FACULTY OF BIOSCIENCE ENGINEERING

Are Heat Shock Proteins a Solution for the Rampant Handling and Transport Mortalities in European Flat Oysters (Ostrea edulis)?

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PREAMBLE

This thesis initially aimed at establishing whether heat shock proteins could be applied to control the excess mortality during the artificial propagation of the European flat oyster. Several challenge tests were to be undertaken, first to establish the optimal concentration of phloroglucinol and the exposure time. Following the establishment of the effective dose, the protective effect of the pre-treatment was to be investigated. However, no experiments could be performed after all the University laboratories were closed due to the current coronavirus (CORVID-19) pandemic. The thesis was changed to a literature study in consultation with my promoter and supervisor. It describes an experimental protocol that can be used to establish whether HSPs can be used to control mortality in the spat of *O. edulis.* The study also expanded to identify the HSP70 genes in the *C. gigas* genome by using several bioinformatics tools. The identification of the proteins was to further our understanding of the heat shock response in oysters.

ABSTRACT

The routine husbandry activities, including handling and transport, inflict stress on the oysters, subsequently resulting in excess mortality. It is believed that controlling stress that results from the regular management activities may help to reduce oyster mortality. Literature shows that enhanced expression of heat shock proteins (HSPs) helps organisms to cope with stress, thereby protecting them from insults in their environment. This thesis describes an experiment that may be used to assess whether HSPs may be used to control mortality during the artificial propagation of the European flat oysters. Plant extracts are recognized as effective clean inducers of HSPs in different organisms. Thus, this protocol is based on using phloroglucinol, a phlorotannin of brown algae, to induce HSP70 in the *O. edulis* spat. At first, the thesis describes the preliminary experiment to establish the cytotoxic effects of phloroglucinol to the spat. This experiment involves treating the spat with a range of concentrations of the compound, from 1 to 10,000µM for 1 to 5 hours. Spat survival is then monitored during a 48h recovery period to determine the lowest toxic concentration. Following the lethality test, a dose-response test to establish the optimal dose and exposure time that induce maximum expression of HSP70 in the spat is described. This challenge test entails exposing the spat to 10^{-1}–10^{-5} of the minimum toxic concentration of phloroglucinol that is established in the lethality test for 1-5 hours. The expression of HSP70 is then determined by immunoblotting at a 2 hour and 2day interval for 8 hours and eight days, respectively. After establishing the optimal dose and the exposure time for phloroglucinol, a three-step experiment to assess the HSP's protective effect is described. In the first step, HSPs are induced in the spat by applying the established optimal concentration and exposure time of phloroglucinol. Then the spat is subjected to a grading simulation when HSP70 expression is at its maximum. The next day after grading, the spat is challenged with 10^6CFU/ml *Vibrio neptunius* by a bath in the last phase of this experiment. Survival is then monitored for six days to find out whether the pretreatment of phloroglucinol before grading improves survival in the spat. This experimental protocol will give proof of whether to incorporate HSP-based therapies in oyster aquaculture.

As an addition to this thesis, HSP70 cDNA were BLAST searched against the Pacific oyster genome to identify the HSP70 genes in that species. A total of eight HSP70 genes, including five cytosolic and three endoplasmic reticulum isoforms, were retrieved. Four of the

corresponding cytosolic proteins are 634 amino acids, while the fifth is 613 amino acids. On the contrary, the proteins for the endoplasmic reticulum isoforms are 656, 633, and 661 amino acids long. All the isoforms exhibit the three unique motifs (IDLGTTYS, IFDLGGGTFDVSIL, and VVLVGGSTRIPKIQK) for the HSP70 family. It is necessary to conduct comprehensive studies to identify all the genes in the different oyster species. Lastly, the development of effective HSP70 therapies will require a clear understanding of these genes' expression patterns under different conditions.

CHAPTER 1

INTRODUCTION

The native European flat oyster (*Ostrea edulis*) supported a vibrant fishery all over Europe during the 18th and 19th centuries. Similarly, the culture of that species grew in the same period to fill the supply gap and meet the excess demand. However, its production plummeted at the end of the 19th century to the current negligible level (Grace et al. 1997). Prolonged intensive harvesting, severe winters, diseases, and eventual replacement with the cupped oysters are implicated for that drastic decline. There is widespread interest in reviving its production through aquaculture and restoration of natural beds by restocking and habitat remediation programs (Laing et al. 2005; Kamermans et al. 2018). The inadequate supply of stocking materials, however, constrains the development of *O. edulis* aquaculture and the restoration programs. Usually, oysters are farmed by collecting spat from the wild and subsequently deploying them in grow-out systems. However, following the decimation of the natural oyster beds, the supply of wild spat from the fragmented residual stocks is highly variable. Besides the limited natural stocks, the supply of *O. edulis* spat is further hindered by the absence of settlement substrates, variable weather conditions, and infections from Bonamia, Marteilia, and *Ostreid herpesvirus* 1 (OsHV-1). Consequently, artificial breeding of flat oysters in the hatcheries remains the practical solution for the reliable large-scale production of spat. Despite the increasing number of hatcheries involved in *O. edulis* propagation in Europe, the production of spat is still limited. The absence of a standardized hatchery protocol and a complex life cycle of the species consisting of hermaphroditism and larval spawning partly explain the low spat production.

The main problem during the artificial propagation of oysters is the excessive mortality that mainly occurs during the critical stage of larval settlement and the transition of young spat from indoor to outdoor facilities. Routine husbandry activities in the oyster hatcheries are known to influence mortality through the infliction of stress from frequent handling, grading, and transport. Therefore, mortality may be controlled by applying strategies aimed at reducing the extent of stress inflicted on the oysters from these management activities. The heat shock response, a conserved cellular process that involves the rapid synthesis and enhanced presence of heat shock proteins (HSPs), helps organisms to cope with stress. The HSPs are molecular chaperones involved in the maintenance of cellular homeostasis by engaging in protein folding, assembly, degradation, and intracellular localization. The enhanced HSPs confer stress tolerance by induction of a transient thermotolerance,

cross-tolerance, and enhancement of the immunity (Clegg et al. 1998; Jackson et al. 2011a). Therefore, it may be possible to control stress and improve survival in flat oyster hatcheries by inducing HSPs before the execution of the husbandry activities. The expression of HSPs in the larvae and spat of oysters has already been shown in different oyster species (Brown et al. 2004; Ueda and Boettcher 2009).

The application of HSP-based therapies will depend on a clear understanding of the expression of HSPs and the development of practical protocols to induce these proteins in commercial hatcheries. The common inducers of HSPs in the laboratory, including thermal shock, heavy metals, hypoxia, and electromagnetism, may not be applicable in the commercial environment. However, there is growing interest in the use of plant extracts like phloroglucinol as cleaner alternatives to induce HSPs (Kumar et al. 2018; Roy et al. 2019). Moreover, plant products have been shown to have antibacterial, antioxidant, and anti-cancer properties, which may be of interest in aquaculture. Despite its proven potency in other species, the effectiveness of phloroglucinol in inducing HSP70 in oysters is not known. Also, the number of HSPs expressed by oysters is not yet confirmed. Although, immunoblotting shows that oysters exhibit three cytosolic HSP70 isoforms of 69, 72, and 77-kDa, the recent availability of the *Crassostrea gigas* genome and transcriptomic studies show that more proteins may be involved. Consequently, different kinds of analyses may be necessary to have a clear understanding of the HSP expression in oysters.

This master thesis will describe a procedure to be used to assess the possibility of controlling mortality resulting from routine husbandry activities of *O. edulis* spat by applying HSPs. To the best of our understanding, this will be the first attempt to use a plant-based extract, phloroglucinol, to induce the heat shock response in oysters. Improving larval and spat survival during the artificial propagation of the European flat oyster is crucial if sufficient production of the spat of this species is to be realized. A reliable supply of adequate stocking material of *O.edulis* will expedite the development of *its* aquaculture and restoration programs. Through a BLAST search, this study will further identify the heat shock protein genes in the recently annotated *C. gigas* genome. Knowing all the HSP70 genes and their corresponding proteins will help to have a clear understanding of their expression patterns under different stress stimuli. This information will help design HSP-based therapies for oyster aquaculture. The specific objectives of this study include:

i. To describe a protocol to be used to determine the optimal dose and sufficient contact time of phloroglucinol that stimulates the maximum production of HSP70 in the spat of

O. edulis. The procedure to assess the toxic effect of phloroglucinol to the spat will also be described.

ii. To describe a grading simulation and bacterial challenge test to be used to examine the protective effect of HSPs in the spat of *O. edulis*

iii. To perform a blast search against the *C. gigas* genome to identify the HSP70 genes

CHAPTER 2

LITERATURE REVIEW

2.1. The production of European flat oyster

2.1.1. Overexploitation led to the decline of European flat oyster

The native European flat oyster (*Ostrea edulis*) had awide natural distribution that stretched from the coast of Norway, through the Iberian Peninsula and south to Morocco (Pogoda 2019). This once-abundant oyster formed an essential part of the diets of many European coastal societies, even during ancient times. The discovery of large piles of oyster shells in kitchen middens in Viking settlements and other earlier communities reveals the historical dietary value of the species (Goulletquer and Heral 1997; Buestel et al. 2009). The harvesting of the oysters for thousands of years was limited to the collection of easily accessible oysters from the intertidal area for individual use. However, about 1000 years ago, communal industrial harvesting and marketing of oysters started (Gercken and Schmidt 2014). Progressively, the harvesting of the oysters increased to meet the growing demand. Population growth and the rise in oyster trade following the advent of railway transport explained the increase in demand for the oysters. Harvesting was further intensified by introducing dredging and trawling to access once unreachable stocks. For instance, estimates show that the Netherlands' annual landings of *O. edulis* in 1889 were between 11 and 18 million oysters (Berghahn and Ruth 2005). Assessments also indicate that London alone consumed over 700 million oysters in 1864 (Helmer *et al.* 2019). The excessive harvesting eventually resulted into destruction of most of the oyster beds- reducing the stock to unprofitable levels all over Europe. For instance, annual landings of flat oysters in France's Brittany (one of the major producing regions) reached its peak of over 100 million oysters by the turn of the 19th century. However, by the end of that century, 18 of the 23 initial beds had been decimated reducing the stocks to unprofitable level (Grace et al. 1997). Also, commercial harvesting of Scotland's Firth of Forth 166km^2 oyster beds started in the 16th century and maintained until the 19th century. However, by 1890 that fishery was no longer profitable following a 99% decline in catches. Today, the stocks at the Firth of Forth are considered as eliminated (Thurstan et al. 2013). The long-term intensive harvesting of *O. edulis* and eventual extermination of the stocks is also well documented in other European countries including Germany, Belgium, Netherlands, Spain, Norway and many others (Grace et al. 1997; Steins 1997; Berghahn

and Ruth 2005; Lotze 2007; Kerckhof et al. 2018). Apart from uncontrolled harvesting, other factors including severe winters, diseases (mainly bonamiasis and marteiliasis), and the introduction of the cupped oysters, particularly the Pacific oyster, *Crassostrea gigas* are partly blamed for the collapse of *O. edulis* stocks (Grace et al. 1997).

2.1.2. Status of the *O. edulis* stocks

Considering the intensive harvesting of *O.edulis*, many European countries intervened in the control and management of that fishery to prevent overfishing in their areas of jurisdiction. Most countries instituted closed areas and seasons, while other countries like the Netherlands and England implemented restocking programs. For instance, fishing from Germany's East Frisian (Wadden Sea) and Helgoland (North Sea) beds was temporarily halted between 1816-1823 and 1879 to 1882, respectively (Gercken and Schmidt 2014). However, despite the control interventions, nearly all beds of flat oyster disappeared from most European producing areas at the end of the 19th century (Goulletquer and Heral 1997). The continuous selective removal of the adult oysters weakened the natural reproduction of the species resulting in the later decline of its stock. Successful reproduction and recruitment were further affected by the removal of natural substratum for spat settlement and freezing winters. Following the destruction of the *O. edulis* beds, the species is classified as a threatened species (OSPAR Commission 2009). Fortunately, recent stock assessments show that small remnants of this previously abundant and widespread *O. edulis* persist in some parts of Europe. Though, most of these stocks are regarded as functionally extinct and fluctuate depending on the environmental conditions and incidences of viral and parasitic infections. A two-year survey (2010-2012) revealed that deposits of *O. edulis* still occur in seven of the previous fifty fishing areas of Ireland. However, the estimations showed a low average density of 0.5 oyster $m^{-2,}$ and one site (the Inner Tralee Bay), seems to contain about 80% of Ireland's existing biomass. The annual production from these areas fluctuates between 100 and 300 tons depending on the total allowable catches (TAC), which are influenced by the prevailing biomass (Tully and Clarke 2012). Persistent stocks have also been reported in the Netherlands, constituting about only 3% of 1970s levels. Natural stocks of *O. edulis* have also been reported in the Nordic countries, notably Norway, Denmark, and Sweden. In Norway, the primary area of occurrence is the Aust-Agder province and, to a less extent, the Helgeland province and near the Swedish border (Gercken and Schmidt 2014). These stocks appear original- genetically, which is mainly ascribed to a lack of introductions. Moreover, the stocks in Sweden are certified to be free of pathogens. Denmark uniquely owns a Marine Stewardship Council

(MSC) certified oyster population in Limfjord. The stock at Limfjord was initially certified to be free of both Bonamiosis and Marteiliosis (Laing et al. 2005); however, the bonamia parasite was detected in that stock in 2015 (Madsen and Thomassen 2015). Reports for the existence of stocks in France, Spain, Scotland, Germany, and other parts of United kingdom are available (Tully and Clarke 2012; Eagling et al. 2015; Long et al. 2017; Kerckhof et al. 2018; Nielsen and Petersen 2019; Thorngren et al. 2019). However, these stocks are variable due to unstable environmental conditions and infections from parasites and the ostreid herpesvirus.

2.1.3. Origin and status of European flat oyster aquaculture

The French pioneered the culture of the European flat oysters in the 17th century (Buestel et al., 2009). Oysters were produced by collecting spat from rocks or dredging them from natural beds and subsequently stocking them in purpose-built ponds for 4-5years. Initially, production was carried out in the saltwater marshes of France's Atlantic coast but later in specially managed ponds in the Marennes-Oléron region (Goulletquer and Heral 1997). Oyster production expanded in the marshland in the 18th century to cover large chunks of salt marsh that were previously under salt production—this followed the replacement of salt as a currency, a practice that began in the medieval period. However, production was then hindered by the decline in the supply of flat oyster spat following a period of long-term, extensive harvesting. This was further exacerbated by decrees banning the harvesting of oysters from the natural beds at the French coast in 1750. The short supply of spat stimulated the development of modern oyster farming, which was then based on the setting of spat on collectors. Initially, in the 1850s, the spat was collected on wooden stakes, a technique copied from Italy before changing to the use of oyster shells and later slates. Limed roof tiles also replaced the slates in 1865, resulting in the abundant and regular supply of spat (Buestel *et al.* 2009). The subsequent shortage of *O. edulis* in 1860 led to the importation of the Portuguese oyster (*Crassostrea angulate*) into different areas in France.

