Extrinsic and intrinsic factors that shape the life history of the short living scallop *Argopecten ventricosus*

Extrinsische und intrinsische Fakoren die die Lebensgeschichte der kurzlebigen Kammmuschel *Argopecten ventricosus* modulieren

Dissertation zur Erlangung des Grades eines Doktors

der Naturwissenschaften

- Dr. rer. nat.-

Fachbereich 2 Biologie/Chemie

Vorgelegt von

Citlali Guerra

Bremen

Dezember 2011

Prüfungsausschuss:

1. Gutachter: PD Dr. Doris Abele (Funktionelle Ökologie, Alfred-Wegener-Institut für Polar- und Meeresforschung, Bremerhaven)

2. Gutachter: Prof. Dr. Allan Cembella (Ökologische Chemie, Alfred-Wegener-Institut für Polar- und Meeresforschung, Bremerhaven)

1. Prüfer: Prof. Dr. Wilhelm Hagen (Marine Zoologie, Universität Bremen)

2. Prüfer: Dr. Alfonso Nivardo Maeda-Martínez (Ecofisiología de Moluscos, Centro de Investigaciones Biológicas del Noroeste, La Paz)

TABLE OF CONTENTS

i. Acknowledgements

I want to thank Prof. Dr. Allan Cembella for evaluating my thesis and for providing me with the opportunity to work at the AWI during the last year of my PhD. Prof. Dr. Wilhelm Hagen I thank for his acceptance as „Prüfer“ within my thesis committee.

A special thank is directed to my supervisors in Germany Dr. Eva Philipp and PD Dr. Doris Abele and in La Paz to Dr. Alfonso Maeda-Martínez. I thank you for giving me the oportunity to work in this subject and for all the discussions and conversations even when I was far away.

During the two years in La Paz I had the opportunity to work with Tania Zenteno who allowed me to conduct my analysis in her laboratory. I worked with Tere Sicard who helped me with the scope for growth measurements, but not only with this, we also had valuable discussions about all aspects of life. Alfredo Hernandez-LLamas, I want to thank you for your kindness and very productive help in statisitics.

I´am deeply grateful to all of the people that supported me during the field and laboratory work at the CIBNOR in La Paz. I especially would like to thank Mario Osuna. When I arrived at the CIBNOR, I did not have a clue about how to grow scallops. Without your knowledge and support, it would have been impossible to rear and maintain the scallops in the hatchery that is for shure! I want to also to thank you for all the field trips that you took with me and for your readiness to drive every month to the field in order to clean nestier trays and sample scallops. I learned a lot from you and we had always such a good time in Rancho Bueno. It was not only work but also joy! Another person that supported me in the field is Ernesto León who helped me to install the nestier trays. I can only be thankful that

you followed all my ideas and always found a way to do things. I never heard from you saying that something is not possible and you always found a solution! Carmen Abundis, you came more or less as casuality to the CIB and I was so lucky that you could work with me! You became not only my right hand in the maintenance of scallops in the lab, but also a good friend. Without you, it would have been impossible to deal with the laboratory system for such a long time! I want to thank also Armando Monge who helped me to install the complete system in the laboratory in order to maintain the scallops. A special thank is directed to Norma Olguín and Orlando Lugo. I never thought that it would be possible to determine a great part of my oxidative stress analysis in the lab, but you made it possible. I will also thank Agustín Meza and Ariel Cruz who helped me in handling with the liquid nitrogen that I needed to freeze my samples. You became crucial for my work!

I thank the students Belisama Carmona and Stephan Koenigstein. I could coach you for your project but your help was indispensable for conducting my PhD.

I thank my working group at the AWI: Julia Strahl, Harald Poigner and Valeria Bers. Especially I want to thank Stefanie Meyer for your unconditional support in the lab.

Kevin and Kristoff, thank you for the productive comments and corrections of the thesis. It was great the help that you was giving me at the end!

To my friends in La Paz and in Bremen, for the happiness and the joy. A special thank to you: mi querida Carmina (la panochita) y mi querida Alex, Yuen mi papipan, Sandino y su humor que sólo de pensar me hace reir. Raul and Christina, los extraño! Rossana, Salvador and Trini for all the campings in the beach!!! My friends in Germany that were there before

and during my PhD and will stay forever: Julia, Kevin, Wiebke, Mari, Simon, Libertad y Jona.

I want to thank all the beautiful people I met during my time in Brest: Geraldine, Matt, Matt, Nono, Ronan, Mathias and Tolotra. Je suis trés contente de vous avoir conue dans cette etape de ma vie!

A very special thank is directed to my family. Pa, Ma, Leo, Gera, Claudita, Franco and the new little member of the family that came during this period, Lila. Gracias por su apoyo incodicional en todas las etapas de mi vida incluyendo este projecto, pero mas que nada, por el amor, el gran amor que me dan. Los quiero!

The partner of my life, mi media naranja. Doudou, gracias por estar a mi lado, eres mi compañero y estoy orgullosa de lo que somos. Me haz hecho crecer como persona durante este tiempo. Te quiero de aqui al cielo Doudou y…. un poquito más allá. Merci aussi a ta faimille: Colette, Alain et Monique pour me recevoir dans votre coeur. Merci.

ii. Abstract

The scallop *Argopecten ventricosus* is characterized by its high swimming activity, fast growth, high reproductive effort and the early age to get first sexual maturity. These traits may be the result of the adaptation to a specific environment that favors an active lifestyle and a short lifespan (2 years). This opens the question of how environmental factors modulate the way a short living marine ectotherm budget energy investments among life history traits and how this modulation impacts the lifespan within a cohort. Temperature and predation are two key environmental factors that affect physiological and cellular responses in marine ectotherms that have been investigated in the present study.

Lifelong investments among life history traits were studied looking at trade-offs among growth, reproduction and cellular maintenance mechanisms under the different environmental conditions. The cellular maintenance mechanisms were studied in different tissues by antioxidant and damage removal capacities. In order to demonstrate the efficiency of cellular maintenance mechanisms, oxidative damage accrual of proteins and lipids and undegradable waste accumulation (lipofuscin) were assessed in parallel. The trade-offs were also investigated within a cohort raised in the field throughout the species record lifespan (2 years).

The long-term elevation of temperature (5°C above the temperature measured in the field) enhanced metabolic rates, reproduction effort but also oxidative damage accrual and high mortality rates despites the conjunctly increase in antioxidant capacities. The high mortality probably exerted a strong selection of better-adapted individuals with less oxidative damage and better growth. Scallops exposed to predator pressure (the blue crab: *Callinectes*

sapidus) developed thicker shells and bigger swimming muscles and at the meantime constrained reproduction investment as indicated by the deferment and the lower investment into gametogenesis. Lower reproductive effort was combined with lower oxidative damage accrual especially in mantle and gill tissues, and may have prevented post-spawned mortalities. When studying the trade-offs in scallops reared in the field, it appears that scallops at their first reproductive event (< 1 year of age) showed the highest levels of oxidative damage (protein carbonyls and lipid peroxidation products). While antioxidant capacities did not appear to prevent oxidative damage, young scallops seem to remove damage before the undegradable waste product lipofusin accumulates. In contrast, older individuals (>1 year of age) failed to prevent lipofuscin accumulation.

For this species, the applicability of evolutionary theories of aging suggest that a rapid growth and early maturation at young age compromise later cellular maintenance. However, species may have a great variety of strategies in order to deal with the oxidative challenges throughout their lifespan, which depend strongly on the environmental conditions and state of life. The results speak for extrinsic factors (temperature and predation) to have potential roles on the lifespan in *A. ventricosus* scallops. This makes aging and oxidative stress mechanisms in short living bivalves an interesting but complex process influenced by a variety of interactive intrinsic and extrinsic processes that should be considered in future studies.

iii. Zusammenfassung

Die Kammmuschel *Argopecten ventricosus* ist charakterisiert durch hohe Schwimmaktivität, schnelles Wachstum, hohen Reproduktionsaufwand und frühes Alter der ersten sexuellen Reife. Diese Eigenschaften können das Ergebnis von Anpassungen an einen speziellen Lebensraum sein, welcher einen aktiven Lebensstil und kurze Lebensspannen (2 Jahre) bevorzugt. Es ergibt sich die Frage inwiefern Umweltfaktoren die Art und Weise, wie kurzlebige marine ektotherme Organismen ihre Energiekosten zwischen lebensgeschichtlichen Eigenschaften einteilen, und wie diese Modulierung die Lebensspanne einer Kohorte verändern kann. Temperatur und Räuberdruck sind in marinen Ektothermen zwei Schlüsselumweltfaktoren, bei denen sich gezeigt hat, dass sie Einfluss auf physiologische und zelluläre Antworten nehmen und wurden in dieser Arbeit untersucht.

Energetische Kosten lebensgeschichtlicher Eigenschaften wurden untersucht, um Austauschbeziehungen zwischen Wachstum, Reproduktion und zellulären Aufrechterhaltungs-mechanismen unter verschiedenen Umweltbedingungen aufzuzeigen. Diese Austausch-beziehungen wurden auch in einer Kohorte verfolgt, die im Feld über die Lebensspanne von 2 Jahren herangezogen wurde. Die zellulären Aufrechterhaltungsmechanismen beinhalten die Messungen antioxidanter Schlüsselenzyme sowie Schädigungs-Abfuhr-Kapazitäten in verschiedene Gewebe. Oxidative Schädigung an Proteine und Lipide sowie das undegradierbare Abfallprodukt Lipofuscin wurden ebenso erfasst. Die lebenslange Temperaturerhöhung (5°C höher als die im Feld gemessene Temperatur) verstärkten Stoffwechselraten, Reproduktionsaufwand, aber ebenso auch den Zuwachs von oxidativen Schädigungen und Mortalitätsraten. Hohe Temperaturen förderten

aber eine stärkere Selektion angepasster Individuen, die niedrigere oxidative Schädigung und besseres Wachstum aufwiesen. Individuen, die einem potentiellen Räuber (die blaue Krabbe: *Callinectes sapidus*) ausgesetzt wurden, entwickelten dickere Schalen und größere Muskeln aber gleichzeitig niedrigere Investition in Reproduktion. Niedriger reprodukiver Aufwand, in Kombination mit verringertem Zuwachs von oxidativen Schädigungen, gerade im Mantel- und Kiemengewebe, führten zu einer Erhöhung der Überlebensraten. Die Untersuchungen der Austauschbeziehungen bei Muscheln, die im Feld aufgezogen wurden, ergaben, dass junge und laichreife Muscheln die höchsten Werte zelullärer Schädingung (Proteinkarbonyle und Lipidperoxidierte Produkte) aufwiesen. Antioxidative Kapazitäten konnten anscheinend die zelluläre Schädingung nicht vermeiden und es scheint dass in *A. ventricosus* die Schädigungsprodukte eher effective entfernt warden, bevor das Abfallprodukt Lipofuscin gebildet wird. Junge Muscheln (jünger als ein Jahr) konnten besser mit den oxidativen Herausforderungen umgehen, indem sie Lipofuscin-Akkumulation verhinderten, im Gegensatz zu älteren Individuen (älter als ein Jahr).

Die Ergebnisse der Arbeit unterstützen evolutionäre Alterungstheorien, die besagen, dass schnelles Wachstum und frühe sexuelle Reife in jungen Jahren die zelluläre Aufrechterhaltung in alten Tieren beeinträchtigen. Arten können jedoch eine große Vielfalt an Strategien besitzen, um mit oxidativen Schädigungen über ihre Lebensspanne hinweg umzugehen, welche stark von Umweltbedingungen und Lebensphase abhängen. Dies macht Alterung zu einem komplexen Prozess, der von interaktiven intrisischen Prozessen innerhalb einer artenspezifischen Umwelt beeinflusst wird.

iv. Abbreviations

CAT	Catalase
CS	Citrate synthase
FMR	Feeding metabolic rate
GSI	Gonadosomatic index
GI	Gonad index
NAD^+	Nicotinamide adenine dinucleotid (oxidized)
NADH	Nicotinamide adenine dinucleotid (reduced)
ODH	Octopine dehydrogenase
ROS	Reactive oxygen species
SFG	Scope for growth
SFT	Simulated field temperature
SMR	Standard metabolic rate
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances

CHAPTER 1: GENERAL INTRODUCTION

General Introduction

1.1. Mechanistic and evolutionary theories of aging

Understanding why some species can attain Methuselah ages while others dwindle in some months or years have been a long-standing question in biology (Orzak 2003; Philipp and Abele 2010). Aging is a nearly universal feature of multicellular organisms that determines the lifespan of species (Krikwood 2005).

