UNIVERSIDADE DE ÉVORA

ESCOLA DE CIÊNCIAS E TECNOLOGIA

DEPARTAMENTO BIOLOGIA

UNIVERSIDADE TÉCNICA DE LISBOA

INSTITUTO SUPERIOR DE AGRONOMIA

”SHIP TRANSPORT OF MARINE INVASIVE SPECIES AND ITS STRESS RESISTANCE”

Filipa Alexandra Paiva Antunes

Orientação: João Canning Clode

Teresa Cruz

Mestrado em Gestão e Conservação de Recursos Naturais

Dissertação

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Recomeça...
Se puderes
Sem angústia
E sem pressa.
E os passos que deres,
Nesse caminho duro
Do futuro
Dá-os em liberdade.
Enquanto não alcances
Não descanses.
De nenhum fruto queiras só metade.

Miguel Torga

Table of contents

i. List of figures and tables

Figures

Tables

ii. List of abbreviations

CSBIO – Committee on Ships' Ballast Operations

Coxph – Cox proportional hazard

df – Degrees of freedom

GAME – Global Approach by Modular Experiments

GISD – Global Invasive Species Database

GT – Gross tonnage

HSP's – Heat-shock proteins

IMO – International Maritime Organization

MS – Mean of squares

NIS – Non-indigenous species

PK – Pyruvate kinase

PSU – Practical Salinity Units

SPI – Stress phase one

SPII – Stress phase two

SS – Sum of squares

T-logger – Temperature logger

UFO – Unidentified Foreign Organisms

iii. Abstract

"Ship transport of marine invasive species and its stress resistance"

In the context of biological invasions, ship transport plays an important role in the transference of species around the world. During this process organisms are exposed to several stressful conditions, which do not prevent them to arrive in areas where they did not occur before. The marine invertebrates *Carcinus maenas* and *Mytilus galloprovincialis* natives in Portugal, have already established stable invasive populations in several regions worldwide. This study exposed organisms of both species to stress conditions similar to what occurs in ballast tanks and on ship hulls. Results showed a high survival of pre-stressed *Mytilus galloprovincialis* individuals when compared to non pre-stressed organisms when exposed to air exposure, hyposalinity and heat events. However when *Carcinus maenas* was exposed to heat stress, there was no significant difference between groups with a different stress history. These findings suggest that transport on ships is not only a vector of dispersal but also a vector capable to increase the resistance to stress conditions in potential species invaders by selection of resistant individuals.

iv. Resumo

"Transporte marítimo de espécies marinhas invasoras e sua resistência ao *stress*"

No contexto das invasões biológicas, o transporte marítimo desempenha um papel importante contribuindo para a transferência de espécies em todo o mundo. Durante este processo os organismos são expostos a variadas condições de *stress* que não os impede de chegar a áreas onde não ocorriam anteriormente. Os invertebrados marinhos *Carcinus maenas* e *Mytilus galloprovincialis* nativos em Portugal, já estabeleceram populações invasoras estáveis em várias regiões do mundo. Neste estudo, submeteram-se organismos de *Carcinus maenas* e *Mytilus galloprovincialis* a condições de *stress* semelhante ao que ocorre em tanques de água de lastro e em cascos de navios. Os resultados mostram uma maior sobrevivência dos indivíduos *Mytilus galloprovincialis* pré – expostos a condições de *stress* quando comparado com organismos sem uma prévia exposição a esses eventos de *stress*, quando expostos ao ar, baixa salinidade e altas temperaturas. No entanto, quando *Carcinus maenas* foi exposto a temperaturas mais altas, não houve diferença significativa entre os grupos com diferentes exposições ao *stress* aplicado. Estes resultados sugerem que o transporte marítimo não é apenas um vetor de dispersão mas também um vector capaz de aumentar a resistência a condições de *stres*s em potenciais espécies invasoras, nomeadamente através da seleção dos indivíduos mais resistentes.

1 Introduction

1.1 Global change

'*The only constant thing in the universe is change'* (Heraclitus).

Since the beginning of the industrialization and agricultural era, climate is changing quickly and its effects are challenging to predict (Vitousek et al. 1997, Carlton 2009, Spielhagen et al. 2011). Half of global atmospheric CO_2 concentrations that are produced due to extensive use of fossil fuels are being absorbed by the ocean and it is related with the increase of global temperature (Vitousek et al. 1997, Easterling et al. 2000, Dupont et al. 2010). In addition, biodiversity loss, species extinctions, habitat destruction, overfishing and pollution represent ongoing global changes with significant consequences to ecosystems (Carlton 1985, Vitousek et al. 1997, Pimentel et al. 2000, Geller et al. 2010). Biological invasions also represent a significant component of global change as they are causing drastic impacts in the natural environment of native populations and risks for human health (Velde et al. 2006, Gurevitch et al. 2011). Thus, since the number of Non-Indigenous Species (NIS) introductions in several biogeographic regions increased in recent years, makes it clear that biological invasions are occurring faster than its understanding (Ruiz et al. 1997, Vitousek et al. 1997, Canning-Clode et al. 2013).

1.2 Biological invasions: Natural vs. Human mediated

Biological invasions occur when a group of individuals are transported from their native range to a novel location. A transport vector is the 'vehicle' that moves a non-native species (or propagule) to its novel location while a transport pathway constitutes the route between source and release region (Lockwood et al. 2007). Vectors and pathways of biological invasions can be both natural and human mediated. The force of nature can lead organisms to spread to new areas, normally predictable due to climate change, through active movement or carried by currents or wind (Carlton 1996, CSBIO 1996, Vermeij 1996, Blakeslee et al. 2010).

Differences between natural and anthropogenic invasions are found in the geographic scope, frequency, and the number of species involved that have grown

enormously as a direct consequence of exploration by transport and commerce by ship since the 16th century. (Mack et al. 2000, Wonham and Carlton 2005, Carlton 2009).

Introductions through anthropogenic vectors can be categorized as intentional or accidental. In the past, one of the more obvious mechanisms for the dispersal of non-indigenous species was via deliberate introductions as food supply, sport, pets and landscape restoration (Vitousek et al. 1997, Pimentel et al. 2000, Geller et al. 2010). However, most marine invertebrates introductions have been accidentally (Carlton 1987, Pimentel et al. 2000, Ruiz et al. 2000a, Blakeslee et al. 2010). For example, the world successful invasion of the European crab *Carcinus maenas* and the zebra mussel, *Dreissena polymorpha*, are among notorious invasions that resulted from human-mediated accidents and significantly altered habitat structure (Vitousek et al. 1997, Roman 2006).

Numerous human-mediated pathways for NIS spread have been recently described. A summary by Hewitt et al. (2004) identified at least 20 present–day vectors and pathways relevant in translocations of species. However, four vectors have been recognized to have a larger influence involving the unintentional introduction of organisms: i) Shipping, which involves ballast water transport and hull fouling (Mack et al. 2000, Ruiz et al. 2000a); ii) Aquaculture (Namboothri et al. 2012); iii) Canals such as Suez, Panama and Kiel (Vermeij 1996, Ruiz et al. 2000a); iv) and marine litter (Wilson et al. 2009). Shipping constitutes the most relevant vector human mediated for new invasions and is still being investigated since it facilitates the transfers not only of several different life cycle stages of marine organisms but also a vast taxonomical range of individuals (Carlton 1985, Lavoie et al. 1999, Gollasch et al. 2002, Ruiz and Carlton 2004). Nowadays shipping is responsible for 90% of goods transportation around the world (Fig. 1) and only in 2006, 7.4 billion tons of goods arrived to ports around the world (WWFInternacional 2009, Kaluza et al. 2010). For example due to this shipping vector, the mussel *Mytilus galloprovincialis* arrived (probably before the 20th century) to the North American Pacific coast, Japan and South Africa, placing this species among the '100 of the world's worst invasive species (Wonham and Carlton 2005, Geller et al. 2010, Lockwood and Somero 2011a). The global movement of ballast water by cargo ships created a large pathway for organism transfer and, beside

the creation of management tools, for example with water discharge in open ocean, invasions are still happening and will continue to occur in coastal environments as long as ballast water is transported and released (Carlton 1996, Ruiz et al. 2000b).

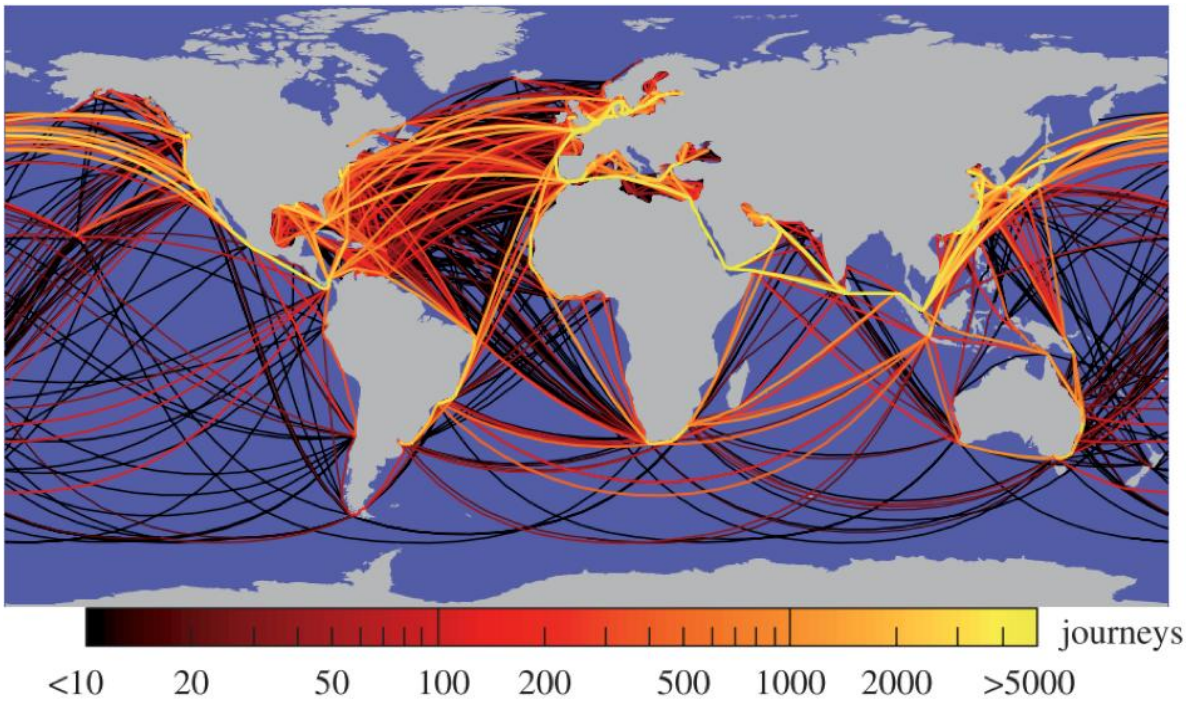

Fig. 1 – Most representative ports and cargo ship trajectories (<10000 Gross tonnage (GT)) in the world during 2007. The color scale indicates the number of journeys along each route. Modified from Kaluza et al. (2010).

Although aquaculture has reduced the number of introduced species in recent years due to the improvement of tanks security, it still constitutes a relevant vector responsible for the introduction of NIS, parasites and pathogens (Grosholz et al. 2012). Moreover, the transference of NIS through canals has facilitated a large number of introductions initially with more than 300 species found coming directly from the Red Sea through the Suez Canal for example (Wonham and Carlton 2005, Galil 2008, Simberloff and Rejmanek 2010). Nowadays however, the implementation of prevention actions reduced the numbers of introductions through these pathways (Simberloff and Rejmanek 2010). Finally, marine debris is too, responsible for the widespread distribution of many marine organisms. Due to their buoyancy and durability, plastic litter can travel substantial distances becoming a mobile home to several animals (Barnes 2002, Derraik 2002).

1.3 Invasion Process

The process of invasion is stepwise and includes several stages responsible for the success on the introduction of a new species (Pyšek et al. 2011). There seems to be no agreement among invasion scientists about the number, designation and order of these stages (See Colautti and MacIsaac (2004)), but all authors include the following seven stages: i) Loading – correspond to the uptake of organisms in the native range; ii) Transport – specimens are transported to a recipient region surpassing barriers that were impossible to overcome without human mediated intervention; iii) Introduction – the transported organisms are released in the new environment; iv) Lag phase – where populations remained small and localized; v) Establishment – if the new conditions are acceptable for NIS it is possible to spread a new population; vi) Spread – if conditions in the new environment are suitable for the reproduction of NIS; and finally vii) Impact – which will determine the economical and ecological impact on the environment where NIS are released (Mack et al. 2000, Allendorf and Lundquist 2003, Colautti and MacIsaac 2004, Blackburn et al. 2011).

1.4 Factors determining invasion success

The ambient inside ballast tanks created by physical and chemical conditions can be very hostile for new organisms causing several impacts in their survival history (Carlton 1985, Gollasch 2006a). These new organisms will have to face changes in water temperature, oxygen concentrations and light, which can be altered by the size and position of a ship (Carlton 1985, Gollasch et al. 2000a). Although the transport process of a population to a new area seems simple, it requires a range of aid to maintain the individuals alive, from the beginning to the end of its process (Ruiz et al. 2000a, Allendorf and Lundquist 2003, Ruiz and Carlton 2004, Gollasch 2006a, Simberloff and Rejmanek 2010). Only some individuals survive from one step to the other and the amount of survivors is always decreasing during the process. So, *what can determine survival on the first stages of introduction*? Three main factors are known to be relevant: i) Propagule pressure; ii) Abiotic; and iii) Biotic conditions of the recipient area.

