertidal zone is a shore zone between high and low tide marks. It is considered to be a unique area in marine environment due to regular exposure to air. Organisms in intertidal habitat would face daily exposure differently depending on type of tide. Daily exposure includes being out of water called emersion and being in water called immersion.

Intertidal communities could be categorized by substrates which are rocky shore communities and soft bottom communities. Each type of intertidal communities would have its own challenges for organisms in those habitats. For example, water loss or desiccate is considered to be main challenges for living in rocky shore area, while sediment types have strongly influences in soft bottom area (Castro and Huber, 1997).

2.1.2 Soft bottom communities

Soft bottoms are unstable and shift with ocean motions like waves, tides and currents. Organisms in this habitat would not have a solid attachment; therefore, most of them are infauna. There are two lifestyle of marine organisms: organisms that live on sediment are called epifauna, while organisms that live in sediment are called infauna.

The sediment types would influence distribution and abundance of soft bottom's organisms regarding with water retention and burrowing. Four types of sediment can be classified by grain sizes: gravel, sand, silt and clay (coarse to fine sediment, respectively). Coarse sediment are found in sandy beach due to waves and current effects. Fine sediment are found in bays, lagoons, estuaries or mangroves (Nybakken and Bertness, 2001).

Living in the sediment is quite challenges for organisms due to their facing problems from several factors exposure. Influencing factors could be considered as vertical impacts and horizontal impacts. Vertical impacts consists of physical factors that affect in vertical directions; temperature and oxygen availability. Horizontal impacts consists of physical factors that affect in horizontal direction; waves and currents. Regarding to near land location, transition between freshwater and marine environment should also be considered as it could alter salinity in this habitat.

With those above factors, zonation of soft bottom could be seen only in sandy beach due to beach slopes. The upper beach is inhabited by amphipods, isopods and some crabs. The middle part of beach would have various species of polychaetes, shrimps, mollusks and crabs. The lower beach is subtidal area where could find sea cucumber.

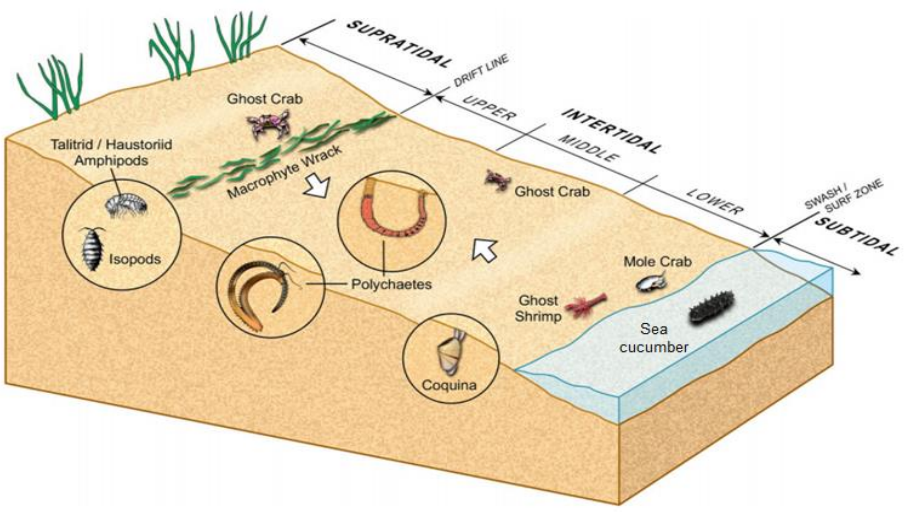


Figure 1 Sandy beach zonation modified from Michel et al. (2017).

As the unique characters of intertidal ecosystem especially soft bottom communities, animals would have changed some physiological state that assist them to live in those kind of environment. Investigations in those animal are needed to fulfill the physiological dimension in the intertidal ecosystem. This explains one of reasons why this study conducted in polychaetes which are inhabited in the intertidal area.

2.2 Environmental stress and physiological responses

2.2.1 Environmental stress and marine environment

Marine environment is all surrounding of living thing in ocean both biotic and abiotic. Marine environment could basically be classified into two levels; macroenvironment, which is an interaction between physical factors and biological factors such as ecosystems, and microenvironment, which is specific for one organism such as habitats. However, the microenvironment is considered to influence environmental physiology of animals. Changes in the microenvironment could result in changes in animals either temporal scale or spatial scale. Although the boundary of microenvironment in organism is unjustified, the activity of organism still undergo in that microenvironment and be part of it eventually. In order to determine the interaction between organism and microenvironment, three factors of environment need to be concerned; the basic stress intensity, the fluctuation in magnitude and time scale, and the energy availability (Willmer et al., 2009).

Environmental stressors are common stress in environment that organism encounters including biotic (other organisms either within or between species) and abiotic factors (physical and chemical parameters). The intensity of stress would depend on habitat and pressure of other organisms. For example, organisms could have a high competition or predation in the stable environment like pelagic zone, while in the extreme environment like hydrothermal vent, the rate of competition or predation could be low. However, both factors on stress intensity lead to negatively effects on animals which require a greater energy to survive. Those negative effects could impact physiological regulation, behavioral changes and competition or defense mechanism in animals.

The fluctuation of stress in timescale may regularly depend on the annual cycle, lunar cycle, tidal cycle, daily cycle and weather, which mostly influence on distribution of organisms. Shoreline and coastal habitats are considered to be representative of the high stress fluctuation in

timescale. While the magnitude of stress fluctuation may link upon size of organism. Smaller animals seem to be highly affected from the microenvironment changes than large animals. Those fluctuations could be used to clarify the tolerance ability of animals.

The energy availability rely on the source of energy which may influence behavior, life-history or morphology of animals. In the extreme environment, the source of food is limit. The animals in the extreme environment normally have low metabolism while in stable environment, the food sources are various which make organism more complex. Bioenergetic and energy budget of the animal in response to the environmental stresses should therefore be concerned.

2.2.2 Physiological Response: Organismic Level

The physiological responses regarding to environmental changes could be considered into different scales; time scales, spatial scales and functional scales (Willmer et al., 2009).

The physiological response to environment in terms of timescale is concerned with the time that animals expose to the changes which may be classified into two levels. Firstly, the short term changes are usually occur within hours, days or weeks. The changes in the physiological state regarding to their energy budget e.g. respiration rate, excretion rate or feeding rates are one of the common responses in the organismic level. Long term effects are also occurred and considered as an evolutionary change that results from natural selection e.g. adaptation of the organisms.

For spatial scales, changes resulting from response to stress could show in tissue-specific (different sites) of organisms such blood, hemolymph, tissues and cells. Since cell has its own compartments, cell composed of intracellular fluid (ICF) and extracellular fluid (ECF). In marine invertebrates, the ECF and blood are similar to seawater. The response sites to stress in marine invertebrates are ECF, cells and within cells. The modification in spatial scale is required in order to response to environmental changes.

In term of functional regulation, the homeostasis of animal is a key factor that keep animals alive. The three strategies play a key part in response to environment of animal; avoider, conformer and regulator. An avoider would have mechanism for running away from environment with contributing to behavior changes or chemotactic response. The conformer would go through those changes which rely on the biochemical and physiological mechanism. While regulator would try to maintain the normal condition as much as possible by changing behavior as a first defense followed by physiological and biochemical adjustments. However, it must be noted that all changes take costs in term of energy and lifestyles.

2.2.3 Physiological Response: Cellular Level

As such those phenomena resulting from environment, protein related mechanism is taken into account as a cellular response. Since the proteins are products of DNA via genetic codes, protein regulation is, therefore, represented responses in the cellular level. In order to react with environmental fluctuation, the changes in protein regulation could be classified into two broad categories as 'coarse adjustment', which is the changes in terms of amount of protein and 'fine adjustment', which is the changes in terms of protein activity.

'Coarse' adjustment of the protein regulation is an adjustment that deal with protein contents via protein synthesis and protein degradation. Mechanism of protein synthesis could be expressed through transcriptional process, translational process and post-translational process. An increasing of all processes in the protein synthesis would, therefore, increase the amount of proteins which is considered as an influx of protein. Contrary to the influx processes, the protein degradation would occurs through mRNA degradation, ubiquitin-proteasome system and lysosomal proteolysis which is considered as efflux of protein contents. The 'coarse' adjustments overview was shown in figure 2.

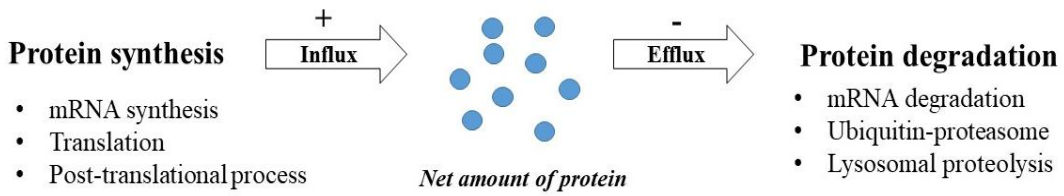


Figure 2 Schematic diagram of coarse adjustment in protein regulation

In order to synthesize proteins in eukaryotes, certain gene in DNA has to be activated and transcription would be then occurred to create mRNA strand using RNA polymerase enzyme. Transcription is controlled by complex protein called gene regulatory protein, which have its own specific site on DNA. There are two types of gene regulatory proteins; activator and suppressor, which cause a positive and negative effect, respectively. Process of activated gene is controlled by three mechanisms including histone methylation, transcriptional factors and enhancers and repressors. Histone is a protein that tightly pack with DNA and form a chromosome in the nucleus. In account for transcription, the chromosome need to be loosed. This is where histone methylation play an important role. Methyl groups are added to histone by histone methyltransferases for giving the access of DNA site to transcriptional factors or enhancers. Transcriptional factors are a protein that bind with specific site on the DNA for controlling expression of genes which could be activated or suppressed. In order to make the transcriptional factor function, this could relate to localization of proteins or signaling pathway. For example, heat shock factor1 (hsf1) relate to signaling pathway of hydrogen peroxide and hsf1 itself is a transcriptional factor of heat shock proteins (hsps), which normally locate in cytosol. The hsf1 would be translocated into the nucleus in order to activate expression of hsps (see more detail in 2.3.3). For enhancers and repressors, they are a non-coding sequence on DNA with activators or suppressors binding site. After binding with activators or suppressors, these complex would likely be fold or loop for binding with promoter to initiate transcription. After encountering the activation process, RNA polymerase enzyme would bind to promotor and transcribed DNA to RNA. Post transcriptional modification would be then occurred. Three mechanisms of RNA

processing are addition of a 5'methyl capping, 3'polyadenylation and RNA splicing. Once the transcribed RNA undergoes those processes, mature mRNA would be given.

Mature mRNAs are then transported out of nucleus to cytosol for translation. The mRNA is coded for amino acids via codon-anticodon system. Translation takes place in a cytosol with two subunits of ribosome, that compose of big and small subunits, and aminoacyl-transfer RNA that are specific amino acid with tRNA. Each amino acid would form polypeptide chains. The polypeptide chains would undergo post-translational modification before becoming mature protein product. Post-translational modification compose of various process depending on the types of proteins. Post-translational processes could be phosphorylation (phosphate-related), glycosylation (carbohydrate-related), lipidation (lipid-related, cleavage peptide bonds (removal of initial methionine), disulfide bond formation (sulfide-related in cysteine) and carbonylation (oxidation of protein that forms carbonyl group). Once the proteins undergo those processes, a structure of protein could occur through protein folding. However, some of post-translational process could cause ROS productions (see 2.3.4.2) in the cell such as protein carbonylation. It should be noted that the related genes could produce the various isoforms of the same protein, which may act differently. Schematic diagram of protein synthesis shown in figure 3.

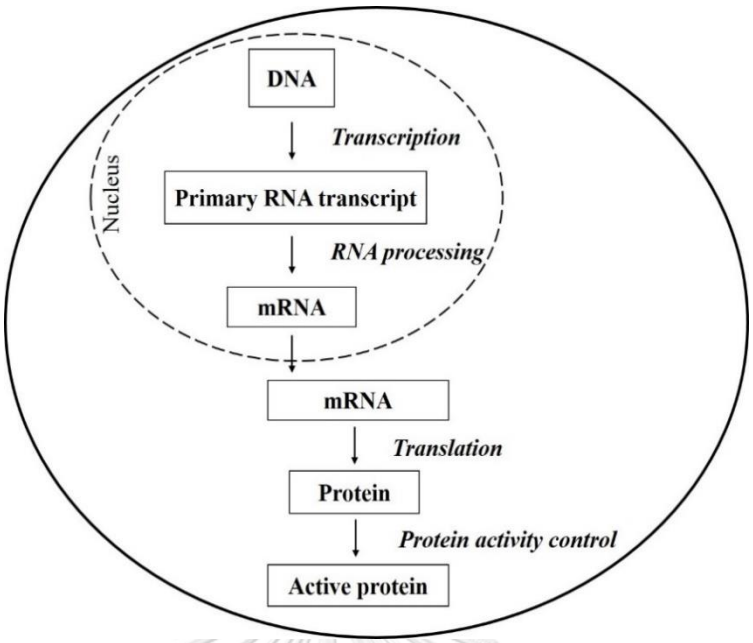


Figure 3 Schematic diagram of protein synthesis modified from Wilmer et al, 2005

However, in order to turn off protein functions, mRNA degradation can occur along with protein degradation processes. Two main pathways are ubiquitin-proteasome and lysosomal proteolysis. Ubiquitin is a small polypeptides that would attach to the proteins as a marker for degradation. The ubiquitin-attached proteins would be then degraded by proteasome which is a protease complex, while the ubiquitin would be reuse again. Lysosome is an organelle inside cell that contains several digest enzymes. The unwanted proteins would be autophagy and taken up into lysosome. The degradation would occur in phagolysosome and is released off cell (Cooper and Hausman, 2004).

For the 'fine' adjustment of the protein regulation, activities of enzymes are controlled by allosteric effects and covalent modification. Small molecules bind onto and change protein conformation called allosteric effects. The allosteric effects may have either negative or positive feedbacks in term of regulation of protein activity along with signaling process.

Covalent modification occurs with enzyme structures and regulate enzyme functions by addition or elimination of some molecules or functional groups. One of the main changes in

functional groups related to the covalent modification is an addition and a removal of phosphate group in protein as also called phosphorylation and phosphate hydrolysis, respectively. Two major enzymes are involved in the processes: protein kinase (phosphorylation) and protein phosphatase (phosphate hydrolysis). Phosphorylation or phosphate removal of protein could activate or inactivate enzyme activity by changing protein conformation or affinity of enzymes. Since signaling process encounter in protein regulation, it should be noted that endocrine system and nervous system may play a part in controlling protein activity.

As the coarse adjustment and the fine adjustment of protein regulation has encountered in cellular level response regarding to environmental stress (schematic diagram of protein regulation shown in figure 4). Both points are necessary to concern in order to investigate the effects of environmental stress as cellular level.