Since the twentieth century, mechanistic (proximate) and evolutionary (ultimate) theories of aging have been linked among each other because their interactions and shared questions contribute to a better comprehension of aging and the evolution of life spans in multicellular organisms (Cohen et al. 2010; McGraw et al.2010). The oxidative stress theory is one, if not the most popular mechanistic theory of aging (Kirkwood 2005; Kregel and Zhang 2006; Salmon et al. 2010) that has provided an essential role in clarifying cellular processes and thus in understanding intrinsic factors that explain *how* organisms age. However, the lack of evidence and the increasing contradictory results have led to the question if this theory can truly explain the complex phenomenon of aging. Nowadays, researchers are tending to conclude that this theory, at least in its original version, is incorrect or at least insufficient (Buffenstein et al. 2008; Perez et al. 2009; Lapointe and Hekimi 2010). Oxidative stress is rather beginning to be integrated into evolutionary theories. These theories bring strengths in understanding the evolutionary forces that shaped the lifespan of species within a specific environment and thus tries to understand *why* organisms age. It is recently that ecologists are giving oxidative stress another meaning

by linking it to specific life history traits such as growth and reproduction rather than to lifespan.

The catarina scallop *Argopecten ventricosus* from the Pacific coast of Baja California Sur, México is an excellent species to investigate the links between proximate and ultimate approaches that conjunctly may provide a better understanding in the forces that shape the life history and the lifespan of this model species.

1.2. The controversy of oxidative stress as a determinant of aging

Since 1990s the free-radical theory of aging was regarded as the dominant mechanistic idea explaining why organisms age (Speakman and Selman 2011). The theory is based on the tenet that oxygen free radicals are spontaneous and natural by-products formed during aerobic metabolism within the powerhouse of the cell, the mitochondria (Harman 1956). With the increase of oxygen in the Earth´s atmosphere, aerobic species have evolved molecular mechanisms that allowed them to use oxygen for a highly efficient energy production (ATP) to fuel all biological processes on which they depend (Monaghan et al. 2009). ATP is produced through a series of redox reactions that involves the transfer of electrons along the cytochrome proteins on the inner mitochondria membrane that ultimately reduce oxygen to water (Kregel and Zhang 2006; Pamplona and Costantini 2011). During this process, single electrons react promiscuously with oxygen leading to the formation of superoxide radical (O_2•-) that can be converted in other ROS such as hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH) (Muller 2000). Parallel to the innovation of using oxygen for energy production, animals have evolved sophisticated and multifaceted

antioxidant defense systems to avoid or minimize the inevitable production of ROS (McCord and Fridovich 1969). Antioxidants include a network of compartmentalized enzymes, primarily superoxide dismutase (SOD), gluthathion peroxidase (GPx) and catalase (CAT) and non-enzymatic antioxidants such as vitamins (E, A and C), tripeptides (gluthation) and trace metals (selenium). These antioxidants are distributed within the cytoplasm and among various organelles and work in a series of integrated reactions to convert ROS to more stable molecules eventually oxygen or water. However, the antioxidant machinery is not 100% efficient in mopping up ROS and some radicals always evade the protection system. When however, the balance between pro and antioxidants is disrupted, ROS escape the antioxidant capacities in a greater extend, and oxidative stress occurs. Under these circumstances, ROS will cause severe oxidation in proteins, membrane lipids and DNA mainly in the formation site, the mitochondria. Peroxidation of membrane lipids result in the loss of membrane integrity and homeostasis (Practicó 2002) while in proteins, ROS can lead to a decrease in the catalytic activity of enzymes, it can cause the protein-protein crosslinkages and protein fragmentation when the backbone is oxidized (Berlett and Stadtman 1997). Damaged mitochondria will favor further ROS production and increase damage creating a vicious cycle (Andreyev et al. 2005). Therefore, damage appears to accumulate slowly with age until it starts to compromise the function of the organism at the whole. According to this theory, the reason why animals ultimately die is because of the accumulated damage that these oxygen free radicals and their derivates cause. In the light of this theory, animals with low antioxidant capacities or higher rates of ROS production are expected to accumulate damage at a faster rate and live a comparatively shorter life.

The oxidative stress theory was immediately attractive because it was suggestive of ubiquitous physiological mechanism as to why organisms age and die and why this happens at different rates. Up to now, there is no doubt that ROS cause oxidative damage, and many advances in the field of aging have shown that oxidative stress (the imbalance between antioxidants and prooxidants) plays some sort of role in the aging process. However, to date no consistence proof exists that unambiguously identifies oxidative stress as the determinant of lifespan (Kregel and Zhang 2006; Buffenstein et al. 2008) and it is still questionable if a complex biological phenomenon such as aging can be explained by a single process or theory (Kirkwood 2005). One of the flaws of the theory is that oxidative stress does not increase with chronological age and that it is rather modulated by life history traits such as growth and reproduction throughout animal´s lifespan independent of age (de Maghalaes and Church 2006; Cohen et al. 2010). Within this context, ecologist are beginning to recognize that oxidative stress might constitute a potential mechanism underlying main life history trade-offs (Kim et al. 2009, Costantini 2010).

1.3. Bivalves and aging

Bivalves represent a special group within the animal kingdom and a challenge to aging theories. This is because this group exhibits a rich diversity of lifestyles with adaptations to diverse environmental conditions (Abele et al. 2009). We find for example sessile species that are attached to the substratum (oysters, mussels) or that burrow deep into the sediment (unioinidae and fresh water mussels) which contrast the active, energetically intensive lifestyles characteristic for mostly epibenthic scallops. Further, we find species that inhabit

Polar Regions (Antarctic clams and scallops) but also others that can survive to the extremely high temperatures of the hot vents such as the scallop *Bathypecten* and the mussel *Bathymodiolus* (Mullineaux et al. 1998). Hence, bivalves possess a high degree of phenotypic plasticity that permits a wide range of responses with respect to growth, age at maturation and record lifespan that are suited to different environmental conditions (Kirkwood and Austad 2000; Buettemer et al. 2010).

Reviewing literature data of the life history of bivalves, a trend towards a longer record lifespan in cold-water compared to warm-water species and vice versa becomes apparent (Philipp et al. 2005 a,b; Philipp et al. 2006). This can be explained to some extent by the free radical theory of aging as temperature directly modulates metabolic rates in ectotherms and thereby ROS production and consequently oxidative damage accrual. High metabolic rates are thought to cause high ROS formation rates and a faster decline in mitochondrial function in a temperate compared to a polar mud clam species (Philipp et al. 2005). In addition, antioxidant activity is higher in polar than in temperate bivalve species (Regoli et al. 2000; Philipp et al. 2005; Camus et al. 2005). These correlates are presumably more decisive for life history in marine ectotherms than mammals that posses a great capacity to adapt to higher levels of ROS so that higher metabolic rates are not necessarily connected to higher ROS production or damage (Costantini 2010). However, although low ROS production and high antioxidant capacities seems to be related to a long life in polar bivalve species, exceptions to the rule shows that, also in bivalves, the oxidative stress theory of aging is not always a straightforward and the only explanation of species specific lifespan. Swimming scallops represent an excellent example of exception. When comparing antioxidant enzyme activity of a shorter-lived and temperate scallop *Aequipecten*

opercularis (record lifespan 8-10 years) and a longer-lived polar scallop *Adamussium colbecki* (record lifespan > 18 years), the shorter-lived scallop had higher levels of the enzyme SOD (Philipp et al. 2006). Moreover, the age pigment lipofuscin and protein carbonyl levels (a marker of oxidative damage to proteins) did not show differences with age and the latter marker is even higher in the longer-lived species (Philipp et al. 2006). A very striking result was that *Aequipecten opercularis* control ROS production at extremely low levels, despites the fact of having higher metabolic rates, a very active lifestyle and a short lifespan (Philipp et al. 2006). That means that other life history traits such as growth and reproduction might modulate ROS and oxidative damage to a greater extent than temperature and lifespan. Moreover, the levels of oxidative damage measurable at a specific time of the life do not necessarily have negative physiological consequences for the animals. This is because oxidative compounds can be recycled or removed from the cell. Cells can be destroyed via apoptosis (programmed cell death), a process that was found to be highly active in the scallop *A. opercularis* (Strahl and Abele 2010). In contrast to necrosis, that is the premature death of cells and tissues by external factors such as infections, toxins or trauma, apoptosis is a naturally, highly regulated energy consuming process in which damaged compounds and cells are fractionated and disposed without causing inflammation (Edinger and Thompson 2004; Peter 2011). These findings gave rise to the question of how scallops repair or "clean" oxidative damage, how efficient are these cellular maintenance mechanisms and how they change in respect not only to age but also to life history.

1.4. Oxidative stress as a life history constraint

Life history studies have been based in how organisms combine definite aspects of their lives such as reproduction, growth and cellular maintenance mechanisms and how such combinations have evolved (Stearns 1992). The study of life history traits has had at its core the idea that energy utilization is the currency that is traded off among life history traits (Alonso-Alvarez et al. 2004). Ecologist and evolutionary biologists have recognized that oxidative stress vary with developmental schedules such as growth rate and reproductive effort (Cohen et al. 2010; Metcalfe and Alonso-Alvarez 2010). This confirmed that oxidative stress is a non-energetic player influencing life history traits and that oxidative stress can be accelerated or reversed either by sensitizing or repairing and removing the damage (Kregel and Zhang 2006). However, the incorporation of oxidative stress into life history questions is starting to emerge and further studies within this area are needed (Monaghan et al. 2009). For example, the direct link between reproductive effort and oxidative stress has mainly been based on changes in antioxidant capacity without directly measuring oxidative damage or damage removal mechanisms (Metcalfe and Alonso-Alvarez 2010). Up to now, there is lack of evidence of the link between life history traits and oxidative stress under realistic environmental conditions (Metcalfe and Alonso-Alvarez 2010). Further, most conclusions are based on studies of flies, birds and mammals and the generality by which oxidative stress impacts on life history evolution remains to be fully explored across a range of other taxa (Dowling and Simmons 2009) such as in bivalves.

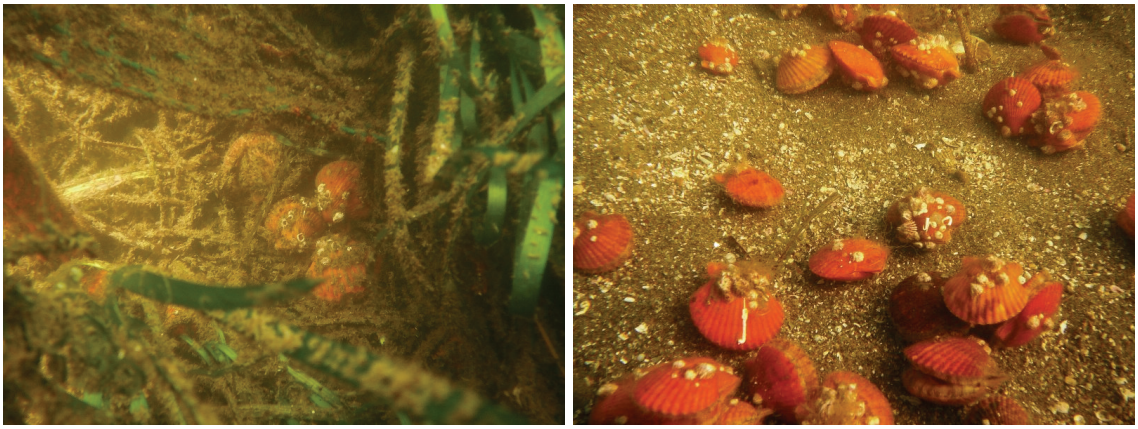
In this thesis, it was possible to combine these missing links using the short-lived catarina scallop as model species. Due to its short lifespan (record lifespan 2 years, Keen 1971), this

scallop is an ideal candidate for the aging research as it is possible to monitor changes in life history traits such as growth and reproduction related to cellular oxidative processes throughout scallops lifetime. Moreover, the explicit knowledge in cultivation of this commercially valuable species (Maeda-Martínez et al. 1997) offers the possibility to rear scallops directly in their natural environment.

1.5. *Argopecten ventricosus*: A short living bivalve model for aging studies

The pacific calico or catarina scallop, *Argopecten ventricosus* is one of the shortest living scallops together with a few other species such as *Donax donax* (record lifespan: 1 year) and *Argopecten irradians* (record lifespan: 2 years) (Powell and Cummins 1985). The lifespan of these species contrasts strongly with lifespan records of some clams such as *Mercenaria mercenaria* and the ocean quahog *Arctica islandica* that live up several 100 years (Ziguanov et al. 2000; Wanamaker et al. 2008). *Argopecten ventricosus* is an epibenthic swimming bivalve that generally resides on the mud-sand sediment at depths ranging between 1-180 m (Maeda-Martínez et al. 1993, 2001). In shallow bays, catarina scallops dwell within eelgrass beds of *Zostera marina,* which serves as primary substrate for the attachment of pediveliger larvae. Even within the eelgrass beds, predator pressure is high and an important cause of mortality particularly in young scallops (Ciocco and Orensanz 2001; pers. observation) (Figure 1.1).