New on-growing techniques including the rearing of juveniles in the oyster box and later on-bottom grow-out in the intertidal area were also developed. Off-bottom culture started around 1900 on the Mediterranean coast and involved raising *O. edulis* cemented onto steel ropes, and later poles hung from mussel rafts. The culture of flat oysters increased considerably in different areas of France, reaching its peak at the beginning of the 19th century. For example, the annual production of *O. edulis* in the Bay of Arcachon between 1908-1912 varied between 15,000 and 20,000 metric tons. However, by 1920, the flat oyster was affected by mysterious mortalities and subsequently in 1950 and 1968

by *Marteilia refringens* and *Bonamia ostreae*, respectively. As a result, the total production of oysters in the 1960s was dominated by the Portuguese oysters at 80% in comparison to 20% of flat oysters (Buestel et al. 2009). The Portuguese oyster was similarly replaced by the pacific oyster (*Crassostrea gigas*) from Canada and Japan following its obliteration by a viral disease.

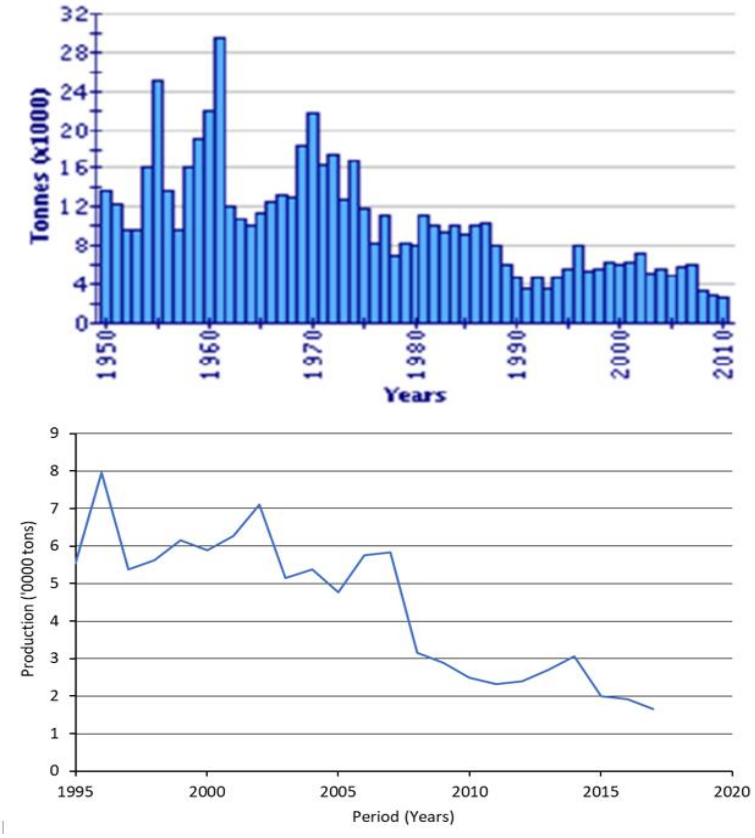

Figure 1 *Evolution of global production of the European flat oyster from aquaculture. The Upper panel-61year period from 1950 to 2010 adopted (Gercken and Schmidt 2014). Lower panel- latest production for 24year-period from 1995 to 2018 data obtained from the Organization for Economic Co-operation and Development (OECD) (https://data.oecd.org/).*

The aquaculture production of *O. edulis* has continued the dramatic downward trend for over 60 years. While in 1961, a maximum of nearly 30 000 tons was still produced, that amount has further declined to about 1,300 tons in 2018 (**Figure 1**) with Spain, France, and Ireland being the primary producers. The occurrence of epidemic diseases and the subsequent shift to the culture of *C. gigas* are implicated in this continued decline. Currently, there is interest in reviving the production of *O. edulis* in different European countries, shown by the numerous regional and national aquaculture and restoration projects. Among the aquaculture projects include NORD-OSTRON (2007-2013), which

aimed at developing flat oyster aquaculture in the Scandinavian nations of Denmark, Sweden, and Norway (Joyce et al. 2013b). Other projects include SEAFARE (2007-2013) and SETTLE (2008) aimed at the optimization of hatchery techniques for that species. Besides, these projects, national institutions, for example, the French Research Institute for Exploitation of the Sea (IFREMER) are extensively studying different issues regarding the aquaculture of *O. edulis* (González Araya et al. 2012; González-Araya et al. 2012; Morga et al. 2012; Gervais et al. 2016). Aquaculture of *O. edulis* is projected to supply seafood to meet the growing demand to supplement the landings from the stagnated and overexploited capture fisheries. Oysters aquaculture is favored because it meets the requirements set in Europe that food production from the sea must be done sustainably. Moreover, shellfish aquaculture does not require feeding or the use of additives nor medication. Apart from the food supply, the culture of *O. edulis* is expected to stimulate economic development in rural coastal communities of Europe through the provision of income-generating opportunities. Among the restoration projects include RESTORE and PROCEED (Germany), FOREVER (France), DEEP (Scotland), ENORI (UK), and "The bivalve project" of Sweden. Several trials were also conducted to restore flat oysters in different areas of the Netherlands, including Voordelta (Didderen et al. 2019), Wadden Sea, and Borkum Stone (https://haringvliet.nu/#/). In Belgium, there is an ongoing project dubbed UNITED aimed at combining restoration and aquaculture of flat oysters in offshore wind farms (https://www.h2020united.eu/). Besides the provision of goods, oyster reefs perform critical ecosystem services which include i) removal of nutrients through harvesting, ii) water clarification through filtration and iii) increasing biodiversity by providing habitats to different organisms (Coen *et al.* 2007; Smaal *et al*. 2015; Pogoda *et al.* 2019).

Although there is heightened interest in the flat oyster, the regular supply of sufficient amounts of spat remains one of the major obstacles hampering its aquaculture and restoration. Historically, the oyster spat was collected in pond-based nurseries (pollers) or estuaries on special collectors (Joyce et al. 2013b). However, the collection of spat from nature is limited to small-scale operations because of the variability in the spat settlement resulting from changing environmental factors. In any case, most of the remaining stocks are infected by ostreid herpesvirus (OsHV-1), and bonamiosis and the healthy ones are strictly protected by respective authorities that limit any form of harvesting. The development of flat oyster aquaculture and restoration programs requires large-scale production of seed in a predictable manner, which can only be achieved through artificial breeding in hatcheries. While protocols for artificial propagation of cupped oysters have been established, those for flat oysters are yet to be standardized and optimized. This is further complicated by the complex lifecycle

of this species, particularly alternation of sex in mature oysters and internal fertilization (Helmer et al. 2019). Although procedures for gametogenesis and conditioning of flat oyster broodstock (González-Araya et al. 2012; Joyce et al. 2013a) as well as the settlement of larvae (Robert et al. 2017) have been described, the meager survival rates of larvae and spat remains a significant constraint for flat oyster artificial breeding. It is reported that over 90% of the spawned larvae in the hatchery do not reach the eyespot stage (Gercken and Schmidt 2014). Therefore, there is a need to study how to increase the survival of larvae and spat in hatcheries and nurseries.

2.1.4. Artificial production of flat oyster spat

The intricate life cycle of the European flat oyster (

Figure 2) makes its domestication and artificial breeding more problematic in comparison to the cupped oysters. *O. edulis* is a protandric hermaphrodite, initially functioning as males and later switching to females before reversing to males (Helmer et al. 2019). Strangely, these sex changes can sometimes occur in a single spawning season. Naturally, they breed in early summer when the water temperature reaches about 15˚C. Unlike the cupped oysters, the flat oysters exhibit internal fertilization. The females release over 1 million eggs in their pallial cavities. The sperm from the water fertilizes the eggs inside the pallial cavity. The fertilized embryos develop inside the female to larvae within 7-10days, depending on the water temperature. The larvae are discharged into the water when about 140-190µm in size. The larvae stay in the water column for roughly 2-3 weeks before they settle on suitable surfaces that may be shells or rocks when they are about 240-300µm. Following the settlement, the larvae metamorphose into miniature oysters called spat of about 0.3-3mm. The spats grow into juveniles of 10-50mm within 1-2years, which takes 2 to 3 years to reach the grownup stage of about 120mm.

A couple of hatcheries in France, Spain, Sweden, Denmark, and the Netherlands are producing *O. edulis* spat. However, the amounts produced are still low and unpredictable to support the development of aquaculture and restoration programs for that species. This is mainly attributed to the low survival of the larvae and spat. Studies are underway to adapt and optimize the hatchery processes for this species. General processes that are used in two *O. edulis* hatcheries each in Denmark and Sweden were described by (Gercken and Schmidt 2014). The principal activities include algae culture, broodstock conditioning, and spawning, larvae culture, and lastly, spat culture. All the stages require an adequate supply of good quality seawater. Before its use, the water is treated to

different degrees of purity depending on the intended use. First, the water is cleared of particulate matter by sedimentation and several phases of filtration in sand filters and drum filters. This is followed by disinfection by Ultraviolet light (UV) and pasteurization. Water intended for algae culture is further autoclaved. Depending on the final use, the water may be heated or chilled to the required temperatures. Obtaining water from greater depths may reduce the intensity of treatment required. Water from lower depths is rich in minerals and has less suspended solids and organic material.

Larval rearing is the most critical activity during the artificial propagation of oysters because the planktonic larvae are susceptible to poor water quality, handling, and infections. This is demonstrated by very high mortality at this stage to the extent that If about 2% of the larvae reach the metamorphosis stage is considered a good result for larvae handling (Gercken and Schmidt 2014). The rearing of larvae is carried out in vertical plastic tubes (**Figure 3**), where they are kept in suspension by air from below. The larvae are fed on different mixtures of microalgae, for example, *I. galbana*, *I. galbana tahiti*, *M. lutheri*, *T. suecica,* and *Rhodomonas salina* (1:1:1:0.1:0.1), at a concentration of 100 cells μL^{-1} *Isochrysis* equivalents. *O. edulis* larvae were also shown to perform well on a diet of two microalgae species, *Chaetoceros neogracile* and *T-iso* (1:1 cell volume) at a concentration of 1500 μm^3 μL^{-1} and water temperature of 25˚C (Robert et al. 2017). Besides feeding, the larvae are regularly size-graded (every after two days), counted, and distributed to different tanks to maintain appropriate stocking densities. After about 10days, the larvae are considered competent for settlement when they start developing eyespots and foots as well as displaying a crawling behavior at this stage, measuring about 300µm. In both Denmark and Sweden, finely crushed (<1 mm) oyster shell particles (micro-cultch) are provided in vessels with perforated bottoms for settlement. Other substrates, including PVC sheets, oyster shells, and Chinese hats, may also be used. Besides the type of substrate, microalgae concentration, and water temperature are reported to influence the success of larval settlement (Robert et al. 2017). The use of chemicals including gamma-aminobutyric acid (GABA), l-3,4-dihydroxyphenylalanine (L-DOPA), epinephrine, norepinephrine, and 3-isobutyl-1-methylxanthine (IBMX) to induce settlement and metamorphosis in flat oysters have already been explored (O'Connor et al. 2009; Mesías-Gansbiller et al. 2013).

Following settlement and subsequent metamorphosis into the spat, the early planktonic spat is initially maintained in vertical, air-perfused tubes and supplied with micro-algae as food. However, when the spats reach about 1mm, they are transferred to round containers with a perforated base (**Figure 3**). These containers are hung in plastic or concrete troughs containing production water with

algae as the food source (**Figure 3**). At about -10 mm, the spat is transferred to floating upweller systems (FLUPSY), which may be anchored in ponds or estuaries. In the FLUPSY, the spat is placed in flat plastic baskets through which algae-rich seawater flow-through from the bottom. The up-flow is generated by water circulation that is created by a propeller. The spat is kept here for about one year until they rich the juvenile stage (50mm), which is transferred to the grow-out system. At all stages, the spat is regularly graded according to their size and split into different holding tanks and baskets to maintain suitable stocking stages. Besides grading, other regular maintenance activities like cleaning of baskets to control clogging are undertaken.

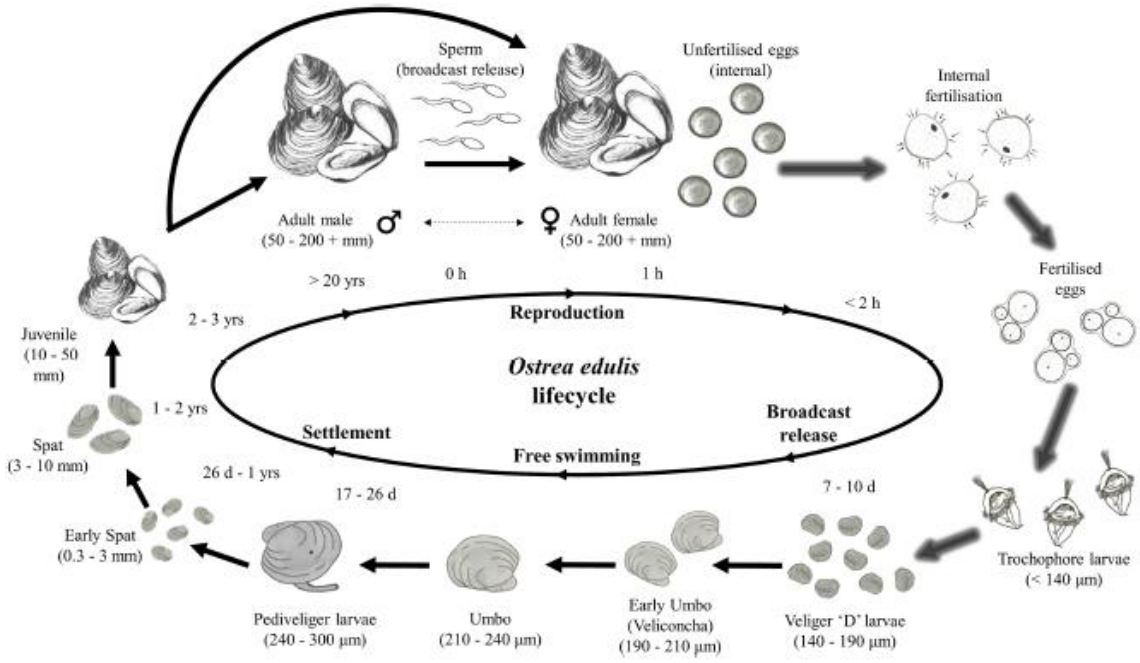

Figure 2 *Lifecycle of Ostrea edulis as adapted from (Helmer et al. 2019). Arrows with glow effect indicate stages that occur inside the mantle cavity of the female, while plain arrows are for external stages. Sperm enters females by way of inhalant current to fertilize the eggs inside the mantle cavity. Fertilized eggs develop into free-swimming Veliger D larvae and are released into the water after a 7-10day brooding period. After 17-26 days, the D-larvae develop to Pediveliger larvae ready for settlement. After settlement and metamorphosis, the spat takes 1-2 years to grow to juvenile stages, which also take 2-3years to grow to the adult stage.*