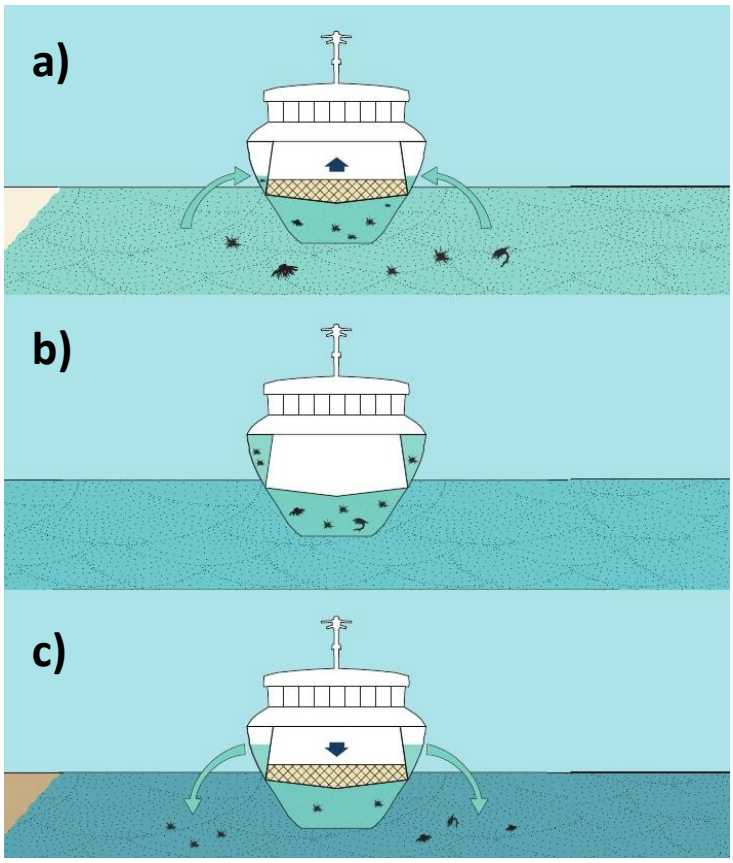

Fig. 2 – Summary of the process of loading the water to the ballast tanks with organisms (a), transport (b) and posterior release of the ballast water in the new region (c). Adapted from Namboothri et al. (2012).

According to Roman and Darling (2007), propagule pressure corresponds to the quantity, richness or frequency of introduced NIS to the recipient area and in which seems to have a great impact on the genetic diversity of the population. Propagule pressure will determine which proportion of genetic diversity from the donor region will be introduced (Wilson et al. 2009, Simberloff and Rejmanek 2010). A population will then establish, grow faster and become species genetic representative if multiple introductions occur helping new individuals responding against the possible population reduction (Alee effect) increasing the survival of the new population (Taylor and Hastings 2005, Verling et al. 2005, Roman and Darling 2007, Wilson et al. 2009).

In the recipient area there are several abiotic factors that can impact the recently transported individuals. For the establishment of NIS, conditions must be suitable to allow the invasion (Alonso and Castro-Díez 2008, Catford et al. 2009). Supported by several hypotheses reviewed by Catford et al. (2009), changes in the resource availability is one of the main key factors because it can increase the survival of the population followed by a fast spread in the new area.

Not only environment itself interplays on the species survival rate. Since organisms are moved to an area outside their native range, they will find new species to compete with (Occhipinti-Ambrogi and Savini 2003). There are two requirements for newly arrived species to survive among local species: (i) enemy release which supports the idea that in the new area, since NIS will not have natural enemies, they will be able to spread without boundaries; and (ii) limiting similarity where a cooperation may occur between species due to a differently functional aspect which decrease competition and new individuals can occupy an empty niche in the new area (Thieltges et al. 2004, Catford et al. 2009).

Some species are able to survive within a range of each of these factors and the capacity for a species to survive within this threshold is known as tolerance range (See Fig.3, Shelford's Law of Tolerance, Shelford (1931)). Some of these changes can occur naturally, for example, the change in the intertidal zone due to salinity variations after a rain period can occur as an external factor, activating the tolerance range for some mussels (Feder and Hofmann 1999). Close to this tolerance limits (upper or lower) individuals can experience stress which can be considered as a key trait determining the ability of a species to become invasive (Van Kleunen et al. 2010).

1.5 Stress in the marine environment

Changes in the environment are increasing and can impose significant stress on natural populations (Vinebrooke et al. 2004). Environmental stress refers to physical, chemical and biological constraints on species productivity and on the development of ecosystems (Rykiel 1985). When the exposure to environmental stressors increases or decreases in intensity, ecological responses result in several changes in individual physiology, affecting fitness, growth, reproduction among other factors (Hoffmann and Parsons 1993, Sørensen et al. 2003, Vinebrooke et al. 2004, Malmendal et al. 2006). Several natural and mediated stressors in the marine environment have been responsible for changes in coastlines, water column and even deep sea floor (Geller et al. 2010).

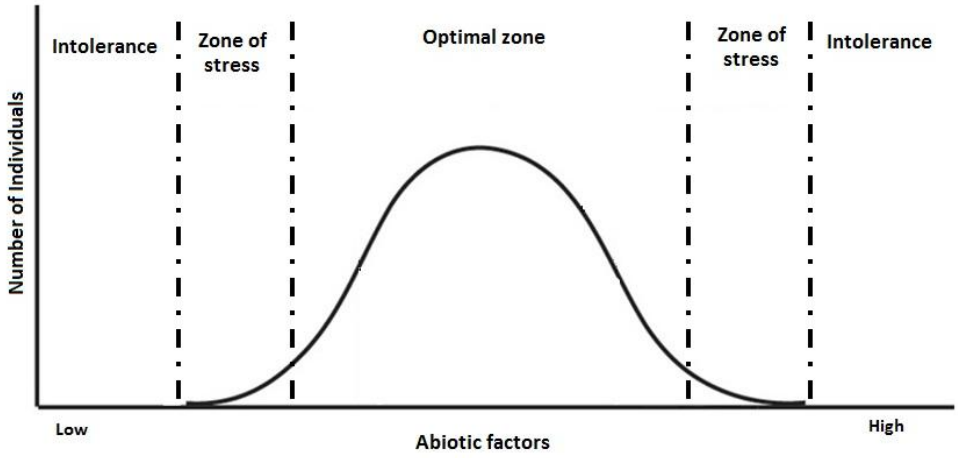

Fig. 3 - Graphic illustration of Shelford's law of tolerance. Plot of number of individuals of a species as a function of abiotic factors (such as heat) generates a curve that present different level of tolerance. Adapted from Krohne (1997).

Natural populations that are regularly exposed to daily fluctuations such as tide changes which can be extremely stressful (sometimes even lethal), need to have an amount of physiological, biochemical and behavioral adaptations that can contribute to overcome such stressful situations and can be useful in an invasion event (Sørensen et al. 2003, Malmendal et al. 2006, Alonso and Castro-Díez 2008, Lenz et al. 2011). Some species are restricted to limited tolerance to stress while others have the potential to be widely distributed with broad tolerance ranges and more ability to invade other ecosystems (Zerebecki and Sorte 2011). Moreover, NIS are generally more resistant to environmental stress than the same species populations in their native environments (Lenz et al. 2011). This could be explained by the selection of the most robust individuals during transport or by introduction processes and genetic diversity of NIS (due to multiple introductions episodes) which can include the most stress resistant ones. In addition, organisms when exposed to periods of high stress (e.g. temperature), will respond by synthesizing an amount of proteins called heat-shock proteins, HSPS (Lindquist 1986, Huang et al. 2007), causing a period of tolerance to stress – stress hardening.

1.6 Objectives of the present study

Survival during transport requires a high tolerance to salinity changes (for example on natural barriers such as the freshwater Gatun lake in the Panama Canal),

temperature ranges (during long distance voyages) and air exposure periods (when ship hulls are expose to air after water discharge) (Alonso and Castro-Díez 2008, Lenz et al. 2011)

The present study was designed to evaluate the impact of transport conditions by ships in the introduction and persistence of NIS in a new environment. In the Lisbon area, Portugal, native individuals of the mussel *Mytilus galloprovincialis* and the crab *Carcinus maenas* were exposed to stress regimes to simulate transport conditions during ship voyages. In particular, *Mytilus galloprovincialis* were exposed to i) air exposure to mimic ship hull conditions when ballast water is discharged, leaving animals to dissection above the waterline, ii) hyposalinity to simulate conditions during transit in a Canal; and iii) heat to simulate conditions in ballast water tanks. Individuals of *Carcinus maenas* were only exposed to heat stress.

Individuals of both species were stress induced on a first phase during a certain period targeting 80% of mortality to select the most robust individuals. Survivors were then divided in two groups: one with a recovery phase and other without a recovery phase to investigate a possible stress hardening event corresponding to an additional stress phase. Finally, survival rates between groups with different stress histories were compared in order to confirm the hypothesis that pre-stressed organisms are more stress resistant than those not previously stressed.

2 Material and methods

All experiments were conducted at Guia Marine Laboratory in Cascais, Lisbon, Portugal from May to September 2012. Guia Marine Laboratory is located in an exposed area of the European west coast on the Atlantic Ocean (38° 41' 42.88'' N, 9° 27' 8.37'' W) (Fig.8).

This study was part of a global project (GAME - Global Approach by Modular Experiments, www.geomar.de/go/game) comparing survival rates between groups with different stress histories of known marine invasive species under stress. Experiments were designed at GEOMAR-Helmholtz Center for Ocean Research Kiel, Germany.

2.1 Study Organisms

2.1.1 *Mytilus galloprovincialis* (Lamarck, 1819)

(Phylum: Mollusca, Class: Bivalvia)

This species is dark blue or brown to almost black with identical shells in quadrangular shape (GISD 2012) (Fig.4). Body sizes ranges between 5-8 cm and exceptionally can grow up to 12 cm (Picker and Griffiths 2011). These mussels can attach firmly to rocks with strong byssal threads (secreted by a mobile foot) forming beds in the intertidal zone. *Mytilus galloprovincialis* is morphologically similar to *Mytilus edulis* and sometimes they can hybridize. Both mussel species can be distinguish by the presence of pointed down curved umbones in *Mytilus galloprovincialis*, posterior to which ventral shell is frequently concave (Hayward et al. 1996). As a bivalve, *Mytilus galloprovincialis* is a filter-feeder that filters a wide range of planktotrophic organisms (GISD 2012). They occur in estuarine and marine habitats, nearby exposed rocky coastline with a high rate of water flow and where nutrient-rich upwelling occurs (Braby and Somero 2006a, GISD 2012).

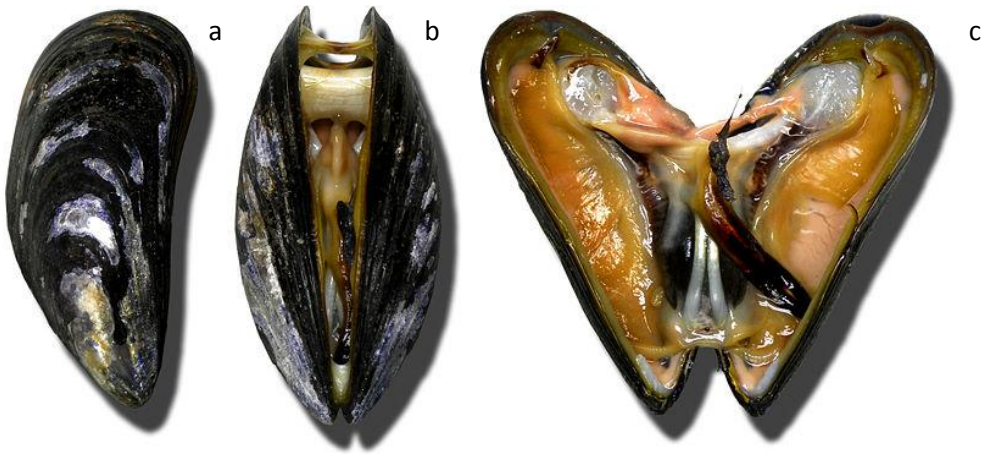

Fig. 4 - Three perspectives of *Mytilus galloprovincialis a*) shell appearance, b) side view of both shells with the visible interior and c) internal appearance of both shells. By Lamiot, 4 november 2006 from wikimedia commons.

Mytilus galloprovincialis lifecycle stages include adult mussels that spawn gametes, after which fertilization of an egg occurs. The egg undergoes gametogenesis, forming a larva that transforms into juvenile which settles and attaches itself using byssal threads after 2 to 4 weeks. Reproduction takes place more than once every year with annual reproductive production (Hayward et al. 1996).

2.1.1.1 Distribution

Mytilus galloprovincialis is considered to be native to the Mediterranean Sea, Black Sea, Adriatic Sea and Atlantic Ocean from Ireland to Morocco (McDonald et al. 1991, Geller et al. 1994, Hilbish et al. 2010) (Fig.5 – green area). It is also present along the coast of France, Britain and Ireland but it is still unclear whether it is native in these regions (Hayward and Ryland 1995, Hayward et al. 1996) (Fig. 5 – yellow area).

The invasion history of this species started in the first half of the 20th century in California where it spread 1100km towards north (Geller et al. 1994). In South Africa this species was found in 1979 in Saldanha Bay, due to an aquaculture accident, and nowadays occupies around 2050km of the coast (Branch and Steffani 2004, Wonham 2004, Robinson et al. 2005). *Mytilus galloprovincialis* has invaded and established populations in several other regions worldwide: Hong Kong, Japan, Korea, Australia, America, Mexico and Canada (Fig. 5 – red area). These locations are associated with shipping routes where the transport of this species occurred in ballast waters or attached to ship hulls (hull fouling), since species had allele frequencies almost

identical to Mediterranean Sea individuals (McDonald et al. 1991, Branch and Steffani 2004, Wonham 2004). The great capability of survival among individuals of *Mytilus galloprovincialis* in extreme conditions of air exposure or temperature in ballast tanks may explain its success in invading different areas on the globe (Geller et al. 1994, Branch and Steffani 2004, Hilbish et al. 2010).