A) B)



C)

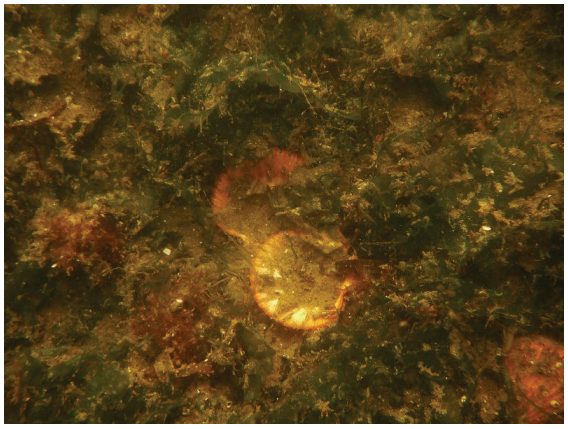


Figure 1.1: (A) 7 months old *A. ventricosus* scallops (size 36 mm) lying on sand bottom in the field within sea grass beds and (B) outside sea grass beds. C) Empty shell of scallop within sea grass beds probably being eaten by a predator.

The catarina scallop is distributed along the Pacific coast from Baja California, México to Paita, Perú (Waller 1991). In Baja California, the interactions of the cold California Current in winter and the tropical North Equatorial Current during summer provoke a high seasonal variation in biophysical settings including temperature and seston quality and quantity (Lodeiros et al. 2001; Luch-Belda et al. 2003a). Here, *A. ventricosus* scallops can attain a size of 6 cm within one year (Maeda-Martínez et al. 1997) and the first sexual maturity at an early age of only 4 months (Cruz et al. 2000). Two main spawning events, one in spring (march-April) and one in summer (August-September) are reported, but mature scallops can be found throughout the year (Felix-Pico et al. 1993; Maeda-Martínez et al. 1993, 2001).

The fast growth and high reproductive effort of the catarina scallop could be an adaptation to the variable environmental conditions combined with high predation pressure. Hence, growing fast could shorten the susceptible juvenile period in order to overcome mortality by predators and an early onset of reproduction may ensure recruitment of the population. According to the life history theory (Kirkwood and Austad 2000), it is more advantageous for species living under high predation pressure to allocate energy towards reproduction early in life and ensure their recruitment before they are consumed by predators. The negative consequence is the deterioration of the soma due to a lack of surplus energy for preventing; repairing or removing ROS and the consequent damage (see Figure 1.2). Hence, the precocious lifestyle of the catarina scallop could be the cause of scallops' short lifespan. The question is how the catarina scallop deal with the constant oxidative "challenge" throughout its lifespan?

In fact, bivalves in general may challenge the life history theory, as the trade-offs between reproduction, cellular maintenance and survival do not necessarily fit with the general theory (Sukhotin and Flyachinskaya 2009). The queen scallop *Placopecten magellanicus* for example, can modulate their energy allocation among life history traits in such a way that expenditure for reproduction may not directly impair current growth and cellular maintenance or constrain future reproductive output (MacDonald and Bayne 1993). This differs from definite growers such as birds, mammals and insects in which growth stops more or less after reaching maturity when senescence sets in (Kirkwood and Austad 2000). Moreover, it was shown that predation in the queen scallop elicit the development of thicker shells, and increase in clapping rates (Lafrance et al. 2003) and so select for traits that evade predators. Similar as already observed by fish (guppies), the extrinsic pressure can select for intrinsic improvement in performance, which may increase individual survival (Reznick et al 2004). This contradicts the theory that high predation will always select for a faster physiological deterioration and a shorter lifespan (Williams and Day 2003, Reznick et al. 2004). Reallocating energy resources into defense mechanisms opens the question of how other life history traits such as somatic growth and reproduction can be affected and whether these trades-offs influence cellular processes linked to aging.

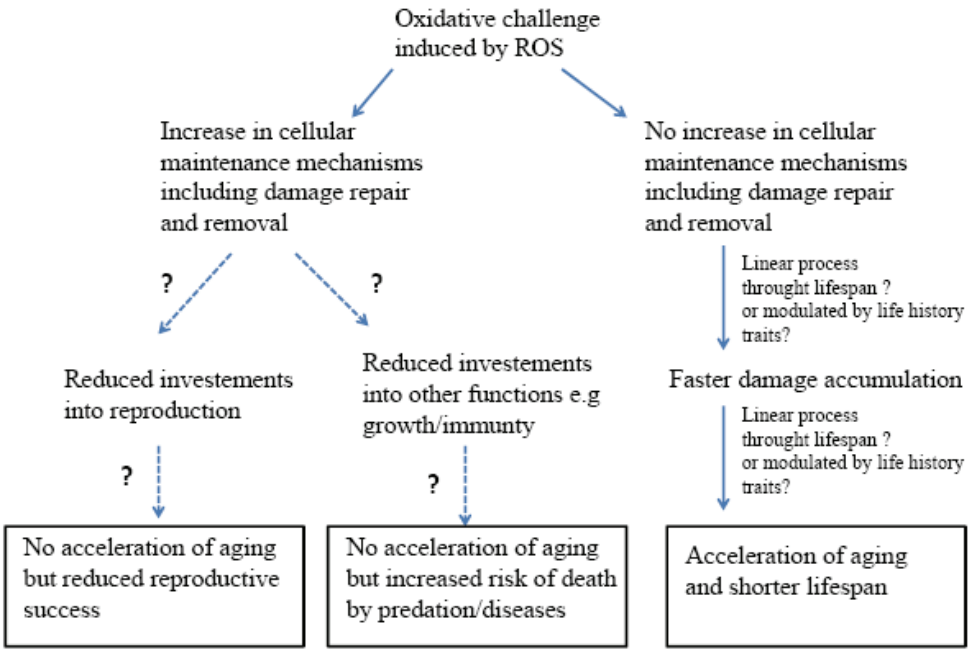


Figure 1.2: Schematic overview of the different possibilities to deal with the oxidative challenge and the consequences for life history traits modified after Monaghan et al. (2009). The consequences for animal fitness are shown in the boxes at the bottom. The questions represent processes that may differ in bivalves from classical models such as birds, mammals and flies.

Growth and survival rates, as well as the age to attain first sexual maturity vary between the different *A. ventricosus* populations living at each site of the peninsula of Baja California. Studies by Cruz et al. (1997, 1998, 2000) demonstrated that one population living in the Gulf of California (Bahía Concepción) had lower growth, survival rates and later onset of first sexual maturity compared to a pacific population living in Bahía Magdalena. When the authors transplanted scallops of both populations to the complementary environment, i.e. the Bahía Concepción population to Bahía Magdalena and *vice versa*, they observed that the formally "better off" Magdalena population showed a lower performance in the Concepción environment compared to the indigenous animals. Simultaneously, the Concepción population transferred to Bahía Magdalena grew faster, reproduced earlier and had lower mortality rates than in its home area. This indicates that populations are adapted

to the specific environmental conditions in their habitat but growth, survival and maturation in other environment can be strongly influenced by extrinsic factors such as food concentrations and temperature. The fact that environmental factors can influence life history traits in this scallop species gives rise to the following questions: How do changes in extrinsic factors such as temperature and predation modulate intrinsic cellular processes within a population of *A. ventricosus*? And how do investments in specific life history traits such as growth, reproduction and cellular maintenance shape the intrinsic aging process throughout the lifespan of this species?

1.6. Aims of the thesis

The aim of the thesis was to obtain a better understanding of intrinsic cellular mechanisms and extrinsic environmental factors that can contribute in shaping the life history of an ectothermic species. The strategy consisted in creating a single population of animals that shared the same chronological age that can be subsequently raised under different environmental settings from the beginning to the end of their life. Controlled laboratory experiments aimed to pinpoint how two extrinsic factors: predation and temperature, modulate the animal´s physiology, life history traits and therefore, the lifespan of populations maintained under different environmental conditions. Intrinsic oxidative stress parameters were monitored across the lifespan of the cohort in the field in order to analyse changes during chronological aging in response to life history traits under natural environmental conditions.

The specific tasks were:

1. Analyze in which way temperature and predation modulate the physiology and survival rates of the species in a long-term experimental setup under laboratory conditions.

Differences in water temperature and food levels have shown to cause differential growth and survival rates and change the time to reach first sexual maturity in *A. ventricosus* populations. The presence of predators can also influence the physiological performance and shell morphology in scallops and can impact somatic growth, reproductive effort and survival. Up to now, the precise influence of environmental factors under controlled laboratory settings is limited to short periods of time and misses the influence throughout a population lifespan and different ontogenetic stages.

Aim: To analyze lifelong changes in animal physiological parameters: somatic and shell growth, condition indices and investments into reproduction and changes in shell morphology in a controlled laboratory set up. Relate the changes to survival rates in order to understand if temperature and predation modulate life history and lifespan of a population.

2. Analyze if temperature and predation induce intrinsic changes in cellular maintainance mechanisms and damage under long-term controlled laboratory conditions

High temperatures can enhance metabolic rates and cellular oxidative damage and might affect energetic balance and compromise individual survival. However, to date, there is a missing link between energy metabolism, oxidative damage accrual and lifespan within individuals of the same species after lifelong exposure to higher temperatures. Further, there is scarce evidence about the cellular mechanisms underlying the effects of predators not only in bivalves but in animals in general. Only a study in damselflies showed that oxidative stress is a cost of predation that could have negative fitness consequences for the prey (Slos and Stocks 2008)

Aim: Analyze metabolic rates and cellular antioxidant defence mechanism against ROS as well formation of oxidative damage in different tissues linked to an increase in temperature and predation pressure.

Principal questions:

(i) Does an increase in environmental temperature enhance metabolic rates and oxidative stress throughout the lifespan of a population that affect energetic balance, growth, reproduction investments and survival rates within individuals of the same population?

(ii) How does predation pressure modulate animal performance, growth and reproduction patterns and does this implicate changes in cellular processes that alter survival of the population?

3. Conduct a high-resolution study of aging through the early maturation phase until the end of the record lifespan (2 years) of catarina scallops reared under natural field conditions.

Oxidative stress has not only been positively related to the aging process but also to periods of intense growth and reproduction. Oxidative stress could thus, constitute a potential mechanism explaining life history trade-offs putting in question how these parameters change with chronological age within a cohort reared in its natural environment.

Aim: Examine cellular damage accumulation over scallop's lifetime by studying degradable and undegradable types of oxidative damage as well as cellular defence mechanisms that include prevention (antioxidant defence) and removal of cellular damage (apoptosis) in order to disentangle if oxidative stress changes with chronological age in the catarina scallop. The fact that scallops grow continuously throughout their life and invest heavily into reproduction allowed to specifically study the effect of growth and reproduction and subsequent recovery on cellular maintenance and oxidative damage in different tissues, and to distinguish the effect of chronological age from the effect of exhaustive reproduction in field-reared specimens.

Principal questions:

(i) Is reproduction and growth traded-off with cellular maintenance mechanisms in this species? And how does this species deal with oxidative stress throughout its short lifespan?

(ii) Is there evidence that the active lifestyle of *A. ventricosus* is related with higher levels of oxidative damage and/or lower levels of cellular defence mechanisms that lead to the comparable short lifespan?

CHAPTER 2: MATERIAL AND METHODS

Material and Methods

In this section is described the acquisition of a single scallop population that was subsequently separated and subjected to different laboratory treatments or maintained in the field. All experiments were carried out at the Centro de Investigaciones Biológicas del Noroeste (CIBNOR), Baja California Sur, México. Analyses were concluded at the CIBNOR as well as at the Alfred-Wegener Institute (AWI), Bremerhaven, Germany. The methods to all experimental work are presented in detail in publication 1 and manuscripts 1-3. Here, I give a summarized and general overview of all methods applied.

2.1. The hatchery

The strategy for the experimental design consisted in acquiring mature *A. ventricosus* scallops from the natural environment that could be transported to the hatchery of CIBONR close before they spawn. The aim was to get a substantial number of larvae from the spawning of the adults in order to get a cohort from which the exact age is known. Subsequently, the cohort was raised and exposed to the different environmental settings.

Mature *A. ventricosus* were found in July-August 2007 in the Pacific Site of the Peninsula of Baja California, México. Hence, 60 mature and close to ripe scallops (4-6 cm) were collected the 27.7.2007 directly from fishermen in Puerto San Carlos. Scallops were transported to the CIBNOR following the method of Maeda-Martínez et al. (2000). The method consists in transporting scallops out of water in a moist, cooled and aerated condition, allowing scallops to maintain their valves closed which diminishes stress caused by desiccation or hypoxia. For this, the scallops are packed within a “sandwich” made of layers of wet sponge and plywood lids placed inside plastic bags and packed in Styrofoam

coolers. Plastic bags were close with rubber bands that can be opened during the transportation to allow the incoming of air. Ice packs were placed within the Styrofoam box to maintain constant temperature at ~ 20°C.

Arriving at the CIBNOR, the mature scallops were induced to spawn following the thermo-stimualtion method, which consist in stimulating sperm and ova release of scallops by thermal shock (Uriate 2001). *A. ventricosus* scallops are found to be more tolerant to thermal shock so that even if individuals may be determined to ripe, gametes release does not always occur directly following thermal stimulation (Sarkis and Lovelatti 2007). Scallops were therefore left over night within a nestier tray suspended within 4 1500L tanks with aerated seawater at a salinity of 33-36 ppt and a temperature of 26°C (Figure 2.1).