Algae culture is an essential activity for any bivalve hatchery. Each development stage in the hatchery that is broodstock, larvae, and spat is fed on a different mixture of microalgae species. In both countries, photobioreactors, plastic bags, and plastic tubes are used for the mass cultivation of algae. However, more sophisticated systems like the electronically operated turbidostats may also be used for that purpose (FAO 2004). Algae is produced by gradually scaling up stock cultures of the different

algae species maintained in the hatcheries. Actual spat production starts with the selection and conditioning of the broodstock. In the two hatcheries, several batches of broodstock comprising of roughly equal numbers of wild and farm-bred oysters are simultaneously conditioned to induce spawning. A brood batch comprising of around 50 oysters are conditioned in a tank by manipulation of water temperature, photoperiod, and nutrition. Flat oysters appear to be most favorably conditioned by respectively increasing water temperature and photoperiod by 1˚C and 2h per week for the first month from 14˚C to 18˚C and from 8h to 16h (Maneiro et al. 2017b)**.** These treatments result in more germinal cells, rapid larval release (10 weeks), and most larval production compared to conditioning at constant temperature (15˚C) and photoperiod (8h). A typical microalga mix for conditioning of flat oysters consists of 10% *Isochrysis galbana*, 10% *Tisochrysis lutea* (T-iso), 10% *Tetraselmis suecica*, 10% *Monochrysis lutheri*, 25% *Skeletonema* spp., 10% *Phaeodactylum* and 25% *Chaetoceros* spp (Maneiro et al. 2017b). In the past, other microalgae assemblages have been evaluated for flat oyster broodstock (González Araya et al. 2012; González-Araya et al. 2012). Besides the microalgae mix, food ration is also a critical factor that influences conditioning. A 6% dry weight algae per dry weight oyster day^{-1} $oyster^{-1}$ food ration appears to be most suitable for the conditioning of flat oysters with the gradients in temperature and daylight described above (Maneiro et al. 2017a). Compared to 3% and 9% food rations, 6% resulted in the rapid development of the gonads and spawning in addition to better larval survival. However, in the latest assessment of the combined effect of both the food ration and water flow rate, a food ration of 3% produced the highest number of viable larvae at 2 Lh^{-1} $oyster^{-1}$ (Maneiro et al. 2020). Following successful conditioning, the positive phototactic larvae collect in a catch basin (**Figure 3**) via an overflow. The collection basins are monitored daily for larvae because spawning of flat oysters is asynchronous. Broodstock can be induced to spawn almost in the same time frame to a limited degree by manipulating both water temperature and photoperiod (Joyce et al. 2013a).

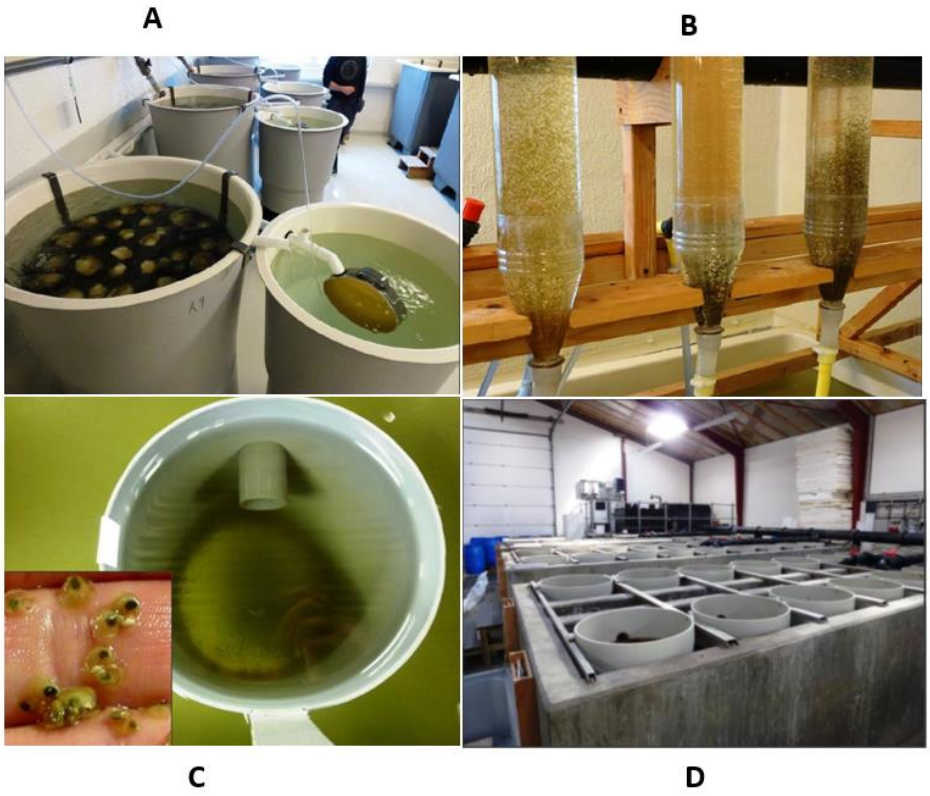

Figure 3 *System structures for O. edulis hatchery.* ***Panel A****: Conditioning tank with an overflow into the larvae collection tank,* ***Panel B****: Vertical tubes for the rearing of planktonic larvae and early spat,* ***Panel C****: Round containers with perforated bottoms for holding young spat,* ***Panel D****: Concrete tanks with microalgae algae with hanging spat containers. Adapted from (Gercken and Schmidt 2014)*

2.1.5. Daily husbandry activities induce stress predisposing oysters to mortalities

The economic loss resulting from oyster mortality is not known despite the overarching nature of the problem. However, the epidemiological aspect of the issue has received more attention from researchers. Generally, the mortality is very high in both the delicate larvae and early spat but declines in juveniles and mature oysters (EFSA Panel on Animal Health and Welfare (AHAW) 2010). For example, (Clegg et al. (2014) conducted an 18month prospective cohort study (2010–2012) on 80 batches located within 17bays to describe the mortality events that occurred in *C. gigas* in Ireland. Cumulative batch mortality (CBM), ranging from 2% to 100%, was observed in oysters deployed at a size of 2 mm. Studies have further shown that the causes of mortalities are multifactorial, resulting from the interaction of environmental, host, and husbandry factors (Burge et al. 2007; Sauvage et al. 2009; Genard et al. 2011; de Kantzow et al. 2016). The most important abiotic environmental factors include variations in temperature and salinity. The biotic factors, on the contrary, include i) viruses, particularly the *Ostreid herpesvirus* 1 (OsHV-1) and it's variant OsHV-1µvar, ii) pathogenic bacteria

(especially *Vibrio sp.*), and iii) parasites (*Marteilia refringens* and *Bonamia ostreae*). The herpesvirus was initially thought to affect only cupped oysters, but it is proven that it also affects the flat oyster (Mirella Da Silva et al. 2008; López Sanmartín et al. 2016). The most important host factors that influence mortality in flat oysters are age and physiological status, mainly influenced by sexual maturation and stress conditions.

Although husbandry practices have not been examined in the mortality of *O. edulis*, they are known predisposing factors in other oyster species (EFSA Panel on Animal Health and Welfare (AHAW) 2015). The nature of management practices undertaken varies depending on the stage in the production chain. That said, the most vulnerable larval and spat stages require intensive management compared to older grow-out stages and are consequently more at risk. Typical husbandry activities necessitate handling and transfer of oysters between different systems. For proper management, oysters must be graded to maintain stocks of uniform sizes. Moreover, grading in hatcheries and nurseries is done more often because of the faster growth rate. Mechanical grading of grow-out oysters at times requires transferring the stocks to onshore facilities. After grading, oysters are redistributed in different holding facilities to maintain appropriate stocking densities. As already discussed in the artificial propagation of oysters, each development stage is transferred to a different system. Transfer of oysters can even be between farms that may be miles apart. In the intertidal area, grow-out oysters may be shifted to deeper waters at the start of winter to protect them against damage from ice. Besides grading and transfer, oysters are handled during cleaning operations. In the hatcheries and nurseries, the tanks and containers must be cleaned and disinfected to control diseases. In the wild, the baskets must be cleaned of fouling matter, which may interfere with filtration. All these management activities involve emersion and exposure of the oysters to extreme temperatures in addition to traumatic stress (Zhang and Li 2006; Qu et al. 2009; Clegg et al. 2014).

Stress resulting from husbandry activities weakens the immune system of organisms, making them more susceptible to infections (Malham et al. 2009; Hooper et al. 2011). Grading exposes oysters to a myriad of stressors that include elevated temperature, physical trauma, starvation, and oxygen depletion. The effect of grading on *C. gigas* was initially demonstrated by measuring the level of blood catecholamine following a grading simulation (Lacoste et al. 2001f). In this experiment, the conditions encountered by oysters during sorting or grading were mimicked by placing the oysters in a 20-liter plastic container (diameter-21cm) rotating on a laboratory agitator for different time periods. The

level of catecholamine representing the magnitude of inflicted stress depended on the intensity and duration of grading. When oysters were subjected to a 15min shaking at 100 rpm, circulating noradrenaline (NA) increased 4-fold from 1.61±0.30 to 6.58±0.56 ng/ml. However, at a higher rotational speed of 300 rpm, NA increased by about 14-folds to 22.07± 0.97 ng/ml. A similar pattern was observed for circulating dopamine- increasing from 0.41±0.05 ng/ml to 1.21±0.11 and 2.24± 0.19 ng/ml for 100 and 300 rpm, respectively. The extent of stress also depends on the type of grader, as shown by comparing three graders (Rotary, Flat Bed, and Inside/Out) that are commonly used in Australia (Qu et al. 2009). The level of NA increased considerably for both the Flatbed and Inside/Out grader from 1.35±0.20 to 3.59 ± 0.29 ng/mL and from 1.57±0.22 to 3.02 ±0.32 ng/mL respectively after five minutes of an on-farm grading experiment. Compared to NA of the two graders above, the use of the rotary grader resulted in a slight increase of NA from 1.40±0.22 to 2.06±0.25 ng/mL. Similar patterns were also observed for the circulating dopamine levels. Consequently, the rotary grader causes the least stress, while the Flat Bed causes the highest stress in the context of Australia. The duration of grading also has an influence on stress, as indicated by the lysosomal membrane stability in the neutral red retention (NRR) assay (Zhang and Li 2006). This was demonstrated in a grading simulation by rolling *C. gigas* in a plastic barrel for 1, 1.5, 3, and 9 min at 50 rpm along the ground in a 15°C room. Hemolymph samples were collected for the NRR assay at increasing time periods post the simulation. As expected, the NRR times decreased with increasing grading minutes, and they took longer to return to the control levels. In the same study, the influence of grading was further exacerbated by starvation, illustrating the action of co-stressors.

Some husbandry practices involve exposing oysters to air, thereby subjecting them to extreme temperatures. The level of stress resulting from emersion depends on the air temperature and duration of exposure. Qu et al., (2009) demonstrated that exposure of *C. gigas* to 27.5°C air temperature for more than 8h considerably increases the blood catecholamine levels compared to 15°C. On the contrary, a 48h air exposure at 15°C did not affect the circulating catecholamine levels compared to the control. While studying the recovery of pacific oysters after a 72h air exposure, Zhang et al., (2006) showed that the NRR times of oysters exposed to 5°C temperature returned to the control level within 24h of recovery compared to 5d and 7d of 15°C and 25°C respectively. The results from the two studies signify that stress from emersion may be minimized by working quickly and at lower temperatures. Also, temperature shock may result from transferring oysters between tanks, farms, or intertidal plots at different water temperatures. In such transfers, it is vital to avoid both sudden and significant temperature changes. Increasing and decreasing water temperature by

2°C from 15°C to 13°C and 17°C respectively did not have any observable effects on the NRR time. However, by continuing to decrease and increase the water temperature to 11°C and 19°C, respectively, considerably decreased the NRR times (Zhang et al. 2006). In the same study, sudden transfer of oysters from water at 15°C to 25˚C and 5°C resulted into rapid reduction in NRR times from 145.0±5.0min to 105.0±5.0min within 0.5h and then down to about 65min in 3h. Complete recovery of these oysters took a minimum of 5 days and seven days for the 25˚C and 5˚C groups, respectively.

The connection between husbandry practices and mortality in oysters is not very clear based on the available laboratory and epidemiological studies. Clegg et al., (2014) conducted an 18months prospective cohort study in Ireland to identify environmental, husbandry, and endogenous oyster factors associated with the cumulative batch mortality (CBM) of 80 batches of *C. gigas* spat in the summer of 2011. A wide variation in the CBM (2-100%) was observed in this study. However, all the considered risk factors relating to farm management, including splitting, grading, and handling frequency were found to be not significant. This is further supported by a recent controlled infection model showing that moderate physical handling through a grading simulation 24h prior to the OsHV-1 challenge does not affect the survival of *C. gigas* (Oliver et al. 2019). Interestingly, a 24h air exposure in the same study seemed to offer protection to *C. gigas* in the subsequent OsHV-1 challenge. It should, however, be noted that a controlled experiment may not be representative of field conditions where different factors could have additive and interactive effects (Pernet et al. 2012). The above studies are, however, contradicted by a field survey done by (de Kantzow et al. 2017). This survey showed that mortality due to OsHV-1µVar in oysters that were handled for routine husbandry seven days before the outbreak was twice as high as non-handled oysters. Handling in this study referred to any routine management procedure applied to the oyster baskets, including grading, movement between leases, and the arrival of oysters onto the farm from a hatchery or another farm.

Despite the unclear relationship between handling and oyster mortality, routine management practices that involve handling like grading and transport inevitably inflict stress on to oysters. Stress is known to suppress the immune system making organisms more susceptible to infection. Mortality of oyster larvae and spat in the hatchery is mainly associated with opportunistic bacterial pathogens, particularly *Vibrio sp* (Dubert et al. 2017). So, stress resulting from several risk factors in the hatchery involving handling increases the incidence of mortalities due to different *Vibrio* including *V. aestuarianus*, *V. coralliilyticus*, *V. ostreicida*, V. alginolyticus, V. tubiashii, *V. splendidus*, *V.*

tasmaniensis, *V. crassostreae* among others (Estes et al. 2004; Prado et al. 2005; Richards et al. 2015; Dubert et al. 2017). Lacoste et al. (2001b) demonstrated that both grading simulation and injection of stress hormones followed by *V. splendidus* challenge resulted in increased mortality and bacterial loads in comparison to the control groups. Consequently, it may be hypothesized that minimizing the amount of stress inflicted on the spat and larval during the routine management may help to control infections and subsequent mortality. The control strategies based on reducing stress would increase the list of eco-friendly alternatives, including Probiotics, Quorum Quenching (QQ), and Phage-therapy in the control of infections in oyster hatcheries. All these strategies are needed to minimize the use of antibiotics, which pose environmental and public health risks resulting from antibiotic resistance and their residues.