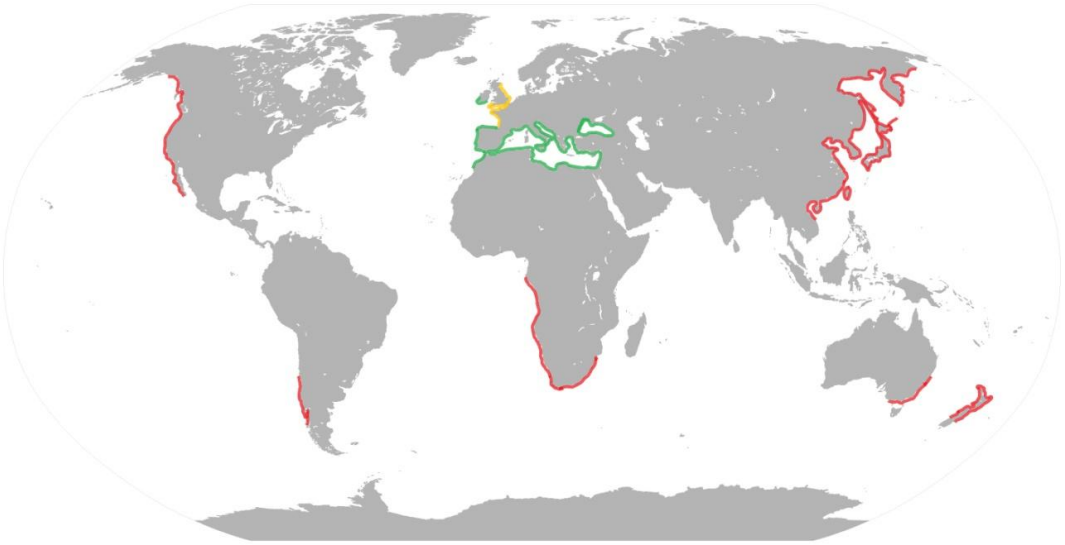

Fig. 5 - Distribution map of *Mytilus galloprovincialis* adapted from GISD. Green area represents native range of species, red area represents the confirmed invaded regions and yellow area represents the possible native range.

2.1.1.2 Impact

Mytilus galloprovincialis has a planktotrophic larval stage and its expansion occurs extremely fast. This trait transforms this species in a space occupier creating beds of individuals that can alter the structure of local communities (Branch and Steffani 2004). Due to their high tolerance to environmental changes it is already reported that their survival is between 20% and 200% greater compared to their natives counterparts in South Africa (Branch and Steffani 2004, GISD 2012). Although some benefits of this species introduction associated to water quality indicators are known (Shumway et al. 2003), *Mytilus galloprovincialis* is both ecologically invasive and a source of 'genetic pollution' and could be responsible for the breaking of heterogeneity of species composition affecting genetic composition (Hilbish et al. 2010).

2.1.2 *Carcinus maenas* (Linnaeus, 1758)

(Phylum: Arthropoda, Class: Malacostraca)

This species has a 5 – 6 cm carapace length that is much broader than long, and minutely granular. Breadth is approximately 7.3 cm and males are slightly larger than females. Color is variable from dark green in adult individuals, with green legs and underside, and in juveniles often varies from speckled, with center white or black triangle or other strong pattern, and often orange bellow (Fig.6). *Carcinus maenas* can be found in splash-zone pools, salt marshes and estuaries, and also in shallow sub littoral down to 200m depth (Hayward and Ryland 1995, Wirtz and Debelius 2003).

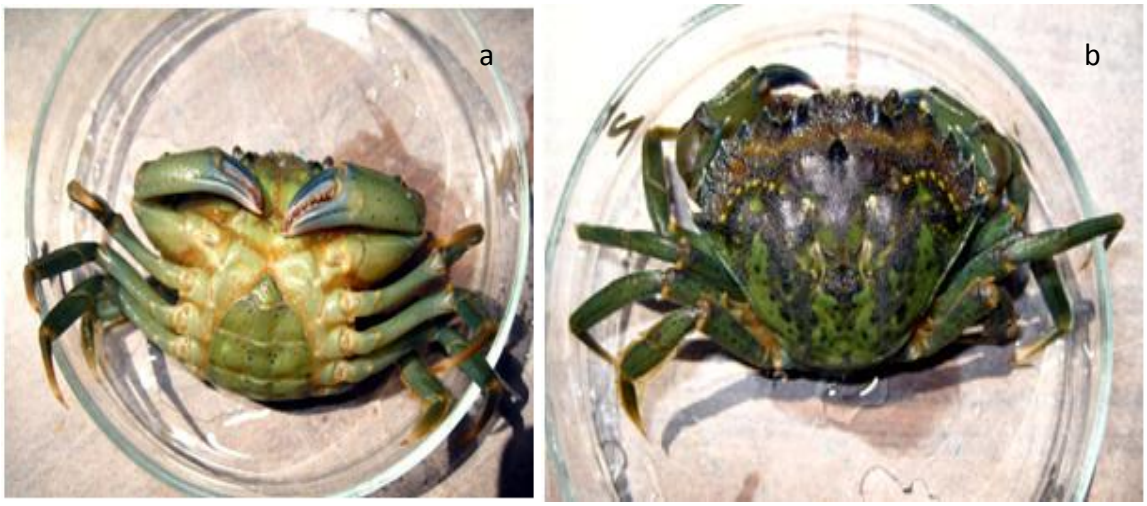

Fig. 6 - *Carcinus maenas:* a) posterior part and b) anterior part

Female green crabs can produce up to 185,000 eggs at a time (Crothers 1967, Neal and Pizzolla 2008) and molt once each year during mating (Vinuesa 2005). The female carries the egg sac for several months and then the eggs hatch into free-swimming larvae, which stay in the water column for 17 days at 25°C to 80 days at 12°C (before settling to the bottom) (Crothers 1967, Vinuesa 2005, Neal and Pizzolla 2008).

2.1.2.1 Distribution

Carcinus maenas is native to Europe and North Africa (Ruiz et al. 2000a, Roman and Palumbi 2004) (Fig. 7 – green area) and it is considered as one of the most successful marine invaders in the world due to its high tolerance to air exposure, starvation and a wide range of temperature and salinities (Yamada and Hauck 2001,

Roman 2006). Such adaptability combined with high fecundity and long planktonic larval stages makes *Carcinus maenas* capable to survive long ocean voyages (Yamada and Hauck 2001, Roman and Palumbi 2004). The invasion history of *Carcinus maenas* started in New York state in 1817 where it was registered for the first time (Roman 2006). During the 1950s *Carcinus maenas* was found in Canadian waters, expanding south (Roman 2006). In the early 1980s it was located in other regions around the world like Tasmania, South Africa, Madagascar, Japan, Brazil and Panama (Fig. 7 – red area) (Boschma 1972, Yamada and Hauck 2001, Carlton and Cohen 2003).

2.1.2.2 Impact

While ecological and economical impacts associated with *Carcinus maenas* have been reported in recent years (Roman and Palumbi 2004, Blakeslee et al. 2010), advantages of its introduction are not known (Cohen et al. 1995, Perry 2010). Generally they can negatively affect other marine invertebrates by preying on them or competing for food resources and for space. For example, the arrival of this species to the Northeast America in the 1950s, had a negative impact in soft-shell clams, young oysters, and native crabs (Neal and Pizzolla 2008). As a consequence, this can have significant impacts on ecosystems species devastating their near shore nurseries (Cohen et al. 1995, Perry 2010).

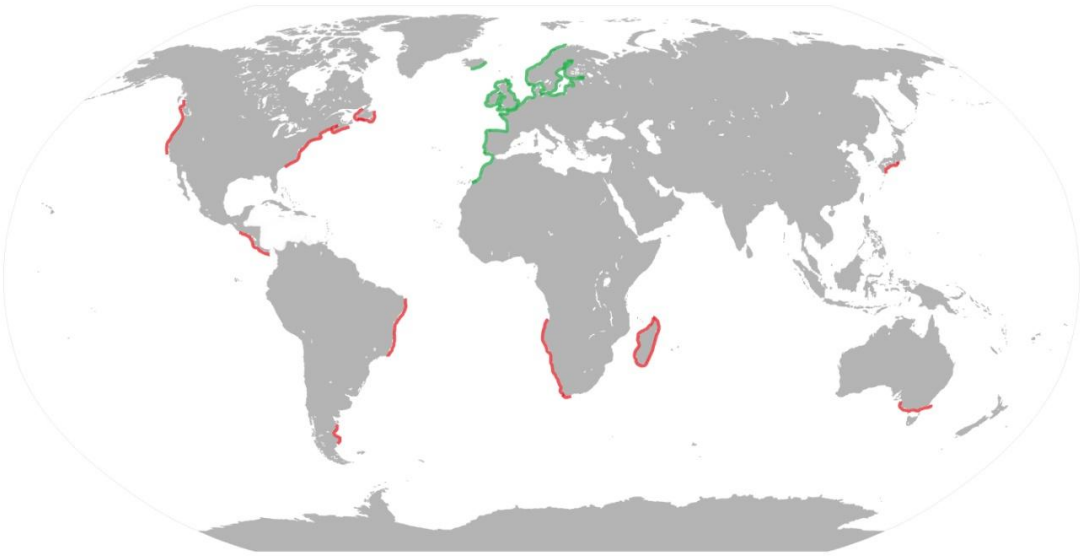

Fig. 7 - Distribution map of *Carcinus maenas* adapted from project UFO (Unidentified Foreign Organisms) – European green crab around the world and GISD. Green area represents native range of species and red area represents the confirmed invaded regions.

2.2 Sampling sites

Individuals of *Mytilus galloprovincialis* were collected along the Cascais coastline, Portugal (Fig. 8b), an extreme exposed area of the European west coast on the Atlantic Ocean (38° 41' 42.88'' N, 9° 27' 8.37'' W) (Fig.8b). Annual water temperature fluctuates between 16.9 ± 3.8 °C and salinity around 36 PSU (Practical Salinity Units) (Pimentel et al. 2012). Adult organisms with a shell length between 3 and 4 cm were collected by hand in May 2012 and June 2012. They were collected into a bucket filled with sea water and transported to the laboratory where they were separated into single containers (0.33 L) provided with air supply.

Individuals of *Carcinus maenas* were collected in the Tagus estuary near Seixal, Portugal (38° 39' 0" N, 9° 6' 0" W) (Fig.8b). Salinity values in this location fluctuate between 20 to 25 PSU and water temperature varies between 17°C ± 4.9°C (Caçador et al. 2012). Crabs were collected with fishing net and placed into a thermal box with seawater, during a period no longer than 40 minutes corresponding to its transport to the laboratory. Once in the laboratory, crabs were separated into individual containers (1.5 L) provided with air supply.

Fig. 8 - Map of Portugal a) showing in detail the sampling locations with blue circles b) Guia, Cascais (38° 41' 42.88'' N, 9° 27' 8.37'' W); b) Seixal, Setúbal (38° 39' 0" N, 9° 6' 0" W). Maps obtained from: a) Google Earth, 10th April 2013, and b) www.maps.google.pt both accessed on 24th March 2014.

2.3 Acclimation and Preparation of Stress Experiments

Collected animals were acclimatized to laboratory conditions one week prior to all pilot studies and main experiments. Temperature was controlled with temperature loggers (T-logger, HOBO Pendant® Temperature Data Logger) in all experiments. T-loggers were set in the same conditions as study organisms depending on the stressor and stress history to keep the values controlled (See section 3.1 – Treatments efficiency). All abiotic factors were kept constant unless manipulated for stress treatments during experiments.

Mytilus galloprovincialis were kept in single 0.33 L containers (1 mussel per container) and fed every other day after water exchange with a nutrition formula (CoralSands® (DT's Premium Blend Live Marine Phytoplankton, CoralSands, Germany, www.coralsands.de). *Carcinus maenas* were placed in 1.5 L containers (1 crab per container) and were fed everyday with a four pellet dose (*Aquatic Shrimp Crab & Lobster Food - Zoo Med*). Individuals of both *Mytilus galloprovincialis* and *Carcinus maenas* were kept in single containers (to avoid pseudo-replication). Air supply was provided to all containers during the whole experiments.

2.4 Stressors

Stressors applied in this study should occur in a real life scenario with animals inside of a ballast tank or exposed on a ship hull. Conditions experienced by organisms inside ballast water were coined by Carlton (1985) and later confirmed by Gollasch et al. (2000a) during a transoceanic voyage. Several factors may affect organisms' survival during ship voyages such as the absence of light, temperature fluctuations and oxygen all depending on the position, size and content of a ship (Carlton 1985). Furthermore, sessile organisms like mussels on ship hulls can experience air exposure, once ships release water from ballast tanks leaving mussels to dissect above the waterline, or hyposalinity events when ships cross canals like Suez, Panama or Kiel.

The present study has focused on three types of stressors: i) air exposure, ii) heat and iii) hyposalinity. *Mytilus galloprovincialis* was exposed to air exposure, hyposalinity and heat while *Carcinus maenas* was only exposed to heat stress (Fig.9).