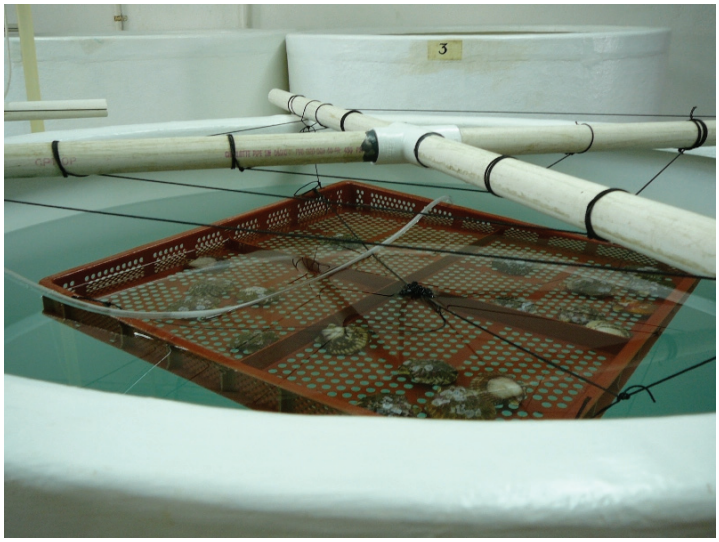


Figure 2.1: Mature *A.ventricosus* scallops suspended within 1500 L tank to induce spawning.

A. ventricosus is a functional hermaphrodite, which liberates sperms and ova into the water, where fertilization takes place (Sarkis and Lovelatti 2007). At the next day, an amount of ~ 7,850,000 ciliated trochophora larvae was obtained. After one day, a thin D-shape shell covered the planktonic larvae. The so-called veliger larvae (Figure 2.2A) develop a foot and an eyespot after 12 days and becomes pediveliger larvae that is ready to attach to the substratum losing the ability to swim (Figure 2.2B). In the laboratory, the pediveliger larvae settled in nylon bags (2.3A). Once the pediveliger larvae have settled, they undergo metamorphosis within 3-4 days (Figure 2.2C and D). The juveniles release then themselves from the primarily substratum, the nylon bags, and began to attach to the walls of the cultivation tanks by byssus formation (Figure 2.3B). Until 19 days, pediveliger larvae were kept in a 1 500 L tank at a salinity of 33-36 ppt and a temperature of 24-26°C.

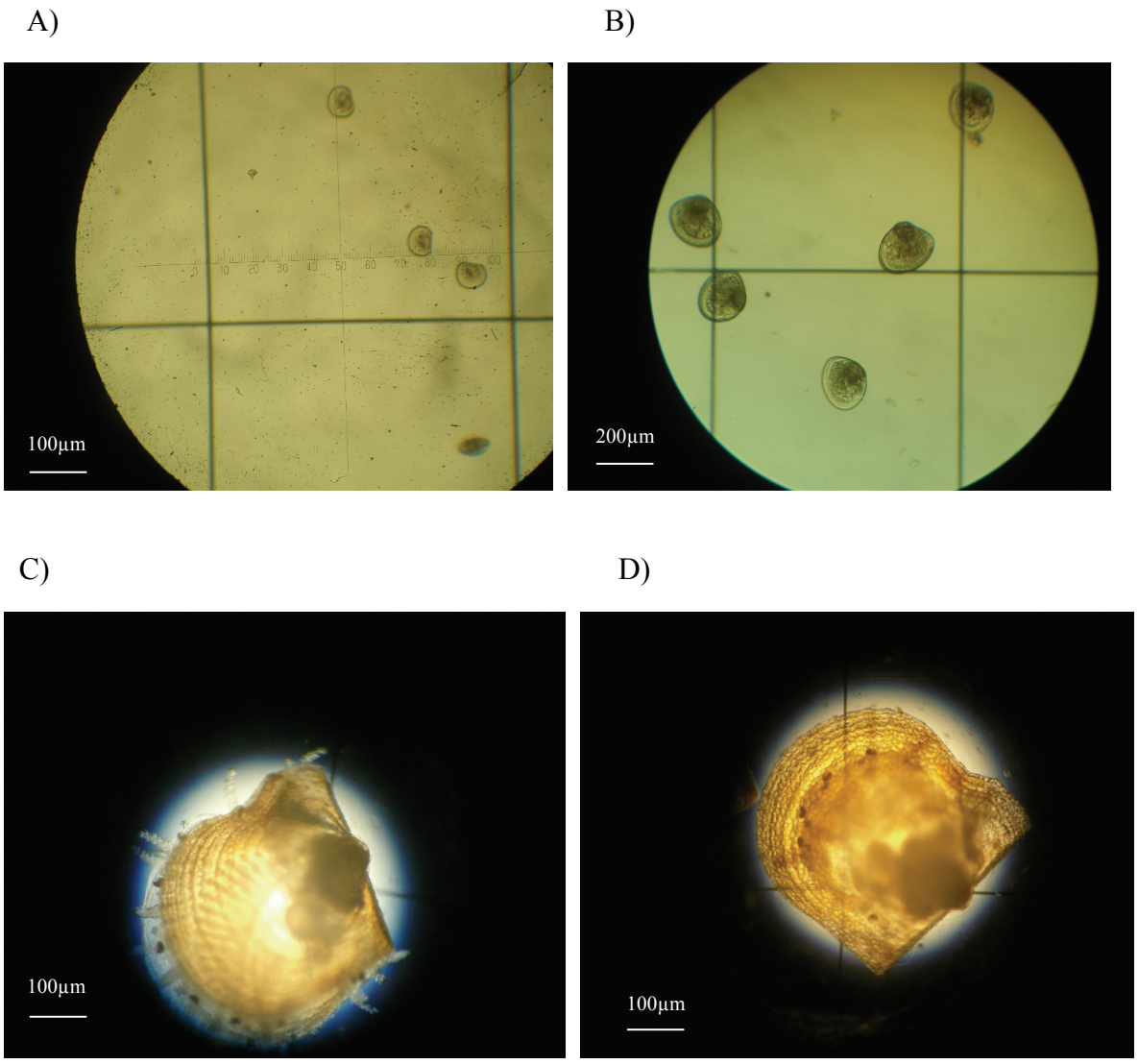
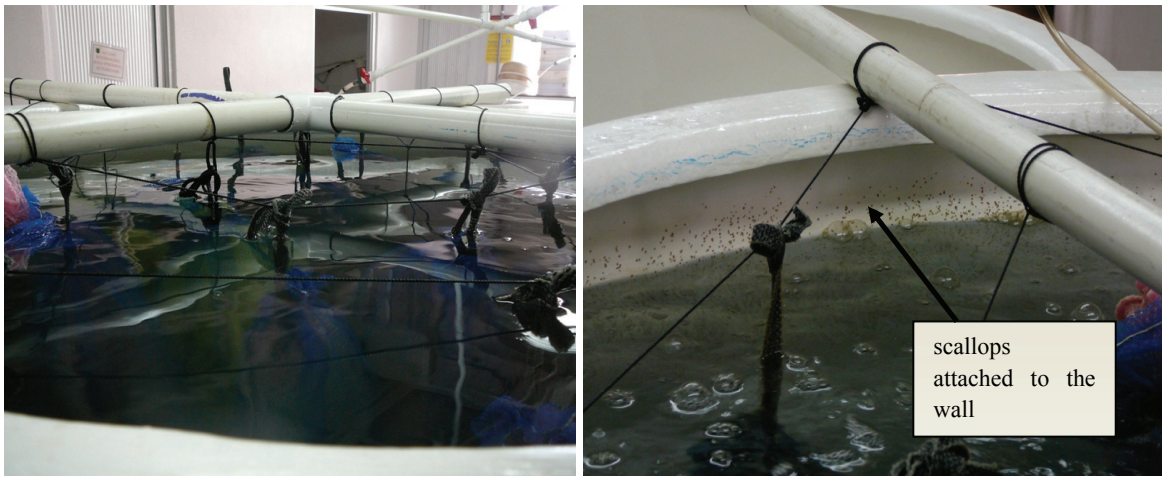


Figure 2.2: (A) *A. ventricosus* veliger larvae (3 days old), (B) pediveliger larvae (12 days old), (C) and (D) juvenile scallop (16 and 19 days old respectively)

A) B)



C) D)

Figure 2.3: (A) Nylon bags that provided a substratum for pedivelifer larvae suspended within 1500L tanks, (B) Juveniles attached to the walls of the tanks, (C) upwelling system, (D) juvenile scallops within sieve of the upwelling system

The trocophora and veliger larvae were fed with the flagellat algae species *Isochrisis galbana* and *Pavlova lutheri* as they can still not digest the silikat shell of diatomic micoraglae. Larvae were daily fed with 8 L of these microalgae in a 1:1 ratio (2.9 x 10^6 cells/L for *Isochrisis galbana* and 3.3 x 10^6 cells/L for *Pavlova lutheri*). Every second day, 100 % water exchange was done and the larvae were sieved to analyze the differences of

sizes. At the second day of being hatched, 60 % of the initial spat were retained in a 60 µm sieve and were kept. The smaller larvae were discarded. As soon as veliger larvae were becoming pediveliver, the diatomae microalgae *Chaetoceros calcitrans* and *Chaetoceros gracilis* were added to the food mix so that the food mix consisted in 15 L/day of a ratio of 2.5:1:1.5:1 (10^8 total cells/L) of *Isochrisis galbana*, *Pavlova lutheri*, *Chaetoceros calcitrans* and *Chaetoceros gracilis.* This microalgal mix has been commonly used in mollusk aquaculture (Brown et al. 1997). The diatom *C. calcitrans* is important for the mollusk diet because of the rich energy content (Shamsudin 1992). However, Lora-Vilchis and Doktor (2001) showed that the best nutritive values for gross growth efficiency in *A. ventricosus* are achieved when a combined diet of *C. calcitrans* an *I. galbana* are supplied because of the complementary fatty acid composition containing in both microalgae that bivalves cannot synthesize and have to get from the food (Lora-Vilchis and Doktor 2001).

Of the initial population, 43 % developed to pediveliger larvae and were kept in the tank until juveniles began to attach to the walls of the tank. From this moment on, juveniles needed to be fed continuously for optimal growth. For this, juveniles were moved to an upwelling system where 500 L of a mixed microalgae rich water (100 000 cells/ml) was used to feed juveniles *ad libitum*. Scallops were kept within sieves (500 µm) that were suspended in the upwelling system (Figure 2.3C and D). Temperature was kept between 23-24.5°C and salinity at 33-36 ppt.

In October 2007, at an age of 3 months and a size of 5-7 mm, the scallop population was divided in two groups. One group of approximately 10 000 scallops was transported into the field whereas the equivalent number of scallops remained for 3 weeks in the hatchery

being transported to the laboratory of mollusk ecophysiology within the CIBNOR for laboratory treatments (temperature and predation).

2.2. Maintenance in the field

The approx. 10 000 scallops were transported to Rancho Bueno estuary in October 2007 (Figure 2.4). The field site chosen in the present study represents a beneficial environment for *A. ventricosus* with high chlorophyll concentrations and the optimal temperature window for growth (Acosta-Ruiz & Lara-Lara 1998, Sicard-Gonzàlez et al. 2006). Scallops were kept in fine mesh bags (2mm) within 20 Nestier trays (55 x 55 x 8 cm) suspended in a long-line system for 2 months until reaching an average size of 24 mm (December 2007) (Figure 2.5A and C). Subsequently, animals were kept without bags in the trays (Figure 2.5B and C). The initial stocking density was set at 500 animals/tray (equivalent to 1700 animals/m^2) for optimal growth (Maeda-Martínez et al. 1997) and adjusted to 150, 90 and 60 animals/tray (495, 297 and 198 animals/m^2) after 187, 337 and 480 days (February, July and December 2008) in order to keep densities optimal for growth. Every month, dead animals were removed and nestier trays were changed to allow proper water and nutrients flow through the trays. The scallops were maintained in the field within the nestier trays until the end of August 2009 when scallops reached the 2^{nd} year of life.