2.2. The heat shock response in oysters

2.2.1. Heat shock response is part of the integrated stress response in oyster

Oysters occupy highly stressful habitats in the estuaries and the intertidal area that are characterized by variations in temperature and salinity, air exposure, pathogens, and anthropogenic pollution. Consequently, oysters have evolved sophisticated systemic and cellular mechanisms to survive in these stressful environments. Stress in this aspect refers to a condition in which the dynamic equilibrium of animal organisms called homeostasis is threatened or disturbed by intrinsic or extrinsic stimuli, commonly defined as stressors (Wendelaar Bonga 1997). At the systemic level, oysters respond to stress via the neuroendocrine-immune (NEI) system like vertebrates (Ottaviani and Franceschi 1996, 1997). The different components of the NEI system including hormones, neurotransmitters, and their receptors coordinate to modulate immune activities, energy allocation, growth, and locomotion in response to various environmental stressors (Ottaviani et al. 1998, 1999; Malagoli et al. 2000; Lacoste et al. 2001d, c, e; Stefano et al. 2002; Adamo 2012; Liu et al. 2018). At the cellular level, oysters respond to stress by a number of mechanisms that include unfolded protein response (UPR), DNA damage response, oxidative stress response, apoptosis, and heat shock response (Fulda *et al*. 2010; Zhang *et al*. 2016). There is growing evidence demonstrating coordination between the operation of the systematic and cellular responses (Lacoste et al. 2001a; Demas et al. 2011; Liu et al. 2016, 2017). The heat shock response is a highly conserved prokaryotic and eukaryotic cellular process that involves rapid synthesis and increased presence of molecular chaperones known as heat shock proteins (HSPs). The increased production of HSPs helps in the conformational folding and unfolding of misfolded and aggregated proteins resulting from the action

of different biotic and abiotic stress stimuli, including temperature and salinity shocks, emersion, pathogens, parasites, pollutants, and physical trauma. The presence of misfolded or aggregated proteins seems to trigger the heat shock response, which functions to restore and maintain the cell balance and function.

There are different classes of HSPs based on their molecular size, expressed in kilodaltons (kDa), and mode of expression. According to expression pattern, there are two classes, including the constitutive HSPs, which are expressed in normal conditions, and the induced HSPs that are only expressed in response to stress. The constitutive group (heat shock cognate/HSC) function as molecular chaperones as extensively reviewed by (Ellis 1994; Hendrick and Hartl 1995; Fink 1999). In summary, they aid in the folding of newly synthesized polypeptides to their functional 3-dimensional conformations. They also maintain cellular homeostasis by preventing both protein misfolding and aggregation. Besides protein folding and refolding, molecular chaperones seem to be involved in other processes, including protein transport (Dierks *et al*. 1993), signal transduction (Richter and Buchner 2001), apoptosis (Beere 2004; Hishiya and Takayama 2008), and immune responses (Noort 2008). The induced HSPs protect the cell from the damage of stressors and play a role in the cell recovery process by refolding proteins and separating aggregated proteins.

There are several size groups of HSPs, but most studied families include 60, 70, 90, and 110-kDa. Members of the HSP70 family are the most conserved HSPs found in both prokaryotes and eukaryotes. This family consists of both the constitutive (HSC70) and inducible (HSP70) isoforms. The functioning of the HSP70 members is based on their ability to recognize exposed hydrophobic amino acid side chains and open polypeptide backbone. They accomplish their roles by binding and releasing their substrates with the regulation of ATP and different cofactors. Similar to the HSP70 family, members of the HSP90 group consist of conserved proteins that are involved in protein structure regulation and signal transduction of all organisms (Li and Buchner 2013). Isoforms of HSP90 are the most abundant chaperones, accounting for 1-2% of constitutive cell proteins (Hoter *et al*. 2018). Unlike HSP70 and HSP90, members of the HSP110 family are only present in mammalian cells where they are believed to collaborate with other chaperones to refold misfolded proteins (Mattoo *et al*. 2013). Compared to the previous groups, the HSP60 family distinctively consists of oligomeric double-ring proteins known as chaperonins (Fabbri *et al*. 2008). Members of HSP60 form assembly complexes for protein folding in all cell organelles except for the endoplasmic reticulum. In addition to the main HSPs, there are small heat shock proteins (sHSP) that fall in the 12 and 43-kDa size range.

Found in the cytosol, nucleus, and mitochondria, their functions are yet to be established. It is thought that they may be necessary for ATP-independent protein chaperoning under stressful conditions (Sun and MacRae 2005; Webster *et al*. 2019).

2.2.2. Expression of heat shock proteins in *O. edulis*

There is minimal information on the expression of 60 and 90-kDa HSPs (Choi et al. 2008; Ivanina et al. 2008, 2009) and non on the small heat shock (sHSP) proteins in oysters. Nevertheless, the 70-kDa family has been extensively studied at both the functional and molecular levels. According to immunoblotting analyses, flat oysters express two constitutive HSP70 isoforms of about 72 and 77-kDa in addition to a 69-kDa inducible isoform as other oyster species. This expression pattern was experimentally demonstrated by (Piano *et al*. 2002) by exposing *O. edulis* to increasing temperatures for one hour. The two constitutive isoforms (72 and 77-kDa) were present in the control groups confirming their housekeeping functions. However, their expression in treatment groups was highly variable, showing slight changes at different temperatures. The 69-kDa isoform was expressed in the mantle and gills tissues of *O. edulis* that were exposed to a temperature higher or equal to 32˚C after 4hrs of post-stress recovery. Similar expression patterns have been revealed in different oyster species, including *Crassostrea virginica* (Ueda and Boettcher 2009; Ueda *et al*. 2009) and *Crassostrea gigas* (Clegg et al. 1998; Jackson et al. 2011b).

The molecular structures of the inducible and constitutive HSP70 genes in *O. edulis* have been described by (Boutet *et al*. 2003a; Piano *et al*. 2005). The gene for the inducible isoform lacks introns, a feature characteristic of all inducible HSP70 genes. The absence of introns is believed to allow for rapid and preferential synthesis of these proteins in response to stress because it evades the step of RNA splicing (Kay *et al*. 1987; Hyun-Bae *et al*. 1996). On the contrary, the constitutive isoform (HSC70) comprised of six exons (184, 206, 168, 553, 401, and 257 bp) interrupted by five introns. The interruption by introns is indicative of constitutive expression in HSP70 (Günther and Walter 1994). Boutet *et al*. (2003b) demonstrated a similar nucleotide structure in *C. gigas*. The amino acid sequences that correspond to the respective HSP70 nucleotide sequences revealed conserved structural and evolutionary features with homologous HSP70 polypeptide sequences from other organisms. The most outstanding structural feature of the HSP70 proteins is their conserved basic structure (***Figure 4***) that consists of (i) a cleavable signal sequence at the N-terminus, (ii) an ATPase

domain; (iii) a peptide-binding domain; and (iv) a G/P-rich C-terminal domain which terminates in the localization signal (Kiang and Tsokos 1998).

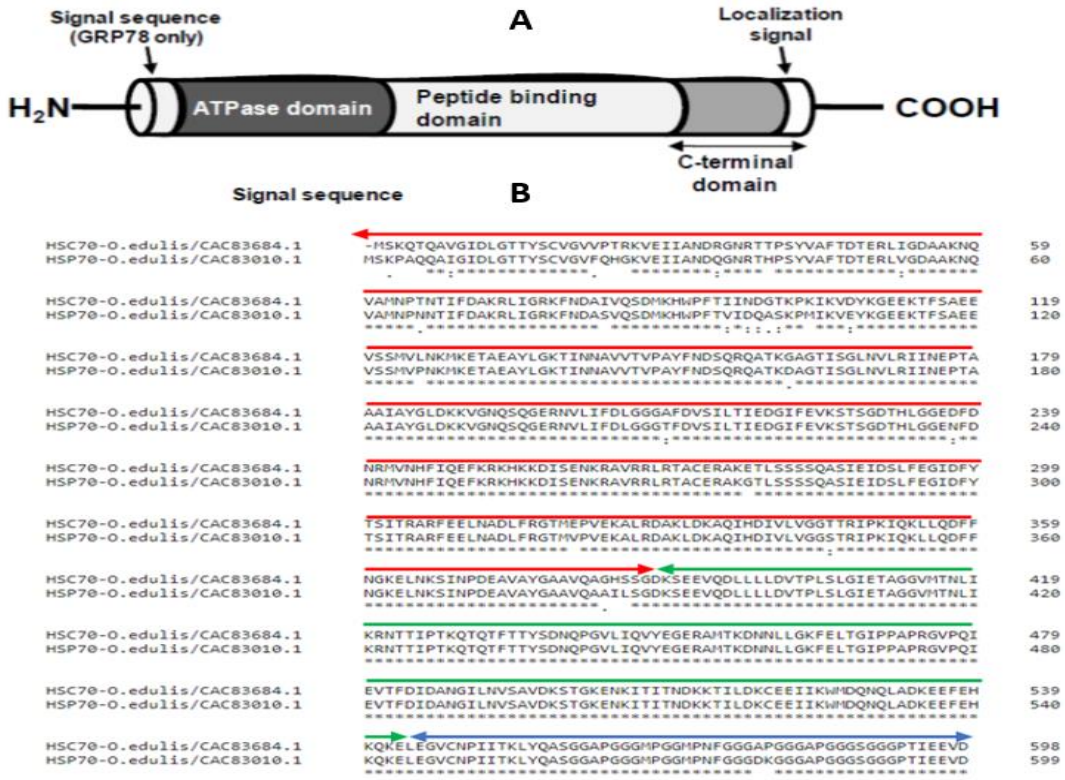

Figure 4 *Heat shock protein 70 (HSP70) in O. edulis. Panel* ***A:*** *Animation drawing illustrating the linear structure of the general domain structure of the HSP70 protein family as adapted from (Fabbri et al. 2008), Panel* ***B****: Alignment of two HSP70 protein sequences form O. edulis showing the position of the major domains (Red arrow: ATPase domain; Green arrow: Peptide binding domain; Blue arrow: C- terminal domain).*

All isoforms of HSP70 from *O. edulis* exhibit the three signature motifs for HSP70 family: IDLGTTYS (residues 11-18) IFDLGGGTFDVSIL(residues 203-216), and IVLVGGSTRIPKIQK (residues 340-354) (Gupta and Singh 1994). So far, no functions have been assigned to these conserved motifs. Additionally, the sequences have the glycosylation domains, NKSI and NVSA, as well as the cytoplasm localization consensus motif EEVD (residues 596-599) at the C-terminal. The ATPase domain is the most conserved section between the inducible and constitutive isoforms across all organisms. Nonetheless, the ATPase domain from cognate isoforms contains an extra tetrapeptide (NQSQ) motif, which is lacking in the inducible isoforms. This pattern is also observed in sequences from other oyster species shown in **Table 1**. Most of the variations between the cognate (HSC70) and the induced (HSP70) isoforms occur within the C-terminal domain. Each of the inducible and constitutive isoforms from different species shares more similarity than both isoforms of the same animal. For instance, a protein BLAST of HSC70 sequence (accession CAC83684) from *O. edulis* indicates a 95.5% homology

to HSC70 protein from *C. gigas* (accession CAC83683) which is slightly higher than 94.7% of HSP70 protein from *O. edulis* (accession CAC83010). In mammals, HSC70 differs from HSP70 by exhibiting two repeats of a tetra-peptide GGMP(Fuertes *et al.* 2004) in their C-terminal domain. Piano et al. (2005) also observed that the GGMP repeats are present in the bivalve constitutive heat shock cognate 70 (Hsc70), while absent in the inducible heat shock protein 70 (Hsp70). Similar repeats have been observed in sequences from other bivalves, including *Pinctada fucata* (Wang *et al*. 2009), and *Mytilus galloprovincialis* (Kourtidis *et al*. 2006). However, in the *C. gigas* (Boutet *et al*. 2003b) and *O. edulis* (Boutet *et al*. 2003a), both of Hsp70 and Hsc70 contained the two repeats of GGMP tetrapeptide. The GGMP motif regulates chaperone interactions and substrate binding in mammals (Gupta and Singh, 1994). It seems to play similar roles in oysters and is the most plausible explanation for the functional differences between constitutive and induced isoforms. Homologous protein alignment also revealed a 60 amino acid deletion in the HSC70 sequence of *O. edulis*. This deletion incorporates the end of the peptide-binding domain and part of the C-terminal domain and seems to be unique for *Ostreidae* (Kourtidis *et al*. 2006).

Table 1 *Complete and partial HSP70 nucleotide sequences that are available in the Genbank*

Species	Isoform	Genbank accession	Reference
Crassostrea gigas	HSC71	AB122064	Unpublished
	HSC72	AF144646	(Gourdon et al. 2000)
	HSP70	AJ318882	(Boutet et al. 2003b)
	HSC70	AJ305315	(Boutet et al. 2003b)
	HSP70	AB122063	Unpublished
	GRP78	AB122065	(Yokoyama et al. 2006)
	GRP94	AB262084	(Kawabe and Yokoyama 2009)
	HSP68	AB122062	Unpublished
Crassostrea ariakensis	HSC70	AY172024	Unpublished
Crassostrea columbiensis	HSP70	DQ294635	Unpublished
Crassostrea hongkongensis	HSP70	FJ157365	(Zhang and Zhang 2012)
	HSP70	KY906021	Unpublished
Crassostrea sikamea	HSP70	LC195299	(Nagata et al. 2017)
	HSP70	JQ844547	Unpublished
Crassostrea virginica	HSP70	AJ271444	(Rathinam et al. 2000)
	HSC70	AJ305316	(Boutet et al. 2003a)
Ostrea edulis	HSP70	AJ318883	(Boutet et al. 2003a)
	HSP70	AF416609	(Piano et al. 2005)
Pinctada fucata	HSP70	EU822509	(Wang et al. 2009)
Saccostrea palmula		DQ294636	Unpublished
Pteria penguin	HSP70	EF011060	Unpublished

2.2.3. Regulation of HSP70 expression in *O. edulis* is not yet studied

Despite the increased understanding of the heat shock response in oysters, little is known about the regulatory mechanisms behind this response. It is believed that both the induction and expression of HSPs involves various proteins and signaling pathways including the hypoxia-inducible factor (HIF)(Kawabe and Yokoyama 2011, 2012) and the mitogen-activated protein kinases (MAPK) (Anestis *et al*. 2007; Patterson *et al*. 2014; Li *et al*. 2017). It is, however, clear that the process is mostly regulated at the transcriptional level by the heat shock factor (HSF) (Wu 1995). Four HSF genes (HSF1 to HSF4) exist in higher animals compared to one gene in invertebrates (Wu 1995; Kiang and Tsokos 1998; Takii and Fujimoto 2016). The family of HSF required for heat shock response differ from organisms; for example, birds use HSF3 contrary to HSF1 in mammals (Takii and Fujimoto 2016). Like mammals, the single gene of invertebrates appears to be HSF1.