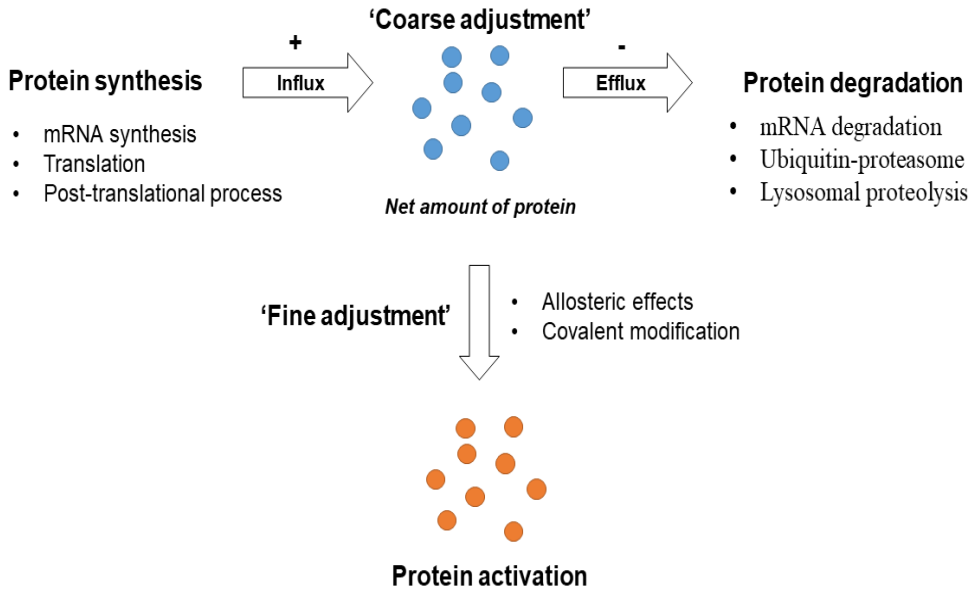


Figure 4 Schematic diagram of protein regulations

2.3 Temperature as an environmental stressor

2.3.1 Temperature stress in marine environment

Temperature is one of the important factors that affect organismic biology. In marine environment, temperature can fluctuate all year rounds depending on geography of that place and seasons. It could be categorized marine ecosystem in term of temperature stability; thermally stable ecosystem (oceanic ecosystem) and thermally unstable ecosystem (intertidal ecosystem). The changes in temperatures are strongly suggested that it had impacted on diversity, abundance, richness in marine ecosystems (Tittensor et al., 2010). Moreover, it also affect species distribution and physiological responses of marine animals (Przeslawski et al., 2008). The temperature in marine environment could also influence on larvae development, which would lead to larval dispersal, growth and survival and thermal tolerance of species (O'Connor et al., 2007).

Organisms have a different ability to adjust their body temperature. The ability to maintain body temperature in organism is thermoregulation. Thermoregulation ability of animals can be classified via different thermal characteristic; source of energy and temperature stability. For temperature stability, body temperature of animals is independent from surrounding environment. These animals can be categorized as homoeothermic organism. In contrast, animals that have vary body temperature are classified as poikilothermic organism. In term of energy source, animals that can create heat to warm their body tend to group into endothermic organisms, while ectothermic organisms use external source of heat instead.

Also, each species is known to have its own specific range of thermal condition. These range could be called as thermal tolerance range which is defined by the upper limit temperature and the lower limit temperature. It is suggested that the range of thermal condition related to the habitat condition throughout year. Intertidal environment is a good representative in thermal study due to their unstable temperatures. According to Madeira et al. (2012), the Critical Thermal Maximum (CT_{max}) was used to clarify the thermal tolerance of intertidal animals. Higher CT_{max}

was found in intertidal and supratidal animals than subtidal or demersal species. This could refer that the intertidal species would be susceptible due to their CT_{max} if the temperature rises. However, there would be the processes in animals that assist them to survive in those kind of environment as mentioned earlier.

2.3.2 Temperature effect on enzyme

Since enzymes are proteins, changes in temperature could alter enzyme performances. However, the modification of protein performances is verified in term of timescale. In order to compensate the change in short term (seconds to hours), hormonal or neuronal mechanism could control the enzyme activity. Those changes include the alterations in enzyme concentration or activity, substrate concentration, and intracellular environment with the high energy requirement. As the longer term (days to weeks), enzyme concentration becomes more significant through protein synthesis and protein degradation. These could be considered as a turnover time of each protein under temperature changes. Finally, the evolutionary changes is the changes in genotype levels which select high suitable protein for thermal range (Willmer et al., 2009).

Apart from mentioned above, temperature changes could influence the function of enzyme in term of enzyme thermostability, which the temperature would have an effect upon enzyme denaturation. Basically, each enzyme has its own optimal temperature. For examples, the optimal temperature of human enzyme is 37°C. The lower temperature would result in low activity of enzyme regarding their kinetics, while the higher temperature would lead to protein denaturation due to breakdown of the weak interactions both intramolecular and intermolecular forces.

2.3.3 Temperature effect on heat shock proteins

Heat shock proteins (Hsps) are a group of conserved proteins that involve in folding of denatured proteins back into their native conformations. Heat shock proteins can be categorized into five families according to their different molecular weights, 15-30 kDa (low molecular

weight HSP), 60 kDa (HSP60), 70 kDa (HSP70), 90 kDa (HSP90) and 100-110 kDa (HSP110) (Lindquist and Craig, 1988). They can be induced by many stresses including temperature changes for minimizing protein aggregations in order to prevent genetic damage as a protective effects (Black and Subjeck, 1990; Fink, 1999).

The regulation of heat shock response has been clarified. Hsps and the heat shock factor (hsf) play an important role as a co-working to unfold proteins. The hsf would active until the hsps occurs. The main hsps are hsp70 and hsp90. After the transcription of hsps, the hsps would also act as a feedback to control the regulation (Hochochka and Somero, 2002) (figure 5).

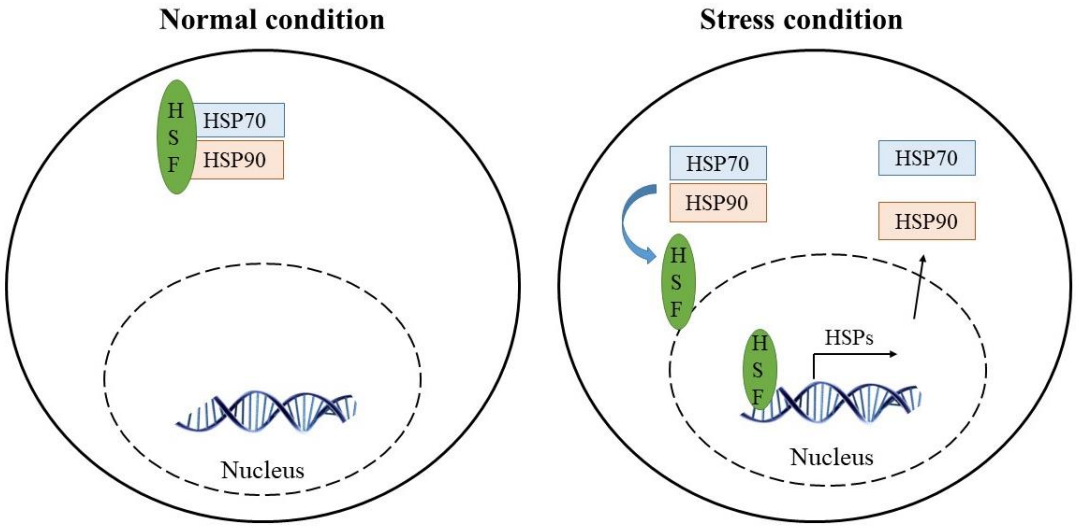


Figure 5 Schematic diagram of heat shock regulation

In this study, hsp70 and hsp90 were chosen to represent the heat shock response as they are abundant and highly conserved in cell.

2.3.4 Temperature effect related to oxidative stress

Temperature changes could induce oxidative challenges as most animals require aerobic metabolism. Since the body temperature of ectotherm related to the ambient temperature, the ectotherm animals are considered to easily suffer from oxidative stress. The oxygen excitation, oxygen solubility and oxygen concentration are main contributing factors that cause reactive

oxygen species (ROS) production. The excited state of oxygen would occur after receiving high energy e.g. heat energy or UV. Meanwhile, the solubility of oxygen is directly related to temperature. In marine environment, the changes in water temperature could impact the solubility of oxygen. The oxygen is less soluble at high temperature and high soluble at low temperature which affect oxygen concentration in the water (Abele et al., 2002).

2.3.4.1 Reactive Oxygen Species (ROS)

ROS are basically a natural byproduct of normal metabolism of oxygen. They play a part in homeostasis and cell signaling. ROS divided into two groups; free radical and non-radicals. The major ROS in organisms are superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$). The oxygen is excited to form singlet oxygen ($^1O_2$). The superoxide anion ($O_2^{\bullet-}$) is then formed via oxygen reduction. The superoxide anion is easily dismutated to hydrogen peroxide. The hydrogen peroxide would undergo the Haber-Weiss Reaction to form hydroxyl radical. In stress conditions, ROS could be increase and lead to cell damages as known as oxidative stress.

Oxidative damages could affected various macromolecules in cells. For lipids, ROS could influence membrane fluidity by alter saturation state of lipid bilayers which cause cell injury. ROS could also cause lipid peroxidation in cells which is a degradation of lipid. Lipid peroxidation in some organelles may lead to cell apoptosis. In term of proteins, ROS is found to have effects upon site of specific amino acid modification and fragmented peptide chains. It could also alter electrical charge and enzyme activity and increase protein degradation. ROS would induce wound in DNA, which may cause the genetic effects such as deleterious and mutation effects. It could damage nitrogenous base, break single-strand and cross-linked proteins (Lesser, 2006).

The ROS in the normal physiological state is considered as a secondary messenger with low level of ROS. This role could results in the expression of various transcriptional factors such

as heat shock factor (hsf), mitogen-activated protein kinase (MAPK) and p53. The ROS also play a part in apoptosis of cell through gene activation (Lesser, 2012).

However, there is a defense mechanism including molecular and enzymatic processes that encounter the environmental changes. In this study, antioxidant defense mechanism will be focused.

2.3.4.2 Antioxidant defense mechanism

The antioxidant defense mechanism could be classified into two categories; enzymatic mechanism and non-enzymatic mechanism. Major enzymes that involved in enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-s-tranferase (GST). Superoxide dismutase can convert superoxide anion into hydrogen peroxide in order to reduce level of free radicals, while hydrogen peroxide is normally produced in cell. Hydrogen peroxide could further break down into hydroxyl radical which is the most reactive of ROS. It could take out electron from polyunsaturated fatty acid and start lipid peroxidation. In order to reduce hydrogen peroxide levels, the catalase and glutathione peroxidase are responsible for changing hydrogen peroxide into water. The schematic diagram of antioxidant defense mechanism shown in figure 6. Meanwhile, major chemicals that involved in the non-enzymatic antioxidants are low molecular weight compound e.g. vitamins (Birben et al., 2012).

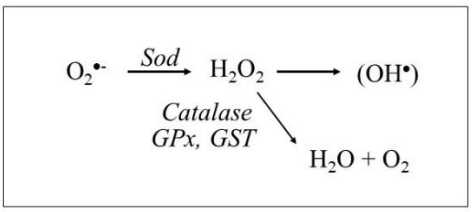


Figure 6 Schematic diagram of ROS and antioxidant enzymes

In this investigation, SOD and CAT were chosen to present the antioxidant defense mechanism as they are easier to function than other enzymes that require co-factors.

Overall, in order to response to temperature via cellular physiological processes, the protein regulation is taken into account. Both the ‘coarse’ and ‘fine’ adjustment of protein should be investigated in term of different points as a representative of a whole picture in protein regulation. Temperature is considered as an important factor in marine environment as it determines thermal window of animal in marine ecosystem. In order to indicate the cellular response to temperatures, heat shock system is concerned as a fast stress response. While the ROS productions could also be induced under the temperature changes. The multi-stress processes would take place at the same time in one animal. The mechanisms that how animal deals with the multi-cellular stresses should be clarified.

2.4 Biology of *Perineris quatrefagesi*

2.4.1 *Perineris quatrefagesi*

The polychaetes, *P. quatrefagesi* is one of the common polychaetes species in Thailand. The general characters of polychaetes composed of three part; prostomium, metastomium and pygidium. The prostomium of this species is a pear-shaped head consisting of mouth, tentacle-like palps, two pair of eyes, a pair of antennae and four pair of tentacular cirri. The metastomium composes of segments with a pair of parapodium for each. The parapodium have notopodium and neuropodium, which consists of setae. The pygidium is the posterior part of body. In order to identify Nereids, the eversible pharynx with paragnaths and the parapodia with setae would be used as the important characters. Also, a number and distribution of paragnath on pharynx and type and distribution of setae could be used for justifying genus and species. The important characters of this species are jaw types, paragnath types and setae types. For this species, jaws are black with 5-7 teeth on cutting edge. There are two kinds of paragnaths in this species: cone and low bars on oral ring and cones on maxillary ring which are found in different area. The first two setigers are uniramous, unlike other setigers that are biramous. Three types of setae can be found in this species: homogomph spinigers, heterogomph spinigers and heterogomph falcigers depending on setigers. It can be distinguished from *P. nuntia* by the length of dorsal cirri and the presence of short tentacle (Hylleberg, 1986). *P. quatrefagesi* is taxonomically classified as shown below with picture of *P. quatrefagesi* (figure 7).

Phylum Annelida

Class Polychaeta

Order Phyllodocida

Suborder Nereiciformia

Family Nereidae

Genus *Perinereis*

Species *P. quatrefagesi*

Figure 7 *P. quatrefagesi* at 5 months old

This species inhabits mostly in sheltered beaches within coarse sand and shells both in Andaman Sea (Phuket) and Gulf of Thailand (Chumpon) with 100 individuals m^{-2} (Hylleberg, 1986). *P. quatrefagesi* feeds mainly on detritus.

2.4.2 Significances of polychaetes

The polychaetes are one of the potential model organisms for environmental and ecotoxicological studies. They are ecologically significant species which play an important part of nutrient cycling between water and sediment (Davey and Watson, 1995). They also have high sensitivity and resistance to some contaminants (Casado-Martínez et al., 2008) and environmental factors (Chapman et al., 1982). The polychaetes are also economically important species. For example, several species of polychaetes have been utilized as food sources for benthic fish and shrimp farms due to their high nutritional values. Moreover, the polychaetes are some of

macrofauna organisms that use in bioremediation for waste water especially in aquaculture system and polluted marine environment (Palmer, 2010). The polychaetes, *Perineris aibuhitensis*, showed high rate of total nitrogen and total phosphorus removal from marine sediment due to their bioturbation and feeding activity (Shen et al., 2016).

2.4.3 Polychaetes and stressors

Since polychaetes dwell in intertidal and estuarine environments, they have to face some challenges due to their naturally fluctuated environments. High degree of temperature fluctuation in environment is thought to be one of possible reasons for the high magnitude of adaptation capacity in animals (Sommer et al., 1997).

In order to response to temperature, the biological organization is adopted to categorize the level of physiological responses. The various suitable indicators would be used to represent the response at each level as reported in previous studies. Growth and metabolism of animals have been used to indicate as organism level. The polychaete, *Neanthes japonica*, showed the significant changes in growth and metabolism due to temperatures (Liu and Xian, 2009; Liu et al., 2009). While the change in biochemical composition and enzyme activities have been indicated as biochemical level. The polychaete, *Nereis diversicolor*, showed a decrease of acetylcholinesterase (AChE) activity regarding the increasing in temperature which represented the higher contamination with higher temperature (Scaps and Borot, 2000). As molecular level, several proteins and genes have been mostly used as indicators for fluctuated temperature conditions such as heat shock system and antioxidant enzyme (Rhee et al., 2012). However, there was few report on temperature effects in antioxidant enzyme activities. The lugworm, *Arenicola marina,* showed the superoxide dismutase (SOD) activities were affected with rising temperatures (Buchner et al., 1996).

CHAPTER 3

Methodology

3.1 Sample preparation

The samples of marine polychaetes, *P. quatrefagesi* (~3 months) were provided from the Coastal Aquaculture Research and Development Reginal Centre 2, Samutsakhon and transported to the Department of Marine Science, Chulalongkorn University. They were maintained and acclimated in aerate seawater about a month at room temperature (29±1°C, salinity 30 psu) and fed 2-5% of body weight with artificial shrimp feeds derived from Siam Priang Farm.