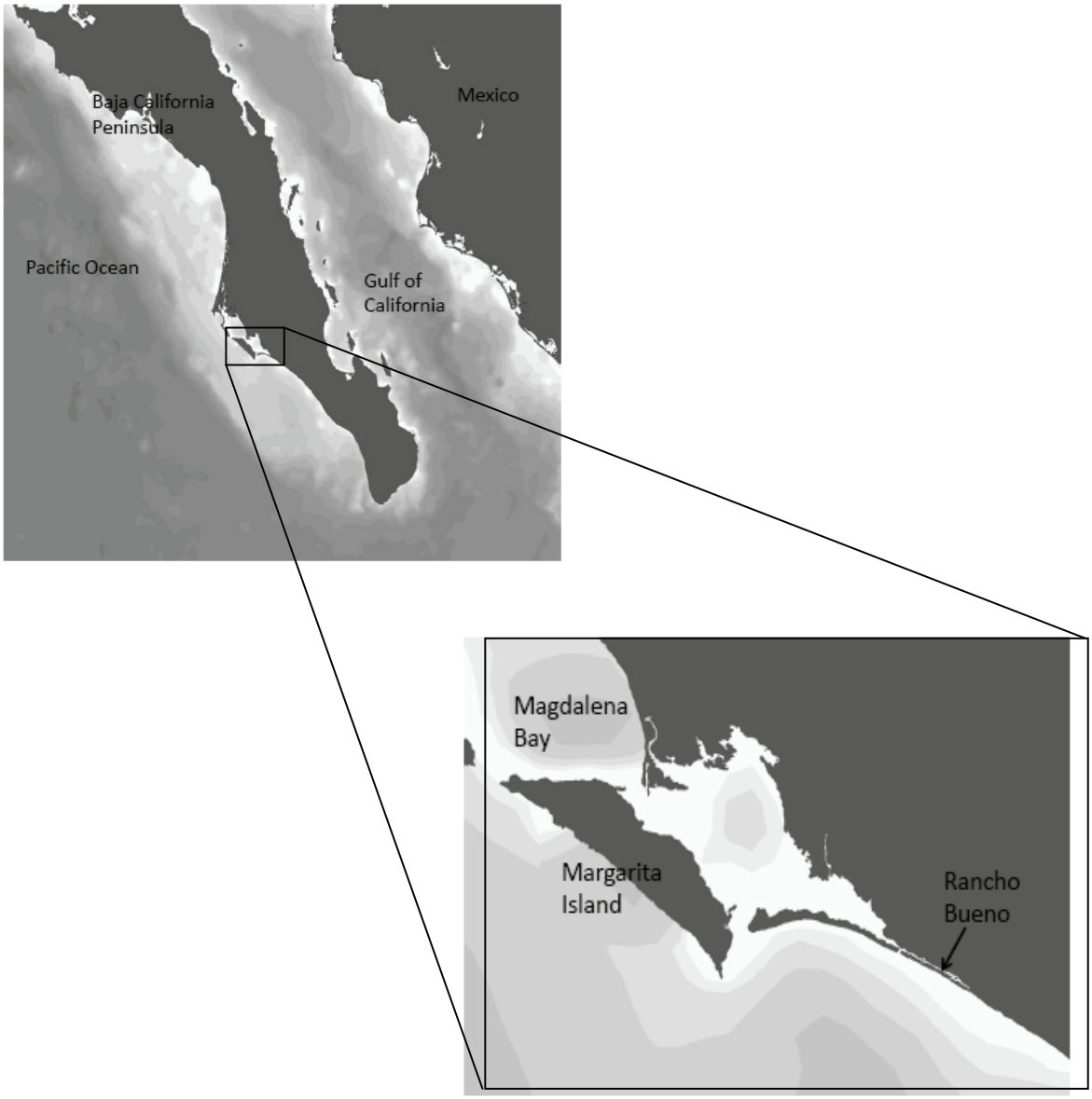


Figure 2.4: Map showing the experimental site in the field (Rancho Bueno). The Rancho Bueno estuary was chosen not only because it represents an optimal site for scallops´ growth, but also because is better protected from hurricanes compared the open sea. Map created with Ocean Data View (http://odv.awi.de 2011).

A)

B)

C)

Figure 2.5: (A) juvenile scallops kept in fine mesh bags within nestier trays until reaching an age of 5 months and a size of ~24 mm shell height, and (B) adult scallops maintained in nestier trays until reaching an age of 2 years and a size of ~ 70 mm shell height. (C) Nestier trays suspended in a long line during 2 years.

2.3. Maintenance in the laboratory

2.3.1. Scallop maintenance

The laboratory study started in November 2007. Scallops (7-9 mm shell height) were kept in a flow-through system of twelve aquaria (70 x 60 x 16 cm) with a constant water flow of 210 L day^{-1}, using ~ 630 scallops per aquarium (Figure 2.6). Initial stocking density was ~ 1500 animals/m^2 in order to keep similar initial densities as in the field. Incoming sea water was filtered over a sand filter (Jacuzzi 225 L, Little Rock AR USA) and a 1 µm gaft filter. The scallops were fed *ad libitum* throughout the experiment using a 1:1 mixture of *Chaetoceros calcitrans* and *Isochrisis galbana* delivered by an automated system containing a mixing tank and a turbidimeter controlled pump (Hach 1720 Loveland, USA). Cell concentrations were monitored using a Coulter Cell Counter (Multisizer 3, Beckham, Coulter, Fullerton, CA, USA). Concentrations varied between 60 x 10^6 and 250 x 10^6 cells/L in the mixing tank. Water outflow from each aquarium was daily checked to ensure that scallops were fed *ad libitum* and a concentration of 20 x 10^3 and 50 x 10^3 cells/L was maintained throughout the experimental time. Salinity was kept at 33-36 ppt similar to values found in the field site (Sánchez-Montante et al. 2007).

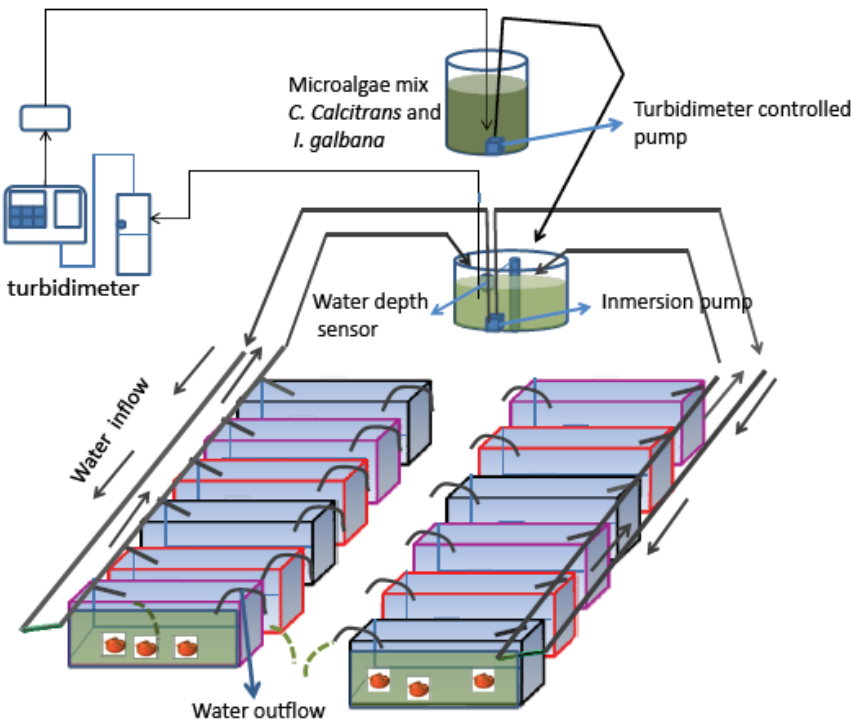


Figure 2.6: Experimental set up in the laboratory showing the micoralgae mixing and supply tank, the aquaria and the turbidimter. The turbidimeter controlled the microalgae concentration in the supply tank. The different aquaria colors represent the different treatments.Black: simulated field temperature treatment (SFT, control group); red: elevated temperature treatment (SFT+5°C); pink: predator exposure treatment (SFT + predator)

2.3.2. Temperature treatment

Of the twelve aquaria, eight were held at simulated field temperature (SFT) and four at 5°C above SFT (SFT+5°C). SFT values were deduced from field measurements. For this, a temperature logger was attached to one of the nestier trays, which recorded water temperature at 60 min intervals (WTA32-5+37, Onset Computer Corp., Bourne MA, USA). The temperature logger were collected and replaced during field trips at monthly intervals. The water temperature in the SFT treatments was adjusted to monthly average field

temperature and controlled using aquarium heaters (Hagen Aquaclear 22952). Thus, the temperature in the SFT treatments represents the mean field temperature of the previous month. The temperature limit was set at 27 ± 1 °C in the SFT+5°C treatment to prevent mortalities linked to high lethal temperature. The SFT treatment was kept at 5°C below the SFT+5°C treatment throughout the experiment except in November 2007, May and June 2008 where only 3-4 °C difference could be adjusted. The SFT experiment lasted from November 2007 to October 2008 and the SFT+5°C from November 2007 to December 2008 (Figure 2.8).

2.3.3. Predator exposure treatment

The SFT + predator experiment started in April 2008 at a scallop age of 8 months. One blue crab (*Callinectes sapidus*) was introduced in four of the eight SFT aquaria (one crab per aquaria). The size of the crabs ranged between 6-9 cm carapax length. *Callinectes sapidus* is a potential predator of *A. ventricosus* in the fied (Ciocco and Orensanz 2001) and crabs induced escape responses in scallops throughout the experiment without any sign of acclimation. Crabs were kept in the aquaria 6 h per day for 5 days a week. The crabs´ pincers were held together by rubber bands to prevent them from eating scallops. We fed the crabs with squid every third night in a separate aquarium (Figure 2.7A, B). This experiment lasted from April 2008 to February 2009 (Figure 2.8).

(A) (B)

Figure 2.7: (A) Maintenance of crabs within a separate aquarium and (B) within experimental aquaria for 6 days and 5 h per day.

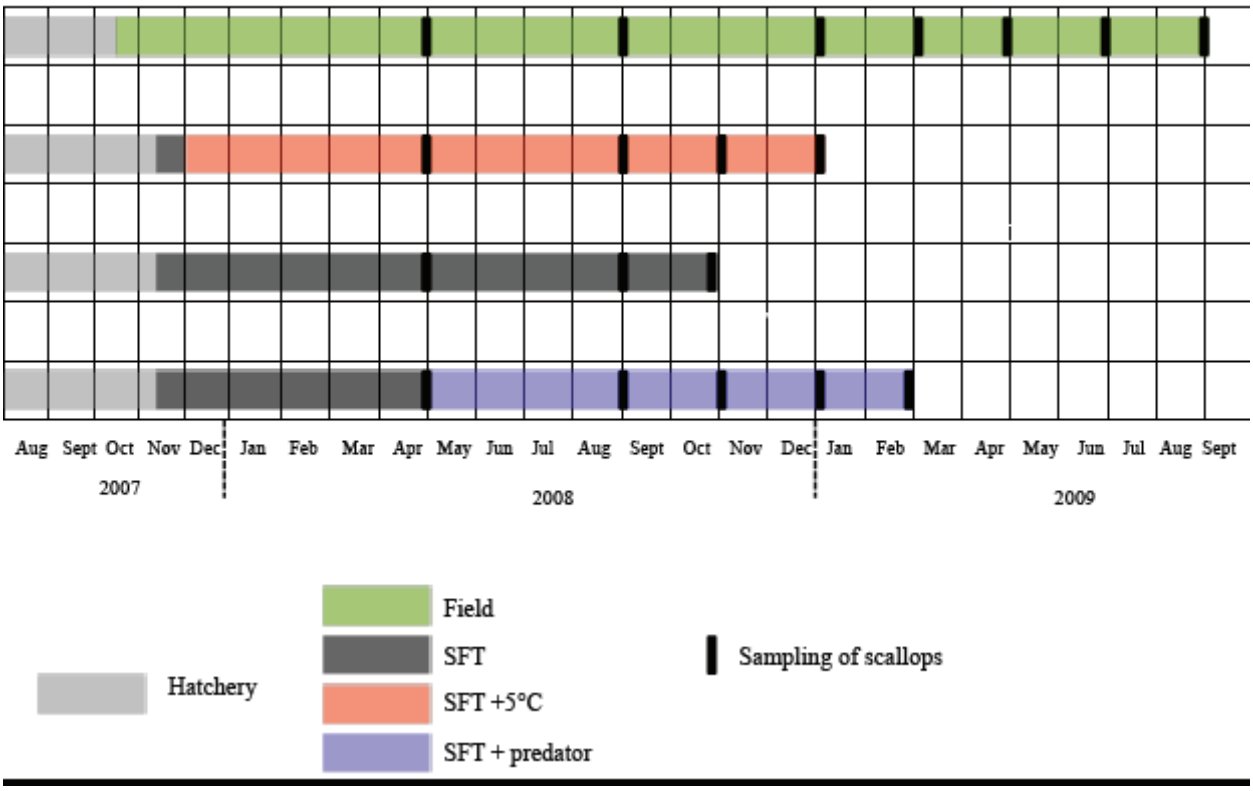


Figure 2.8: Schematic diagram of the experimental protocol and sampling periods.

2.4. Sampling of scallops

At each sampling date, 20-25 scallops were collected from the field and from each laboratory treatments. In 8-10 individuals, whole animal parameters were determined, that means metabolic rates, ingestion and excretion rates as well as tissue condition indices. In the other 8-10 scallops, biochemical parameters in mantle, muscle and gills were determined. The shell height (distance from hinge to distal shell margin) was measured at monthly intervals in each treatment.

2.5. Measurement of physiological parameters

2.5.1. Standard metabolic rate

Standard metabolic rate (SMR) represents the basal maintenance requirements to keep an organism alive (Clark and Fraser 2004). To estimate SMR we measured the metabolic rates of resting, unstressed individuals that are not digesting food and are at a stable temperature within their optimal range (Rolfe and Brown 1997). The method consists in measuring oxygen consumption of animals that were placed in hermetic chambers connected to a multi-channel flow-through system (Figure 2.9 and 2.10). Respiration rates were calculated from the difference in oxygen between the in- and outflowing water. Following the measurements, scallops were dissected and all tissues were dried in order to relate metabolic rate to g tissue dry weight. More details of the method for measurement of SMR are presented in Manuscript 1, 2, 3.