The HSFs mainly regulate HSP genes by binding the heat shock elements (HSEs) to the promoter of the target genes to initiate transcription and subsequent translation. The HSFs are triggered by changes in their conformational structures resulting from stress insults, including temperature and cellular pH variations and reactive oxygen species (ROS). Also, variations on the levels of cellular HSPs have been shown to activate the HSF (Jacquier-sarlin and Polla 1996; Zou *et al*. 1998; Zhong *et al.* 1999; Hentze *et al*. 2016). Under normal conditions, the latent HSFs localize in the cytosol bound to HSPs (HSP70/90). During stress, however, the HSFs undergo a series of transformations, starting with the detachment of the HSPs and then phosphorylation by several protein kinases. The phosphorylated HSFs then uniquely oligomerize into trimers, which possess high DNA-binding affinity. The HSF trimers bind to the HSEs, which are multiple adjacent and inverted repeats of the pentanucleotide 5'-nGAAn-3' in the promoter regions of the HSP genes (Wu 1995; Kiang and Tsokos 1998). The HSFs is further phosphorylated to initiate transcription of the target genes and further translation in the cytosol. The HSFs return to the cytosol and reattach the HSPs to restore its inactive form.

Despite the existence of different HSF families in eukaryotes, they all share a typical core structure that consists of the DNA-binding domain (DBD), Oligomerization domain, and transcriptional activation domain (**Figure 5**). The amino acid sequences of DBD and oligomerization domain (hydrophobic heptad repeat-A/B; HR-A/B) are highly conserved (Wu 1995; Takii and Fujimoto 2016). Moreover, the nuclear localization signals and other similar short sequences in these proteins are also conserved. In comparison, the transcriptional activation domains of the HSF families differ in

terms of location and sequences. The DBD is located near the N-terminal of the protein, and as the name suggests, it is where HSF bind to the DNA. The hydrophobic heptad repeat-A/B (HR-A/B) constitutes the oligomerization (also known as the "trimerization") domain. The HR-A/B is separated from DBD by a 10-20 amino acid flexible spacer. At a secondary structure, this domain forms two α-helices (HR-A and HR-B) containing repetitive seven amino acid sequences. These heptad repeats include hydrophobic amino acids such as leucine, isoleucine, and valine, which help in the formation of HSF trimers. The second hydrophobic heptad repeat HR-C near the C-terminus regulates the trimerization of HSF during normal conditions. Another mysterious hydrophobic heptad repeat DHR down-stream of the HR-C seems to be restricted to vertebrate HSFs (Nakai *et al*. 1997). There are two recognized nuclear localization signals (NLSs), NLS1, and NLS2 surrounding HR-A/B domains. These mediate the entry into the nucleus of HSFs. The activation domains may be in the C-terminal (CTA) and or in the N-terminal (NTA). Their sequences are not evolutionally conserved though their activity is suppressed in unstressed conditions by a conserved motif CE2 that exists near the activation domain.

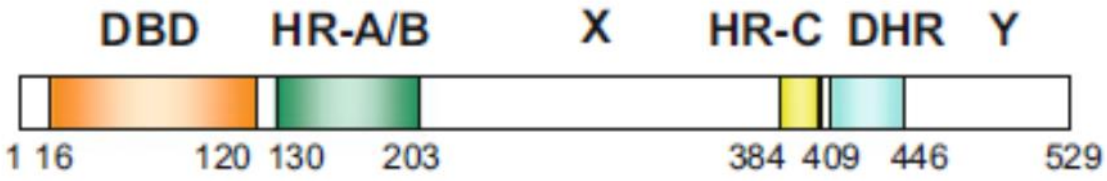

Figure 5 *Diagrammatic representation of the structures of human HSF1. The values below show the number of amino acids. DBD DNA-binding domain; HR hydrophobic heptad repeat; DHR down regulation of HR-C*

Despite the recognized heat shock response of bivalves, the transcriptional regulation of that putative response has not yet received much attention. To date, the only studied bivalve HSF is from the Pacific oyster (Kawabe and Yokoyama 2011; Liu et al. 2019, 2020). Like mammals and other invertebrates, *C. gigas* display HSF1. Cloning of *C. gigas* HSF1 full-length cDNA revealed that it encodes a 463 amino acid protein of 52-kDa estimated molecular weight (Kawabe and Yokoyama 2011). The DBD of the novel *C. gigas* HSF1 is situated between the 5th and 107th amino acids of the polypeptide. The two hydrophobic heptad repeats, HR-A/B and HR-C constitute amino acids 126 to 209 and 350 to 396, respectively. Comparison of the full-length amino acid sequence of *C. gigas* HSF1 showed low homology (42-31%) to HSF1 from other animals, as shown in **Table 2**. However, (Kawabe and Yokoyama 2011) observed up to 74% homology between oyster and mollusk DBD and HR-A/B

sequences. As expected, the HR-C and the other amino acid sequences of HSF1 showed much variation. Moreover, the HR-C domain of *C. gigas* was discovered to exceptionally contain three different insertions: one 48bp long the other two of 42bp each. Previously, only two insertions in *Drosophila* (Fujikake *et al*. 2005) and one in both mammals (Goodson and Sarge 1995) and fish (Råbergh *et al*. 2000) had been reported. Kawabe and Yokoyama (2011) further demonstrated that through alternative splicing, the *C. gigas* single HSF1 gene produced eight different HSF1 isoforms (HSF1a to HSF1h) consisting of varying combinations of the insertions. As earlier noted that HR-C regulates trimerization through its interaction with HR-A/B (Rabindran *et al.* 1993), and trimerization is needed for the attainment of transcriptional activity of HSF1. The variations in the HR-C sequences and the different HSF1 isoform expression patterns might contribute to the observed species and tissue-specific heat shock response.

Table 2 The level of homology between C. gigas HSF1a isoform and HSF1 sequences from other organisms

Organism	**Identity (%)**	**Accession Number**
Pomacea canaliculate	38	PVD20998
Carassius auratus	40	AHN60082
Haliotis asinine	42	ABR15461
Oncorhynchus mykiss	31	BAD10988
Ctenopharyngodon Idella	42	ALK27859
Homo sapiens	37	AAA52695
Xenopus laevis	34	AAA99999
Danio rerio	42	ABR15461

There is a need to understand the regulation of the heat shock response in *O. edulis* to optimize HSP-based therapies in the bid to control mortality. Oysters respond to stress conditions by increased synthesis of protein chaperones, mainly members of the HSP70 family. The HSF1 proteins are the master regulator of the heat shock response in all organisms. The gene that encodes the HSF1 was for the first time cloned and sequenced in a bivalve the *C. gigas* and its involvement the regulation of HSP70 in that species has been recently confirmed (Liu *et al*. 2019, 2020). The *C. gigas* HSF1 gene varied greatly in the HR-C domain with three insertions, which resulted in eight HSF1 isoforms under air exposure. It would be essential to establish whether the *O. edulis* HSF1 gene displays similar insertions. The expression pattern of the HSF1 isoforms in the different animals appears to be tissue specific. Of the two isoforms HSF1-α and HSF1-β in mammals, the former seems to be preferentially expressed in the heart and brain of mice while the former in the testis (Goodson and Sarge 1995). In

the orange-spotted grouper (*Epinephelus coioides*), the two isoforms HSF1a and HSF1b are mainly expressed in the brains, eyes, and fin compared to other tested tissues like the heart, gills, and spleen (Wang *et al*. 2016). In *C. gigas*, mRNA of all the eight isoforms was detected in both the heart and the gills. However, their expression in the other tissues, including the adductor muscle, mantle, body trunk, and labial palps, was variable (Kawabe and Yokoyama 2011).

The HSF1 isoforms also appear to have different regulatory functions during the stress response and normal cell functioning. In *C. gigas*, HSF1a, the isoform without any insertion seems to regulate constitutive expression of HSP70, whereas the other isoforms responsible for the inducible members of HSP70. The level of HSF1a mRNA from oyster gills remained noticeably unaffected after 72-hour immersion in hypoxic water. Nevertheless, the mRNA levels of the other isoforms, especially HSF1b-d and HSF1f, more than doubled after 48hour treatment (Kawabe and Yokoyama 2011). Interestingly, there was a corresponding increase in the level of HSP70 mRNA just after the induction of the mRNA of the Hsf1 isoforms. Differential response of HSF1 isoforms was also observed in fish, including the orange-spotted grouper (Wang *et al*. 2016) and the zebrafish (Airaksinen *et al*. 2003). Heat shock (36˚C) increased the level of HSF1b transcripts in grouper larvae, while cold shock (16˚C) increased that of HSF1a. Also, while both isoforms were present in the eggs of the grouper, the level of HSF1b considerably increased during the larval stage while that of HSF1a remained consistently low. The expression pattern in the zebrafish is like that of the grouper. Heat shock in this species upregulates HSF1a while HSF1b is downregulated, leading to a 10-fold increase in the HSF1a/HSF1b ratio. The expression pattern of the *O. edulis* HSF1 isoforms in response to different stress stimuli that need to be examined.

2.2.4. Potential to use heat shock response to control mortality in oysters

The heightened interest in HSPs-based therapies stems from the observation that sublethal stress induces thermotolerance, cross-tolerance, and enhances immunity in different organisms (Zügel and Kaufmann 1999; Sung et al. 2011). Induced thermotolerance and cross-tolerance respectively defined as enhanced tolerance to lethal stress resulting from exposure to sublethal stress and attainment of protection from stressors other than the initial stress, have been demonstrated in several organisms including Artemia (Sung et al. 2008), shrimp (Sung et al. 2018; Aishi et al. 2019), mussels (Aleng et al. 2015), fruit flies (Krebs and Feder 1998) and fish (Sung et al. 2012, 2014). Thermotolerance resulting from heat shock response was first demonstrated in the Pacific oyster by (Clegg et al. 1998). That study showed that heat shock of 37°C for one hour enabled Pacific oysters

to survive an otherwise lethal heat treatment of 43° to 44°C for one hour. Moreover, the acquired thermotolerance persisted for at least two weeks of the study. This acquired protection has subsequently been shown in other oyster species, including eastern oysters (Encomio and Chu 2007; Jackson et al. 2011b), Olympia oyster (Brown et al. 2004; Bible et al. 2020) and the European flat oyster (Piano et al. 2002). A 1h exposure of *O. edulis* to heat shock of ≥32˚C enhances the expression of HSP70 that lasts for at least 14 days. The enhanced levels of HSPs confer cytoprotection by repairing damaged proteins and protecting oysters from subsequent stress. The transient protection may be used to control stress resulting from routine husbandry activities in oyster hatcheries, thereby improving survival.

Three approaches have been used to test the potential of applying HSPs-based therapies to control mortality in different organisms. The first approach involves selective breeding aimed at improving thermotolerance (Samain et al. 2007; Lang et al. 2009). Tan et al. (2020) proved that selective breeding to increase the expression of cognate HSP70 genes could improve the survival of juveniles and larvae of the hard clam, *Mercenaria mercenaria,* in a thermal shock challenge at different temperatures (24 to 33˚C). Also, in a search for a controlled challenge method that could result in the highest selection response towards increased OsHV-1 resistance in *C. gigas*, Camara et al. (2017) compared a heat shock challenge (as a proxy for the virus challenge) against an OsHV-1 lab challenge. Although the heat shock challenge exhibited a lower narrow-sense heritability (h^2) of 0.15 as compared to 0.45 of the OsHV-1 challenge tests, it showed higher variability in the survival of the families in that study. The second approach involves genetic manipulations, like gene knockdowns, to show the role of molecular chaperones HSP70 in protecting organisms against abiotic and biotic stressors. At the moment, this method has been limited to studies in crustaceans, particularly Artemia and shrimp (Iryani et al. 2017; Aishi et al. 2019). The third and most popular method involves stimulation of HSP production using different inducers followed by a challenge test with a stressor of interest to show whether the enhanced HSPs indeed protects the organisms from the factor.

The protective effect of enhanced HSP70 expression on subsequent challenge tests has been tested in oysters under both field and laboratory conditions. Encomio (2005) assessed the role of HSPs in protecting eastern oysters from Dermo disease caused by the protozoan, *Perkinsus marinus*. In that study, moderately infected oysters (10^4-10^5 *P. marinus* cells/g wet weight) were heat-shocked for 1 hour at 40˚C. Following the heat shock, the oysters were exposed to a lethal heat shock of 44˚C for one hour. The sublethal shock improved the survival of the infected oysters compared to the control

group. Moreover, the role of HSPs in protecting oysters from the effects of ocean warming has been tested in two species, the flat oyster, *Ostrea angasi*, and the Sydney rock oyster, *Saccostrea glomerate* (Pereira et al. 2020). The oysters were initially heat-shocked before deployment in Lake Macquarie, Australia, in a location where the seawater is warmed to about 37.5˚C by exhaust water from two nearby power plants to mimic the ocean warming conditions. There were negligible differences in the survival, shell growth, and condition index of treatment and control groups after a 7months field placement. However, no verdict can be taken with certainty on the protective effect of HSP70 in controlling mortality in oysters based on the nature of this study. First, thermotolerance in oysters has been shown to last for about 14 days, so, after seven months, the protective effect of the HSPs could have already receded. Also, many other factors other than ocean warming could influence the monitored parameters in the field where the experimenter has no control. Increasing thermotolerance through selective breeding may be the right approach to improve survival in the face of ocean warming.

The use of HSPs to control mortality resulting from husbandry related activities has recently been examined in the eastern oysters (Casas and La Peyre 2020). The study examined whether heat shock before harvest could reduce the elevated mortality that limits cold storage and transportation of eastern oysters in summer and early fall from the Northern Gulf of Mexico. Market size oysters were heat-shocked (41 °C, 1 h) in the laboratory, and their level of HSP70 and cumulative mortality monitored during a 21day cold storage in a walk-in cooler maintained at about 4 °C. There was no difference in the cumulative mortality between the treatment and the control groups even though the heat shock increased the HSP70 levels in the treatment groups by more than two times. The role of HSPs in controlling oyster mortality associated with routine brief handling and transport during oyster culture may not be ruled out based on the above study. The immense physiological demands put on the oysters by prolonged cold storage and transport may be above the protection of heat shock proteins. The technique of “hardening” involving the prolonged placement of juvenile oysters in the stressful upper intertidal area before transfer for on-growing has been used for decades to improve survival in Japanese and Korean oyster farms (Fujiya 1970; Ventilla 1984; Mondol et al. 2016). Although the role of heat shock response in these stunted oysters is not established, prolonged exposure of the juvenile oysters to air, starvation, and temperature variations during the hardening process may induce thermotolerance, thereby improving their immunity. Consequently, it is crucial to assess the protective effect of prior stress in controlling mortality associated with routine husbandry activities, particularly handling and transport in flat oyster hatcheries and nurseries.