3.2 Culturing system

The 12 plastic trays (0.50 m length, 0.34 m width and 0.19 m depth) were prepared with added PVC drain pipe and valve above the bottom of tray for 1 inch. After setting the trays in the experimental shelf, the running water system was individually built for each treatment. For each set, a PVC pipe size with a diameter of 3-4 inches was used as a water trough. The big PVC pipe was cut into four connected sections; the first three sections were connected to the drain pipe of the tray and the last section was connected to storage plastic tank which was located under the shelf (figure 8A). The water trough was tightly locked to the shelf with wires. In the storage plastic tank, there were filtered fiber mats, shells and submersible pump (1500 L/hr) as shown in figure 8B. The rubber or silicon tubing size 1 inch was joined between the pump and the small trough above the trays in order to pump the water up and release to the trays. The small trough was a PVC pipe size with a diameter of 1 inch at the length around 80-100 cm with the PVC pipe covered at one side. The three holes were drilled with driller in the small trough. The plastic straight tube fitting with controller was put into the hole. The small silicon tubing was then connected to the fitting as an offlet of pumped water to the tray. In order to control temperature, a heater was put in the storage tank of the system for heating water temperature. While the water

temperature was lowered by joined the rubber tubing between the pump, an aquarium chiller and the small trough. The system was run with water and checked for leakage.

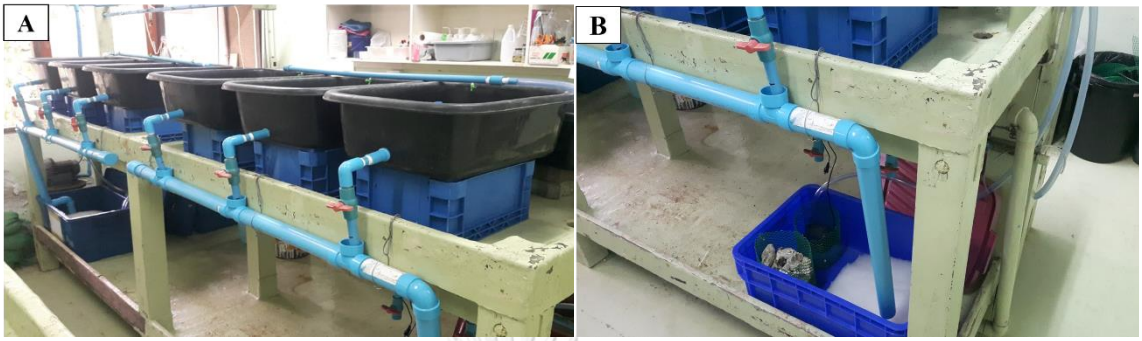


Figure 8 Culturing system A) the overall of the culturing system B) the storage plastic tank

The sand was prepared after setting the running water system by washing with water and exposing to the sun until dry. The washed sand were then put into each tray about 10 cm depth. The seawater with 30-31 psu salinity was filled into the system. The air was provided to each tray and storage tank through the air stone at the end of air tubing which jointed to air pipe from an air pump. The complete system was fully run for a week before the experiment (figure 9).

Figure 9 Culturing system with sand

During the experiment, temperature in each tray was measured with thermometer. New seawater (salinity 30-31 psu) was filled up into the storage tank every two days throughout the period of experiment. The air stone and air tubing was sometimes taken out for cleaning. The left feed and feces were daily taken out using a siphon.

3.3 Experimental design

The experiment was designed as Randomized Completely Block Design (RCBD). There were four different temperature treatments in this experiment (27°C, 29°C, 31°C and 33°C) based on organism's environmental temperature range (27°C, 29°C and 31°C) and high temperature scenario (33°C) with three replications for each treatment (figure 11).

27°C	29°C	31°C	33°C
27°C	29°C	31°C	33°C
27°C	29°C	31°C	33°C

Figure 10 Experimental designs

3.4 Animal culture

The acclimated polychaetes were sorted and put into the tray about 220 individuals (approximately density 1,300 individual per m^2). They were all acclimated for 5 days prior the temperature treatments. Some polychaetes was sampled from the system as the initial samples (baseline samples) for comparing enzyme activities and gene expression analyses. The polychaetes were then exposed to temperature treatment with varies exposure times (12 hrs, 1 d, 3 d, 7 d, 14 d and 28 d). During the experiment, animals were daily checked for mortality rate, and fed 2-5% of body weight twice with artificial shrimp feeds derived from Siam Priang Farm.

3.5 Sample collections

P. quatrefagesi were collected at varies exposure times (12 hrs, 1 d, 3 d, 7 d, 14 d and 28 d) from the culturing system. The polychaetes were picked up after sieving sands out until 12 individuals were sampled per tray. The samples were kept in -80°C for further analysis after weighting them.

3.6 Growth parameters

The samples were weighted at the beginning of the experiment and prior to the collections. This would indicate a growth in the polychaetes. All weight data could represent the pattern of growth in polychaetes. The initial weights and the final weights would be used to calculate specific growth rate (SGR). Specific growth rate (SGR) were calculated as follow:

$$SGR\ (\%d^{-1}) = In\ W_1\text{-}InW_0 / T$$

W_0 is the initial wet weight of polychaetes.

W_1 is final wet weight of polychaetes.

T is the spent time in experiment.

The number of polychaetes were counted after the end of experiment for determining the survival rate of the polychaetes throughout experiment. Survival rate (SGR) were calculated as follow:

$$Survival\ rate\ (\%) = (N_1/N_0) \times 100$$

N_0 is the number of polychaetes at the beginning of experiment.

N_1 is the number of polychaetes at the end.

3.7 Tissue extraction

The samples were individually homogenized in ice cold 0.1 M Tris/HCl, pH 7.4 containing 1% Triton X-100 (see Appendix A) by using pellet pestles. Homogenates were then centrifuged at 14,000 g, for 5 min at 4°C. The supernatant of each sample were collected and stored at -20°C for a short period prior to the experiment.

3.8 Protein determination

The total protein concentration was determined with Bradford method using a 96 well plate. The bovine albumin serum (BSA) was used as a standard. The concentrations of BSA were prepared at 0, 0.10, 0.250, 0.50, 1.00, 1.50, and 2.00 mg/ml. The extraction's buffer was applied as a blank. The protein standards, blank, and the extracted tissue samples were added into separate wells about 5 µl. Then, Bradford Reagent (see Appendix A) were added about 250 µl and mixed in each well. The plate was incubate at room temperature for 5 min. The absorbance was performed at the wavelength of 595 nm with Tecan's Sunrise absorbance microplate reader by Tecan's Magellan^{TM} software. The standard curve was then plotted after obtaining absorbances with the concentration of standard protein as X axis and the optimal density at the wavelength of 595 nm as Y axis. The correlation of standard curve was estimated. The acceptable R^2 of standard was in range 0.9-1.0. The concentration of protein in samples was therefore calculated regarding to the standard curve.

3.9 Superoxide dismutase activity assay

Superoxide dismutase (SOD) activity were determined with Superoxide Dismutase Activity Assay Kit (Catalog number 19160-1KT-F, Lot#BCBV5418, Sigma-aldrich, USA) using a colorimetric method in a 96 well plate. The 50% inhibition of SOD can be detected by measuring the decrease in the color development at the absorbance at 440 nm. The WST working solution was added at amount of 200 µl of each well. There were three blanks in this assay. The Blank #1 consisted of 20 µl ddH_2O and 20 µl Enzyme working solution. The Blank #2 consisted of 20 µl extracted tissue sample and 20 µl Dilution buffer. The Blank #3 consisted of 20 µl ddH_2O and 20 µl Dilution buffer. The extracted tissue sample was added about 20 µl to the WST working solution. The Enzyme working solution was then added about 20 µl into the sample wells. The plate was incubated for 20 min at 37°C. The measurement of SOD activity was then

performed with Tecan's Sunrise absorbance microplate reader by Tecan's Magellan™ software. The obtained data from measurement was calculated as follow:

$$\text{The SOD activity (\% inhibition rate)} = \frac{[(\text{Ablank1}-\text{Ablank3})-(\text{Asample}-\text{Ablank2})]}{(\text{Ablank1}-\text{Ablank3})} \times 100$$

Ablank1 is the absorbance of Blank 1

Ablank2 is the absorbance of Blank 2

Ablank3 is the absorbance of Blank 3

Asample is the absorbance of sample

3.10 Catalase activity assay

Catalase (CAT) activity (EC1.11.1.6) was determined following Regoli et al. (2011). The spectrophotometric analyses were performed at absorbance 240 nm. The 100 mM KPi Buffer (see Appendix A) was added about 980 µl into a quartz cuvette in order to set blank. The 1.2 M H_2O_2 was then added into the cuvette about 10 µl and mixed it well. Before adding extracted tissue sample about 10 µl, the absorbance of 1.2 M H_2O_2 was recorded. Then, the sample was added about 10 µl and mixed vigorously. The absorbance was continually noted every minute after adding the sample. The result was expressed in µ mol H_2O_2 min^{-1} $protein^{-1}$ of wet tissue. The CAT activity was calculated as follow:

$$\text{The CAT activity} = [(\Delta \text{ Abs} / -0.04) \times \text{sample dilution}] / \text{proteins}$$

Δ Abs is the change in absorbance per minute,

-0.04 is the extinction coefficient (mM^{-1} cm^{-1})

Proteins refer to the protein concentration in sample

3.11 mRNA Extraction

The total RNA of polychaetes were extracted by using RNeasy Mini Kit (Catalog number 74104, Qiagen, USA). The individual sample was lysed and homogenized in 600 µl Buffer RLT with pellet pestle. The samples was then centrifuged at maximum speed for 3 min. The supernatant was taken into the new tube about 500 µl. The volume of 500 µl 70% ethanol was added up and mixed well with the supernatant. The sample was immediately transferred to RNeasy Mini Spin column in 2 ml collection tube and centrifuge at 8,000 g for 15 sec. The buffer RW1 was added about 700 µl after discarding the flow through and centrifuged at 8,000 g for 15 sec. The sample was washed with 500 µl Buffer RPE twice by centrifuging at 8,000 g for 15 sec and 20 sec. The RNeasy Mini Spin column was placed into a new collection tube. The RNA free water was then added about 30-50 µl into RNeasy Mini Spin column and centrifuged at 8,000 g for 1 min to elute RNA. The extracted RNA was collected and kept in -20°C.

3.12 Quantitative measurement of RNA

The extracted RNA was measured in 10x dilution using spectrophotometer at the optimal density (O.D.) of 260 and 280 nm (see Appendix B). The acceptable range of O.D. 260/280 was 1.8-2.2. The concentration of RNA was calculated as follow:

$$\text{The concentration of RNA} = O.D._{260} \times \text{coefficient} \times \text{dilution}$$

The coefficient of RNA at $O.D._{260}$ equals 40 µg single-stranded RNA in solution (ml).

3.13 Agarose gel electrophoresis

The agarose gel electrophoresis was performed in order to check bands of the extracted RNA. 0.8% agarose gel was prepared for gel electrophoresis. The sample was mixed with 6X loading dye before loading into gel in 1X TBE buffer at 80 volts for 35-40 min. The gel was then stained in Ethidium bromide for 5 min and destained for 20 min. The RNA bands was visible under the U.V. light (see Appendix B).

3.14 Primer design

Primers for qRT-PCR was designed by IDT's PrimerQuest. In order to design the specific primers, the criteria was applied with the following conditions; T_m in range 55-65°C, GC in range 45-55% and size in range 22-25 nt. While the size of amplicon was considered with the number of nucleotide in each gene (see Appendix B) that obtained from GenBank with different accession numbers. Selected genes and primer sequences with amplicon length and accession number are given in table 1.

Table 1 Primer sequences for qRT-PCR

Gene	**Primer sequence 5'-3' (forward and reverse)**	**Amplicon Length**
Heat shock protein 70 *(hsp70)*	GATGTCCTTCTCGTGGATGT	100
	TGTTAGGGATTCGGGTGTTC	
Heat shock protein 90 *(hsp90)*	CATCAAAGCTGGACGCAAAG	124
	CCCAGGTTGTTCACCATATCA	
Catalase *(cat)*	GTTGGAATCTTCTGGAGAGAGG	92
	TCTTGGGCGTTCCTTTGTAG	
Superoxide dismutase *(sod)*	GGGAAACCAATGCAAAGAT	107
	CCTTTACATTTCCCAGGTCTC	
Actin	AGGTCATCACCATCGGAAAC	87
	GGATACCAGCAGATTCCATACC	

3.15 Real-time Quantitative PCR

Real time PCR was performed using One Step SYBR® PrimeScript™ RT-PCR Kit II (Perfect Real Time) (Catalog number RR086A, Takara, Japan). The specific primers were used in order to synthesize cDNA with reverse transcriptase. The PCR amplification was then performed. One reaction consists of 10 µl 2X One Step SYBR® RT-PCR Buffer IV, 0.8 µl PrimeScript™ 1 step Enzyme Mix II, 5.6 µl RNase Free water, 0.8 µl Forward Primer, 0.8 µl Reverse Primer and 2 µl extracted RNA. The final volume of the reaction was 20 µl. Then, qRT-PCR was performed with the CFX96 Real-Time System - C1000 Thermal Cycler (Bio-Rad) at Department of Botany, Faculty of Science, Chulalongkorn University. Thermocycling was run following conditions: 1 cycle of 42°C for 5 min and 95°C for 10 sec, followed by 40 cycles of 95°C for 10 sec, 52°C or 57°C for 10 sec and 60°C 30 sec. PCR products were subjected to an amplification curve (C_q values) (see Appendix B). Control reaction was conducted with RNase free water to determine levels of background. *Actin* was used as the reference gene. C_q values of each gene were normalized with C_q value of reference gene and then reported as the relative mRNA expression by using $2^{-\Delta\Delta}$ formula (Livak and Schmittgen, 2001). The $2^{-\Delta\Delta}$ formula was calculated as follow:

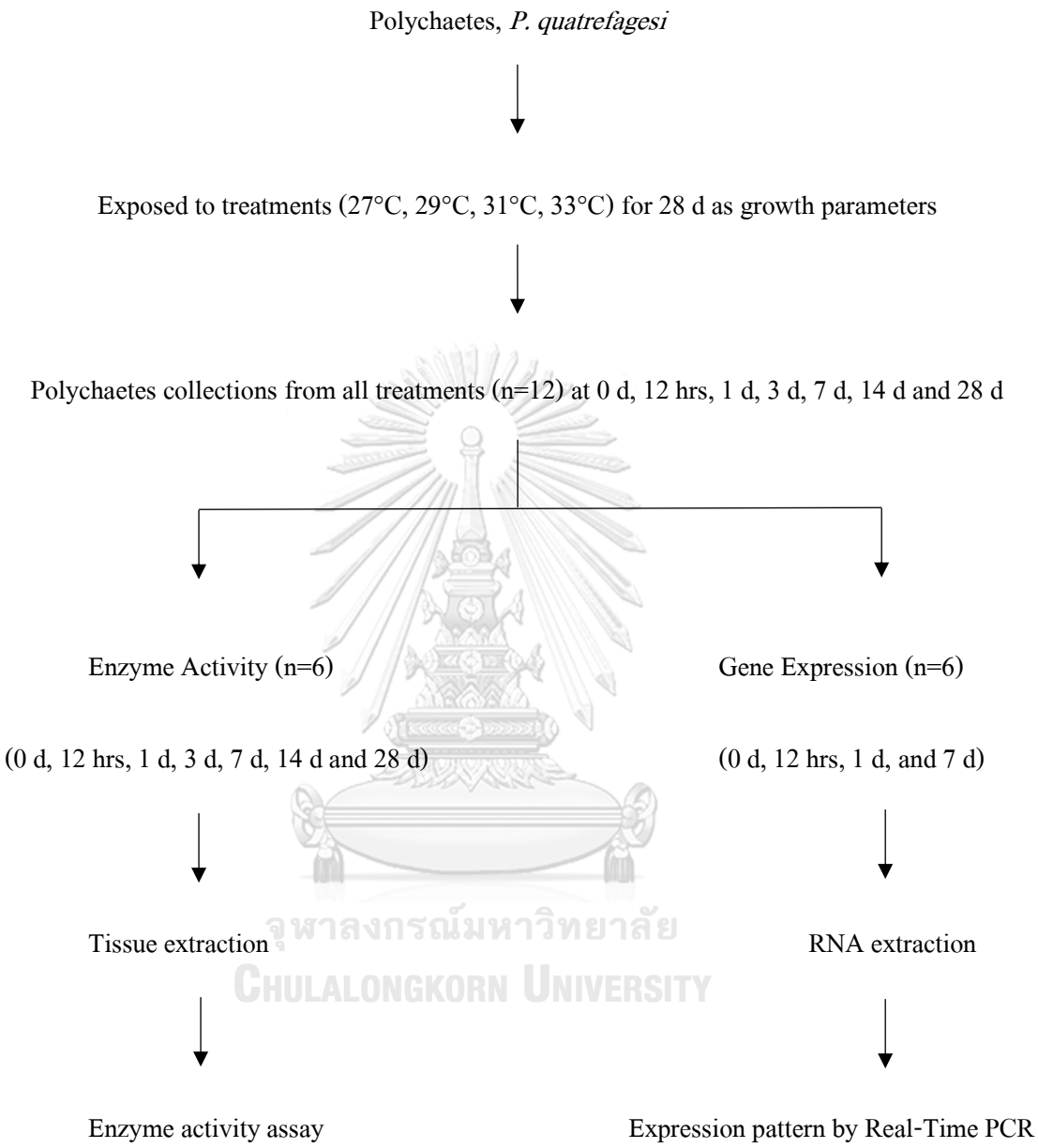
Δ = C_q target gene - C_q reference gene