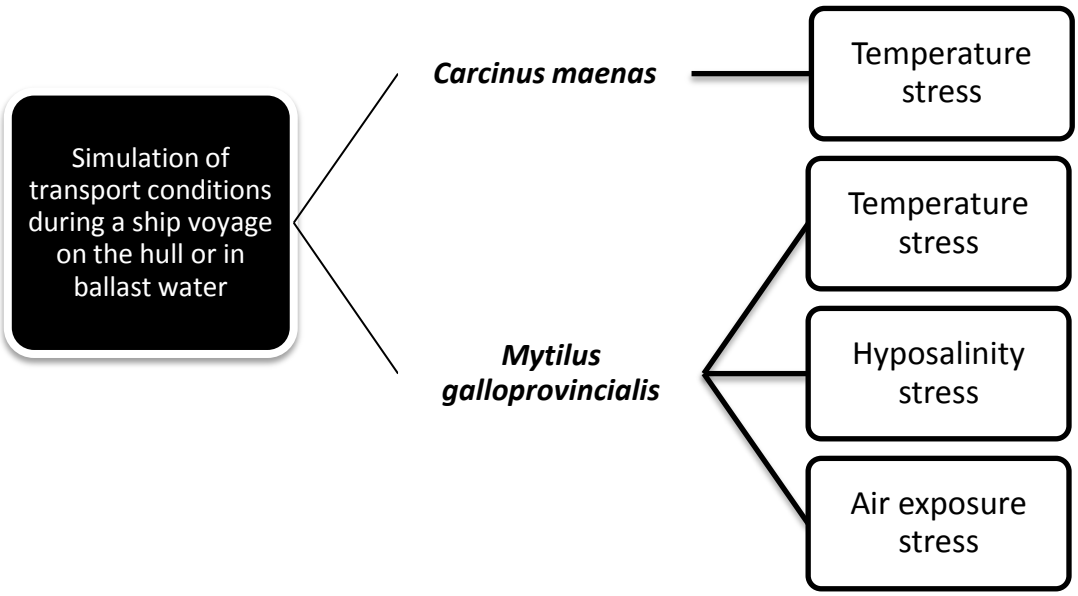

Fig. 9 - Overview of the stressors used in each species taking into account the target of the experiments

2.5 Experimental design

This study was conducted following an experimental design with 3 treatment groups (except for air exposure in *Mytilus galloprovincialis,* which will be explained bellow) and a reference group. The reference group was used as a control to check background mortality and influence of laboratory conditions since it was not exposed to any stressor during the experiments. From a total of 210 individuals per selected species (i.e., 210 individuals of *Mytilus galloprovincialis* + 210 individuals of *Carcinus maenas*), 160 experienced an initial stress phase – hereafter designated stress phase one (SPI), 25 served as a reference group and 25 were later exposed to a posterior stress phase (Fig.10). This first stress was applied to generate 80% of mortality, creating a 'selection' between the most resistant individuals from SPI. Theoretically, the 20% individuals surviving this first stress would be more robust. The 80% mortality mentioned above was previously calculated with pilot studies (See section 2.5.1 - Pilot studies). SPI was terminated when approximately 80% mortality was reached.

After SPI, the remaining 20% of the 160 animals that survived were divided into two groups: A and B for all the stress experiments, except for air exposure with *Mytilus galloprovincialis* where group B did not exist due to the results of the pilots which proved the impossibility to maintain a B group under air exposure conditions (See section 3.2.1 – Air exposure in *Mytilus galloprovincialis)*. Group A consisted of a group

of animals that after SPI had a two week recovery phase at ambient water temperature, while group B did not experienced any recovery phase. Animals in group B were immediately exposed to stress phase 2 (SPII). After a 2 week recovery period, individuals from group A were exposed to SPII. At the same time, 25 organisms kept at normal conditions until here, were set as group C and were exposed to their first stress phase. The rationale for the creation of these groups was to test whether organisms with a stress background were more resistant than the other group without any previously stress.

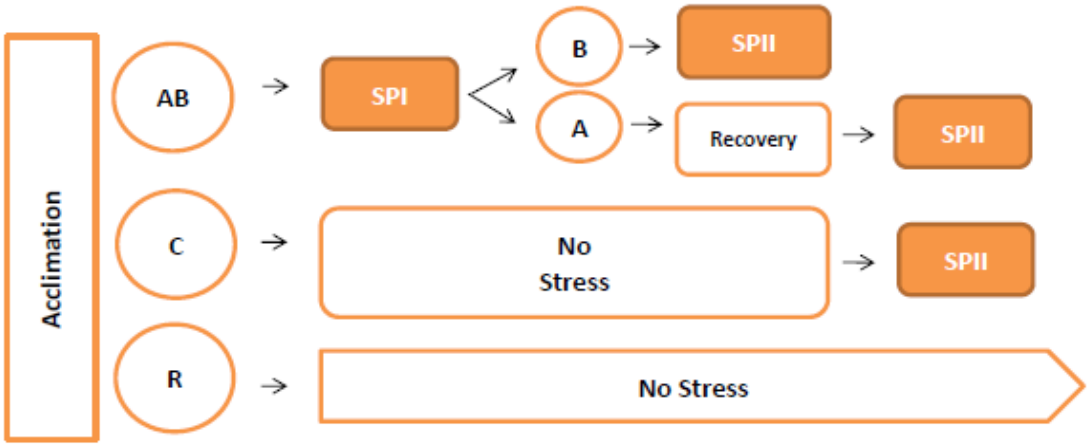

Fig. 10 - Diagram resuming the experimental design used in this study. Acclimation (corresponding to the acclimation to laboratory conditions), A – Group A (group with recovery phase), B – Group B (group without a recovery phase), C – Group C (group with one stress phase), R – Reference (group without any stress exposure during the whole experiment), "Recovery" (period referent to the recovery of the individuals after first stress exposure), SPI – Stress phase one, SPII – Stress phase two and "No stress" – any stress was applied during this period.

2.5.1 Pilot studies

Four pilot studies with *Mytilus galloprovincialis* (2x air exposure, hyposalinity and heat) and one with *Carcinus maenas* (heat) were conducted to optimize stress levels to apply in the main experiments. To optimize this value it was necessary to maintain values that can occur in a real scenario and also to "control" individuals' mortality since the experiment was based in 2 stress phases where survivors from the first phase were transferred to the second. In all experimental set ups, containers were

randomly positioned to account for confounding effects of gradients of temperature and light regime in the laboratory.

2.5.1.1 Air Exposure in *Mytilus galloprovincialis*

For this pilot, 225 *Mytilus galloprovincialis* individuals were placed in single containers (Volume=0.33 L) filled with seawater and kept under laboratory conditions. One hundred individuals were placed outside the laboratory and additional 100 under laboratory conditions, while 25 served as a reference. Water was then discharged and during five days animals were exposed to air conditions.

2.5.1.2 Hyposalinity in *Mytilus galloprovincialis*

For the hyposalinity pilot experiment a total of 35 individuals divided by 7 different treatments were exposed to different PSU values. The treatments consisted in reducing the PSU values from 36 PSU (normal salinity) decreasing 5 PSU per day until a maximum level of 5 PSU (low salinity). Salinity was beforehand decreased in a big tank adding fresh water to sea water until reaching the salinity level wanted. Water was then replaced in each container (Fig.11a)).

The starting value was 36 PSU and during 6 days was decreased stepwise. Five organisms stayed at a salinity of 36 PSU and reduced 5 PSU per day. At each level 5 organisms were left from the initial number to check induced mortality. This pilot ended when the 5 animals at 5 PSU and the 5 animals at 10 PSU all died.

2.5.1.3 Heat in *Mytilus galloprovincialis*

The heat stress pilot experiment consisted in a water bath (used also in the main experiment, 150x100x25 cm each) previously arranged with 2 heaters (Tetra® HT Aquarium Heater), containers and a water pump, to circulate the water in the water bath (Fig.11b)). Water from containers and water bath were never mixed. To this pilot study 2 water baths were used. One bath was prepared with 2 heaters while the other served as a reference. Thirty individuals were exposed to SPI, where temperature was gradually raised every day 2°C while 10 animals were kept under normal conditions. After 20 days at SPI a temperature of 29°C was reached. This value was kept during 2 days and 75% mortality was obtained. At this mortality temperature was decreased stepwise to normal values. Four animals survived SPI and stayed during 2 weeks on

recovery phase. From the reference group left in the beginning (n=10), 6 stayed as a control while 4 were, at the same time as the survivors from SPI, exposed to heat stress.

2.5.1.4 Heat in *Carcinus maenas*

For the *Carcinus maenas* pilot study, 28 individuals were kept in water baths prepared with the same conditions as *Mytilus galloprovincialis*. Nineteen of 28 animals were exposed to SPI of heat stress. Temperature was increased in both baths 1°C everyday. A maximum of 29°C was reached and persisted until achieved a mortality value close to 60%. The stress experiment was conducted for a 20 day period and was not possible to test a second stress phase due to limited space and time. In this pilot study feeding experiments were also tested, first with dead mussels used as food and afterwards with pellets.

Fig. 11– a) Methodology used to create hyposalinity in *Mytilus galloprovincialis* stress by mixing fresh water with salt water before in a bucket and afterwards spread on each container inside of the blue box; b) Experimental set up used in *Mytilus galloprovincialis* experiment using heat as a stressor.

2.5.2 Main experiments

2.5.2.1 Air exposure in *Mytilus galloprovincialis*

Based on the two pilot studies on air exposure in *Mytilus galloprovincialis* the main experiment was conducted under laboratory conditions. For the experiment with air exposure 210 individuals were collected. After the acclimation period the water was removed from 160 organisms while 50 remain under normal conditions. Container's walls were dried with paper towels to remove any trace of water that could be attached. After 3 days from the start of the experiment the containers were

refilled. Survivors were kept during one week under recovery. After this recovery period survivors were again exposed to air conditions and at the same time 25 individuals from the 50 organisms left as a reference, become group C and were also exposed to stress (See Table 1 section 3.1).

2.5.2.2 Hyposalinity in *Mytilus galloprovincialis*

Pilot studies with hyposalinity in *Mytilus galloprovincialis* indicated 10 PSU as a suitable limit of stress intensity. Instead of reducing 5 PSU per day like in the pilot study, values were reduced 10 PSU from the normal values until a minimum of 10 PSU. After 3 days, 160 organisms were exposed to 10 PSU. After 6 days mortality was 69.9% and PSU values restored to 36 PSU (see Table 1, section 3.1). Survivors were divided and half were kept under recovery until the next stress phase which also involved 25 organisms named as group C, and the other half was exposed to a second stress phase without a recovery period. Before the begging of SPII byssus threats (silky fibers used by mussels to attach Fig. (13 b)) were removed with scissors in all groups (including reference) in order to evaluate possible differences between groups on the production of this fibers after SPII.

2.5.2.3 Heat stress in *Mytilus galloprovincialis*

The set up for the heat stress main experiment included 5 water baths in which 4 included 2 heaters and 2 water pumps in each bath. From a total of 210 of *Mytilus galloprovincialis*, 160 were exposed to heat stress for the first time while 50 stayed under normal conditions (25 as Reference and 25 as group C). Heaters were regulated every day from one to two degrees in each water bath. The pilot study determined 29°C as maximum temperature value. To proceed with water exchange, a major tank was beforehand heated until the temperature wanted every day. After this procedure water was provided in each container and placed randomly inside the water bath. After achieved 82.9% of mortality during the SPI, temperature started being decreased until ambient water temperature (see Table 1 section 3.1). The length of SPI was 21 days. After achieving ambient temperature, survivors were divided in 2 groups, one with a recovery phase (A) and other that was right after the end of SPI, stressed again (group B). After that, A and C (group with 25 individuals kept under normal conditions

until now) were stressed at the same time. SPII had a length of 18 days to group B since all animals died at this time.

2.5.2.4 Heat stress in *Carcinus maenas*

In addition to improve experimental set up, pilot studies served to verify if water exchange could be done every second day to reduce the contact with individuals which was changed in the main experiment. Organisms were disposed in single containers and disposed in water baths (Fig.13 c) and d)). Water temperature in tanks was increased 1-2°C every day until reaching 29°C. As with *Mytilus galloprovincialis* experiments, water was previously heated in a major tank and just after distributed in each container. After a mortality of 69.2% the temperature was decreased until the ambient temperature gradually as before (see Table 1 section 3.1). SPI lasted 21 days and after this period while group B was immediately stressed after the SPI, group A was in recovery for two weeks at ambient sea water temperature. After this, group A survivors were stressed at the same time with group C.

Fig. 12 a) Experimental set up for *Mytilus galloprovincialis*, b) detail of byssus threats attached to the container, c) single container with *Carcinus maenas* individuals; d) Experimental set up of *Carcinus maenas* with the arrangement of the containers in the water bath.

2.5.2.5 Response Variables

Survival was the main response variable measured everyday in all experiments. In case of *Mytilus galloprovincialis,* mussels were considered dead if it fell off the container wall (byssus des-attachment) and if valves of the 2 shells were opened without resistance. In C*arcinus maenas,* individuals were considered dead when they did not respond to tactile response and when water had an intense smell. In both cases, dead individuals were removed immediately from containers and not replaced.

Beside survival, byssus production was also measured during hyposalinity in *Mytilus galloprovincialis* which consisted in counting the production of byssus created by

mussels to get attached to substrate. Byssus were counted after total removal before SPII in all groups.