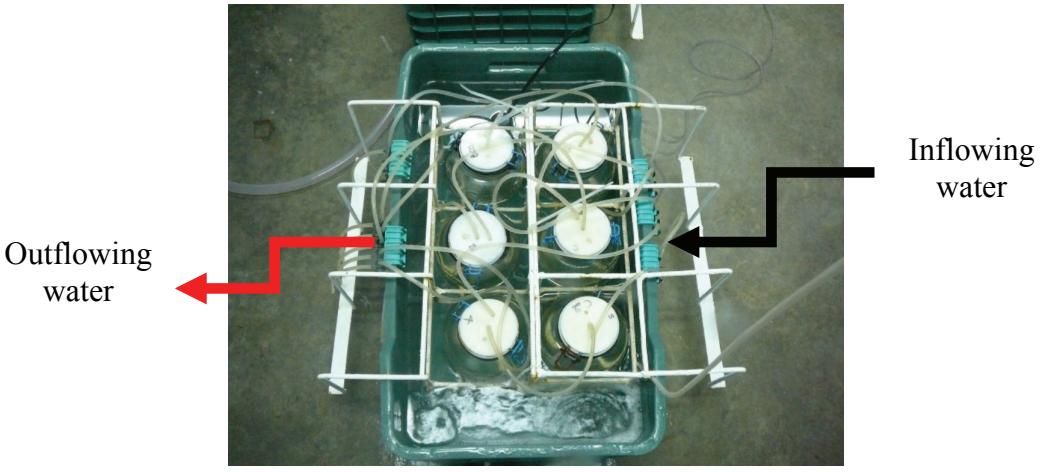


Figure 2.9: Flow-through respiration system. Oxygen saturated water was pumped into 700 ml hermetic chambers (black arrow). Each chamber contained one scallop, or when scallops where too small, 3-5 scallops were introduced into one chamber. The difference between in- and outflow water (black and red arrow, respectively) represents the oxygen consumed by the scallops.

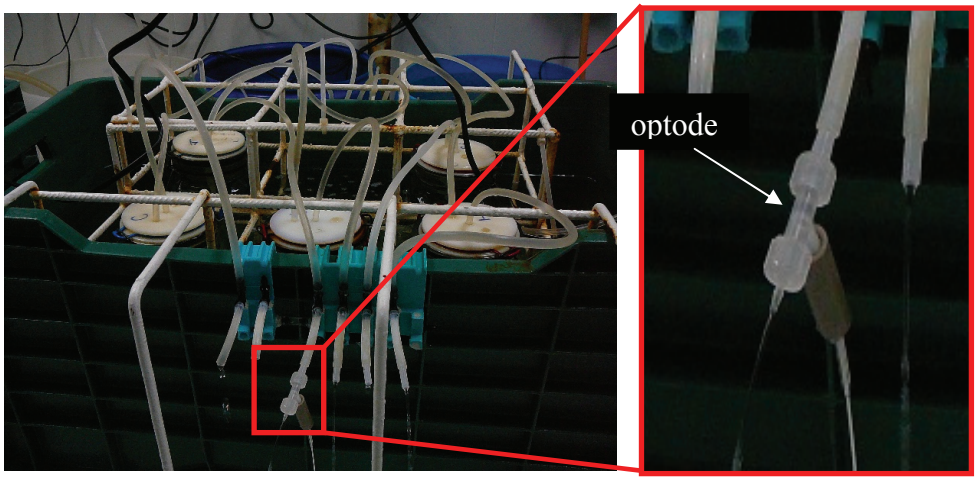


Figure 2.10: Oxygen concentration of the water was measured with an oxygen needle optode that was connected to the exit of outflow water. Oxygen saturated water from the inflow was injected with a syringe through the optode.

2.5.2. Energetic balance and the scope for growth

Metabolic rate typically increases following the ingestion of food associated with physiological activities linked with digestion (Jobling 1981). The total metabolic expenditure of these activities is determined as specific dynamic action (SDA). As SDA is an important component of organism energy budget, its quantification is valuable to determine the scope for growth (SFG). SFG is essentially an energy balance measurement of an individual, determined from the difference of the energy absorbed from ingested food and the energy expenditure via respiration and excretion. It provides an estimation of the production for somatic growth and is a measurement of the quantitative energy status of the animal (Widdows 1976, Widdows and Johnson 1988).

The physiological rates that integrate the scope for growth, speak ingestion-, respiration- and excretion- rates were measured in the same animals within the multi-channel flow-through system after measurement of SMR. For that, the inflowing water was enriched with microalgae during three hours. Three hours after feeding, difference in oxygen between the in- and outflowing water were determined again. Ingestion rates were calculated from differences in microalgae concentration between the in- and outflowing water. Excretion rate was calculated as the difference of nitrogen content in the chambers respective to a blank chamber. More details of the method for measurement of SFG are presented in Manuscript 1.

2.5.3. Condition indices

The condition index of an individual can be determined by the ratio of total tissue weight related to shell weight (Lucas and Beninger 1985). The condition index expresses the "fatness" of an individual and is useful to characterize the apparent health of the individual (Etim 1997, Sarkis et al. 2006). In addition to the condition index, the proportion of gonad, muscle, gill and mantle weight in respect to the total tissue weight were calculated separately to determine the condition of each tissue. The gonadosomatic index indicates the reproductive status of an individual. Hence, a high gonadosomatic index is indicative for mature animals and a low index reflects spend gonads or the initiation of gametogenesis (Sarkis et al. 2006). As changes in muscle mass are also associated with gametogenesis, it is difficult to assess if changes in the gonadosomatic index reflects changes in gonad or muscle weight. To rectify the validity of the gonadosomatic index, gonad and muscle weight were also related separately to shell weight. In this case the gonadosomatic index is defined as gonad index (publication 1, manuscript 2 and 3).

2.6. Measurement of biochemical assays

2.6.1. Citrate synthase and Octopine dehydrogenase

Both enzyme activities were measured in homogenates of muscle tissue at 25°C using a spectrophotometer. Citrate synthase (CS) catalyzes the transfer of sulfydryl groups from CoASH to 5´,5´-dithio-bis(2-nitro)benzoic acid (DTNB). The absorbance increase of DTNB was measured at 412 nm (Sidell et al. 1897).

Octopine dehydrogenase (ODH) catalyzes the oxidation of NADH to NAD^+. The decrease of NADH can be followed at 340 nm (Ballantayne et al. 1981). The activities of both enzymes were expressed in international units (µmol substrate converted to product min^{-1} × g^{-1} tissue wet mass). More details of the method for measurement of CS and ODH activities are presented in manuscript 2.

2.6.2. Catalase and Superoxide dismutase

Both enzyme activities were measured in homogenates of gill, mantle and muscle tissues at 25°C using a spectrophotometer. Catalase (CAT) converts hydroperoxide to water and oxygen ($2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$) (Aebi et al. 1984). The method consists in recording decrease of H_2O_2 at 240 nm.

For the measurement of superoxide dismutase (SOD), the xanthine/xanthine oxidase (X/XO) system was used to generate $O_2^{\bullet-}$ which reacts with nitroblue tetrazolium (NBT). The inhibition of NBT formation by superoxide dismutase can be detected at 560 nm (Susuki 2000). The activities of both enzymes were expressed in international units (µmol

substrate converted to product × g $^{-1}$ tissue wet mass). More details of the method for measurement of CAT and SOD activities are presented in manuscript 1, 2, 3.

2.6.3. Apoptosis

Apoptosis intensities were determined in homogenates of gill, mantle and muscle tissues modified after Lui (2004). The method was assessed using a Caspase-Glo 3/7 assay kit (Promega, Madison USA). The assay provides a luminogenic caspase-3/7 substrate that is quenched by caspases in the samples´supernatant. The quenched luminogenic substance (amino-luciferin) is a substrate for luciferase. The resulting luminescence signal is proportional to the amount of caspase activity present in the supernatant. More details of the method for measurement of apoptosis intensities are presented in manuscript 3.

2.6.4. Protein carbonyls and TBARS

Protein carbonyls and TBARS (lipid peroxides) were measured in homogenates of mantle, muscle and gill tissues. Protein carbonyls are formed by the interactions of ROS and products of lipid peroxidation (aldehydes) with proteins. The carbonyl groups react with the carbonyl specific reagent 2,4-dinitrophenylhydrazine (DNTP). After precipitation with trichloroacetic acid (TCA) carbonyls can be measured spectrophotometrically at 360 nm (Levine et al. 1990, Stadtman and Levine 2000). Amount of protein carbonyls was assessed as nmol mg^{-1} protein. The protein content was measured in each sample using the Bradford method (Bradford 1976).

Lipid peroxides are known to produce a variety of intermediate substances including malondialdehyde (MDA). MDA reacts with thiobarbituric acid (TBA) under acidic pH and elevated temperatures (90°C). Lipid peroxides were measured using the generation of

MDA/TBA adducts (thiobarbituric acid reactive substances, TBARS) to quantify MDA formation at 560 nm and were presented as nmolar equivalents g^{-1} fresh weight (Persky 2000). More details of the method for measurement of protein carboyls and TBARS are presented in manuscript 1, 2 and 3.

2.6.5. Lipofuscin

Lipofuscin is an undegradable age pigment with autofluorescence. Lipofuscin can be extracted in a hydrophobic chloroform-methanol solution following extraction method modified after Vernet et al. (1988). The fluorescence intensity of each sample was determined at an emission maximum of 536 nm for gills, 434 for mantle and 431 for muscle (Vernet et al. 1988). Lipofuscin concentrations were expressed as relative fluorescent intensities (RFI). More details of the method for measurement of lipofuscin concentrations are presented in manuscript 3.

CHAPTER 3: PUBLICATION

Publication

The general concept of this study was developed by Dr. Eva Philipp together with PD Dr. Doris Abele and Dr. Alfonso N. Maeda-Martínez. The project was funded by the DAAD grant nr. D/06/47197 and the SAGARPA- CONACyT project nr. 11947 for field and laboratory work in Mexico. The last year in Germany was funded by the Alfred Wegener Institute for Polar and Marine Research, Bremerhaven.

Publication 1

Citlali Guerra, Alfonso Nivardo Maeda-Martínez, Alfredo Hernandez-Llamas, Maria Teresa Sicard-González, Stefan Koenigstein, Doris Abele, Eva E. R. Philipp. The influence of temperature and presence of predators on growth, survival and energy allocation for reproduction in the Catarina scallop *Argopecten ventricosus*. **Publisched in: Aquaculture Research doi:10.1111/j.1365-2109.2011.02885.x**

I designed the experimental setup together with the last author. I carried out maintenance and sampling of *A. ventricosus* scallops with help of Mario Osuna, Carmen Abundis and the fifth author in the hatchery and in the laboratory. Mario Osuna and Ernesto León supported me with the sampling and maintenance of animals in the field. I performed experimental and analytical work as well as the evaluation of the data with contribution of the third, the fourth and the fifth author. I wrote the manuscript, which was revised by the sixth and the last author.

3.1. Publicaton 1: The influence of temperature and presence of predators on growth, survival and energy allocation for reproduction in the Catarina scallop *Argopecten ventricosus*

Citlali Guerra, Alfonso N. Maeda-Martínez, Alfredo Hernandez-Llamas, Maria T. Sicard-González, Stefan Koenigstein, Doris Abele, Eva E. R. Philipp

Published in Aquaculture Research doi:10.1111/j.1365-2109.2011.02885.x

ABSTRACT

Environmental factors are known to modify the life history of marine ectotherms. In a 16 month laboratory experiment we investigated the influence of temperature and presence of predators on life history parameters including shell growth, survival and the energy investment in reproduction and body mass, of the short-lived (~2 years) scallop *Argopecten ventricosus*. In parallel *Argopecten ventricosus* was maintained in the field at the Pacific coast of Baja California, México to compare growth, survival and reproductive effort under natural conditions. For the laboratory treatments, scallops were reared at simulated field temperatures (SFT), 5°C above SFT and in the presence of predators. Elevated water temperatures caused higher growth and gonad production although at the cost of increased mortality. Presence of predators induced energy allocation to muscle rather than gonad growth, deferred spawning and extended survival. Field scallops exhibited higher growth, higher reproductive investment and were able to reproduce twice, whereas all laboratory

scallops died after the first spawning. The natural variability of environmental parameters such as food and temperature may thus support optimal growth in the field and, when animals are protected from predators, reproduction in the second year of life.

Keywords: *Argopecten ventricosus*, temperature, predation, energy allocation

INTRODUCTION

Bivalves offer a rich diversity of lifestyles and adaptations to environmental conditions and are ideal model organisms to study the influence of intrinsic and extrinsic variables on life-history parameters such as growth, reproduction, fitness and senescence (Abele et al. 2009; Philipp and Abele 2010). The short lifespan of 2 years (Keen 1971) and the long-standing use of the Catarina scallop (*Argopecten ventricosus)* in aquaculture (Félix-Pico 1993; Maeda-Martínez et al. 1997; Sicard-González et al. 1999; Steller and Cáceres-Martínez 2009) render this species an ideal model to investigate the influence of environmental factors on life history parameters in laboratory experiments covering the species lifespan.