$\Delta\Delta$ = (C_q target gene - C_q reference gene)$_{tx}$ – (Mean C_q target gene – Mean C_q reference gene)$_{t0}$

3.16 Statistical Analysis

Statistical analyses were performed by One-way ANOVA (Turkey's post hoc test) using SPSS 22.0 in order to compare each variable factors in this experiment with a significant difference between treatment and control at $p<0.05$. Values are presented as means ± SE

Overview of Experiment



CHAPTER 4

Results

4.1 Growth parameter of *P. quatrefagesi*

The initial wet weights of polychaetes *P. quatrefagesi* were within the range 0.07-0.11 g. While other time points also showed no significant difference between the treatments in terms of wet weight as shown in figure 11. At the end of experiment, the final wet weight was significantly affected by temperature ($p \leq 0.05$). The high temperature treatments of the longest exposure time showed the lowest wet weight in 33°C about 0.1256 ± 0.018 g, while in 31°C the wet weight was about 0.2270 ± 0.012 g which was still lower than the other treatments (0.38-0.47 g).

The specific growth rate (SGR) of *P. quatrefagesi* revealed a decrease pattern in response to high temperatures, with the highest growth rate at 29°C (5.53 ± 0.60 %/day) and the lowest growth rate at 33°C (0.23 ± 0.40 %/day). While there were no significantly difference between 27-31°C, except at 33°C ($p \leq 0.05$) as shown in figure 12.

The survival rate of *P. quatrefagesi* in response to different temperatures was also shown the similar trend as in SGR with the highest rate at 29°C and the lowest at 33°C, respectively (figure 13). There were significantly difference only at 33°C ($p \leq 0.05$).

However, it should be noted that the subjected animals were found death at 33°C after two weeks until the end of the experiment (personal's observation).

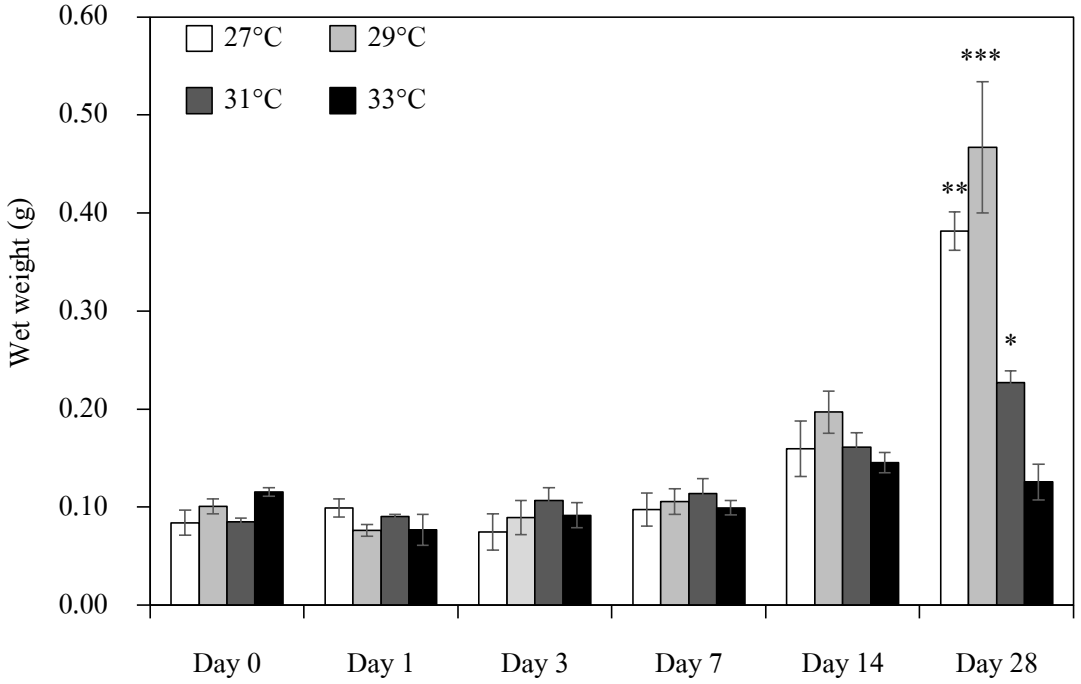


Figure 11 Wet weight of *P. quatrefagesi* under different treatments. White, light gray, dark gray and black bars stand 27°C, 29°C, 31°C and 33°C, respectively. Values are expressed as means ± SE (n=12). Asterisk above the bars represent the level of significance ($p \leq 0.05$).

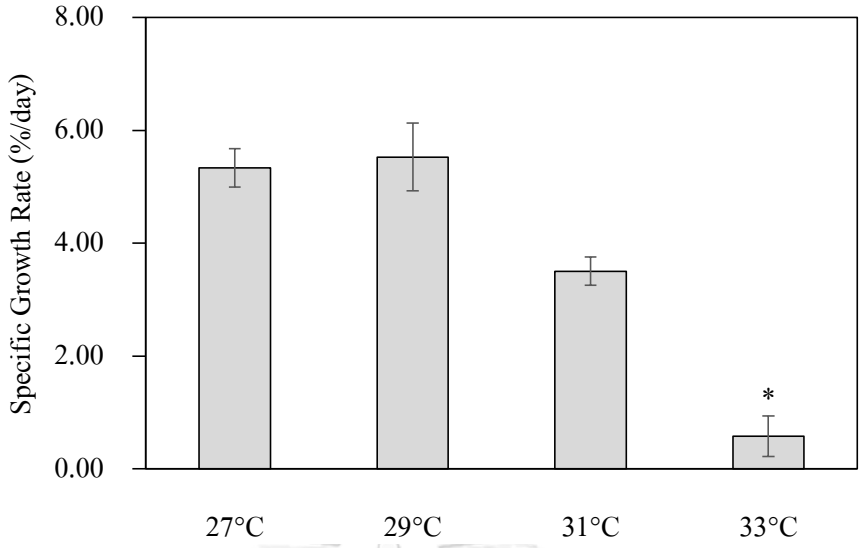


Figure 12 Specific growth rate of *P. quatrefagesi* under different treatments. White, light gray, dark gray and black bars stand 27°C, 29°C, 31°C and 33°C, respectively. Values are expressed as means ± SE (n=12). Asterisk above the bars represent the level of significance ($p \leq 0.05$).

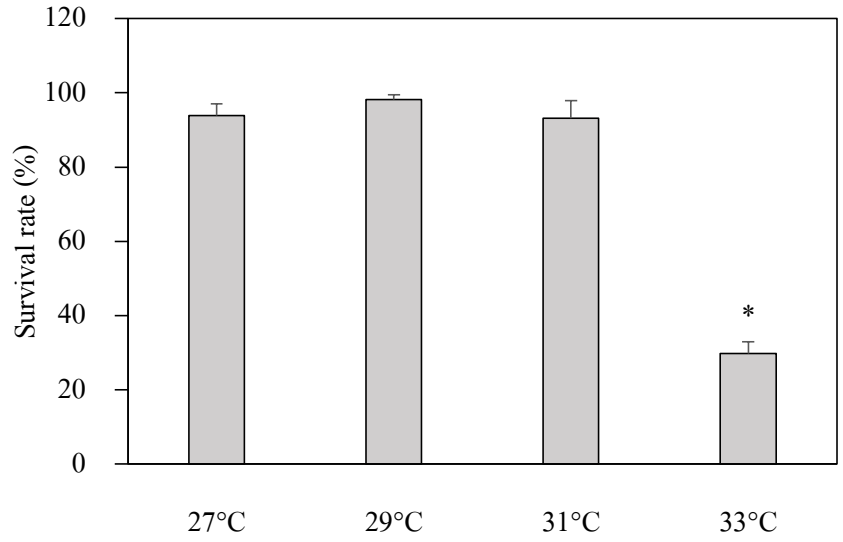


Figure 13 Survival rate of *P. quatrefagesi* under different treatments. White, light gray, dark gray and black bars stand 27°C, 29°C, 31°C and 33°C, respectively. Values are expressed as means ± SE (n=12). Asterisk above the bars represent the level of significance ($p \leq 0.05$).

4.2 Enzyme activity of exposed *P. quatrefagesi* to various temperatures

4.2.1 Total protein concentration

The total protein concentration was varied regarding to treatments through times (figure 14). The minimum total protein concentrations of *P. quatrefagesi* was found in 29°C at day 28 (5.40 ± 0.08 mg/ml), while the maximum total protein concentrations of *P. quatrefagesi* was found in 27°C at day 7 (11.45 ± 0.66 mg/ml). The significant changes of the total protein concentrations ($p \leq 0.05$) were revealed only in day 3 and 14 with the different patterns.

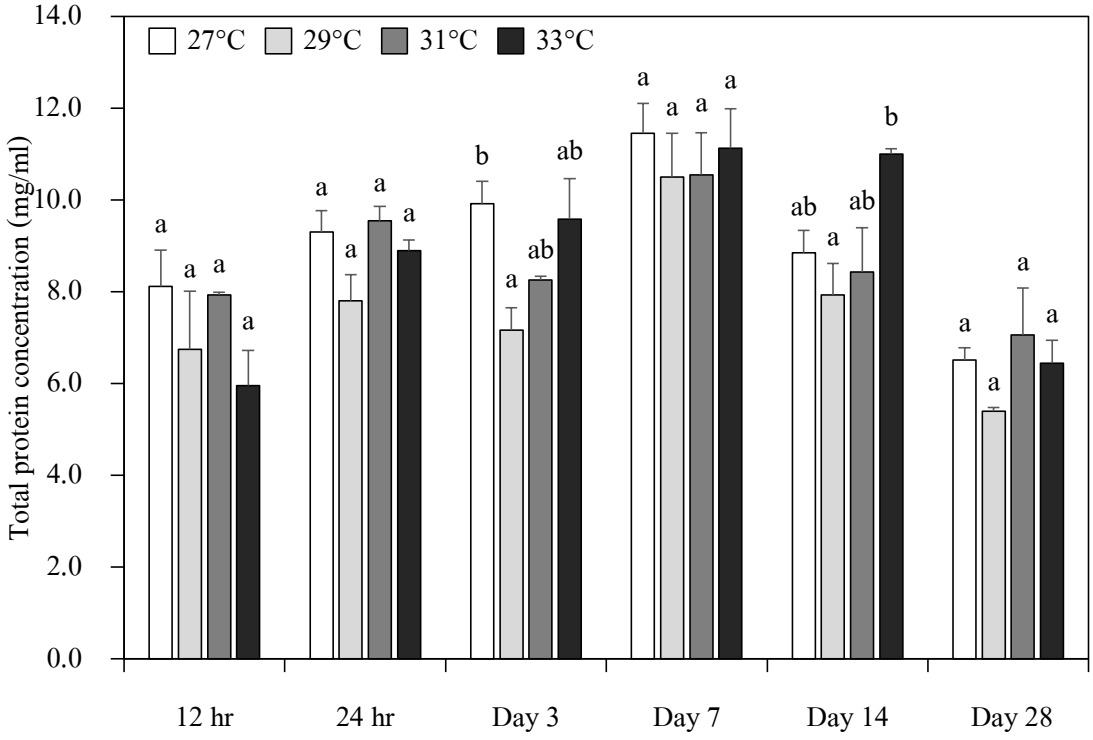


Figure 14 Total protein concentration of *P. quatrefagesi* under different treatments. White, light gray, dark gray and black bars stand 27°C, 29°C, 31°C and 33°C, respectively. Values are expressed as means ± SE (n=3). Letters above the bars represent the level of significance ($p \leq 0.05$).

4.2.2 Superoxide dismutase activity

The superoxide dismutase (SOD) activity was reported in tern of % inhibition rate due to the ability to inhibit 50% of superoxide (figure 15). An increasing pattern of SOD activity was found at the first 12 hours after exposed to temperatures with the highest SOD activity at 33°C (84.47 ± 4.34 % inhibition rate), and a decreasing pattern of SOD activity was then detected at 12 hours later with the lowest SOD activity at 33°C (68.65 ± 2.26 % inhibition rate). The high activity above 90% of SOD activity was demonstrated at the longest exposure time of experiment. However, the significant changes of sod activity were revealed at day 7 and day 14 only with the different trends of sod activity ($p \leq 0.05$).

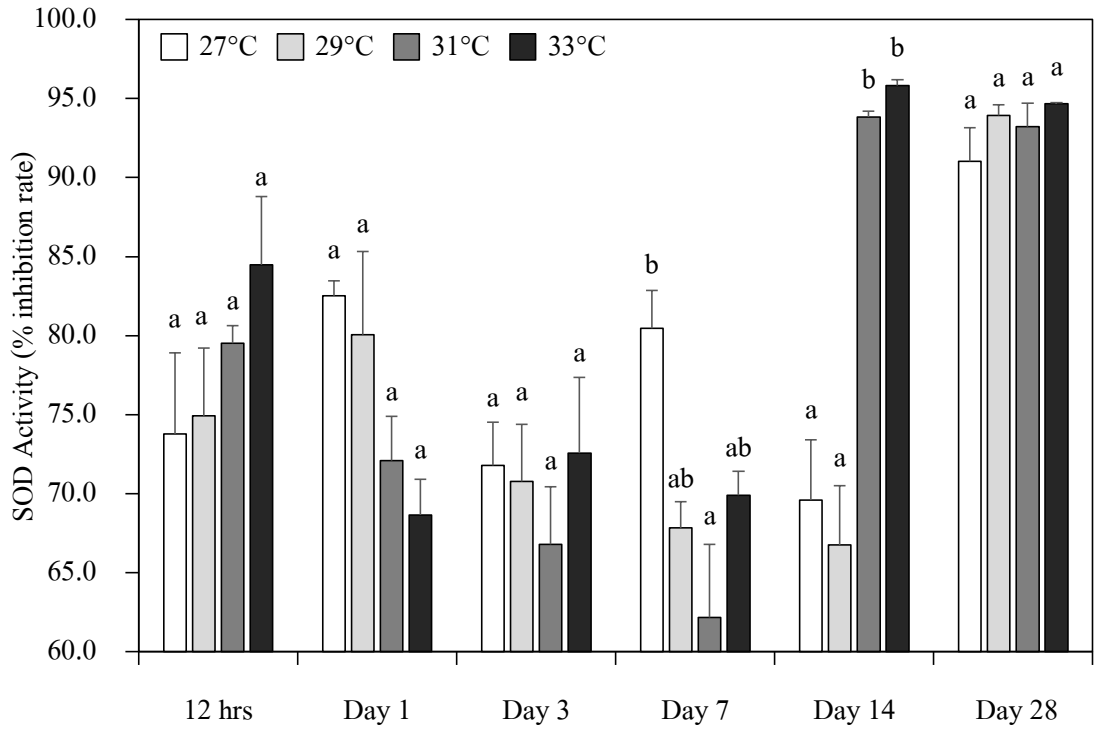


Figure 15 SOD activity of *P. quatrefagesi* under different treatments. White, light gray, dark gray and black bars stand 27°C, 29°C, 31°C and 33°C, respectively. Values are expressed as means ± SE (n=3). Letters above the bars represent the level of significance ($p \leq 0.05$).