2.6 Statistical Analysis

All statistical analyses, including graphs, were performed using R Version 2.14.2 (R Development Core Team 2012). The level of significance for all statistical analysis was set at $p<0.05$ (95%). Prior to analysis, data distribution was illustrated using box-whisker plots. Data distribution was tested for normality with Shapiro-Wilk W-tests and for homogeneity of variances with Fligner Killen tests. Significant differences in median size among groups of individuals with different stress history were identified using a Kruskal-Wallis test when distribution was not normal. Data from reference group was not included in the analysis except for the analysis of byssus production. The analysis of the differences in survival between groups was performed with Cox proportional hazard (Coxph) and the survival analysis using "survival" package in R (Therneau & Lumley 2009), taking shell length as a continuous independent variable (covariate). The hazard ratio, as an output from Coxph, describes the relationship between the influence factor of each group and survival. The significant influence of length on survival was showed as a scatterplot and analyzed with linear regression, showed the R^2 as a measure to access the goodness of fit of the regression line. To achieve normal distribution and homogeneity of variances for byssus production, data was square root transformed. A One-way ANOVA was then performed with the transformed data. Significant differences of survival between groups during heat in *Mytilus galloprovincialis* and byssus production were also determined by one-way ANOVA and post-hoc test Tukey HSD. Kaplan-Meier-curves were used for survival analysis in SPII. The graphic illustration as a result of this analysis shows in the y- axis 'survival' and in the x- axis 'time' and compares percentages of mortality between groups. Therefore, the time it takes until the event of death occurred in each individual during stress phases was measured (Fig.14).

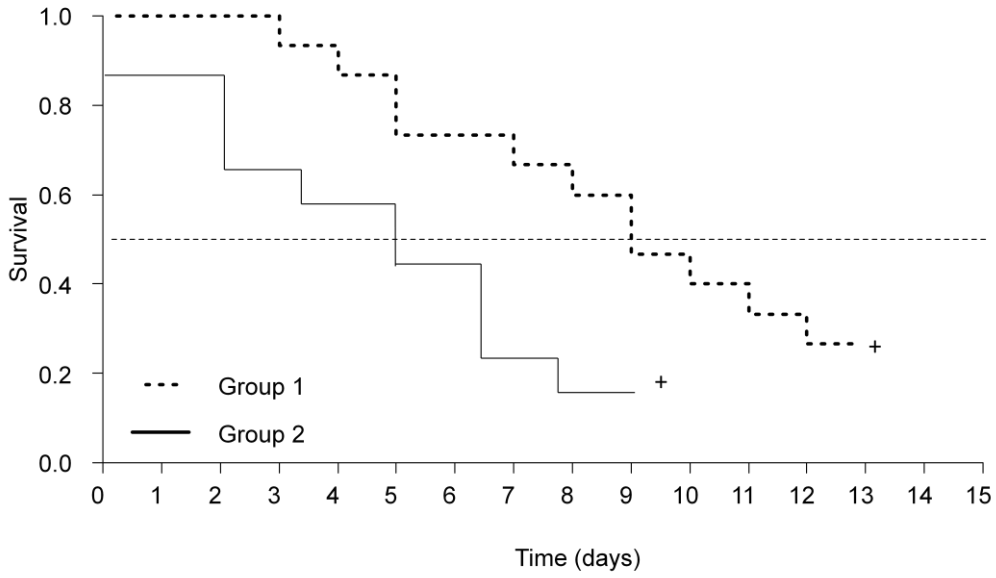

Fig. 13 - Kaplan-Meier curve as a demonstration for results. This graph was generated with artificial data in order to explain differences in the results section. X- axis represents time in days and y- axis represents survival. All individuals are alive at 1.0 in y- axis. Dotted line shows 50 % of mortality, as in y-axis achieve 0.5. Plus signs (+) shows that individuals survived. Differences between groups are statisticially significant when p-value < 0.05

3 Results

For a better understanding of the results section, groups were defined with permanent colors to all graphs. Group A – green, group B – red, group C – blue and reference – black.

3.1 Summary of survivals between stress phases

Table 1 shows the percentage of mortalities achieved in each stress phase as well as the mortality registered during recovery periods and in the reference group. The percentage in SPI was monitored to achieve values close to 80% since it was necessary to induce a type of selection of the most resistant individuals.

Table 1 - Summary of mortalities in SPI, Recovery and SPII for *Mytilus galloprovincialis* (air exposure, hyposalinity and heat) and for *Carcinus maenas* (heat).

		Group A	Group B	Group C	Reference
Air exposure	SPI	70,0%		0	0
Mytilus	Recovery	0		0	0
galloprovincialis	SPII	0		28,6%	0
Hyposalinity	SPI	62,9%		0	0
Mytilus	Recovery	3,0%	0	0	0
galloprovincialis	SPII	48,4%	77,4%	68%	0
Heat	SPI	82,9%		0	0
Mytilus	Recovery	7,7%	0	0	0
galloprovincialis	SPII	100%	100%	100%	0
Heat	SPI	68,8%		12,5%	13%
Carcinus	Recovery	12,9%		8%	8%
maenas	SPII	23%	20%	28,6%	27,1%

3.2 Treatments efficiency

Experiments involving temperature stress (air exposure and heat) were controlled with T-loggers in order to record temperature variations along the experiment. Per each stress experiment 4 T-loggers were set (one per each group). For

Mytilus galloprovincialis and *Carcinus maenas* heat experiments, temperature for groups A, B and C never exceed 29.5°C both in SPI and SPII (Fig.14 a) and b) respectively). In the recovery period temperature values were maintain as the reference group between 20 and 22°C in *Mytilus galloprovincialis* and in *Carcinus maenas*. During air exposure experiments with *Mytilus galloprovincialis* air temperature achieved maximum values of 30°C for a 3 day period while reference group was maintain at ambient se water temperature between 22 and 23°C during the entire experiment (Fig.15).

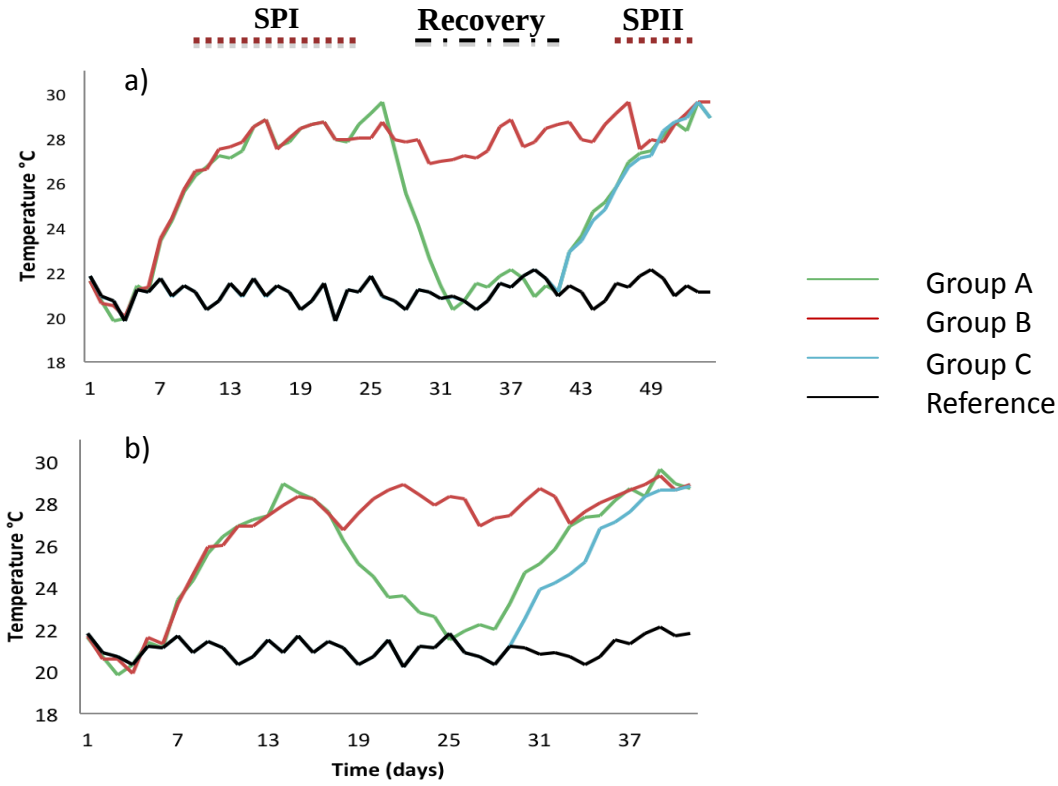

Fig. 14 – Data from T-loggers during heat exposure *in* a) *Mytilus galloprovincialis* and in b) *Carcinus maenas* representing group A (group with recovery phase), group B (group without recovery phase) group C (group with one stress phase) and reference. The 2 types of traced lines indicated above the graph refers to periods of stress and recovery (SPI, Recovery and SPII).

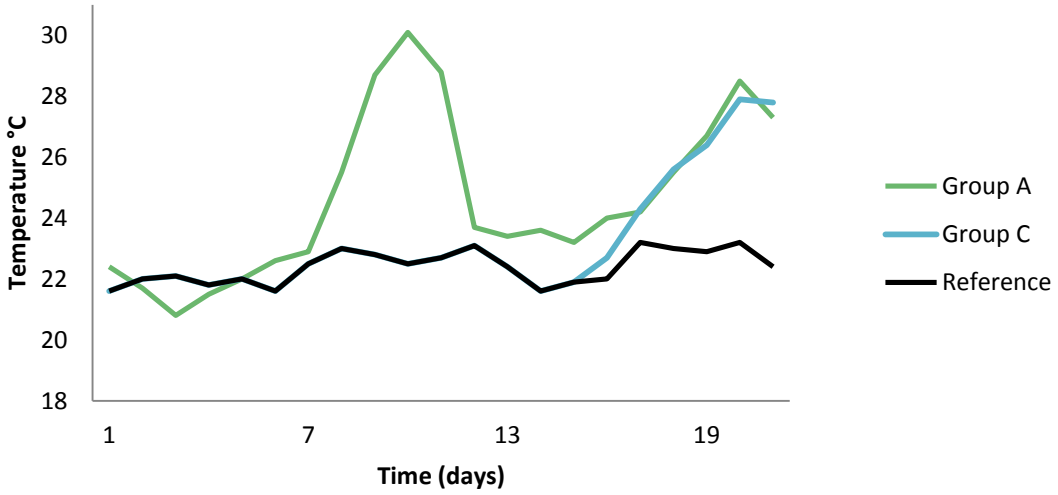

Fig. 15 - Data from T-loggers present on air exposure *in Mytilus galloprovincialis* representing group A (group with recovery phase), group C (group with one stress phase) and Reference.

3.3 Pilot studies

3.3.1 Air exposure in *Mytilus galloprovincialis*

Pilot studies conducted with air exposure as a stressor were taken under laboratory conditions and also outside the laboratory. In the end, only 1 main experiment was conducted using air exposure as a stressor and was conducted under laboratory conditions since outside individuals would be exposed to other stressors that could not be controlled (e.g. rain, predation). To test mortality under air exposure mussels were exposed during one week outside and inside the laboratory without water. After this period all organisms died. This outcome suggested using animals without water only for a 3 day period for the main experiment.

3.3.2 Hyposalinity in *Mytilus galloprovincialis*

Seven different salinity levels were used to evaluate the mortality in each PSU level in order to find a suitable level to obtain 80% mortality. Mortality occurred in 2 levels only, 5 and 10 PSU (Fig.16). This result indicated the use of 10 PSU treatments more appropriate for the main experiment after analysis of differences on survivals between groups.

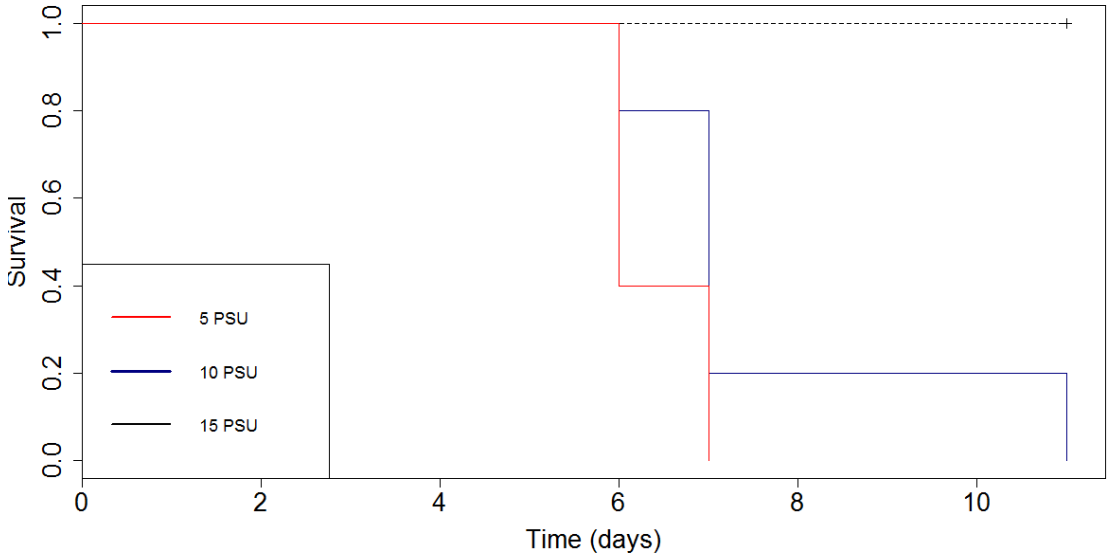

Fig. 16 - Kaplan meier curves for *Mytilus galloprovincialis* after exposing them to hyposalinity stress. *O*nly three salinity levels are showed 5 PSU (n = 5), 10 PSU (n = 5) and 15 PSU (n = 5).

3.3.3 Heat in *Mytilus galloprovincialis*

This pilot study was conducted with 3 groups of animals: one exposed to heat stress 2 times with recovery, one exposed to one stress phase and one as a reference (no stress applied). This experiment showed differences between the group exposed 2 times and the group with 1 stress phase. Animal achieved 75% mortality after 2 days at 29°C which suggested using a maximum of 29°C for the main the experiment.