Enhancing the cellular level of HSPs before undertaking any stressful activity could make the spat more robust, thereby withstanding farming conditions, thus improving the overall survival.

2.2.5. Possibility of using phloroglucinol to induce heat shock protein 70-kDa (HSP70) in the spat of *O.edulis*

The artificial production of the European flat oyster spat is limited by the excess mortality in the hatcheries and nurseries. It is believed this mortality is caused by the weakening of the immune system of the larvae resulting from excess handling during regular husbandry practices. To control such mortality, farmers mainly rely on the application of different antibiotics and chemotherapeutics agents. However, the use of the above agents has got a negative reputation because of the widespread resistance, contamination of food, and their negative effect on the environment. The control of mortalities in oysters is further complicated by the absence of vaccination because oysters do not have acquired immunity (Wang et al. 2018). Consequently, alternative strategies have been proposed in the control of mortalities, including the application of HSP therapies. The application of HSPs- based control strategies are based on thermotolerance and cross-tolerance that are induced by sublethal stress. The successful application of heat shock therapies will depend on the development of practical protocols that can be effectively used in commercial production systems to induce the heat shock response.

The traditional inducers of HSPs in the laboratory may have limited applications in the commercial setting. For instance, heavy metals, including cadmium, mercury, zinc, and copper, have been demonstrated to successfully induce HSP70 production in oysters both in the laboratory and the environment (Piano et al. 2004; Choi et al. 2008; Ivanina et al. 2008, 2009). However, they cannot be applied for commercial purposes because most are carcinogenic and can bioaccumulate in the animal tissue. Hypoxia and electromagnetic fields have also been demonstrated to induce HSP70 in different bivalve species, including mussels; however, their effect in flat oysters is not known. Unlike other stressors, the effect of thermal shock to the expression of HSP70 has received much attention in *O. edulis* (Fisher et al. 1987; Piano et al. 2002, 2004). It has been demonstrated that sub-lethal heat shock increases the levels of constitutive and inducible HSP70 isoforms in the tissues of *O. edulis*. At temperatures below 32˚C, *O. edulis* expresses two constitutive HSP70 isoforms of about 72-kDa and 77-kDa. However, above 32˚C, a third isoform of about 69-kDa is induced. The synthesis of this protein is observed after 4hours and reaches its maximum at 24hours after the shock application. It should, however, be noted that this effective temperature is so high to be applied to valuable oyster

stocks, including breeders and larvae. Indeed, Piano et al. (2002) observed 50% mortality of the oysters exposed to temperatures of 38˚C and a decrease in the expression of the 69-kDa isoform above that temperature. Moreover, Eymann et al. (2020) has recently established the lower lethal temperature for *O. edulis* to be 36˚C, which is close to the temperature which induces HSP70 in oysters. Consequently, the high temperature and the associated mortality may limit the use of heat shock to induce HSP70 in commercial situations.

There is growing interest in using plant extracts as alternatives for induction of the expression of HSP70 besides their general application in disease control. Preliminary results indicate that some of the plant products can provoke the heat shock response independently while others co-induce the same with other stressors. The products appear to be non-toxic, and their effective doses depend on the species studied. The most studied plant product so far is Tex-OE®, a patented extract from a tropical cactus, *Opuntia ficus indica*. The immersion of angelfish *Pterophyllum scalare* in TEX-OE® boosts both Hsp70 and Hsp90 synthesis in the liver, muscle, and gills and protects that fish against other toxic compounds (Roberts et al. 2010). It has also been shown to be an effective HSP70 inducer in other aquatic organisms, including *Artemia fransiscana* (Baruah *et al*. 2012), *Cyprinus carpio* (Sung et al. 2012) and *Danio rerio* (Boerrigter et al. 2014). Pro-Tex®, a soluble variant of TEX-OE®, is commercially applied in salmon and sea bream culture to reduce the stress involved in both smoltification and transport (Roberts et al. 2010). Wieten et al. (2010) demonstrated that overnight exposure of mammalian cells to as high as 1mM of carvacrol, a major component of oil in many *Origanum* species, could not enhance HSP70 production. However, pre-incubation of the cells for two hours with 0.2mM of carvacrol (optimal concentration) immediately followed by heat shock (42.5˚C, 1hour) enhanced the HSP70 levels. Also, pyrogallol optimally induced HSP70 expression in *Artemia franciscana* at an optimal concentration of 595µM. Moreover, incubation of the artemia larvae with 1185µM pyrogallol (the highest tested concentration) for two hours was not toxic to the artemia larvae (Baruah et al. 2015). Other plant-based extracts that have been demonstrated to induce HSP expression include Paeoniflorin (Yan et al. 2004), Curcumin (Teiten et al. 2009), Celastrol (Westerheide et al. 2004), and Schisandrin B (Ip et al. 2001).

The application of plant extracts to provoke the expression of HSP70 in oysters is yet to be investigated. The polyphenol plant-based compounds, including phloroglucinol, present enormous potential in the oyster aquaculture. Phloroglucinol (1,3,5-trihydroxy benzene) is a monomer of phlorotannins (tannins of seaweeds) mainly produced in brown algae (Alamgir 2018). Structurally,

this compound consists of three hydroxyl groups attached to a central benzene ring, as shown in **Figure 6**. The interest in the use of phloroglucinol in aquaculture is based on its known antioxidant, anti-inflammatory, and anti-cancer properties (Kim and Kim 2010; Quéguineur et al. 2012; Pinhatti et al. 2013; So and Cho 2014). The antioxidant activity of this compound seems to be based on its ability to scavenge reactive oxygen species (ROS) including hydrogen peroxide, hydroxyl radical and superoxide anion (Kang et al. 2006, 2010). Recently, (Kumar *et al*. 2018) demonstrated that phloroglucinol could effectively induce HSP70 in the larvae of aquatic organisms *Artemia franciscana* and *Macrobrachium rosenbergii*. The optimal doses for HSP70 induction were 30µM and 5µM for the Artemia and Macrobrachium larvae, respectively. The compound did not show any signs of toxicity at the highest concentrations of 100µM and 30µM for Artemia and Macrobrachium, respectively.

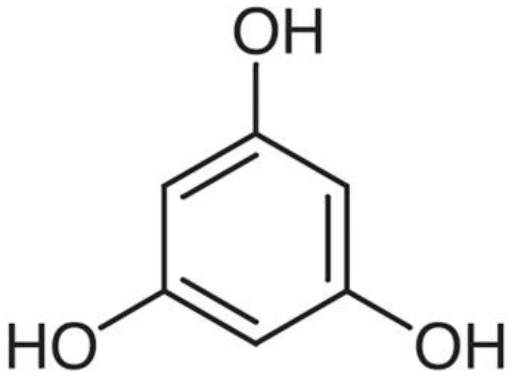

Figure 6 *Chemical structure of phloroglucinol (1,3,5-trihydroxybenzene)*

The mechanism for induction of HSP70 production by phloroglucinol is linked to the generation of reactive oxygen species (ROS) as the case with other polyphenolic compounds (Niu et al. 2014; Baruah et al. 2015). The pro-oxidant activity of phloroglucinol was recently confirmed in a model of the crustacean, *Artemia franciscana* (Kumar et al. 2018). In this model, the brine shrimp larvae treated with phloroglucinol showed higher expression of HSP70 compared to larvae co-treated with both phloroglucinol and antioxidant enzymes (catalase and superoxide dismutase). Further evidence for the prooxidant effect of phloroglucinol was revealed by the level of malondialdehyde (MDA), a product of lipid peroxidation in the larvae. The larvae that were pretreated with phloroglucinol had substantially higher MDA content/g Artemia when compared with the negative control. It is, however, not yet clear how phloroglucinol could exhibit two opposing properties of ROS generation and scavenging. Nonetheless, Wu et al. (2008) demonstrated that cupric ions (Cu^{2+}) enhance the prooxidant activity of two phloroglucinol compounds garcinielliptone HF and garcinielliptone FC from *Garcinia subelliptica.*

Given its proven potency in inducing HSP70 by prooxidant activity and its purported lack of toxic effects, make phloroglucinol an attractive plant extract to induce heat shock response in the spat of *O. edulis*. Moreover, phloroglucinol and its derivatives have known anticancer, antioxidant, and antibacterial properties, which may have secondary benefits in the hatcheries (Kang et al. 2006, 2010; Pádua et al. 2015; Wu et al. 2020). The increased cellular HSP70 protects the cells by helping during the repairing and folding of proteins that are damaged by different stress factors. Routine husbandry activities, including size-grading, handling, and transport, impose stress on the oysters that make them more susceptible to subsequent bacterial, viral, and parasitic infections. The survival of flat oysters in the hatcheries may be improved by enhancing the expression of HSP70 before undertaking stress-inflicting husbandry activities. Increased HSP70 levels by phloroglucinol could make the delicate larvae and spat more robust to withstand the stressful activities involved in the daily management of the hatcheries and the nurseries. A plant-based extract is already used to improve survival during smoltification and transport of salmon and seabream (Roberts et al. 2010). Consequently, this study will first establish the dose of phloroglucinol and the exposure time that can optimally induce HSP70 in the spat of European flat oysters. The toxic effect of the extract to the spat will also be assessed. Then grading simulations and bacterial challenge tests will be conducted on spat pre-treated with phloroglucinol to examine the protective effect of HSP70 from husbandry related mortality.

2.3. Heat shock detection and quantification

The expression of heat shock proteins in oysters is primarily analyzed by western blotting (Piano et al. 2002, 2004; Cruz-Rodríguez and Chu 2002; Ivanina et al. 2009; Ueda and Boettcher 2009; Ueda et al. 2009). Basically, the proteins are first denatured before they are separated according to their size by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following the separation, the proteins are then transferred to a membrane by the application of a current. The membrane can then be processed with primary antibodies specific for HSP70. Next, secondary antibodies attached to enzymes are applied, and finally, a substrate that reacts with the secondary antibody-bound enzyme is added for the detection of the antibody/protein complex (Mahmood and Yang 2012). Despite its extensive use, this protocol is time-consuming and labor-intensive. Also, the antibodies used to detect HSP70 in oysters are obtained from other organisms, especially rats and rabbits, making their sensitivity and specificity questionable. Western blotting shows that oysters express three isoforms of HSP70, including two constitutive proteins of 72 and 77-kDa and one inducible protein of 69-kDa. However, the heat shock response seems to involve more proteins that cannot be

detected by the antibodies. We retrieved eight different HSP70 genes in a blast search of reference sequences against the *C. gigas* genome (Table 3). This may be explained by duplication of immune genes that have been demonstrated in different bivalves (Takeuchi et al. 2016; Cheng et al. 2016; Guerin et al. 2019; Peng et al. 2020).

It is necessary to have a comprehensive understanding of the pattern of expression of all the HSP70 genes in the influence of the different stress stimuli. This knowledge is essential in the development of effective therapies that are based on the heat shock response. Therefore, other analytical techniques may need to be used in addition to western blotting to study heat shock protein expression. Molecular-based techniques, particularly real-time reverse transcription-polymerase chain reaction (RT-qPCR) is gaining recognition in the study of heat shock protein expression in oysters (Ivanina et al. 2009; Lim et al. 2016; Yang et al. 2017; Liu et al. 2019). compared to immunoblotting, RT-qPCR is a quick technique and more specific. However, its application requires prior knowledge of the sequences of the different genes to design the necessary primers. With the right primers, this technique can be used to study differences in the expression pattern of different HSP70 proteins. This study will combine both western blotting and RT-qPCR to analyze the expression of heat shock proteins. The expression of genes differs depending on the type of stress applied with some genes being expressed while others not. Also, not all transcribed messenger ribonucleic acid (mRNA) is subsequently translated into polypeptides due to gene expression regulation where some mRNA is degraded or not exported to the cytoplasm where translation occurs. Therefore, both proteomic and transcriptomic analyses complement each other to have a clear understanding of the expression of all the genes in different conditions.

CHAPTER 3:

MATERIALS AND METHODS

3.1. Identification of HSP70 genes of *Crassostrea gigas*

The availability of the *C. gigas* reference genome (GenBank GCA-000297895.1), has expanded frontiers for studying the different life processes in this species, including the HSP response. Initially, several HSP70 cDNA sequences had been cloned and sequenced (**Table 1**). These sequences were retrieved from the GenBank and used to identify the HSP70 genes in the *C. gigas* genome using several bioinformatics tools. The *C. gigas* HSP70 genes were obtained by BLAST searching each of the cDNA sequences against the genome using *Ensembl Metazoa* (Howe et al. 2019) to identify homologous sequences. From the BLAST search hits, we selected those that occurred at scaffolds overlapping with already annotated genes. The rest of the hits were short to make full genes, while others were introns and, therefore, not useful for further analysis. For the hits that occurred in the annotated genes, the corresponding protein sequences were retrieved from the ensemblGenomes database (http://ensemblgenomes.org/) and analyzed to confirm whether they were HSP70 proteins. This was undertaken by aligning them in *ClustalOmega* http://www.clustal.org/ with other HSP70 proteins from the GenBank. Proteins were proved to be HSP70 if they contained the three unique signature motifs (IDLGTTYS, IFDLGGGTFDVSIL, and IVLVGGSTRIPKIQK) for that family (Gupta and Singh 1994). The confirmed HSP70 proteins were retained for further analysis, including multiple alignments and phylogenetic analysis using *ClustalOmega*.

3.2. Induction of HSP70 in *O. edulis* spat by phloroglucinol

3.2.1. Cytotoxic effects of phloroglucinol to *O. edulis* spat

This preliminary lethality test will be performed to screen for the concentration range and exposure times of phloroglucinol to be used in the subsequent tests. Fifty spats will be immersed in 1, 10, 100, and 1000, 10,000µM solutions of phloroglucinol for increasing periods of 1, 2, 3, 4, and 5h. To avoid diluting the chemical, the oysters will not be fed during the treatment. After the treatment, the spat will be rinsed repeatedly to remove the compound and returned to a recovery system at 22˚C and 30-35ppt salinity, their normal rearing conditions (Gercken and Schmidt 2014). To avoid the accumulation of the chemical from the metabolism of the oysters, the recovery will be in a flow-through system. Spat maintained under standard rearing conditions that will not receive the

phloroglucinol treatment will serve as the control group. During the recovery, the spat will be maintained on an algal mix of *Tetraselmis sp., Isochrysis sp*., and *Chaetoceros sp*. at a ration of 6% dry weight algae $spat^{-1}$ day^{-1} according to (SARF 2014). Mortality will be monitored every 4h for a total of 48h by identifying gaped oysters that do not display any muscular response to probing. Three replicates will be maintained for each treatment and control group. The mean survival of the spat in the control and the treatment groups will be analyzed with two-way ANOVA to check for significant variations. The concentrations and exposure time that give survivals that are like the control group will be used for further tests.