4.2.3 Catalase activity

An increasing trend of the catalase activity due to exposed to temperature was obviously revealed at the first 12 hours with significant differences ($p \leq 0.05$) as shown in figure 16. The highest catalase activity was about 0.96 ± 0.09 μ mol min^{-1} mg^{-1} proteins in 33°C at 12 hr. While the other exposure time showed catalase activity in range 0.41-0.63 μ mol min^{-1} mg^{-1} proteins.

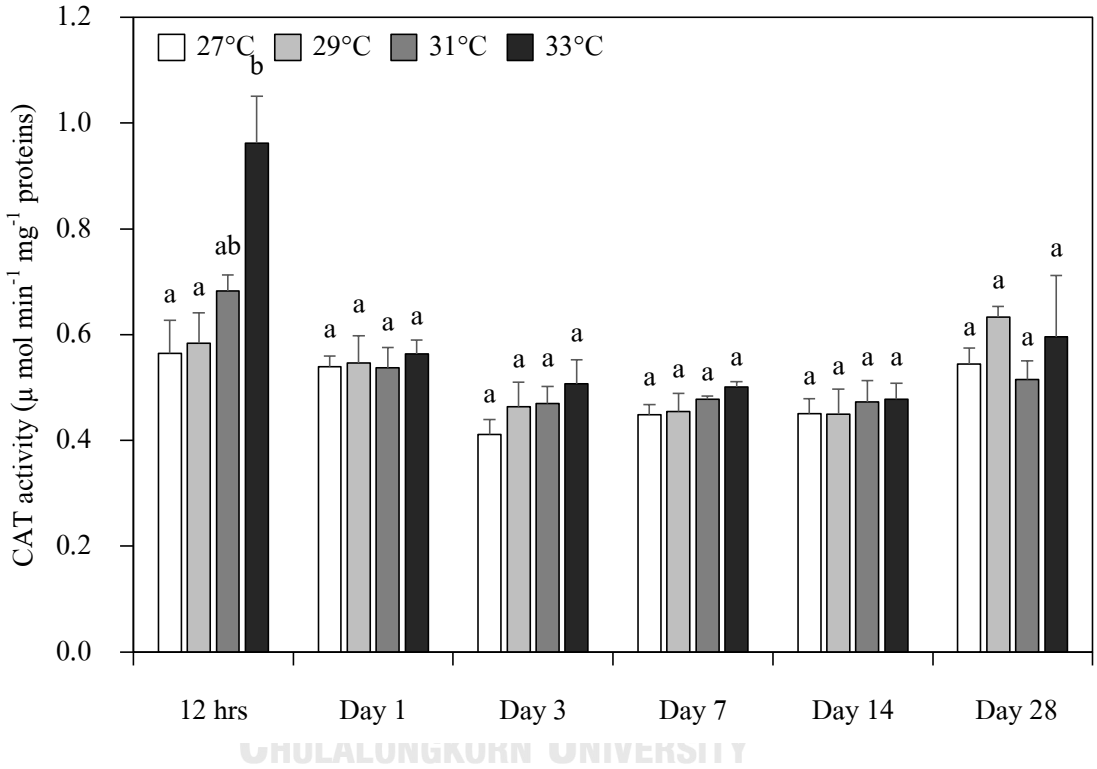


Figure 16 Catalase activity of *P. quatrefagesi* under different treatments. White, light gray, dark gray and black bars stand 27°C, 29°C, 31°C and 33°C, respectively. Values are expressed as means ± SE (n=3). Letters above the bars represent the level of significance ($p \leq 0.05$).

4.3 Gene expression of exposed *P. quatrefagesi* to various temperatures

4.3.1 *Hsp70* expression

The *hsp70* of *P. quatrefagesi* showed a various level of relative mRNA expression (figure 17). The highest expression of *hsp70* was found at 33°C compared with the other exposure times. There was found a significantly increase pattern of *hsp70* at 12 hours exposure ($p \leq 0.05$). At day 1, a slightly increasing of *hsp70* expression was found in 29°C, while other treatments increased the expression about 0.5-1 times. At day 7, there were both the increase and decrease of treatments. The increase of *hsp70* was expressed in 29°C and 33°C, while the decrease of *hsp70* was expressed in 26°C and 32°C.

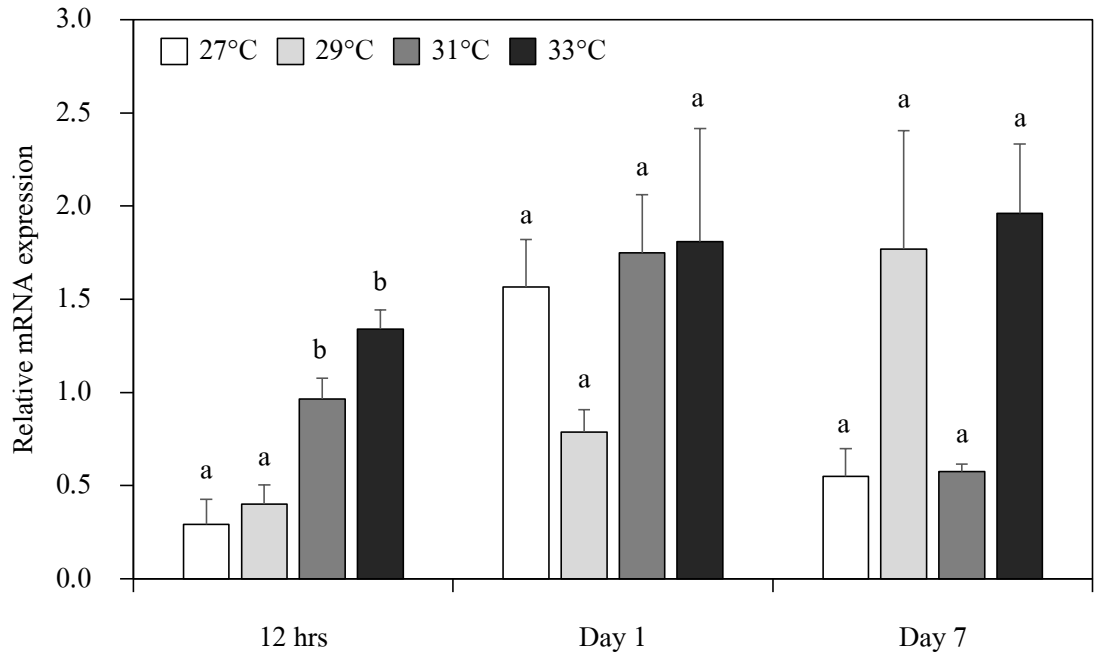


Figure 17 Relative expression of *Hsp70* in *P. quatrefagesi* under different treatments. White, light gray, dark gray and black bars stand 27°C, 29°C, 31°C and 33°C, respectively. Values are expressed as means ± SE (n=3). Letters above the bars represent the level of significance ($p \leq 0.05$).

4.3.2 *Hsp90* expression

The different levels of *hsp90* expression was shown in figure 18 with no significant changes at all treatments. The highest values of *hsp90* expression were given in 33°C of 12 hours and day 1 exposure. There were found a decrease of *hsp90* expression over time in high temperatures (31°C and 33°C), while the other treatments were found in consistent with the *hsp90* expression at all exposure times.

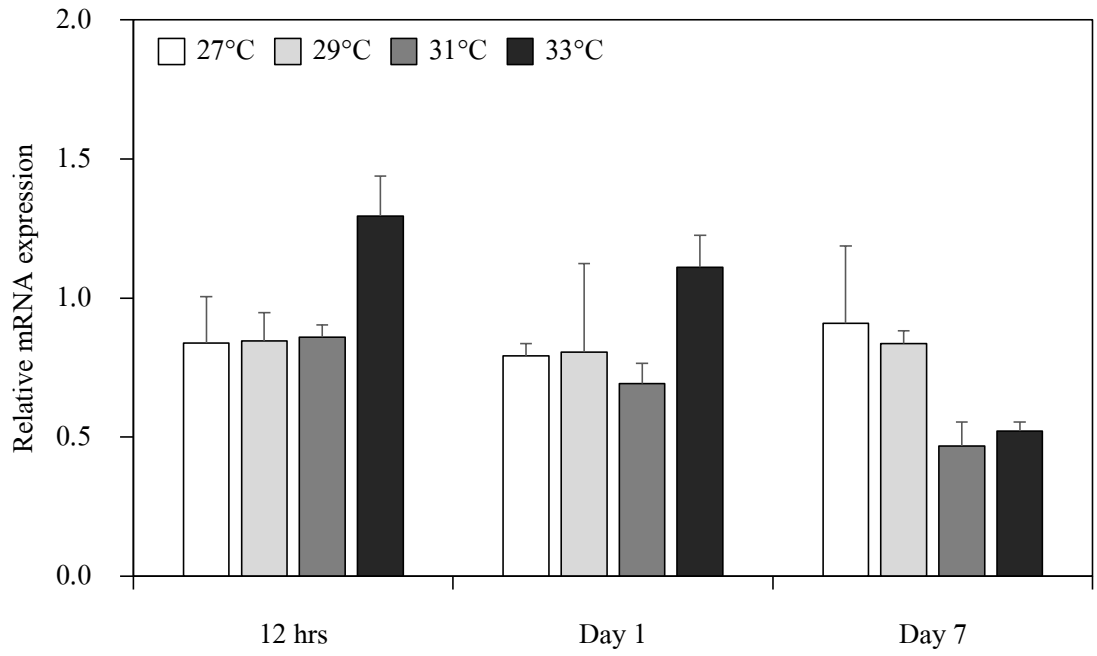


Figure 18 Relative expression of *Hsp90* in *P. quatrefagesi* under different treatments. White, light gray, dark gray and black bars stand 27°C, 29°C, 31°C and 33°C, respectively. Values are expressed as means ± SE (n=3).

4.3.3 *Superoxide dismutase* expression

The expression of *sod* in polychaetes *P. quatrefagesi* was demonstrated in figure 19. The first 12 hours after exposed to temperature, there was no *sod* expression detected. The extremely high expression of *sod* was significantly found later in high temperatures (31°C and 33°C) ($p \leq 0.05$). The significantly upward trend of *sod* expression was revealed at day 7 at 33°C ($p \leq 0.05$).

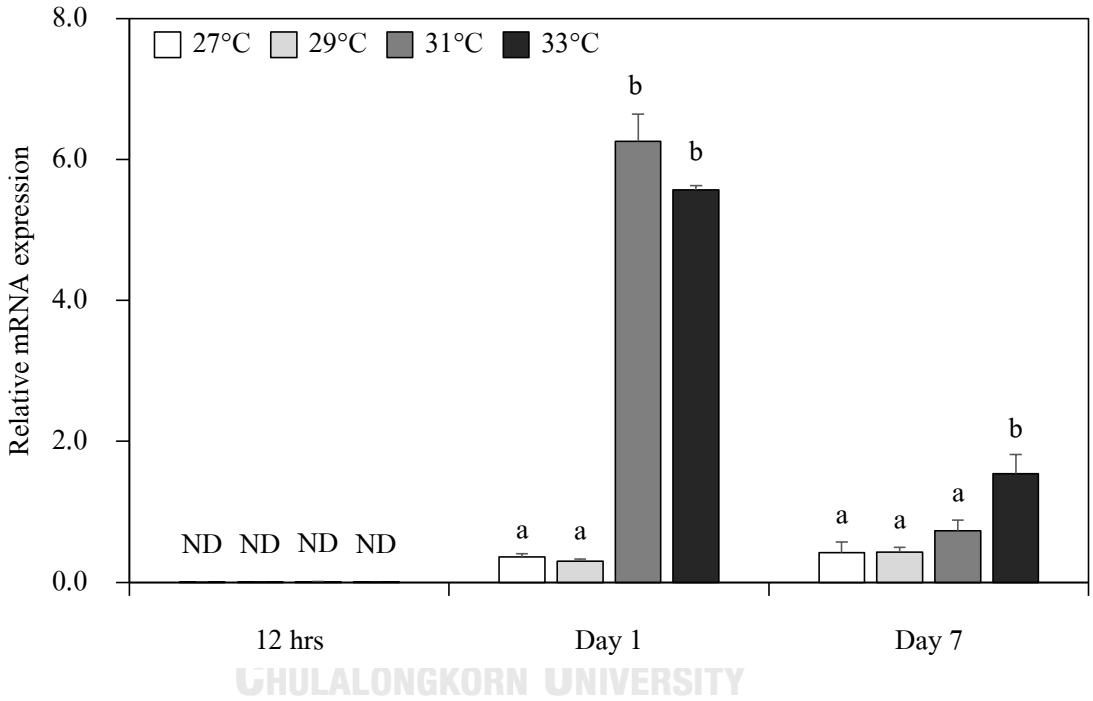


Figure 19 Relative expression of *Sod* in *P. quatrefagesi* under different treatments. White, light gray, dark gray and black bars stand 27°C, 29°C, 31°C and 33°C, respectively. ND stands for the non-detectable values. Values are expressed as means ± SE (n=3). Letters above the bars represent the level of significance ($p \leq 0.05$).

4.3.4 *Catalase* expression

The expression of *cat* in polychaetes *P. quatrefagesi* was shown in figure 20. The highest value of *cat* was expressed in 33°C at 12 hrs. The decreasing trend of *cat* expression was found in 26°C and 33°C over time. There were no significantly different found in *cat* expression.