Differences in food levels and water temperatures have been suggested to cause differential maturity, growth and survival of *A. ventricosus* populations when cultivated at different sites on the coast of the Baja California Peninsula, México (Cruz et al. 1998; 2000). Villalaz (1994) found that gametogenesis in *A. ventricosus* maintained in controlled laboratory conditions occurs primarily at low phytoplankton densities, whereas high phytoplankton concentrations increased muscle growth. However, the author did not observe a relationship between reproductive condition and water temperature.

The presence of predators can also influence growth, reproduction and physiological performance of a species. Anti-predator responses, including changes of shell morphometrics (length and width) and thickening of different shell sections, such as the umbo or the lid, are observed when exposing bivalves or gastropods to waterborne predator cues or caged predators (Leonard et al. 1999; Delgado et al. 2002; Cheung et al. 2004). Lafrance et al (2003) observed wild scallops (*Placopecten magellanicus)* to have stronger shells and perform a more intense escape response compared to cultured individuals, which they attributed to the presence of starfish in the natural environment. If the energy budget does not suffice for the mechanical and physiological arms race with the predator, reproduction can be altered. Hoverman et al. (2005) showed that the occurrence of predators deferred reproduction and accelerated growth of the freshwater snail *Helisoma trivolvis*.

Although many studies have shown the effect of one or more environmental factors on physiological parameters in laboratory experiments, most investigations are limited to short periods of time. These studies miss the influence of environmental factors over longer time periods and through different ontogenetic stages. Our study presents a long-term investigation of the effects of elevated temperature and the presence of predators on growth and survival rates, and on the alternating energy investment into reproduction and body mass over the lifetime of Catarina scallops. For a better understanding of the natural conditions, scallops produced in the same cohort were reared in the field and investigated in parallel to the laboratory experiment.

MATERIAL AND METHODS

Brood stock and larvae culture

Sixty mature adult *A. ventricosus* were purchased from local fishermen in August 2007 in Bahía Magdalena. Animals were transported as described by Maeda-Martinez et al. (2000) to the hatchery station of CIBNOR. Spawning of mature scallops was induced by the thermal shock method following Uriate et al. (2001). Larvae settlement in the tanks occurred naturally. Larvae were maintained at 25 ± 1°C in 1500 L tanks and fed a 1:1 ratio of *Isochrisis galbana* and *Pavlova lutheri* during their first 13 days. On day 14, the diatoms *Chaetoceros calcitrans* and *Chaetoceros gracilis* were added to the food mix to an end concentration of 3:1:2:1, respectively. After 47 days, the scallops were transferred to tanks with a continuous upwelling flow of 5000 L • d^{-1} using the same microalgae species for feeding. In October 2007, the animals reached the harvest size of 5-7 mm shell height (Maeda-Martínez 1997). At the time, the spat was divided into 2 groups one of which was returned to the field while the other group was kept for laboratory treatments (temperature and predation).

Field study

Approximately 10000 scallops of 5-7 mm shell height were transported to Rancho Bueno estuary, in the vicinity of Bahía Magdalena in October 2007 (geographical position: N 24´19´ 17, 3´´ W 111´ 25´ 37, 3´´). The field site chosen in the present study represents a beneficial environment for *A. ventricosus* with high chlorophyll concentrations and the optimal temperature window for growth (Acosta-Ruiz & Lara-Lara 1998, Sicard-Gonzàlez

et al. 2006). Scallops were kept in fine mesh bags (2mm) fitting within 20 Nestier trays (55 x 55 x 8 cm) suspended in a long-line system for 2 months until reaching an average size of 24 mm (December 2007). Subsequently, animals were kept without bags in the trays. Initial stocking density was set at 500 animals/tray (equivalent to 1700 animals/m^2) for optimal growth (Maeda-Martínez et al. 1997) and adjusted to 150, 90 and 60 animals/tray (495, 297 and 198 animals/m^2) after 187, 337 and 480 days (February, July and December 2008) in order to keep densities optimal for growth.

Laboratory study

The laboratory study started in November 2007. Scallops of 7-9 mm shell height were kept in a flow-through system of twelve aquaria (70 x 60 x 16 cm) with a constant water flow of 210 L • day^{-1}, using ~ 630 scallops per aquarium. Initial stocking density was ~ 1500 animals/m^2 in order to keep similar initial densities as in the field. No density adjustments were carried out in the laboratory, as densities were always lower than in the field. Incoming sea water was filtered over a sand filter (Jacuzzi 225 L, Little Rock AR USA) and a 1 µm gaft filter. Of the twelve aquaria, eight were held at simulated field temperature (SFT) and four at 5°C above SFT (SFT+5°C). SFT values were deduced from field measurements. A temperature logger recorded water temperature at 60 min intervals (WTA32-5+37, Onset Computer Corp., Bourne MA, USA) attached to one of the nestier trays. The temperature logger was collected and replaced during field trips at monthly intervals and the water temperature in the SFT treatments adjusted to monthly average field temperature and controlled using aquarium heaters (Hagen Aquaclear 22952). Thus, the

temperature in the SFT treatments represents the mean field temperature of the previous month (Fig. 1).

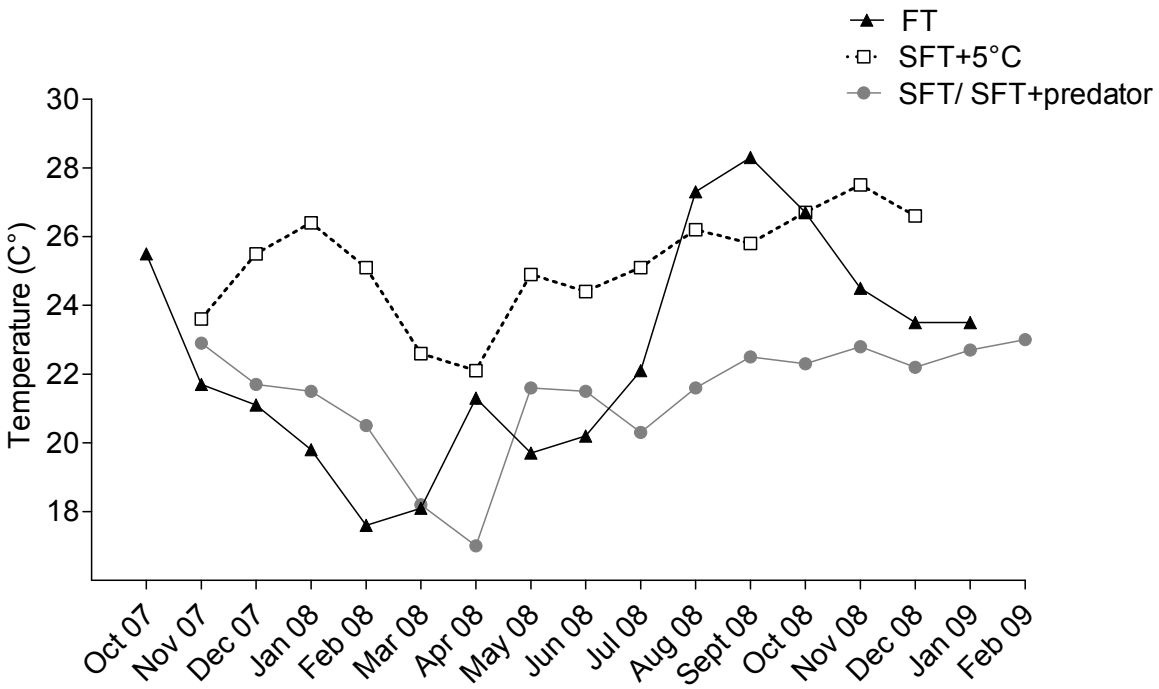


Fig. 1: Mean values of temperatures during the experimental period. The monthly average FT was simulated in the laboratory 1 months later (see "Material and methods"). SFT, simulated field temperatures; SFT+5°C, 5°C above SFT; SFT+predator, SFT with presence of predator and FT, field temperatures.

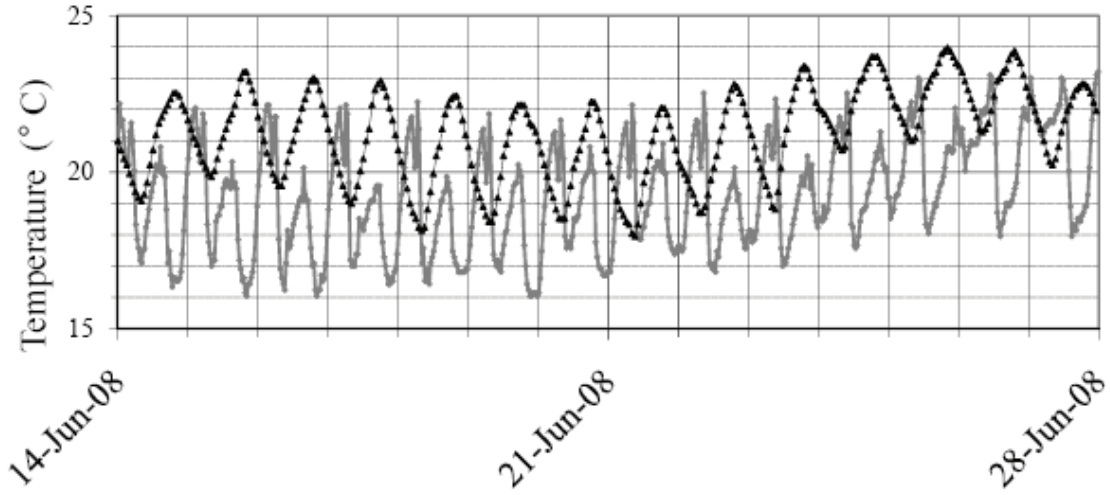


Fig. 2: Example of daily temperature fluctuations in the laboratory (black line) and in the field (grey line) during two weeks in June 08. Each vertical line represents 1day.

Temperatures in the laboratory fluctuated daily by about 3°C with one peak day^{-1}. In the field, two distinct peaks within a day were recorded and a wider thermal window of oscillation of about 5°C (Fig. 2) was observed. In fall (Aug 2008 – Oct 2008), FTs ranged close to species specific lethal temperature of 29°C (Sicard-Gonzáles et al. 1999). During this period, the temperature limit in the laboratory was set at 27 ± 1 °C in the SFT+5°C treatment to limit mortalities linked to high temperature exposure. The SFT treatment was kept at 5°C below the SFT+5°C treatment throughout the experiment except in November 2007, May and June 2008 where only 3-4 °C difference could be adjusted (Fig. 1).

The SFT + predator experiment started in April 2008. Predators were introduced in four SFT aquaria (one crab aquarium^{-1}). Crabs were kept in the aquaria 6 h day^{-1} for 5 days week^{-1}. The crabs´ pincers were held together by rubber bands to prevent them from eating scallops. Crabs were fed squid every third night in a separate aquarium.

The scallops were fed *ad libitum* throughout the experiment using a 1:1 mixture of *Chaetoceros calcitrans* and *Isochrisis galbana* delivered by an automated system containing a mixing tank and a turbidimeter controlled pump (Hach 1720 Loveland, USA). Cell concentrations were monitored using a Coulter Cell Counter (Multisizer 3, Beckham, Coulter, Fullerton, CA, USA). Concentration varied between 60000 and 250000 cells mL^{-1} in the mixing tank and between 20 and 50 cells mL^{-1} in the outflow water of each aquarium throughout the experimental time ensuring that scallops were fed *at libitum*. Salinity was kept at 33-36 ppt similar to values found in the field site (Sánchez-Montante et al. 2007).

Data collection and analysis

Shell height growth

Shell height (distance from hinge to distal shell margin) was measured monthly using calipers. For SFT scallops, measurements were undertaken from October 2007 to October 2008, for SFT+5°C from November 2007 to October 2008, for SFT + predator from April 2008 to October 2008 and for the Field scallops (FT) from October 2007 to October 2008. Scallops were randomly selected at each sampling date: Field: 2 animals Nestier $tray^{-1}$ (a total of 40 animals); Laboratory: 10 animals $aquarium^{-1}$ (a total of 40 animals). The growth of scallops was subsequently calculated using the model proposed by Ratkowsky (1986) which is an alternate parameterization of the von Bertalanffy growth model with close-to linear behavior. This model was shown to be more reliable than the conventional von Bertalanffy equation, because it allows nonlinear regressions to converge easily and to

conduct tests for parameter invariance more precisely (Hernandez-Llamas & Ratkowski 2004):

$$H_t = h_i + (h_f - h_i)\,(1-k^{m-1}) / (1-k^{n-1}) \quad (1)$$

where H_t is the calculated height of scallops at time t, h_i the initial height, h_f the final height, k the growth coefficient, n the number of data points, and m the time modified according to:

$$m = 1 + (n-1)\,(t-t_i) / (t_f - t_i) \quad (2)$$

where t_i is the initial time and t_f the final time.