3.2.2. Dose-response test/HSP70 expression

This experiment will assess the influence of phloroglucinol concentration and exposure and recovery time on the production of HSP70 in the spat of *O. edulis* **Figure 7**. The expression of HSP70 mRNA will not be assessed in this study because sufficient primers cannot be designed given the lack of comprehensive transcriptome databases for this species. The HSP70 will be induced in the spat that is acclimated for seven days at 22˚C. To stimulate HSP70, 200 spat will be immersed in baths of 1 to 5log reductions of the toxic concentration of phloroglucinol determined from the lethality test above. To assess the effect of exposure time, 40spat will be randomly selected from each of the 5 baths at 1h interval (5 samples in total) depending on the exposure time selected from the lethality test. The spat will be rinsed repeatedly to remove the compound and then left to recover in a flow-through system at 22˚C as described in the lethality test above. To assess the change in the expression of HSP70 over time, 5spat will be randomly selected from each of the 25 recovery basins at 2, 4, 6, and 8h and then 2, 4, 6, and 8days. The spat that will not be pretreated with phloroglucinol will serve as the negative control group. Also, spat treated with a non-lethal heat shock (NLHS) of 32˚C for 1h, according to (Piano et al. 2002), will be a positive control. Three replicates will be maintained for both the treatment and the control groups.

The expression of HSP70 proteins will be determined by immunoblotting (Western blotting). Given the small size of the spat, HSP70 will be determined for a pool of 5 whole spats as Ueda and Boettcher (2009). The oysters will be killed by dipping in liquid nitrogen and then stored at -80˚C while waiting for the analysis. The samples will be processed on ice using a hand-held 5-mL homogenizer in 10mM sodium phosphate buffer (pH 7.4) at a ratio of 1:4, weight of the sample to buffer volume as described by (Piano et al. 2002). The buffer will be made of 1% Nonidet-P40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). To slow down protein degradation, a protease inhibitor cocktail

at a final concentration of 1µg/mL pepstatin A, E-64, bestatin, leupeptin, and aprotinin plus 0.25 µg/mL phenylmethane sulfonyl fluoride will be added to the spat-buffer mixture. The homogenates will subsequently be centrifuged at 14,000×g for 10 min to separate the supernatant and the tissue debris. The supernatant will then be carefully transferred to new Eppendorf tubes and recentrifuged for an extra 10 min and the supernatant transferred to another Eppendorf before storage at −80°C for further analysis. The total protein content in the samples will be measured by the Bradford method (Bradford 1976) using Bovine Serum Albumin (BSA) as a standard for the calibration curve.

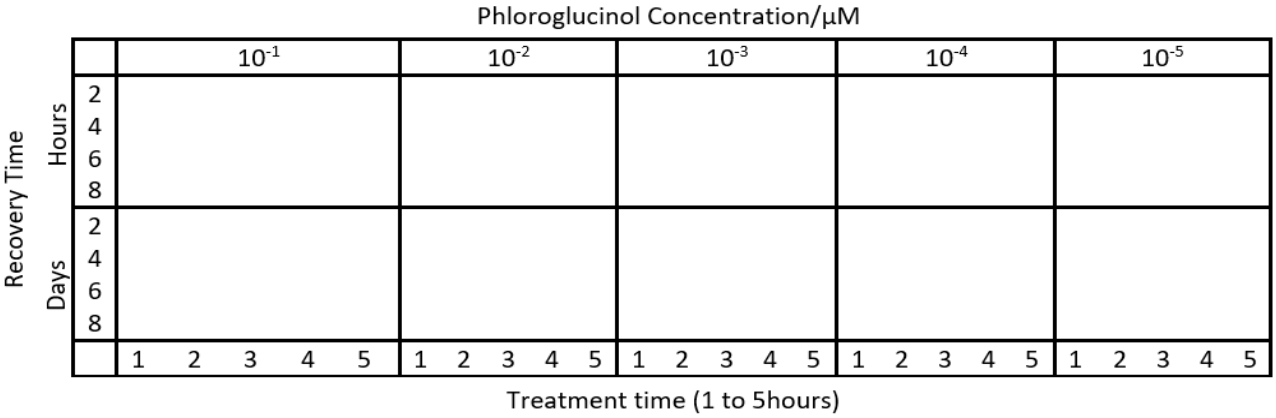

Figure 7 *The experimental design to determine the optimal concentration and exposure time of phloroglucinol and the recovery time of O. edulis spat. The concentration range will be between 1log to 5log reductions of the lowest toxic concentration from the preliminary lethality test. The treatment time will be between 1 to 5 hours. The HSP70 will be sampled after 2, 4, 6, and 8h and then 2, 4, 6, and 8days after a 2-hour recovery.*

The measurement of HSP70 production will start with SDS-PAGE. Here, 20µg protein samples of each supernatant will be prepared by the addition of a loading buffer (SDS sample buffer + mercaptoethanol). The prepared samples will be vortexed, followed by 5min boiling at 95˚C to denature the proteins. Subsequently, 10µl of the boiled sample will be resolved on a 10% polyacrylamide gel for 150min at 130V. Six micrograms (6µg) of heat-shocked HeLa cells (Enzo Life Sciences, USA) will also be separated on the gel to serve as a standard. The separated proteins will then be transferred to a nitrocellulose membrane at 100V for 60 minutes. The membranes will be probed for 1h with anti-HSP70 rat monoclonal antibody (1:10,000 dilution) as the primary antibody at 22˚C. For the secondary antibody, anti-rat immunoglobulin G (IgG) conjugated with horseradish peroxidase (1:6000 dilution) will be used for 30min at 22˚C. Then the immunoblots will be washed several times with 20mM Tris phosphate buffer (pH 7.4) and 1% polyoxyethylene sorbitan monolaurate (Tween 20). Finally, the immunoblots will be incubated with a chemiluminescence reagent (clarity western ECL substrate) for 5min and then detected by the ChemiDoc MP imaging

system (BioRad, Belgium) as described by (Kumar et al. 2018). The whole experiment will be repeated two more times to obtain reliable data. The measured HSP70 from the triplicates of the three experiments will be used to calculate the average expression level. The mean HSP70 levels will be subjected to a 3-way analysis of variance (3- Way ANOVA) to test for significance. Statistical differences will be accepted at $P < 0.05$. The lowest concentration and exposure time of phloroglucinol that induces the highest HSP70 in comparison to the positive control will be selected for the challenge test.

3.3. Grading simulation and the challenge test

This challenge test will be performed to establish whether pretreatment of *O. edulis* spat with phloroglucinol prior to handling could offer them protection against opportunistic *Vibrio* pathogens. This trial will be accomplished in three steps, as shown in **Figure 8**. At the start, 320 spats will be split equally into two groups (n=160, 2tanks). One group (Treatment group) will then be treated with an optimal concentration of phloroglucinol for the optimal contact time as determined from the dose-response time to induce HSP70 production. After the treatment, the spat will be washed to remove the compound and then left to recover at 22˚C for an optimal duration when HSP70 expression is at its highest as determined in the dose-response test. The second group (Control group) will not be subjected to any treatment. Ten oysters from the treatment group will be used first to confirm whether the phloroglucinol pretreatment successfully induced HSP70 expression before the grading challenge. The HSP70 level will be measured as described above in the dose-response test. The HSP70 level from 10 untreated spat (control group) will be used as the control.

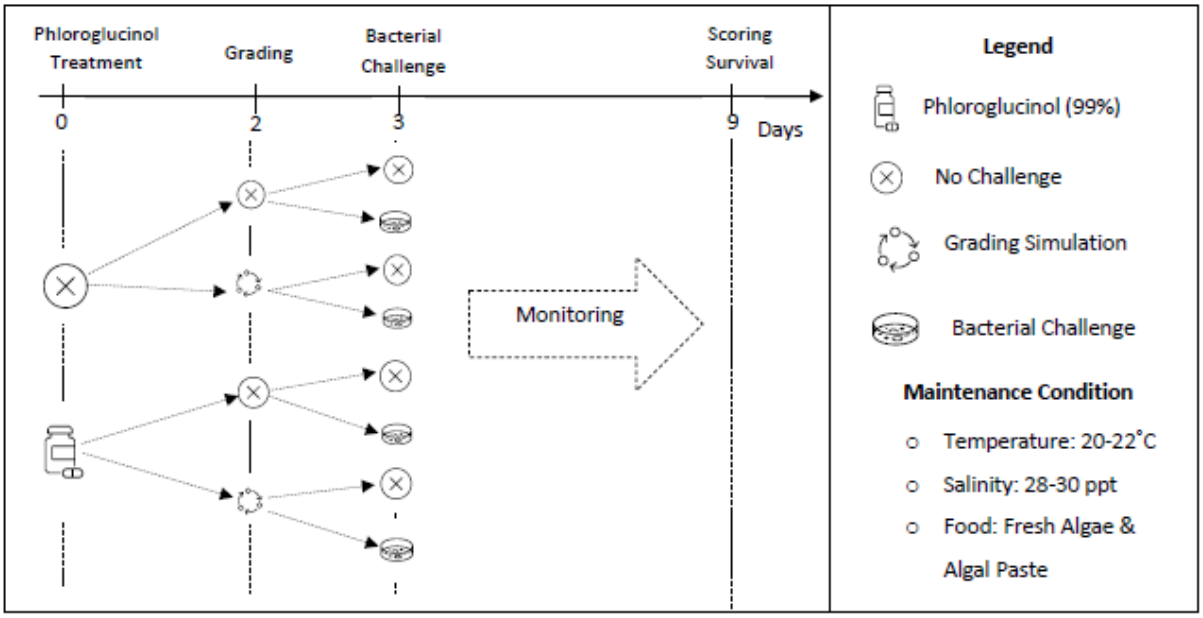

Figure 8 *The experimental design for the challenge test. Spat pre-treated with phloroglucinol is the Treatment group (n=150 spat), while untreated spat is the Control (n=150 spat). After 24h, half of both the groups (n=75spat) is subjected to a 15min grading simulation. After 24h, a sample of 30 spats from each sub-group is distributed to 2 new tanks maintained at usual conditions. One of the two tanks is then inoculated with Vibrio neptunius at a bacterial density of 106CFU/ml. The second tank is not inoculated with the pathogen. Spat will be maintained under standard rearing conditions, and final survival recorded 6days after the inoculation.*

In the grading simulation, the remaining spat (150) for both the treatment and control groups will be split equally into two sub-groups (n=75, 4tanks). One of the sub-groups will subsequently be exposed to mechanical disturbance to mimic grading conditions. The grading will be simulated as earlier described by (Lacoste et al. 2001f). Briefly, the spat will be placed in a plastic container and afterward rotated on a laboratory agitator for 15minutes. After the simulation, the spat will be returned to their respective tanks. The second sub-group will not be subjected to any disturbance.

Lastly, a bacterial challenge test will be performed the next day after the grading simulation. Prior to this challenge test, a sample of 30 spat from each of the 4 sub-groups from the previous experiment will be redistributed to new tanks (n=30, 8tanks). For the two tanks from each sub-group, one tank will be subjected to a *Vibrio neptunius* challenge at 10^6CFU/ml seawater as described by (Prado et al. 2005). The other tank will not be subjected to the bacterial challenge. The survival in all the tanks will be continuously monitored for six days after inoculation. The dead spat will be identified by gaping (opening of shells) and lack of mobility following a mechanical disturbance. During the challenge, the spat will be maintained on an algal mix of Tetraselmis sp., Isochrysis sp., and Chaetoceros sp. at a ration of 6% dry weight algae spat-1 day-1 as described in the lethality test. All the treatment and the

control groups will be maintained in triplicates, and the entire experiment will be repeated three times.

The data will be processed to get the mean spat survival of the treatment and control groups of the three experiments. To check for significant differences and variations between and amongst the mean survival of the spat, One-way ANOVA and a t-test will be used at a significance level of 0.05 (*α=0.05)*.

CHAPTER 4:

RESULTS

4.1 The heat shock protein 70-kDa (HSP70) genes of *Crassostrea gigas*

In the blast search against the *C. gigas* genome, we retrieved four cytosolic HSP70 proteins (Table 3). All the deduced polypeptides are 634 amino acids long. Multiple alignments (**Figure 10**)revealed that these proteins display the three signature motifs of the HSP70 family, including IDLGTTYS (residues 10-17), IFDLGGGTFDVSIL (residues 198-211) and VVLVGGSTRIPKIQK (336-350). They also have the two glycosylation domains NKSI and NVSA between residues 362-365 and 489-492, respectively. The cytosol localization domain at the C-terminus ends of proteins CGI_10003417, CGI_10010646, and CGI_10010647 is EEMD while protein CGI_10002594 exhibits EEID. On the contrary, these proteins lack the extra NQSQ tetrapeptide motif in the ATP-binding domain and the GGMPGGMP double tetrapeptide repeat in the C-terminals.

A fifth gene (CGI_10002823), which is almost identical to the above cytosolic isoforms, was also retrieved. The deduced polypeptide (NP_001295853.1) is 613 amino acids. The gene exhibits all the signature motifs for HSP70 family that include IDLGTSFS (residue 10-17), VYDLGGGTFDVSIL (residue 198-211), and VVLVGGSTRIPKIQT (residue 336-350). However, the glycosylation domains in this protein are NVTA and NRSV instead of NVSA and NKSI presented by the four cytosolic proteins. Like the above four proteins, this protein lacks the extra NQSQ and GGMPGGMP domains in the ATP-binding domain and at the C-terminus, respectively. Moreover, this protein lacks the C-terminal end that houses the cytosol localization domain.

We also retrieved three HSP70 genes, including CGI_10028167, CGI_10027395, and CGI_10015492. The corresponding polypeptides from the database consist of 633, 656, and 661 amino acids and are labeled as Glucose-Regulated Protein, 78kD (GRP78). However, two of the predicted proteins XP_011456187.2 and XP_011420631.2 lack the ER localization signal tetrapeptide KDEL (***Appendix 1***). Despite that, all the three proteins display the three conserved HSP70 signature motifs (IDLGTTYS, I(V/I)LVGGSTRIPK(V/I)QQ, V(F/I)(D/N)(L/M)G(G/A)(G/S)TFDVSLL). The pairwise alignment showed that the protein (NP_001292291.1) from gene CGI_10015492 is identical to protein, BAD15288.1, already available in the GenBank.

The phylogenetic analysis (**Figure 9**) of the proteins shows that the retrieved proteins form one cluster with the inducible HSP70 isoform of that species.