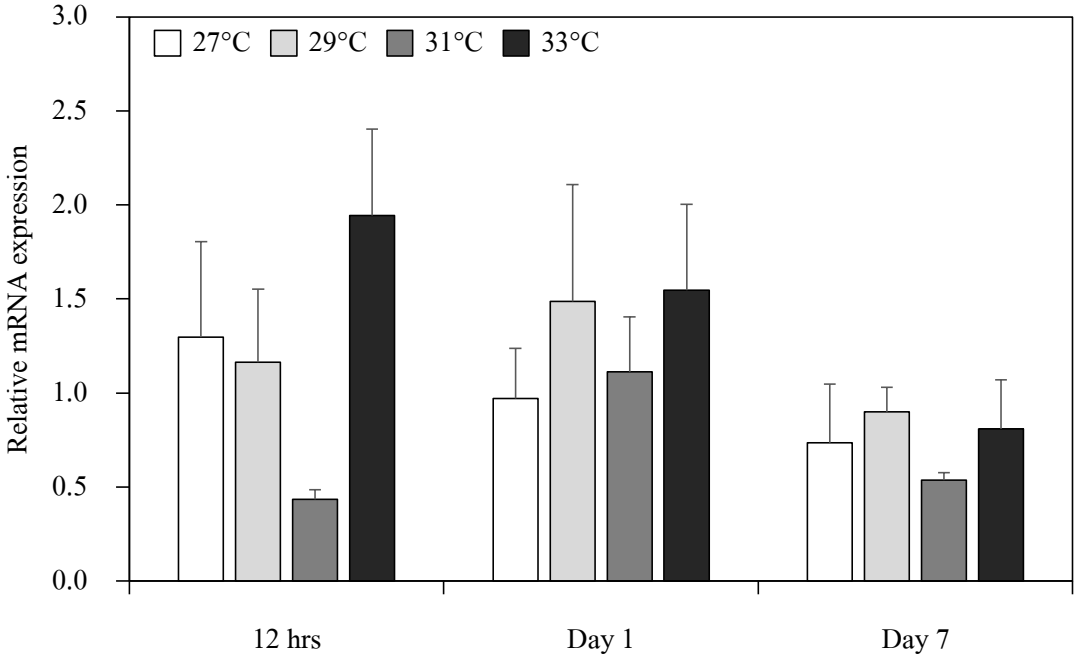


Figure 20 Relative expression of *Cat* in *P. quatrefagesi* under different treatments. White, light gray, dark gray and black bars stand 27°C, 29°C, 31°C and 33°C, respectively. Values are expressed as means ± SE (n=3).

CHAPTER 5

Discussions and conclusions

Growth parameter

An increasing in wet weights over time of *P. quatrefagesi* was found in every treatment except in the 33°C treatment. The similar pattern of the increasing in terms of final wet weight, specific growth rate and survival rate has also been revealed in this study. The results in the survival rate of *P. quatrefagesi* showed the optimal range of temperature in culture of this species which was within the range of 27-31°C. While at the highest temperature level, the tested animals could survive only in the short period of time (in this study, less than three weeks) with no significant changes in their growth rates. The results of these growth parameters supports the idea of temperature-dependent growth which stated that the growth rate could increase when temperature rises until it reaches to the upper limits of temperature, then the declining in growth rates could be detected (Madeira et al., 2012). As the growth rate depends on food intakes and metabolism, feeding rates and oxygen consumption rates of the animals could be altered in order to response to temperatures (Brett, 1979). A drop in the growth rate, the feeding rate and metabolism were found at the higher temperature in many species of polychaetes e.g. *Neanthes japonica* (Liu and Xian, 2009; Liu et al., 2009) *Sabellastarte magnifica* and *Hermodice carunculata* (Sander, 1973) and *Nereis* spp. (Kristensen, 1983). A drop point is considered as a turning point that define the upper limit of temperature tolerance for the animals (Liu and Xian, 2009). Moreover, temperature sensitivity or tolerance of animal at the highest temperature was considered to link with the bioenergetic of animals (Freitas et al., 2010). The energy budget is the value of food consumed by the animal that could be used for growth, excretion, metabolism, and fecal productions (Carfoot, 1987). In marine invertebrates, the major costs of the total energy budget is metabolism (An et al., 2007; Liu and Xian, 2009). Since the rate of metabolism would increase and food consumption decrease at the high temperature, animals may not have enough

energy to compensate the energetic costs under temperature stress condition. However, the animal may reduce their assimilation rate and maintenance costs for growth to sustain their energy (Freitas et al., 2010).

While in this experiment, the tested animals were only fed until satiation for their food requirements. The left feeds were found at about 40% for each feeding at the 33°C condition with inactive feeding behavior during feeding time after 14 days (personal observation). This could be the preliminary information to support the drop in feeding rate and maintaining costs of survive in this polychaetes species. However, in order to determine the turning point, feeding rate and metabolism of the worms are required for further investigation.

Cellular response

Heat shock response

The upregulation of *hsp70* is considered as a basic function of protein synthesis and its activity during the stress conditions (Dong et al., 2008; Ji et al., 2008), which have been reported in various aquatic animals e.g. coral *Acropora millepora* (Rodriguez-Lanetty et al., 2009), copepod *Tigriopus japonicus* (Rhee et al., 2009), bivalves *Mytilus edulis* (Chapple et al., 1997), oyster *Crassostrea gigas* (Farcy et al., 2009), and abalone *Haliotis tuberculata* (Farcy et al., 2007), shrimp *Litopenaeus vannamei* (Zhou et al., 2010), goby *Gillichthys mirabilis* (Buckley and Hofmann, 2002; Dietz, 1994)

An increasing of *hsp70* mRNA expression at the first 24 hours after the exposure has been shown to associate with lower level of hsp70 protein levels (Oksala et al., 2014) and high amount of misfolded protein over time (Lewis et al., 2016). While *hsp70* mRNA expression at day 7 was various, both increasing and decreasing expression had been observed. The changes in *Hsp70* expressions could be relevant to the amount protein denaturation and levels of hsp70 protein (Axenov-Gribanov et al., 2014). Levels of hsp70 proteins is therefore considered as 'molecular thermometer' that higher stock of hsp70 proteins would block the activation of *hsp70*

genes as negative feedback mechanism (Craig and Gross, 1991). Therefore, longer the exposure time to the stressors can increase hsp70 protein accumulations which could result in both increasing and decreasing *hsp70* expressions (Lewis et al., 2016).

A decreasing in *hsp70* expression may indicate metabolic depression or stress because of the ATP-dependent function of hsp70 (Chapple et al., 1997). According to Hofmann and Somero (1995), the thermal stress affected energy budget of animals due to high energy cost requirements in protein mechanism including protein maintenance, protein synthesis and protein degradation. On the other hand, any decreasing in *hsp70* expression could be relevant to heat shock factor (hsf1) which is known to activate HSPs. The change in hsf1 resulting from the phosphorylation signaling of hsf1 could increase HSPs expressions (Buckley and Hofmann, 2004)

The upregulation of *hsp90* expression during thermal stress is concerned to maintain structure of proteins which are damaged (Dietz and Somero, 1992). Not only to maintain the structural protein, but it also increases the thermal tolerance of animals and corrects the misfolding protein under stress conditions (Zhao et al., 2011). Many studies have informed that *hsp90* were upregulated in several species of aquatic animals; copepods *T. japonicus* (Rhee et al., 2009), goby *G. mirabilis* (Dietz, 1994; Dietz and Somero, 1992) and *G. seta* (Dietz and Somero, 1992) sea cucumber *Apostichopus japonicus* (Zhao et al., 2011), and mollusks *H. tuberculata* (Farcy et al., 2007) and bivalve *Paphia undulata* (Lin et al., 2018)

Changes in *hsp90* expression in this experiment during heat stress over times showed an increasing of *hsp90* expression to the highest peak following a decrease trend. These changes have been suggested to be related to energy consumption. During thermal stress, more energy would be required to fulfil the demands as shown at the highest peak in the results, while the decreasing trend could indicate an incapable of compensation for high energy cost which would have an effect upon protein synthesis, development and growth of animals (Lin et al., 2018;

Nakamura et al., 2012; Zhao et al., 2011). The changing *hsp90* expression could also linked to hsf1 as in the similar way to the *hsp70* expression (Prodromou, 2016).

It should be noted that each animals has a different level of endogenous hsp, while hsp itself has its own half-life and turnover time (Hofmann, 2005; Hofmann and Somero, 1996). Simultaneously, the plasticity of hsps induction could reflect thermal history of animals which has its own threshold induction and set points (Buckley et al., 2001; Dietz, 1994). It is also suggested that the acclimation of animals may play a part in hsp70 induction by their prior accumulation of hsp70 before the experiment started (Buckley and Hofmann, 2002; Buckley et al., 2001). In this study, the acclimation of animals was done at ambient temperature about a month before sorting worms into each tray. After that, the acclimation still continued about 5 days until starting temperature exposures.

As mention earlier in the literature review (chapter 2), it seems like both hsp70 and hsp90 are responsible for protein homeostasis during stress, but their roles needs to be addressed whether it is consistence with this results. In order to response to heat stress, heat shock response is conceived as the co-ordinating of hsp70, hsp90 and hsf1 for maintaining protein homeostasis (fig 4). According to heat shock response model, as a high misfolding proteins in stress condition, hsf1 that normally binds to hsp90 would be separated to form trimers. Phosphorylation could occur with the trimers and then activate hsps transcriptions. However, since the long exposure time in stress, these chaperone system would be eventually out of hand and lead to apoptosis (Proctor and Lorimer, 2011). In this results, it showed high consistent level of *hsp90* expression comparing with *hsp70* expression at the first 12 hours. This could indicate the release of hsf1 under stress conditions as the activator of *hsp70* expression, which would result in the increase of *hsp70* expression at the 12 hours later. At day 7, the chaperone system still showed the good sign of their function which could point out to the level of stress at this exposure time as moderate stress. While there was no observation on expressions after two weeks, which is considered as high stress because the dead animals were found.

However, it is worth noted that the heat shock response is a protein mechanism which depends on transcription, post-transcription, translation and post-translational modification. As every step may have the fluctuations that alter the results in different organisms.

Antioxidant responses

As the expression and enzyme activity due to thermal stress had been detected, the total protein concentration here could not show the quantity of specific proteins or the proteins that had been degraded, but can represent only the protein baseline in fresh tissues especially for enzyme assay. Both enzyme expression and activity could represent the antioxidant response in different aspects. The gene expression is considered as the amount of enzyme production. The enzyme activity is determined the actual functioning of the enzyme.

The *sod* expression was non-detectable at the first 12 hours, while SOD activity was found with an increasing trend to temperature. This could indicate that there was enough sod enzyme in cell that ready to act with superoxide. An increase trend of sod may infer that there was a high level of superoxide at high temperature which sod needed to handle. Meanwhile, catalase expression and activity had significant changes only in the highest temperature at this time. This could refer the role of catalase as a scavenger of excess hydrogen peroxide from sod activity. The similar response of sod and catalase activity exposed to high temperature was reported in shrimps, *Palaemon elegans* and *P. serrata* (Vinagre et al., 2014b), where it was also found to be tissue-specific in Antarctic fish *Notothenia coriiceps* and *N. rossii* (Klein et al., 2017), mussel *Perna viridis* (Verlecar et al., 2007), and rock goby *Gobius paganellus* (Vinagre et al., 2014a).

After 12 hours after the exposure, there was 5-6 folds level of expression in sod at the high temperature which show the significant need for synthesizing new sod proteins in response to temperature. Meantime, the activity of sod had showed decrease trend to temperature that confirm the requirement of new sod production. According to Lushchak and Bagnyukova (2006),

the upregulation of *sod* under the high temperature may be resulted from an increase of lipid peroxidation products and lipid peroxide.

An increased trend of sod expression over each temperature treatments at day 7 was contrast to the case of activity of sod, which the highest sod activity was found in the lowest sod expression. This could point to the process of sod during the thermal stress.

The high levels of sod activity after two weeks in high temperature and all treatments could indicate extremely high of superoxide and inhibitory effects of sod to reactive oxygen species (ROS) accumulation (Birnie-Gauvin et al., 2017; Verlecar et al., 2007). High sod activity was considered to associate with the oxidation of haemoglobin under the redox stress as shown in lugworm (*Arenicola marina*) and clam (*Astarte borealis*) (Abele-Oeschger and Oeschger, 1995) and hydrothermal worm (Genard et al., 2013). However, one of the concerning factors for the longer exposure time is the recovery time of sod which had been reported in ark shell under thermal stress that the sod recovery time was much longer than 6 hours (Zhao et al., 2016). It has also been reported that half-life of sod was quite short and various depending on type of sod. The range of sod half time was about 10 mins to 6 hours (Fukai and Ushio-Fukai, 2011).

It is suggested that the isoforms of sod may alter the result of expression and activity. As each isoforms would appear to be found in different locations (tissue-specific) of organism with having distinct sensitivity to environmental stressors. In polychaetes, *A. marina*, MnSOD was a high sulphide resistant dominated in blood, where CuZnSOD was common in body tissue (Abele-Oeschger, 1996). This evident could represent the relevance between sod and sulfide which normally found in natural environment for benthic marine invertebrate and at the end of this study (personal observation). While the sod activity in this experiment was a total sod activity with a partial coding sequences for primer's design.

It should be noted that the duration of stress, the extreme of stress and the proportion of ROS might have effects on the sod. In some tissue, the sod activity would increase at the long

exposure (Verlecar et al., 2007). While the extreme of stress in this case refer to CT_{max} which is the upper limits of temperature in animals. At the CT_{max}, some aquatic animals showed changing level of sod activity but not to the peak (Vinagre et al., 2014b). The proportion of ROS could directly relate with the responsible enzyme activity during stress.

The process of ROS occurring in cell is basically linked to aerobic metabolism, which superoxide appear to be toxic and cause the damage in cell such as lipid peroxidation and DNA damage (Wang et al., 2018). While hydrogen peroxide could act as the signaling molecules that could change the regulatory functions of proteins such as the oxidation of cysteines (Finkel, 2011). Although there are many responsible enzymes for hydrogen peroxide degradation; catalase, glutathione peroxidase and peroxiredoxins with different enzyme structure and sensitivity (Finkel, 2011). As mentioned in this work, it is noticed that there are several factors in term of signaling process that might alter the expression and activity of antioxidant enzymes. According to Zhao et al 2016, the relevance of antioxidant enzyme activity and thyroid hormones, which need to be further investigated in invertebrates, were discussed. Meanwhile, expression of antioxidant enzymes could be influenced from RNA interference or RNAi, which may alter results in expression part.

Two systems in cellular responses under thermal stress had been presented in this study. Theory was that the temperature stress could increase the aerobic metabolism and lead to oxidative stress. The results showed a faster response to temperature stress is heat shock response rather than antioxidant enzyme process. This could entirely support the theory that oxidative stress may occur after animals were exposed to stress for a period of time. It has also showed the coordination between each response to stress as mentioned in Feder and Hofmann (1999).

Related studies of two cellular response in polychaetes was limited in order to compare the differences between species or experimental conditions. Most of the previous studies in polychaetes in terms of sod and catalase activity had been done in terms of toxic accumulations

such as copper (Geracitano et al., 2002), cadmium (Yuan et al., 2010), petroleum hydrocarbon (Sun and Zhou, 2008), the anoxic or hypoxic condition (Abele-Oeschger and Oeschger, 1995; Rosa et al., 2005) and hydrogen sulfide exposure (Abele-Oeschger, 1996; Genard et al., 2013) based on their habitat in sediment or hydrothermal vents. While fundamental factors like temperature or salinity have been neglected. However, a similar work in term of molecular study was only reported in Rhee 2012 that represented the potential of heat shock response and antioxidant defense system as biomarker in polychaetes for environmental risk assessments.

Natural condition in real environment needs to be concerned in response to environmental factors of animals. As environmental factors changes, animals may obviously response through behaviors by escaping the threatened conditions which can be considered as a proximate responses. In the same time of behavioral changes, physiological changes may occur, but it could not be obviously seen without the proper approaches. Moreover, it must be noted that this study was conducted under laboratory conditions which focused only a single factor of real environmental factors. In their wild condition, multi-environmental factors plays a part to polychaetes. Regarding to their habitat, sandy beaches, both vertical factors (temperatures, oxygen availability, and grain size) and horizon factors (waves and currents) would change through times including biological factors like density or competition. Thus, the best way to conduct the experiment under laboratory on polychaetes in response to environmental factors is to choose one or two environmental factors as a main interest and control the rest of factors close to the real nature to prevent confounding factors and effects.

Finally, the whole work can be summarized and presented in the schematic diagram on figure 21. The highlight of this study clarifies the optimal temperature range in polychaetes, *P. quatrafagesi* and how this organism response to temperature stress via cellular response.