3.3.4 Heat in *Carcinus maenas*

A group of 18 individuals of *Carcinus maenas* were exposed to heat stress (max 29°C) for a 20-day period and compared with a reference group (no stressed). After 20 days, stressed animals registered a 60% mortality at 29°C indicating this temperature appropriate fo the main experiment (Fig.17). Although there was also mortality in the reference group, the main experiment was conducted in the same conditions as the pilot study.

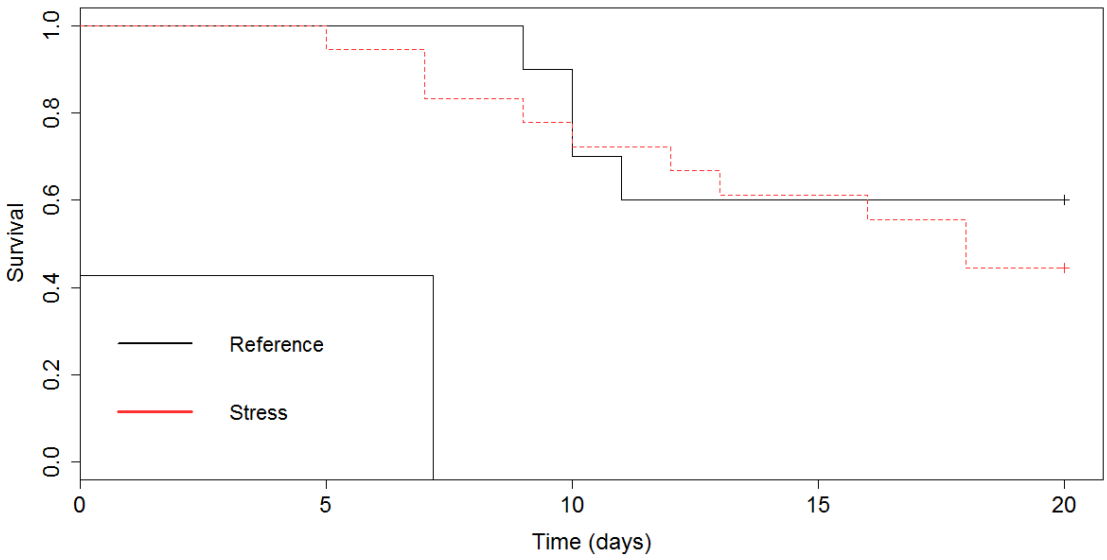

Fig. 17 Kaplan Meier curves for *Carcinus maenas* after exposing them to heat stress during pilot study conducted before the main experiment. Two groups were kept under laboratory conditions: Stress ((n = 18) which was exposed to heat stress of 29°C) and Reference ((n = 10) group that was not exposed to stress conditions).

3.4 Main experiments

3.4.1 Air exposure in *Mytilus galloprovincialis*

The effect of air exposure stress on *Mytilus galloprovincialis* showed a significant difference in survival between the two groups (group B was not present in this stress experiment) studied during stress phase II (Fig.21) (Kruskal-Wallis rank sum test; $X^2 = 8.0091$, df = 1, p-value < 0.05). After SPII, group A (group stressed two times with a recovery period) showed a survival of 100% while group C (group with one stress phase) showed a survival of 80%.

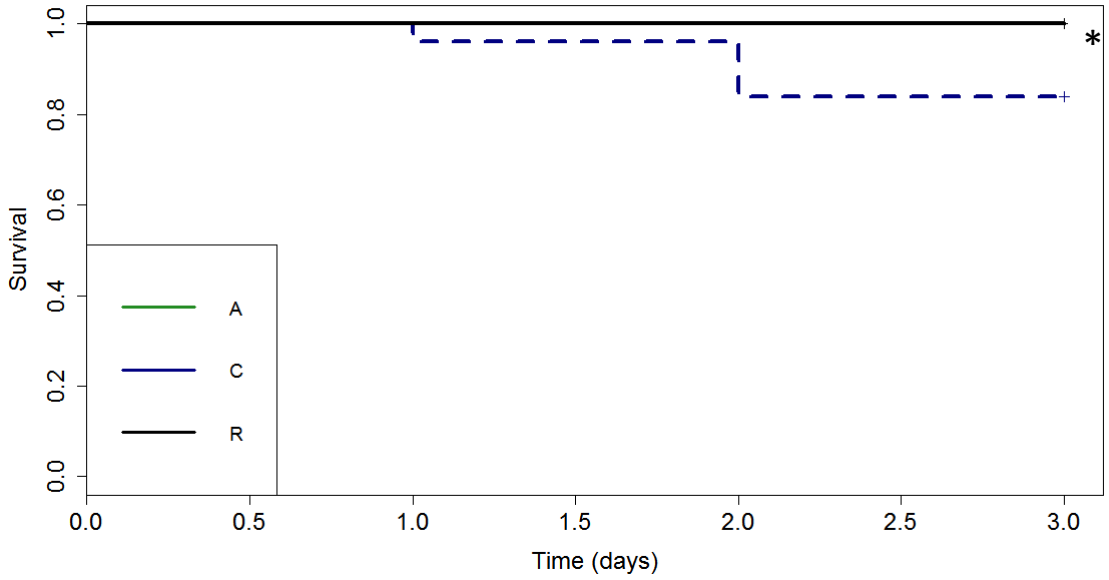

Fig. 18- Kaplan Meier curves for *Mytilus galloprovincialis* after exposing them to air exposure during SPII (Stress phase II) where group A (n=48, group with recovery phase) and group C (n=25, group with one phase of stress) were exposed to air exposure stress during 3 days (P-value ≤ 0.05). *Group A and reference (R) are overlapping since both did not show mortality during SPII.

3.4.2 Hyposalinity in *Mytilus galloprovincialis*

The survival of individuals between groups with different stress histories during hyposalinity in *Mytilus galloprovincialis* showed significant differences (Table 2). Groups differed significantly in their ability to survive (Fig.19) (Kruskal-Wallis test: X^2 = 10.4686, df = 2, p-value < 0.05). Individuals of group A survived significantly longer when compared with groups B and C.

Table 2- Results from Cox-Model analysis from the survival of *Mytilus galloprovincialis* under hyposalinity stress comparing group B (n=31, group with no recovery phase) and group C (n=25, group with one phase of stress) with group A (n=31, group with recovery phase). It is also present the values from the influence of the maximum stress in shell length. (0.95 CI – confidence interval).

Variable	Hazard ratio	95% CI	P-value
Group B	2.586	1.350 – 4.954	0.00419 **
Group C	2.077	1.034 – 4.171	0.03988 *
Shell length	1.132	1.030 – 1.244	0.00988 **

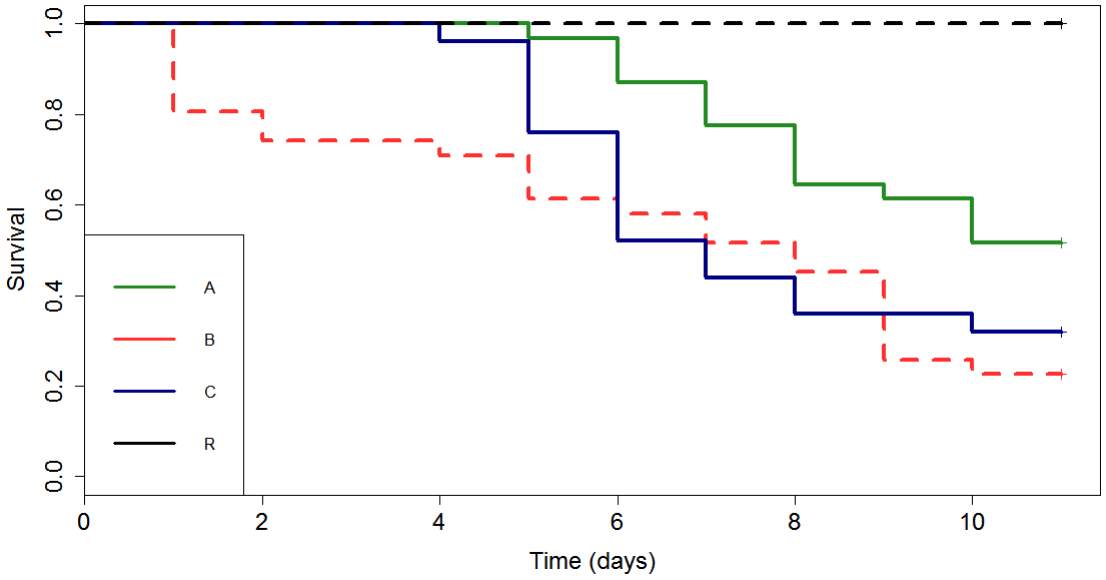

Fig. 19 Kaplan Meier curves for *Mytilus galloprovincialis* under hyposalinity exposure during SPII (Stress phase II) of group A (n=31, green line, with recovery phase), group B (n = 31, red line, with no recovery phase), group C (n=25, blue line, group with one phase of stress) and Reference (R) (n=25, black line, no stress applied). A maximum stress intensity of 10 PSU was applied to groups A, B and C which differed in mortality.

Shell length in mussels had an influence on survival of individuals on group B, showing that the capability of survival decreased with increasing shell length (R^2: 0.31, p-value ≤ 0.05) (Fig.20).

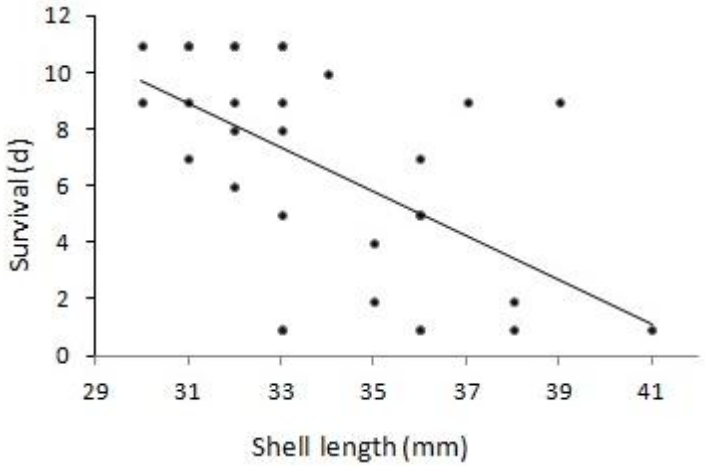

Fig. 20 – Survival analyses of group B (group with no recovery phase) and connection with the lower capability of survival of the larger individuals when stressed at 10 PSU.

3.4.2.1 Byssus production analyses

Mussels in group B (group with no recovery phase) showed a lower production of byssus when compared with group A (group with recovery phase), four times lower (6.69 ± 5.29, mean byssus production ± SD) than group A (22.77 ± 11.61), three times lower than group C (group with one stress phase) (18.88 ± 11.53) and the reference group (18.72 ± 10.33) with regard to the median (Fig.21, Table 3).

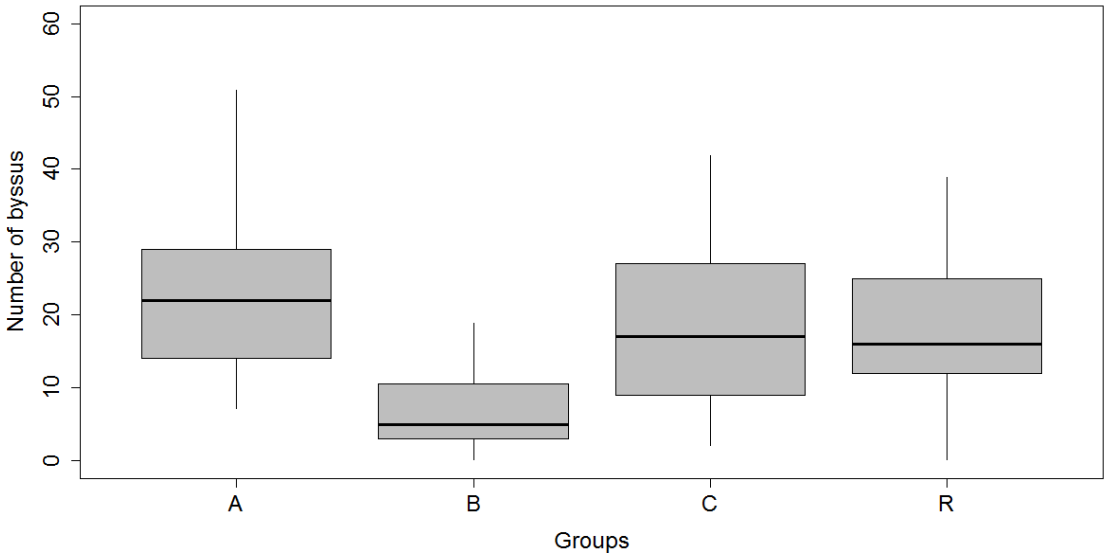

Fig. 21– Box-Whisker plots comparing byssus production between groups A, B, C and Reference during hyposalinity in *Mytilus galloprovincialis.* Medians are indicated as black horizontal lines. Group B is significantly different from other groups: Tukey HSD: $p \leq 0.05$.

Table 3 - Influence of stress history on byssus production in *Mytilus galloprovincialis* (SS = sum of squares, df= degrees of freedom, MS = mean of squares).