Table 3 Cytoplasmic and Endoplasmic HSP70 genes and their corresponding proteins in the C. gigas genome

Gene ID	Protein Accession	Number of amino acids	Description
Cytoplasmic			
CGI_10002594	XP_011436292.2	634	Heat shock protein 68
CGI_10003417	XP_011435905.1	634	Heat shock protein 70 B2
CGI_10010646	XP_011455958.3	634	Heat shock protein 68
CGI_10010647	NP_001295842.1	634	Heat shock protein 70
CGI_10002823	NP_001295853.1	613	Heat shock protein 68-like
Endoplasmic			
CGI_10027395	XP_011420631.2	656	GPR78
CGI_10028167	XP_011456187.2	633	GPR78
CGI_10015492	NP_001292291.1	661	GRP78

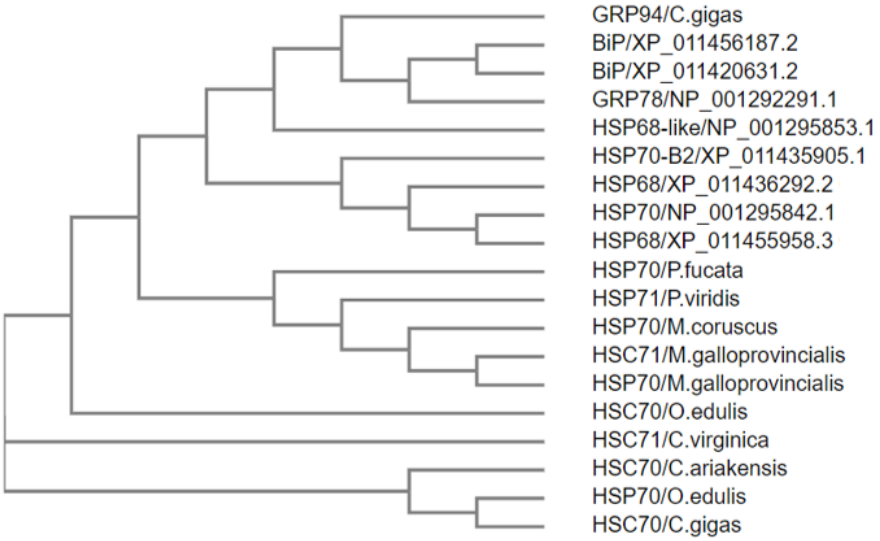

Figure 9 *The phylogenetic relationship of HSP70 members from various bivalves*

Figure 10 *Multiple alignments of HSP70 members from the different bivalve species. HSP heat shock proteins, HSC- heat shock cognate. Dashed black sections; the three signature motifs for the HSP70 family, Blue; extra domain in the ATP-binding domain of Ostreidae, Yellow; the two glycosylation domains, and Red; double tetra-polypeptide domain.*

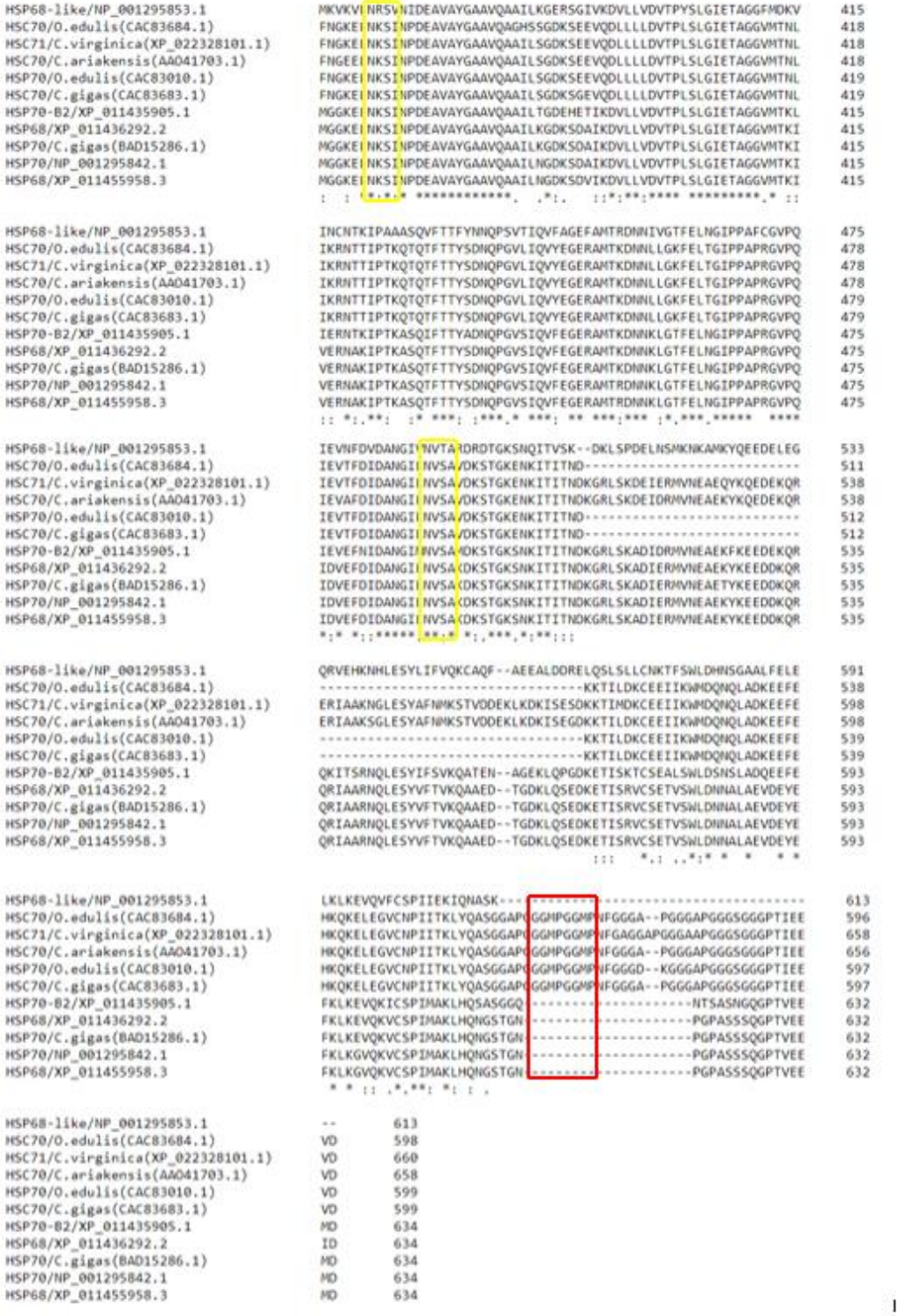

Figure 10-continued, Multiple alignments of HSP70 members from the different bivalve species. HSP heat shock proteins, HSC- heat shock cognate. Dashed black sections; the three signature motifs for the HSP70 family, Blue; extra domain in the ATP-binding domain of *Ostreidae,* Yellow; the two glycosylation domains, and Red; double tetra-polypeptide domain.

CHAPTER 5:

DISCUSSION AND RESEARCH PROSPECTS

5.1. Discussion

This discussion will be limited to the cytosol HSP70 proteins, which are the focus of this study. More importantly, there is limited information on the expression of the endoplasmic reticulum HSP70 isoform. The HSP70 are highly conserved proteins in both prokaryotic and eukaryotic organisms. They are comprised of four groups depending on the subcellular location, amino acid composition, and expression pattern. They include the two cytoplasmic groups comprised of the inducible (HSP70) and the constitutive (HSC70) members. The other two groups are the glucose-regulated protein 78/GRP78 (also known as immunoglobulin binding protein/BIP) and glucose-regulated protein 75 (GRP75). The former group functions in the endoplasmic reticulum (ER), whereas the latter in the mitochondria. These proteins are under the control of different genes whose expression pattern is species dependent. For instance, *Artemia franciscana* exhibits at least five genes (Junprung et al. 2019) compared to thirteen genes of humans (Radons 2016). The expression pattern of HSP70 members in *C. gigas* is not yet clear. All proteome-based analyses seem to agree that *C. gigas* expresses one inducible protein (69-kDa) and two constitutive isoforms of 72 and 77-kDa (Clegg et al. 1998; Boutet et al. 2003b; Hamdoun et al. 2003; Meistertzheim et al. 2009). However, the selectivity and sensitivity of the HSP70-antibodies used in these studies are questionable because they are from other animals, particularly rats and rabbits. Although these proteins are highly conserved, some variations may influence the sensitivity of the HSP70 antibodies induced in other organisms. Clues for low sensitivity of the antibodies may be obtained from the recent transcriptomic studies that have revealed more HSP70 transcripts during the heat shock response of C. gigas. Liu *et al*., (2019) reported five HSP70 genes in *C. gigas* that are regulated by the HSF1 during heat shock. However, Liu *et al*., (2020) identified four genes, of which three are likely to be inducible isoforms.

We BLAST searched *C. gigas* HSP70 cDNA sequences (**Table 1**) against the reference *C. gigas* genome to identify the HSP70 genes. Members of the HSP70 family have three unique motifs IDLGTTFS, IFDLGGGTFDVSIL, and VVLVGGSTRIPKIQK (Gupta and Singh 1994). All the proteins that were retrieved in this study exhibit these three motifs, which confirms that they are indeed HSP70 members. The presence of specific motifs determines the location of a given HSP70 member within a cell. The localization motifs for the cytosol and the endoplasmic reticulum isoforms are EE(V/M)D

and KDEL, respectively. The presence of EEMD in proteins CGI_10003417, CGI_10010647, CGI_10002594, and CGI_10010646 confirms that they are cytosolic. There is no consensus on how to distinguish between cytosolic cognate and inducible HSP70 isoforms physically. However, it is accepted that most of the variability between these isoforms are observed in the C-terminus. This domain is thought to be responsible for chaperone binding and substrate binding regulation (Fabbri et al. 2008). Unlike the inducible isoforms, their cognate counterparts in humans and other species express an insertion that contains a tetrapeptide repeat GGMPGGMP in the C-terminus domain (Piano et al. 2005; Kourtidis et al. 2006; Wang et al. 2009). However, both HSP70 and HSC70 isoforms of *O. edulis* express this insertion (Boutet et al. 2003b, a). Regardless, the HCS70 from Ostreidae seems to uniquely possess an extra domain NQSQ in the conserved ATPase domain (Kourtidis et al. 2006). The absence of the above extra motifs in the retrieved genes implies that they may be inducible isoforms. This conclusion can also be drawn from the phylogenetic analysis in figure 8. All the cytosolic HSP70 proteins retrieved in this study form a separate cluster different from the cognate cluster of the other bivalves. Moreover, a recent transcriptomic study by (Liu et al. 2019) has demonstrated the inducible expression pattern in four of the genes. In that study, the mRNA of genes CGI_10002594, CGI_10010646, CGI_10010647, and CGI_10002823 increased 20 times following a 30min thermal shock of *C. gigas* at 35°C.

5.2. Research Prospects

Widespread mortality is unarguably a significant factor affecting oyster aquaculture at all levels of the production cycle. The increased mortality is partly attributed to stress resulting from excess handling and variation in the water quality parameters including, temperature, salinity, and hypoxia. Stress makes oysters more vulnerable to infections from viruses, bacteria, and parasites. Like other organisms, oysters respond to stress by increased production of acute-phase proteins, especially heat shock proteins (HSPs) and metallothioneins (MTs). The HSPs are examples of the several immune effectors that help organisms to modulate stress response and protect them from environmentally induced cellular damage (Wang et al. 2018). They function as molecular chaperones, facilitating the folding of proteins and preventing protein aggregation. The involvement of HSPs in the immune response of oysters has been demonstrated by several studies (Li et al. 2007; Nikapitiya et al. 2014; De-la-Re-Vega et al. 2017; Liu et al. 2017). Besides its participation in the immune response, studies have also confirmed that enhanced HSPs following exposure to sub-lethal stress give oysters resistance to subsequent lethal stress, a condition known as thermotolerance. For instance, Pacific

oysters survived a 1-hour exposure to the lethal 44°C following a prior 1hr shock of 37°C (Clegg et al. 1998). Similar results have been observed in other bivalves, including scallops (Brun et al. 2009) and mussels (Aleng et al. 2015). Another important observation is that heat shock response due to a specific stressor is believed to confer protection to oysters from a different stressor in what is known as cross-resistance. Consequently, HSP-based therapies may be developed to control stress and mortality in aquaculture of many species, including oysters as alternatives to antibiotics.

Identifying all the oyster HSP70 genes and having full knowledge of their regulation and expression patterns under different stress conditions is vital if effective HSP therapies are to be developed. The availability of the *C. gigas* genome presents more opportunities to understand the heat shock response in oysters. At least this study has retrieved eight HSP70 genes through a BLAST search. Comprehensive studies may be necessary to identify all the genes in this species. Besides, literature shows that the expression of the HSP70 genes varies depending on the stress stimulus. Therefore, it may be necessary to undertake a combination of transcriptomic and proteomic studies to demonstrate the response of different genes to specific conditions. Sequencing the genomes for the other oyster species, including *O. edulis,* will help to understand the heat shock response in those species clearly. It may also be essential to use the *C. gigas* genome to establish the genotypic differences between stress-resistant and vulnerable oyster strains based on the HSP70 genes. Such information may be used to breed for hardy strains that can withstand the harsh culture conditions and environmental stressors.

HSP70 expression is mainly studied by immunoblotting. However, this technique is time-consuming, given the several steps involved, which may limit its application for routine testing in commercial settings. Moreover, these studies use antibodies whose selectivity and sensitivity are questionable because they originate from different organisms, especially mice and rabbits. Several studies have successfully used the enzyme-linked immunosorbent assay (ELISA) to quantify HSP70 levels in oysters (Boutet et al. 2003b; David et al. 2005; Meistertzheim et al. 2009; Collin et al. 2010). Although ELISA does not show the specific HSP70 isoforms expression, it may be used for the quick quantification of total HSP70 in aquaculture environments. Consequently, it may be necessary to develop commercial ELISA kits that are specific for oysters. The availability of kits will make measuring HSP70 in oyster farms during the application of HSP70 therapies easy. Likewise, it may be necessary to ascertain whether the commercial antibodies used in western blotting and ELISA can detect all the HSP70 isoforms. One of the approaches could be to separate the proteins by two-dimensional gel

electrophoresis (https://www.appliedbiomics.com/Services/2d-wb-membrane.html). After the electrophoresis, the proteins on all differentially produced spots may be sequenced to confirm that the spots are indeed HSP.

CHAPTER 6:
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CHAPTER 7:

APPENDICES

Appendix 1. Endoplasmic reticulum (ER) HSP70 from *Crassostrea gigas*

Appendix figure 1 Binding immunoglobulin protein (BiP)/ GRP78 of C. gigas showing the main domains. Solid black rectangles: three signature motifs for HSP70 family; Red solid rectangle: the ER localization signal