Since the basic biology of this species in response to temperatures was fulfilled and discussed, it is suggested that this study could be significantly applied in aquaculture or the use of polychaetes as the water treatment. However, in order to carry on the works in the biological responses of polychaetes under environmental stressors and polluted areas, further investigations on both metabolic and molecular mechanisms would be needed.

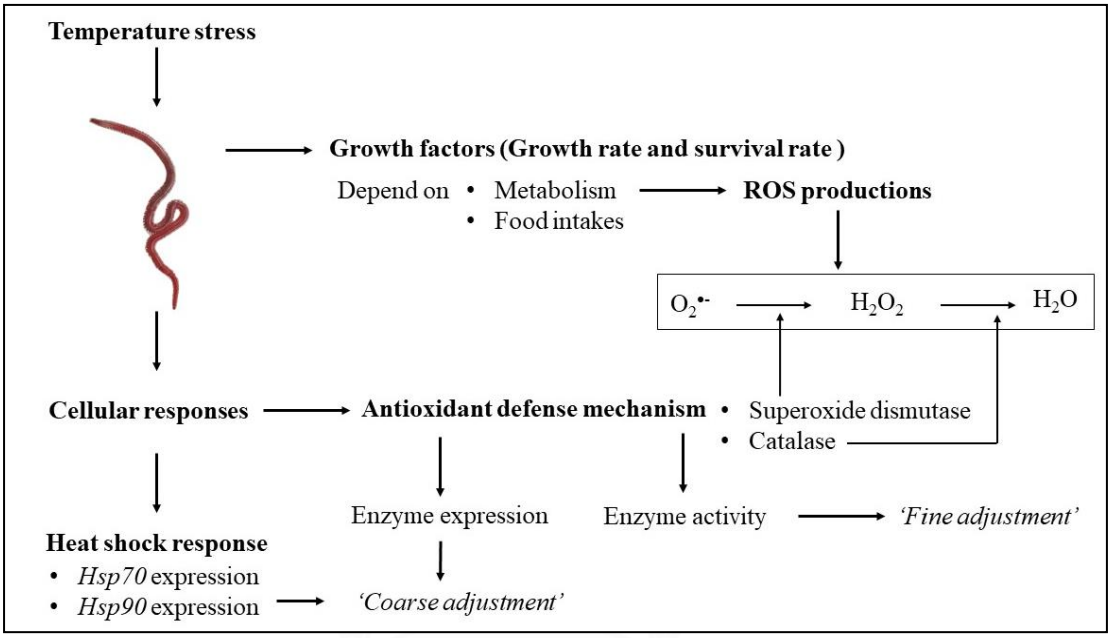


Figure 21 Schematic diagram of this study. The temperature stress was added into the model organism, *P. quatrefagesi*. The growth rate and survival rate were determined as growth factors. Since growth rate depends on food intakes and metabolism that could cause ROS productions. As the cellular response, heat shock response and antioxidant enzymes were observed. The gene expressions would be considered as 'coarse adjustment of protein', while the enzyme activity would be considered as 'fine adjustment of protein'.

Conclusions and Highlights

1. The optimal range of temperature for culture polychaetes, *P. quatrefagesi* was within the range of 27-29°C.
2. The specific growth rate and survival rate was ranked from the highest to the lowest rates in the treatment at 29°C, 27°C, 31°C and at 33°C, respectively.
3. The total protein concentration for the whole experiment was in range 5-12 mg/ml, which was calculated as a baseline for enzyme assay.
4. The sod activity showed the increase trend at the first 12 hours exposure and the decrease trend at 12 hours later, while the high peak of sod activity was found at the end of experiment for all treatment.
5. The catalase activity demonstrated a rise only in a short period (at the first 12 hours).
6. The highest upregulation of *hsp70* was expressed in 33°C, while the other treatment showed the various levels of expression. An increase trend with temperatures was also found at the first 12 hours exposure.
7. The highest upregulation of *hsp90* was expressed in 33°C at 12 hours and day 1 exposure, while a decrease trend with temperature was found at day 7.
8. The first 12 hours exposure, *sod* expression was non-detectable. The highest upregulation of sod was expressed at day 1 in high temperatures (31°C and 33°C), while an increase trend with temperature was found at day 7.
9. The highest upregulation of *cat* expression was found in 33°C at 12 hours exposure.

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APPENDICES

APPENDIX A

1. Preparation for extraction buffer

The ice cold 0.1 M Tris/HCl, pH 7.4 containing 1% Triton X-100

Weight Tris (hydroxymethyl) aminomethane 12.1 g in 1000 ml DI water for 0.1 M Tris, take 100 ml of 0.1 M Tris, add 84 ml 0.1 M HCl, bring the final volume to 200 ml, adjust pH to 7.4 and add 1% triton x-100.

2. Preparation for protein determination

Bradford Reagent

Weight coomassie brilliant blue 25 mg dissolve in 25 ml methanol, add 50 ml 85% (w/v) phosphoric acid, and bring the final volume to 500 ml with distilled water. Filter with whatman paper #1 and keep in the light protected storage bottles.

3. Preparation for catalase activity

The 100 mM KPi Buffer

Weight 0.68 potassium phosphate monobasic in 50 ml distilled water.

APPENDIX B

The concentration of extracted RNA and 260/280

Table 2 Concentration of extracted RNA and the ration of absorbance at 260 to 280

Exposure times	Treatments - replication	Ab260	Ab280	Ab260/Ab280	RNA conc.
Day 0	1	0.100	0.049	2.0	80.0
	2	0.174	0.096	1.8	139.2
	3	0.163	0.083	2.0	130.4
12 hrs	27-1	0.131	0.068	1.9	104.8
	27-2	0.325	0.173	1.9	260.0
	27-3	0.083	0.041	2.0	66.4
	29-1	0.356	0.183	1.9	284.8
	29-2	0.257	0.137	1.9	205.6
	29-3	0.209	0.101	2.1	167.2
	32-1	0.146	0.076	1.9	116.8
	32-2	0.262	0.142	1.8	209.6
	32-3	0.110	0.056	2.0	88.0
	35-1	0.199	0.105	1.9	159.2
	35-2	0.101	0.049	2.1	80.8
	35-3	0.136	0.070	1.9	108.8
Day 1	27-1	0.062	0.032	1.9	49.6
	27-2	0.188	0.101	1.9	150.4
	27-3	0.169	0.096	1.8	135.2
	29-1	0.182	0.095	1.9	145.6
	29-2	0.103	0.054	1.9	82.4
	29-3	0.067	0.035	1.9	53.6
	32-1	0.133	0.074	1.8	59.2
	32-2	0.107	0.058	1.8	85.6
	32-3	0.078	0.043	1.8	62.4

Table 3 continue

Exposure times	Treatments - replication	Ab260	Ab280	Ab260/Ab280	RNA conc.
Day 1	35-1	0.032	0.017	1.9	25.6
	35-2	0.079	0.042	1.9	63.2
	35-3	0.245	0.139	1.8	196.0
Day 7	27-1	0.290	0.160	1.8	232.0
	27-2	0.166	0.089	1.9	132.8
	27-3	0.287	0.160	1.8	229.6
	29-1	0.321	0.175	1.8	256.8
	29-2	0.313	0.172	1.8	250.4
	29-3	0.346	0.192	1.8	276.8
	32-1	0.284	0.156	1.8	227.2
	32-2	0.117	0.061	1.9	93.6
	32-3	0.144	0.077	1.9	115.2
	35-1	0.140	0.073	1.9	112.0
	35-2	0.169	0.140	1.9	135.2
	35-3	0.129	0.070	1.8	103.2

The agarose gel electrophoresis

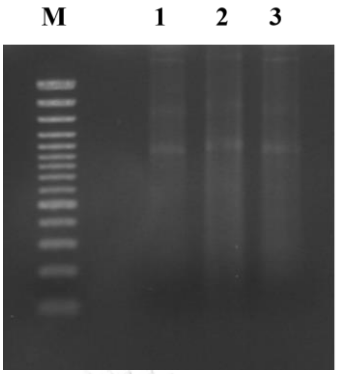

Figure 22 Extracted RNA of *P. quatrefagesi* at day 0. Lane M contained 50 bp DNA ladder and other lane contained each replication of the treatments.

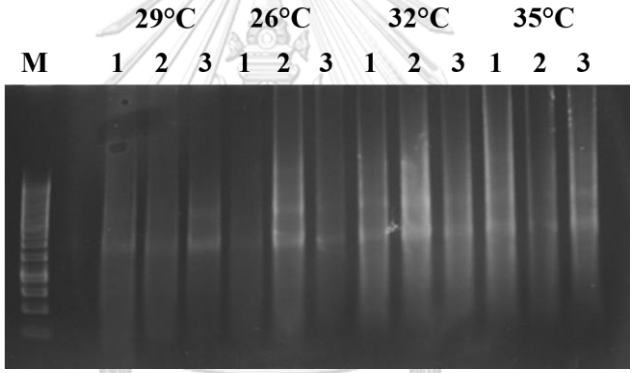

Figure 23 Extracted RNA of *P. quatrefagesi* at 12 hours exposure. Lane M contained 50 bp DNA ladder and other lane contained each replication of the treatments.

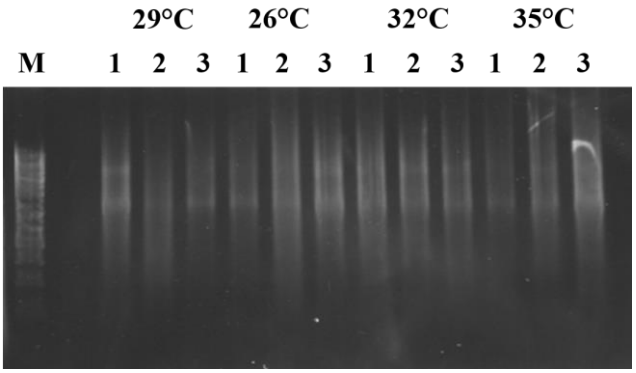

Figure 24 Extracted RNA of *P. quatrefagesi* at day 1 exposure. Lane M contained 50 bp DNA ladder and other lane contained each replication of the treatments.

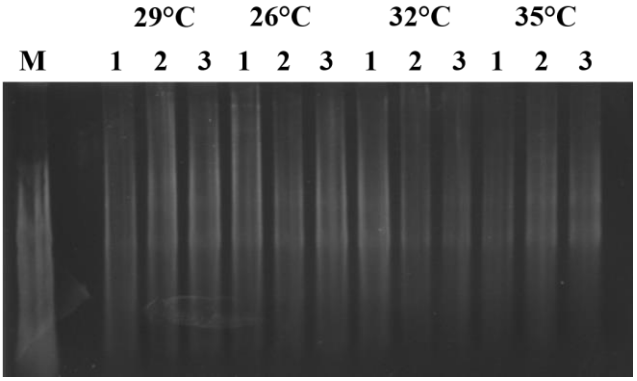

Figure 25 Extracted RNA of *P. quatrefagesi* at day 7 exposure. Lane M contained 50 bp DNA ladder and other lane contained each replication of the treatments.

The number of nucleotides in each gene

Table 4 Number of nucleotides in each gene

Gene	Acc. No.	Number of nucleotides
Heat shock protein 70 *(hsp70)*	HM625717.1	219
Heat shock protein 90 *(hsp90)*	HM625726.1	654
Catalase *(cat)*	HM625832.1	255
Superoxide dismutase *(sod)*	HM625833.1	465
Actin	JN609588.1	751

The amplification curve of Real Time PCR

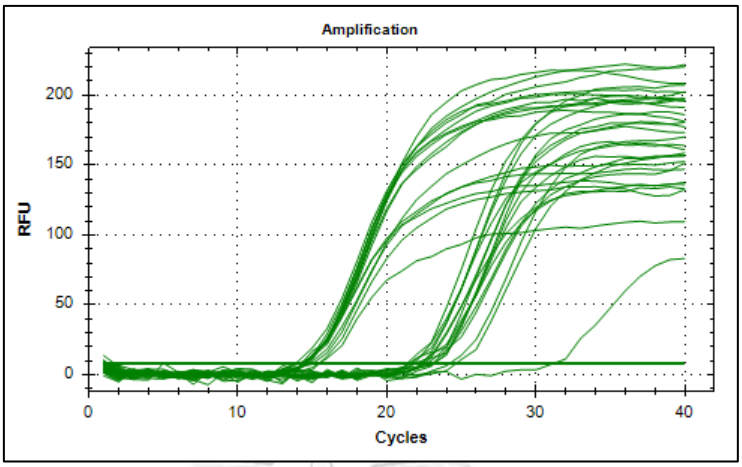

Figure 26 Amplification curve of *hsp70* and *actin* in 0 and 12 hours samples

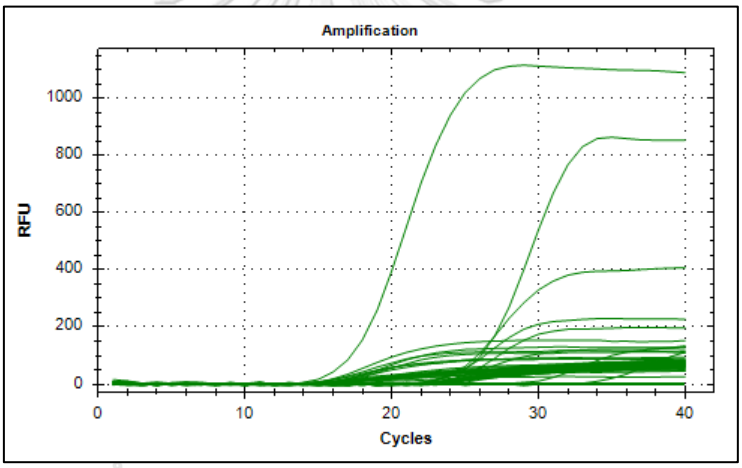

Figure 27 Amplification curve of *hsp70* and *actin* in 0, 1 and 7 days samples

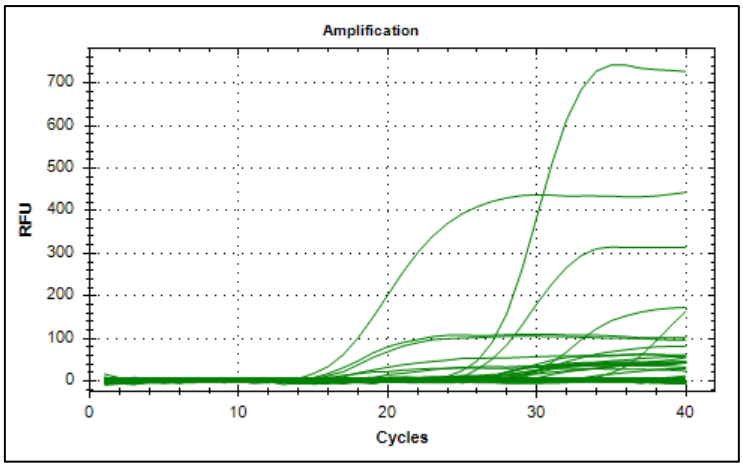

Figure 28 Amplification curve of *sod* and *actin* in 0 and 12 hours samples

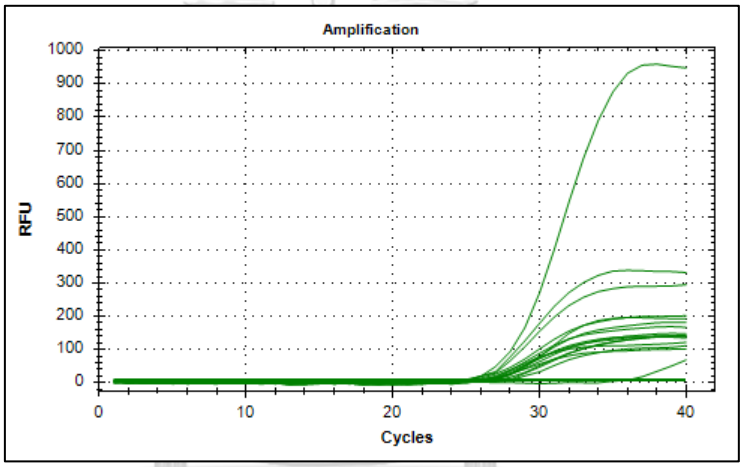

Figure 29 Amplification curve of *sod* in 0 and 1 day samples

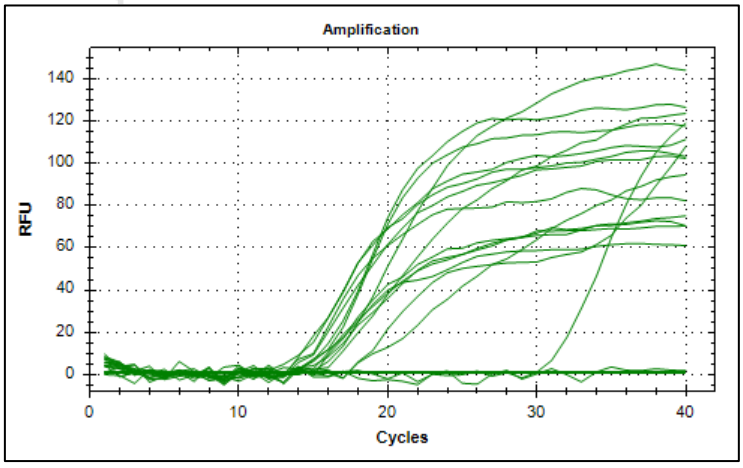

Figure 30 Amplification curve of *actin* in 0 and 1 day samples for calibrating sod