	SS	df	MS	F value	p-value
Stress history	75.49	3	25.16	15.11	$3.51e^{-8}$ ***
Residuals	166.57	100	1.67		
Total	242.06	103			

3.4.3 Heat in *Mytilus galloprovincialis*

Heat stress experiment showed significant results between groups (Fig.22, Table 4) (ANOVA; $F = 6.28$, $df = 2$, $p < 0.05$). Group B (group with no recovery) showed a higher survival during SPII when compared to group A (group with recovery)

and C (group stressed one time) (Tukey's HSD: p-value < 0.05) (Fig.24). At the end of the experiment mortality was 100% in all groups.

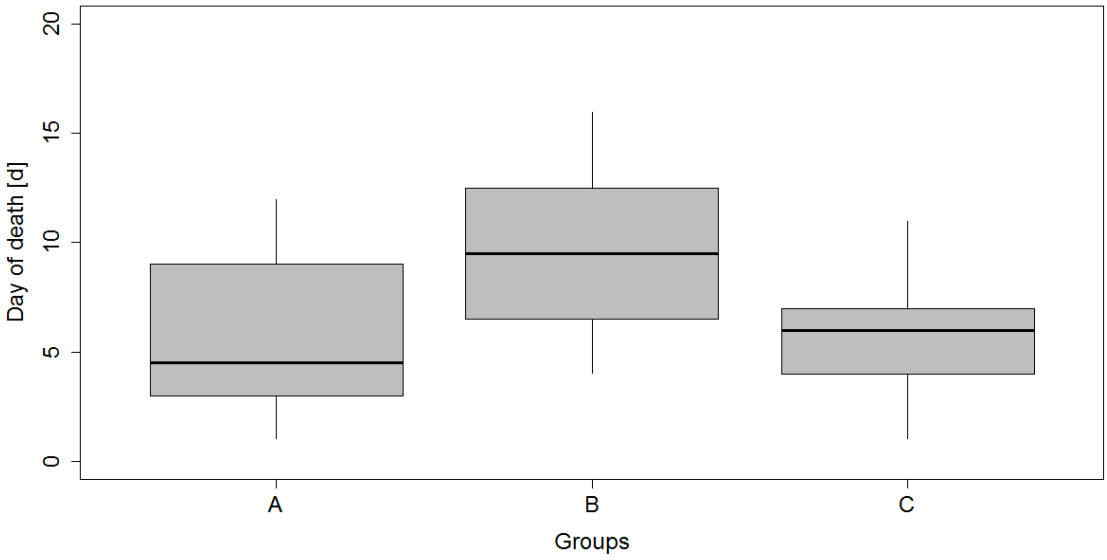

Fig. 22 - Differences between groups on heat stress experiment in *Mytilus galloprovincialis*. Black lines correspond to medians (Group A: n= 12, median = 4.5; Group B: n = 12, median = 9.5; Group C: n = 13, median = 6.0). Groups A and C significantly differs in their mean values comparing with group B (Tukey's HSD: p-value < 0.05).

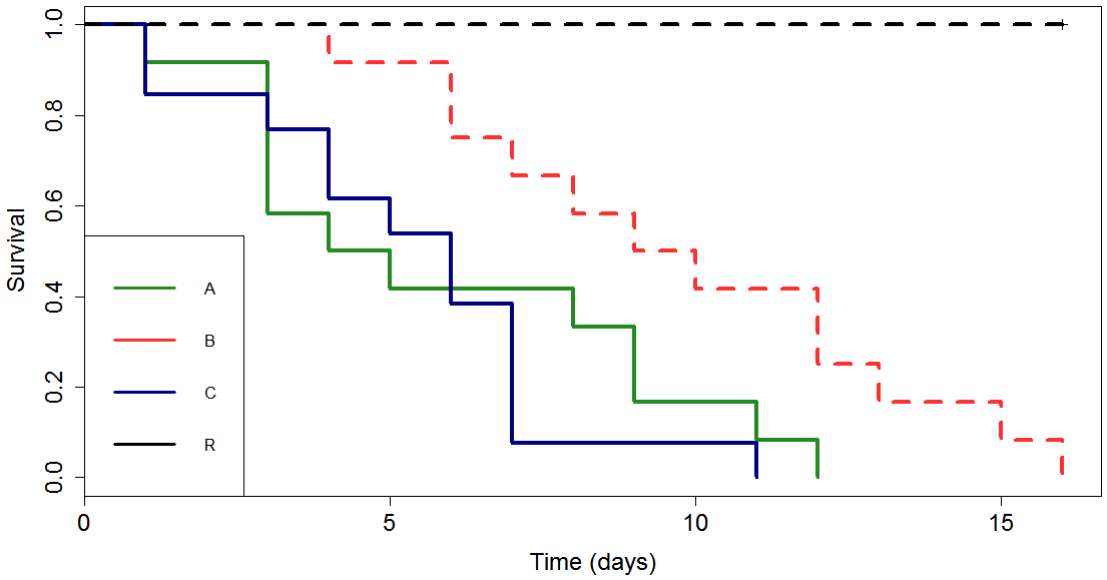

Fig. 23 - Kaplan Meier curves for *Mytilus galloprovincialis* during SPII (Stress phase II; Stress=heat stress of 29°C sea water) of group A (n=12, green line, with recovery phase), group B (n = 12, red line, with no recovery phase), group C (n=13, blue line, group with one phase of stress) and Reference (R) (n=25, black line, no stress).

Table 4 Results of the Cox model for the comparison of survival of *Mytilus galloprovincialis* in groups B (n = 15, group with no recovery phase) and C (n = 25, group with one phase of stress) with group A (n = 13, group with recovery phase), with renewed heat stress of 29 °C and for the influence of shell length as a covariate on the survival of the individuals.

Variable	Hazard ratio	95% CI	P-value
Group B	0.283	0.107 – 0.749	0.011*
Group C	2.077	0.467 – 2.860	0.754
Shell length	1.132	0.805 – 1.083	0.362

3.4.4 Heat in *Carcinus maenas*

Carcinus maenas individuals were exposed to 29°C but the median survival of individuals in SPII between groups was not significantly different (Table 5, Fig.24) (Kruskal-wallis test: $X^2 = 0.3371$, df = 2, p-value = 0.8449).

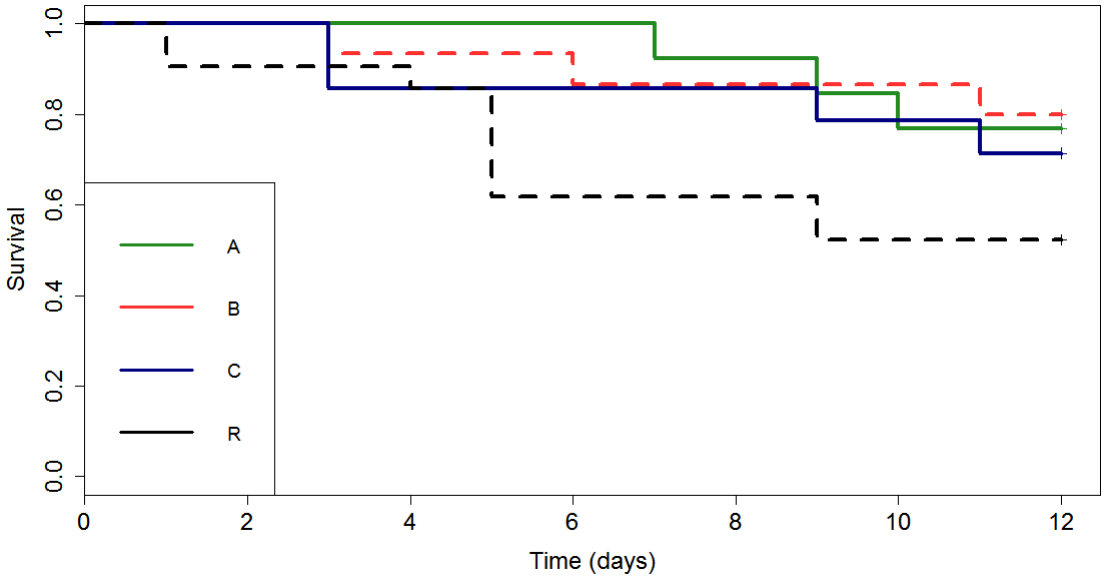

Fig. 24 - Kaplan Meier curves for *Carcinus maenas* during SPII (Stress phase II; Stress=heat stress of 29°C sea water) of group A (n=13, green line, with recovery phase), group B (n = 15, red line, with no recovery phase), group C (n=14, blue line, group with one phase of stress) and Reference (R) (n=21, black line, no stress) when exposed to a heat stress of 29°C sea water.

Table 5 Results of the Cox model of the comparison of survival of *Carcinus maenas* between group B (n = 15) and C (n = 14) with group A (n = 13).

Variable	Hazard ratio	95% CI	P-value
Group B	0.939	4.773 – 0.185	0.940
Group C	2.086	9.915 – 0.439	0.355

4 Discussion

4.1 Summary

The main objective of this study was to evaluate stress resistance by survival in individuals of two successful invasive species – the mussel *Mytilus galloprovincialis* and the crab *Carcinus maenas* – when exposed to simulated transport conditions due to human mediated transport by ships.

This study showed differences in *Mytilus galloprovincialis* survival between groups previously stressed and not previously stressed during stress phase II. Depending on the stressor, survival of *Mytilus galloprovincialis* was different between group A (group with a recovery phase after SPI) and group B (group without a recovery phase after SPI), but it was never lower when comparing with group C (group stressed once). Survival of group A was higher than group B when *Mytilus galloprovincialis* was exposed to air exposure and hyposalinity, and group B was higher than group A when exposed to heat stress. In addition to significant differences between groups in the survival of *Mytilus galloprovincialis* during the hyposalinity stress, it was possible to relate a lower survival when shell length was higher in group B and also a significant decrease in byssus production in the same group. The stress applied to *Carcinus maenas*, did not show any significant differences between groups during stress phase II.

4.2 Definition of stress levels

Pilot studies were conducted to determine stress levels for main experiments, to understand how to handle both species and to choose appropriate laboratory conditions. Stress tests were based in real stress events that can occur in ship voyages that could affect survival of organisms. For both species, it was key to investigate further its invasion history, habitat preferences, biology, ecology and behavior. Stress levels were based in other studies focusing on similar stress experiments with both species (e.g. Cuculescu et al. (1998), Branch and Steffani (2004), Anestis et al. (2007)). These studies as well as the pilot experiments performed here indicated suitable maximum stress levels to each stress case. The aim was to get 80% of mortality in a

first stress phase in order to allow selection of the most resistant genotypes to move towards to the next stress phase.

Mussels are part of a range of organisms that live in the intertidal zone that daily are exposed to periods of dryness and heat at low tide which can lead to dehydration affecting oxygen availability (Babarro et al. 2007). Similar conditions can occur when these organisms are attached to ship hulls. Episodes of air exposure can occur for undetermined time when a discharge of water from ballast tanks take place and expose to air the before submersed individuals (Darbyson et al. 2009). The difference between both natural and 'induced' events is that the punctuality that occurs in the first event does not occur in the second. *Mytilus galloprovincialis* was tested before by Anestis et al. (2010) to air exposure periods where after 3 different temperatures of acclimation they were exposed to periods of desiccation during a maximum period of 50 hours. In this study, after 20 hours of air exposure, mussels exposed to 32°C showed a 50% survival. In a different study with mussels, Branch and Steffani (2004) showed high tolerance of *Mytilus galloprovincialis* to air exposure when compared to *Perna perna, Choromytilus meridionalis and Aulacomya ater,* after 7 days of continuous exposition to air (high tide level) showed a 92% of survival, while others showed 78, 37 and 10% respectively. Both these studies were used as a baseline for the two pilots on air exposure conducted in the present study: 5 days to air exposure between 18 and 24°C under laboratory conditions and 5 days to air exposure at between 21 to 29°C outside the laboratory. Mortality was 100% in both experiments after 5 days. These results determined the air exposure main experiment duration of 3 days under laboratory conditions since the mortality target was 80%.

Freshwater inputs from periods of rainfalls and rivers can occur naturally in the marine environment and can vary significantly from 4 to 38 PSU (Hamer et al. 2008, Gröner et al. 2011). This environmental stress can affect species abundance and their fitness which can also affect other levels from genes to community levels (Hamer et al. 2008). Mussels for example can surpass this type of events by closing siphons, which help to maintain the composition of body fluids in its mantle cavity and will help to keep the maintenance of the organism metabolism (Braby and Somero 2006b, Hamer et al. 2008). During transport on ship hulls animals are also exposed to low salinity

levels when ships go through canal passages as Panama, Suez or Kiel Canals. Ship Transit at these pathways, including stays, can range from hours to several days (Gollasch et al. 2006, Gröner et al. 2011). Hamer et al. (2008) exposed a population of *Mytilus galloprovincialis* from the Adriatic Sea to different levels of salinity to test the impact of salinity stress on these individuals. Organisms showed a survival of 20% after a 14 days period of stress involving values of 10 and 11 PSU. The present study used Hamer et al. (2008) findings as a baseline considering that mortality rates achieved after 14 days with a lower salinity concentration, was the amount wanted with the first stress phase of the present experiment.

The marine intertidal zone is formed at the same time by two areas: marine, during high tide, and terrestrial, during low tide (Stillman 2002). Thermal limits and adaptation to different tidal conditions have been studied in *Mytilus galloprovincialis* and *Carcinus maenas* (e.g. Cuculescu et al. (1998) Zerebecki and Sorte (2011)). Gollasch et al. (2000a) further investigated real environmental conditions occurring inside ballast tanks and how this influenced the survival of plankton organisms. These authors evaluated salinity, pH, oxygen content and temperature fluctuations during a 23-day voyage from Singapore to Bremerhaven. They showed a significant variation on temperature depending on sea water surface indicating that the upper parts of the side tanks when exposed directly to sun action registered higher temperature values (31°C) for several days. In laboratory experiments in order to evaluate differences between Mytilus species in heart rates when exposed to heat stress, Braby and Somero (2006b) showed that *Mytilus galloprovincialis* is better able to cope with higher temperatures than either *Mytilus trossulus* or *Mytilus edulis*. A critical heart rate was achieved at a temperature of 30.9 °C. Above this value mussels can suffer irreparable damages (Braby and Somero 2006b).

Carcinus maenas shows a vast distribution around the world as this species can survive from -1°C to 22°C sea water temperature (Cohen et al. 1995). First signs of heat stress in *Carcinus maenas* were reported by Cuculescu et al. (1998). These authors reported critical thermal maximum values in *Carcinus maenas* ranging from 31 to 36°C. An additional study showed a loss of metabolism efficiency in *Carcinus maenas* at temperatures around 30°C (Taylor et al. (1977)).

The above mentioned studies were key to find a suitable experiment considering mimicking a real voyage duration, temperatures ranges inside of ballast tanks and to determine the temperature threshold without harming individuals. Based on these reference studies as well on pilot experiments for both species, a maximum temperature level of 29°C for heat stress was implemented in the present study.

4.3 Ecological Implications of ship transport

The littoral zone represents a highly variable environment associated with a tidal cycle that impacts the metabolism of several marine invertebrates such as mollusks causing the exposure to air and consequently, intermittent anoxia that cause important changes in the energy metabolism of animals (Almeida and Bainy 2006, Babarro et al. 2007).

Survival in air, suggested as a useful tool to reflect differences at glycogen content and feeding rate (Thomas et al. 1999), is depending on mechanisms for surviving thermal stress and oxygen deprivation (Babarro et al. 2007, Anestis et al. 2010). *Mytilus galloprovincialis* is able to behave partially aerobic at rates of approximately 6 – 17% of aquatic aerobic values when emersed even to 26 °C (Widdows et al. 1979). Complex biochemical and physiological mechanisms facilitate survival of organisms like mussels (Almeida and Bainy 2006). In a short time study involving *Mytilus galloprovincialis,* Malagoli et al. (2007a) presented results of high production of immunocytes after 30 min and 120 min of air exposure and an increase in phagocity activity after 120 min of exposure but not less than 120 min. An additional study with mussels conducted during 24 - 48h showed a more activated metabolism under anoxia in oxygen free sea water in *Mytilus galloprovincialis* (Babarro et al. 2007). Anaerobiosis occurs in air exposure episodes causing acidification of body fluids and tissues in marine bivalves (Michaelidis et al. 2005). To fight these events, organisms show a similar behavior like in hyposalinity exposure by closing their valves conserving water during long intertidal periods (Widdows and Shick 1985). Progressive reduction of body fluid oxygenation might contribute to trigger the expression of heat-shock proteins (HSP's) in *Mytilus galloprovincialis* since these proteins assist organism on coping with stress of both internal and external nature (Sørensen et al. 2003, Anestis et al. 2010). This response is universal and it is activated by a variety of stressors including extreme temperature,

gases, and heavy metals (Lindquist 1986, Feder and Hofmann 1999). A stress hardening event is an increased tolerance to stress after a pre-conditioning at low doses of that stress and it is associated with the induction of these proteins (Kultz 2005). Findings by Anestis et al. (2010) confirm the presence of HSP's after a 10h period of air exposure in *Mytilus galloprovincialis*. A variety of anaerobic pathways can operate in Mytilus species but there are a range of parameters that change from individual to individual inside populations that can increase or decrease their survival to stressors like air exposure. In the results obtained in the present study during air exposure in *Mytilus galloprovincialis,* SPI could have selected the most resistant ones in group A corresponding to 30%, since these individuals passed to the SPII and ended the last stress phase with 0% mortality. In this case a stress hardening event could have happened but it could also indicate that the recovery time after SPI was not long enough to catabolize the protein products generated after the first exposure to the stressor.

It is hypothesized that invasive species are physiologically ready to be adjusted to new habitats by being particularly more tolerant to environmental stressors (Braby and Somero 2006b, Lenz et al. 2011). Mytilus species are osmoconformers which means that they adjust the intracellular concentration of solutes to match the surrounding environment (Lockwood and Somero 2011b). Therefore, ambient salinity will lead to the dilution of the internal fluids and perturbation of ionic composition of the cells (Braby and Somero 2006b). To cope with this stress, cells simultaneously decrease the concentration of solutes in the cytoplasm to avoid uncontrolled cell swelling (Lockwood and Somero 2011b). Results presented here during hyposalinity stress experiments in *Mytilus galloprovincialis,* show a significant difference in survival between groups A and C when organisms were exposed to low salinities (10 PSU). This suggests a metabolic adaptation required to handle episodes of stress which could have been reached after the first stress phase. The higher survival of group A when compare to group C on the second stress phase can conduct to a higher resistance of the individuals and consequently a higher chance of invade.

Basic mechanisms and molecules of the invertebrates stress response are fundamentally comparable to those known in vertebrates (Ottaviani and Franceschi

1997). Decrease in heart rate associated with the valve closure and changes in DNA integrity are some of the changes that occur in *Mytilus galloprovincialis* intern system contributing to the better resistance to salinity fluctuations (Braby and Somero 2006b, Malagoli et al. 2007b, Hamer et al. 2008).

Considering group B, group with two stress phases with any recovery phase between both, we can infer that the length in total could have been too long to allow an advantage on these individuals to be visible when compared with A and C groups. Moreover, it was possible to see a significant effect of hyposalinity on mortality considering shell length. Qiu et al. (2002) found a significant difference on survival between adults and juveniles organisms from two Mytilus species when exposed to salinities of 10 PSU. While juveniles showed mortality lower than 7%, adults showed values up to 60%. This is consistent with the data showed here referring to *Mytilus galloprovincialis* since individuals with bigger shells died earlier than smaller individuals at 10 PSU salinity exposure.

Byssus, or byssal threads are strong, silky fibers located in the posterior part of the foot and are made from proteins that are used by mussels to attach to rocks and other substrates like ship hulls (Winkle 1970). In group A since a higher survival was reached after SPII, it was expected that byssus production should also be higher when compared with groups B and C. Although there were no significant differences between groups A and C, it is still possible to visualize a trend of a slight higher ability to survive in the group A compared with group C. Group B showed a reduced byssus production which can be related to an allocation of resources. Since organisms were exposed to an extended exposure without a recovery phase it is expected that the finite resources availability to organisms must be allocated to several different daily processes including growth, maintenance and of course survival (Cronin 2001). It is also expected that individuals in group B find a strategy to allocate energy to survive when the exposure to stress is long and so the production of byssus would decrease as in this case. In the case of a real life event, a low production of byssus would result in a dropout of mussels from the ship hull since they would also be submitted to other forces (e.g. hydrodynamic conditions). The fact that the reference group (R) showed a lower production of byssus could be explained by the absence of hydrodynamic movements or other stress induced under laboratory conditions which is supported by

an experiment conducted by Young (1985) that showed more production in field than in laboratory on a group of individuals from *Mytilus edulis* species.

Thermal tolerance studies are one important tool to understand impacts of climate change (e.g. Canning-Clode et al. (2011)). At the same time, biological invasions is also occupying the same relevance in terms of changes in ecosystems and also used thermal tolerance research to understand species tolerances in the environment that they were carried to. *Mytilus galloprovincialis* invasion success led to several questions involving temperature tolerances. In comparison with other species, *Mytilus galloprovincialis* presents a superior adaptation to warmer waters (Schneider 2008, Lockwood and Somero 2011a). During prolonged thermal stress, bivalves may readjust their metabolism and energy budget to meet the energy demand for HSP's synthesis as in air exposure episodes (Anestis et al. 2007). In general, the stress temperature for HSP's induction is correlated with the typical temperatures at which species lives (Feder and Hofmann 1999). Presence of HSP90 and HSP72, types of HSP's induced by environmental stressors, were activated after an exposure to 26 and 28°C in an experiment conducted by Anestis et al. (2010). Beside the synthesis of these proteins, a periodic reduction of energy income while the organisms is exposed to heat stress it is also present (Anestis et al. 2010). This metabolic depression caused by stress plays a significant role afterwards in its invasion potential (Schneider 2008). In the end of the experiment that involved heat as a stressor none of the organisms submitted to stress survived but there were significant differences on survival between groups. During the first heat stress exposure the protective proteins could have been triggered. Evaluating the survival of group B individuals probably did not have these proteins working during SPII or otherwise could have increased their survival. In contrast, group A individuals' proteins seemed to be developed in SPI allowing the transition to the next stress phase of the survivals. But probably due to the recovery phase the amount of these proteins dropped and this could explain the difference of 4 days in the mortality compared with group B. Although it was the same intensity and the same conditions as in SPI in SPII these proteins were probably not activated which may indicate that group A and group C were weaker than group B. Finally, laboratory conditions too, including feeding, disturbance due to air stones intensity and the

necessary handling because of the manual water exchange, could have influenced the viability of the organisms.

Co-existing in the same area as mussels, *Carcinus maenas* presents a similar behavior when they are exposed to different environmental conditions. Heart rate adjustments, HSP's expression and cellular restructuring are some of the costs related to the thermal adaptation to warm regions (Somero 2002). In this experiment conducted with this species pilot experiments were crucial to know how to be prepared to individuals' behavior due to its intense activity. Beside the efforts to keep the organisms' alive results indicated always to some deaths and no differences on survival between groups. These non-significant results may partially be explained by the short acclimation period that did not provide the individuals time enough to allow recovery. Stressors associated with the catching, transport and laboratory conditions could have been too intense for a one week recovery. On the other hand results can indicate that stress intensity was not higher enough to induce mortality, since results lead to differences on survival that may not be associated with the stress applied. However, maximum temperature value of 29°C was chosen at the present study to keep and maintain viability of *Carcinus maenas*.

4.4 Conclusions

The 20th century started with a deep change in the global maritime commerce passing through a change that eventually set the stage for a vast network of invasions (Carlton 1996). Global shipping became fundamental to world trade and it moves nowadays more than 90 percent of the world's commodities (CSBIO 1996, Gollasch 2006b, Kaluza et al. 2010). Records indicate that an estimated 10 billion tones of water is carried on the major tank of a ship (Gollasch et al. 2000b) and inside of these tanks are transported between 7000 to 10000 species every day (IMO 2009). The problem is that in addition to the amount of species transported, routes are increasing each year and they are also becoming more frequent. This will potentially help transported NIS on spreading due to multiple introductions and these events could favor a mixture of genetic pools from original genetically differentiated populations and allow the emergence of new genotypes (Dutech et al. 2010).

In general, ballast water and hull fouling communities at the end of a voyage represents only a subset of the original population, and how well organisms survive in each situation is depending on several aspects (Verling et al. 2005).

Environmental stress tolerance varies widely depending on each species and it is extremely important on phenomena involving transport of species (Kultz 2005). During a voyage inside of a ballast tank there is a wide range of stressors that can activate differently the tolerance to stress. Events of higher resistance side by side with enemy release and resource availability, will affect the success of NIS in the new environment during the first steps. Stress hardening events cause an increased tolerance to stress at low doses of one stress, but when several stressors occur at the same time a cross tolerance event can happen and the resistance to one stress can be increased by a preconditioning by another (Kultz 2005, Verling et al. 2005). At the same time when organisms are being loaded and then exposed to high temperature levels and decrease in oxygen availability can select the most resistant ones and make the weaker individuals died. This would mean that if the most robust individuals arrive to the new area, the potentially invasive population would be permanently resistant. The results presented here may indicate that in events of air exposure and hyposalinity could have existed a genetic selection but only with the posterior generations of the successful individuals would be possible to confirm that transport under these conditions can increase the resistance of individuals.

4.5 Global replication

This project was integrated in the global project GAME (Global Approach by Modular Experiments). GAME is an international student training and research programme founded in 2002 by Prof. Dr. Martin Wahl. Each year GAME addresses a marine ecology topic and the experiments are design at the GEOMAR | Helmholtz Centre for Ocean Research in Kiel, Germany, one of the world's leading institutes in the field of marine sciences.

This specific study was part of global replication that involved 13 experiments conducted simultaneously in 5 different locations spread worldwide (Brazil, Chile, Finland, Indonesia and Portugal). From temperate areas to tropical areas and

depending on the conditions in each location native species were chosen and the experimental set up was adjusted in each situation. Heat stress was the common stressor to all experiments. In 5 experiments from 13, group A (group with recovery phase) had a higher significant survival during SPII (Table 6). In none of the cases survival of pre-stressed groups was lower when compared to group C. On a global scale, 40% of the conducted GAME experiments indicated that transport conditions might increase resistance to stress.

Table 6 – Summary of the global study showing differences between group A (group previously stressed) and group C (group not previously stressed). Significance is representative by plus signs (+) and not significant by minus signs (-).

Study side	Organism	Stressor	Significance
Brazil	*Perna perna*	Heat	**–**
	Pachygrapsus transversus	Heat	**–**
Chile	*Semimytilus algosus*	Heat	**+**
	Romaleon polyodon	Heat	**–**
Finland	*Mytilus trossulus*	Heat	**–**
	Idotea baltica	Heat	**–**
Indonesia	*Perna viridis*	Heat	**–**
	Penaeus vannamei (hours)	Heat	**+**
	*Penaeus vannamei (*days)	Heat	**+**
Portugal	*M. galloprovincialis*	Heat	**–**
	Carcinus maenas	Heat	**–**
	M. galloprovincialis	Air exposure	**+**
	M. galloprovincialis	Hyposalinity	**+**

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