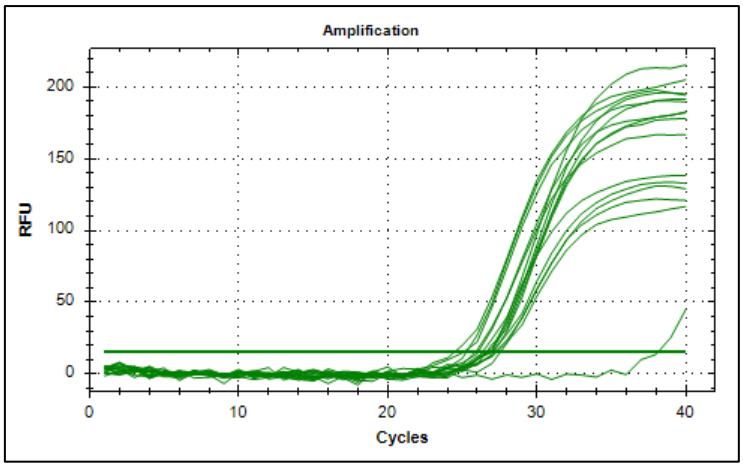

Figure 31 Amplification curve of *sod* in 0 and 7 days samples

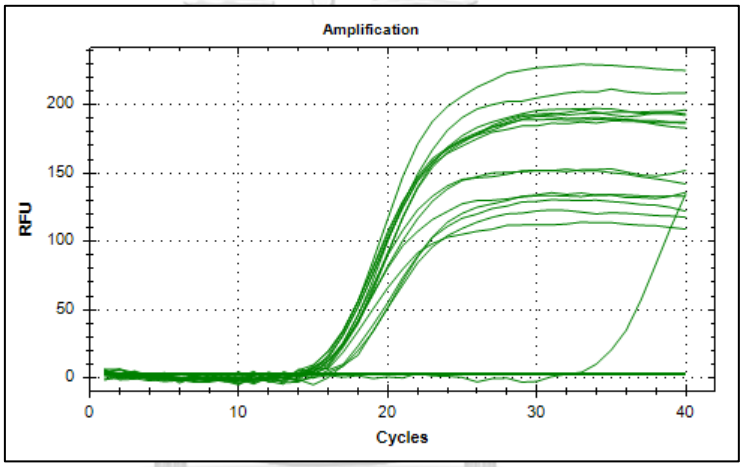

Figure 32 Amplification curve of *actin* in 0 and 7 days samples for calibrating sod

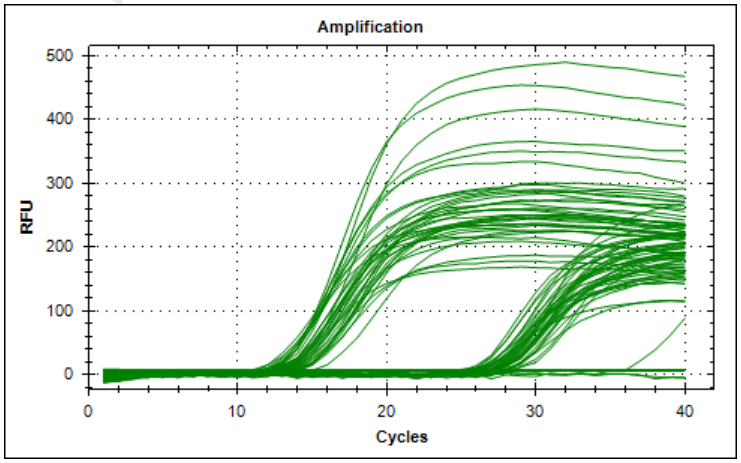

Figure 33 Amplification curve of *cat* and *actin* in 0, 12 hrs, 1 day and 7 days samples

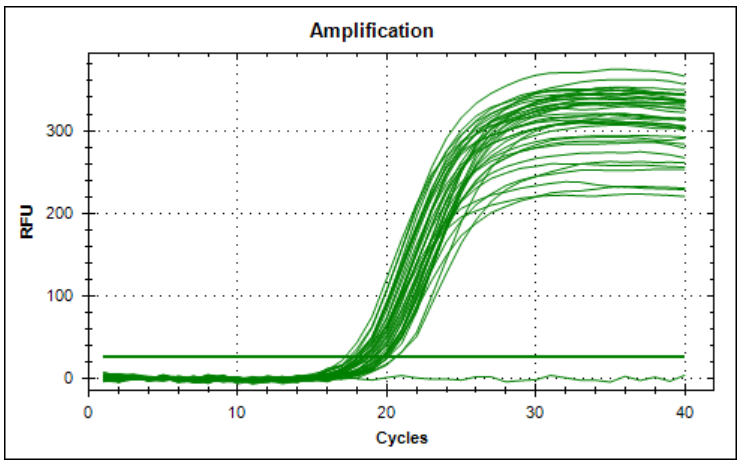

Figure 34 Amplification curve of *hsp90* and *actin* in 0, 12 hrs, 1 day and 7 days samples

APPENDIX C

Table 5 Wet weight, specific growth rate and survival rate of *P. quatrefagesi* under different treatments conditions. Values represent as mean ± SE. Letters in superscript represent the significant difference between treatments ($p<0.05$).

Parameters	Treatments (°C)			
	27	29	31	33
Initial weight (g)	0.0723 ± 0.001 – 0.1086 ± 0.020			
12 hrs weight (g)	0.0841 ± 0.008[a]	0.1007 ± 0.013[a]	0.0850 ± 0.004[a]	0.1155 ± 0.004[a]
3 d weight (g)	0.0747 ± 0.017[a]	0.0893 ± 0.018[a]	0.1069 ± 0.013[a]	0.0918 ± 0.013[a]
7 d weight (g)	0.0974 ± 0.013[a]	0.1057 ± 0.017[a]	0.1137 ± 0.015[a]	0.0993 ± 0.007[a]
14 d weight (g)	0.1595 ± 0.021[a]	0.1968 ± 0.028[a]	0.1611 ± 0.015[a]	0.1452 ± 0.010[a]
Final weight (g)	0.3818 ± 0.067[b]	0.4670 ± 0.020[bc]	0.2270 ± 0.012[ab]	0.1256 ± 0.018[a]
SGR (%/day)	5.33 ± 0.34[a]	5.53 ± 0.60[a]	3.50 ± 0.25[a]	0.23 ± 0.40[b]
Survival rate (%)	93.86 ± 3.21[a]	98.21 ± 1.26[a]	93.14 ± 4.81[a]	29.71 ± 3.19[b]

Table 6 Total protein concentration of *P. quatrefagesi* under different treatments conditions in unit mg/ml. Values represent as mean ± SE. Letters in superscript represent the significant difference between treatments ($p<0.05$).

Exposure times	Treatments (°C)			
	27	29	31	33
0 hr	9.90 ± 2.00			
12 hrs	8.12 ± 0.79 [a]	6.74 ± 1.27 [a]	7.93 ± 0.06 [a]	5.96 ± 0.76 [a]
Day 1	9.30 ± 0.46 [a]	7.80 ± 0.58 [a]	9.55 ± 0.31 [a]	8.90 ± 0.23 [a]
Day 3	9.92 ± 0.48 [b]	7.17 ± 0.48 [a]	8.25 ± 0.09 [ab]	9.58 ± 0.88 [ab]
Day 7	11.45 ± 0.66 [a]	10.50 ± 0.96 [a]	10.55 ± 0.92 [a]	11.13 ± 0.86 [a]
Day 14	8.84 ± 0.49 [ab]	7.93 ± 0.68 [a]	8.43 ± 0.97 [ab]	11.00 ± 0.12 [b]
Day 28	6.51 ± 0.27 [a]	5.40 ± 0.08 [a]	7.06 ± 1.02 [a]	6.44 ± 0.49 [a]

Table 7 SOD activity of *P. quatrefagesi* under different treatments conditions in unit % inhibition rate. Values represent as mean ± SE. Letters in superscript represent the significant difference between treatments ($p<0.05$).

Exposure times	Treatments (°C)			
	27	29	31	33
0 hr	77.21 ± 3.00			
12 hrs	73.78 ± 5.13 [a]	74.91 ± 4.29 [a]	79.50 ± 1.11 [a]	84.47 ± 4.34 [a]
Day 1	82.53 ± 0.95 [a]	80.04 ± 5.28 [a]	72.09 ± 2.81 [a]	68.65 ± 2.26 [a]
Day 3	71.77 ± 2.76 [a]	70.78 ± 3.60 [a]	66.79 ± 3.65 [a]	72.56 ± 4.78 [a]
Day 7	80.45 ± 2.40 [b]	67.83 ± 1.67 [ab]	62.14 ± 4.63 [a]	69.88 ± 1.54 [ab]
Day 14	69.59 ± 3.82 [a]	66.76 ± 3.72 [a]	93.84 ± 0.37 [b]	95.82 ± 0.38 [b]
Day 28	91.04 ± 2.10 [a]	93.93 ± 0.67 [a]	93.24 ± 1.46 [a]	94.66 ± 0.09 [a]

Table 8 CAT activity of *P. quatrefagesi* under different treatments conditions in unit μ mol H_2O_2 min^{-1} mg^{-1} proteins. Values represent as mean ± SE. Letters in superscript represent the significant difference between treatments ($p<0.05$).

Exposure times	Treatments (°C)			
	27	29	31	33
0 hr	0.51 ± 0.23			
12 hrs	0.56 ± 0.06 [a]	0.58 ± 0.06 [a]	0.68 ± 0.03 [ab]	0.96 ± 0.09 [b]
Day 1	0.54 ± 0.02 [a]	0.55 ± 0.05 [a]	0.54 ± 0.04 [a]	0.56 ± 0.03 [a]
Day 3	0.41 ± 0.03 [a]	0.46 ± 0.05 [a]	0.47 ± 0.03 [a]	0.51 ± 0.05 [a]
Day 7	0.45 ± 0.02 [a]	0.45 ± 0.03 [a]	0.48 ± 0.01 [a]	0.50 ± 0.01 [a]
Day 14	0.45 ± 0.03 [a]	0.45 ± 0.05 [a]	0.47 ± 0.04 [a]	0.48 ± 0.03 [a]
Day 28	0.54 ± 0.03 [a]	0.63 ± 0.02 [a]	0.51 ± 0.04 [a]	0.60 ± 0.12 [a]

Table 9 Relative *Hsp70* expression of *P. quatrefagesi* under *different treatments conditions.* Values represent as mean ± SE. Letters in superscript represent the significant difference between treatments ($p<0.05$).

Exposure times	Treatments (°C)			
	27	29	31	33
0 hr	1.01 ± 0.21			
12 hrs	0.29 ± 0.14 [a]	0.40 ± 0.10 [a]	0.96 ± 0.11 [b]	1.34 ± 0.10 [b]
Day 1	1.57 ± 0.25 [a]	0.79 ± 0.12 [a]	1.75 ± 0.31 [a]	1.81 ± 0.51 [a]
Day 7	0.55 ± 0.15 [a]	1.77 ± 0.63 [a]	0.57 ± 0.04 [a]	1.96 ± 0.37 [a]

Table 10 Relative *Hsp90* expression of *P. quatrefagesi* under different treatments conditions. ND refer as non-detect values. Values represent as mean ± SE. Letters in superscript represent the significant difference between treatments ($p<0.05$).

Exposure time	Treatments (°C)			
	27	29	31	33
0 hr	1.03 ± 0.19			
12 hrs	0.84 ± 0.17 [a]	0.85 ± 0.10 [a]	0.86 ± 0.04 [a]	1.30 ± 0.14 [a]
Day 1	0.79 ± 0.04 [a]	0.81 ± 0.32 [a]	0.69 ± 0.07 [a]	1.11 ± 0.12 [a]
Day 7	0.91 ± 0.28 [a]	0.84 ± 0.05 [a]	0.47 ± 0.09 [a]	0.52 ± 0.03 [a]

Table 11 Relative *Sod* expression of *P. quatrefagesi* under different treatments conditions. Values represent as mean ± SE. Letters in superscript represent the significant difference between treatments ($p<0.05$).

Exposure times	Treatments (°C)			
	27	29	31	33
0 hr	0.86 ± 0.13			
12 hrs	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]
Day 1	0.36 ± 0.05 [a]	0.30 ± 0.03 [a]	6.26 ± 0.38 [b]	5.57 ± 0.06 [b]
Day 7	0.42 ± 0.15 [a]	0.43 ± 0.07 [a]	0.73 ± 0.15 [a]	1.54 ± 0.28 [b]

Table 12 Relative *Cat* expression of *P. quatrefagesi* under different treatments conditions. Values represent as mean ± SE. Letters in superscript represent the significant difference between treatments ($p<0.05$).

Exposure times	Treatments (°C)			
	27	29	31	33
0 hr	1.19 ± 0.50			
12 hrs	1.30 ± 0.51 [a]	1.16 ± 0.39 [a]	0.44 ± 0.05 [a]	1.94 ± 0.46 [a]
Day 1	0.97 ± 0.27 [a]	1.49 ± 0.62 [a]	1.11 ± 0.29 [a]	1.55 ± 0.46 [a]
Day 7	0.74 ± 0.31 [a]	0.90 ± 0.13 [a]	0.54 ± 0.04 [a]	0.81 ± 0.26 [a]

VITA

NAME	Sucharat Suksai
DATE OF BIRTH	10 October 1992
PLACE OF BIRTH	Phuket, Thailand
INSTITUTIONS ATTENDED	Bachelor of Science in Biology (First Class Honors) from Faculty of Science, Prince of Songkla University in 2015
HOME ADDRESS	6/76 Pasason ParkVille, Vichitsongkram Road, Kathu, Phuket
PUBLICATION	Suksai, S. and Pairohakul, S. (2018). Growth and Survival rates of Perineris quatrefagesi (Grube, 1878) in response to temperatures. The 44th Congress on Science and Technology of Thailand (ST44), Bangkok International Trade and Exhibition Centre (BITEC), Bangkok during 29-30 October 2018. Number A003PF, p 1-5.
AWARD RECEIVED	Science Achievement Scholarship of Thailand since 2011 to present. Chulalongkorn University Graduated School Thesis Grant