Shell weight to shell height ratio

In order to investigate whether the scallops develop heavier shells under predation pressure, shells of the SFT and SFT + predator group were used to determine the shell weight/shell height ratio.

Survival

Animal survival in the laboratory was measured by monthly counts of dead animals in each experimental aquarium over scallop lifetime: SFT from December 2007 to October 2008 (150-410 days of age), SFT+5°C from December 2007 to December 2008 (150-480 days of age), and SFT + predator from May 2008 to February 2009 (280-540 days of age). Survival in the field was calculated in February, July and December 2008 when scallop´s number had to be adjusted in the nestier trays (see "Field Study").

Tissue indices

For gonadosomatic, muscle, mantle, gill and condition indices scallops were sacrificed and tissue fresh weight, total fresh weight, as well as shell weight was determined using a digital balance (Precisa XT 320M, Precisa Instruments AG, Dietikon, Switzerland). The indices were calculated as follows:

Tissue Index = (weight of component tissue / total tissue weight) * 100

Condition Index = (total tissue weight / shell weight) * 100

Statistical analysis

Differences in shell growth parameters between the different treatments were calculated with nonlinear regression analysis using GraphPad Prism (version 5.0 for Windows, GraphPad Software Inc., San Diego California USA). Significant differences indicate differences in growth among treatments using the least restrictive invariance test, analyzing the parameters h_i, initial height; h_f, final height and k, growth coefficient acting together (for references of the method see also: Hernandez-Llamas et al (1995), Osuna-García et al (2008)).

Differences in survival between laboratory treatments were assessed with the Kaplan-Meier method. Kaplan-Meier survival curves were constructed using the known birth and death dates of each individual. This method allows comparison of two or more survival curves over time and the calculations take into account censored observations due to samplings for analysis. The log rank test (log), as implemented in the program PASW Statistics 18

version 18.0.0 (SPSS Inc., Chicago, IL, USA) was used (for references of the method see also Motulsky, 1995). One-way ANOVA and Tukey´s post hoc test (unequal sample size) or student- *t* test was used for analysis of indices after testing for normality with Kolmogorov-Smirnov-test (GraphPad Prism 5.0 software). Index values were arcsin transformed and are presented as back-transformed means.

RESULTS

Shell growth

Treatment affected final shell height (*hf*) (Fig. 3, Table 1). Applying Eq. (1) to growth data yielded significant differences between SFT and SFT+5°C as well as between SFT and FT treatments ($p < 0.0001$, Table 1). Exposure to predators did not influence shell growth but resulted in significantly heavier shells in October 2008 after exposure to predators for 6 months (Table 2).

Survival

Using the Kaplan-Meier model, lower survival rates for the SFT+5°C treatment compared to SFT animals were detected (log, $\chi^2_{df=1} = 71.247$, $P < 0.001$) (Fig. 4a). Around October 2008 (410 days of age), almost all scallops in the SFT and SFT+5°C laboratory treatments died after a massive spawning event. The remaining scallops in the SFT+5°C treatment lived for another 2 months and the last individuals (2.6 % of initial population) were sacrificed in December 2008 for analysis (480 days of age). Individuals in the SFT

treatment had a lower survival rate compared to individuals of the SFT + predator treatment (log, $\chi^2_{df=1} \geq 30.193$, $P < 0.001$, Fig. 4b). The difference was due to the high mortality following the spawning in October 2008 in the SFT treatment. Survival rates before the spawning event did not differ between both treatments ($\chi^2_{df=1} \geq 0.4$ p = 0.5). The last scallops of the predator-exposed group were sacrificed in February 2009 (540 days of age). We could not compare survival rates of SFT *vs* FT with the Kaplan-Meyer model because of the small number of samplings in the FT treatment. However, in December 2008, when all SFT animals were already dead, 10% of the initial population in the field was still alive and field scallops lived up to August 2009.

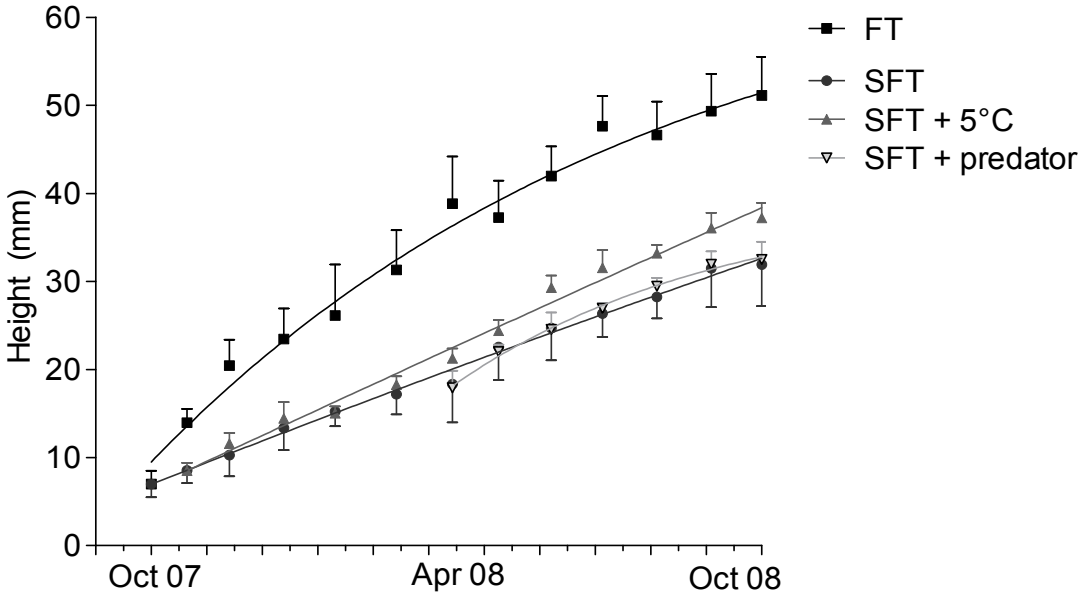


Fig. 3:.Shell growth (in height) of *A. ventricosus* in different treatments.Values are means ± S.E.M. *N* = 40 for each treatment. Fitted growth curves correspond to Eq (1). SFT, simulated field temperatures; SFT+5°C, 5°C above SFT; SFT + predator, SFT with presence of predator and FT, field.

Table 1: Growth parameters of *A. ventricosus* using Eq. 1.

Treatment	h_i	h_f	k	P	*Time period*
SFT	6.9 ± 1.5	31.9 ± 4	0.93 ± 0.01	< 0.0001	October 07-October 08
FT	6.9 ± 1.5	51.2 ± 4.4	0.89 ± 0.01		
SFT	8.5 ± 1.4	31.9 ± 4	0.98 ± 0.03	< 0.0001	November 07-October 08
SFT+5°C	8.5 ± 1.4	37.2 ± 1.7	1.0 ± 0.02		
SFT	18.3 ± 1.6	31.9 ± 4	0.97 ± 0.1	0.672	April 08-October 08
SFT+ predator	17.9 ± 2	32.5 ± 2	0.92 ± 0.07		

Significant *P*-values indicate differences in growth among treatments using the least restrictive invariance test (i.e. testing the parameters acting together). N = 40 for each treatment. SFT, simulated field temperatures; SFT+5°C, 5°C above SFT; SFT + predator ,SFT with presence of predator and FT, field; h_i, initial height (mm); h_f , final height (mm); k, growth coefficient

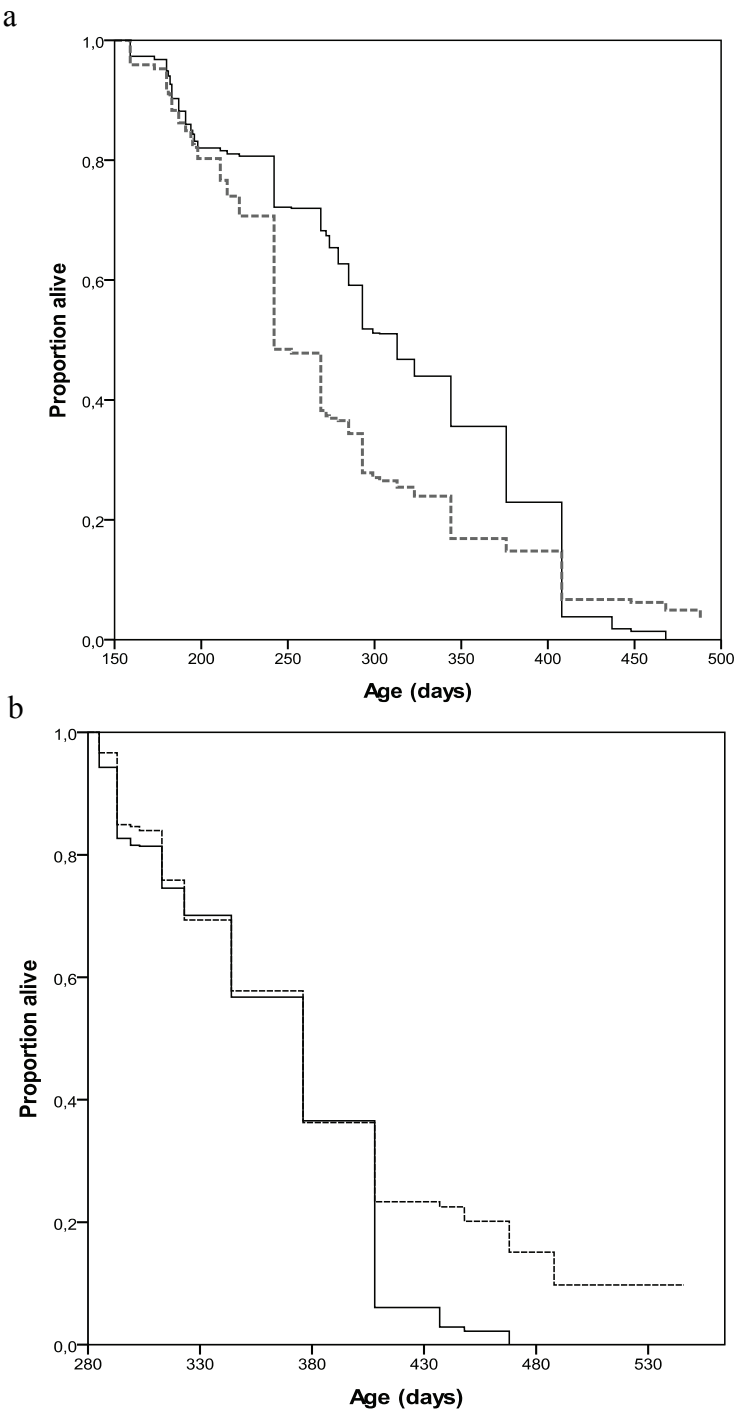


Fig. 4: Survival analysis using the Kaplan-Meier method and the log-rank test for comparison of curves. a) SFT (solid line) vs. SFT+5°C (dashed line) (initial N = ˜635). b) SFT (solid line) *vs* SFT + predator (dashed line) (initial N = ~ 250). SFT, simulated field temperatures; SFT+5°C, SFT 5°C above SFT; SFT + predator, SFT with presence of predator.

Table 2: Shell weight to shell height ratio of *A. ventricosus* in different treatments

Treatment	April 2008	August 2008	October 2008
SFT	0.144 ± 0.008[A]	0.14 ± 0.009	0.13 ± 0.012
SFT + predator	0.15.± 0.009[A]	0.16 ± 0.01	0.2 ± 0.015*

* Indicate significant differences between treatments in the respective month (t-test, $P < 0.05$).
[A] Introduction of predators. Values are means ± S.E.M (N = 10-16).
SFT, simulated field temperature; SFT + predator, SFT with presence of predator.

Tissue indices

Tissue indices were determined to evaluate the alternating energy investment into reproduction [gonadosomatic index (GSI)] and other body mass components (muscle, mantle and gill) over time. GSI differed significantly between field-reared and laboratory SFT animals (Fig. 5a). The highest GSI within the field treatment was measured in August 2008 (ANOVA, $p < 0.0001$) after mean temperatures rose from 22°C to 27°C in only one month. The scallops spawned thereafter which is indicated by the decrease in GSI. In the SFT group, the highest GSI was observed two months later in October 2008 (ANOVA, $p = 0.0026$) simultaneously with the SFT+5°C scallops (ANOVA, $p < 0.0001$). After reaching the maximum gonad index, SFT and SFT+5°C scallops spawned, and almost all animals died. Peak gonad indices in FT, SFT+5°C and SFT were 12.57 ± 2.4%, 10.15 ± 1.5% and 7.09 ± 1.31%, respectively, and significantly higher for FT and SFT+5°C individuals compared to SFT scallops (FT vs. SFT: t test, $p < 0.001$; SFT vs. SFT+5°C: t test, $p = 0.006$). In the SFT + predator group, the maximal gonad index was measured in December 2008 (5.83 ± 1.2%) (ANOVA, $p < 0.0001$) and was significantly lower than the maximal

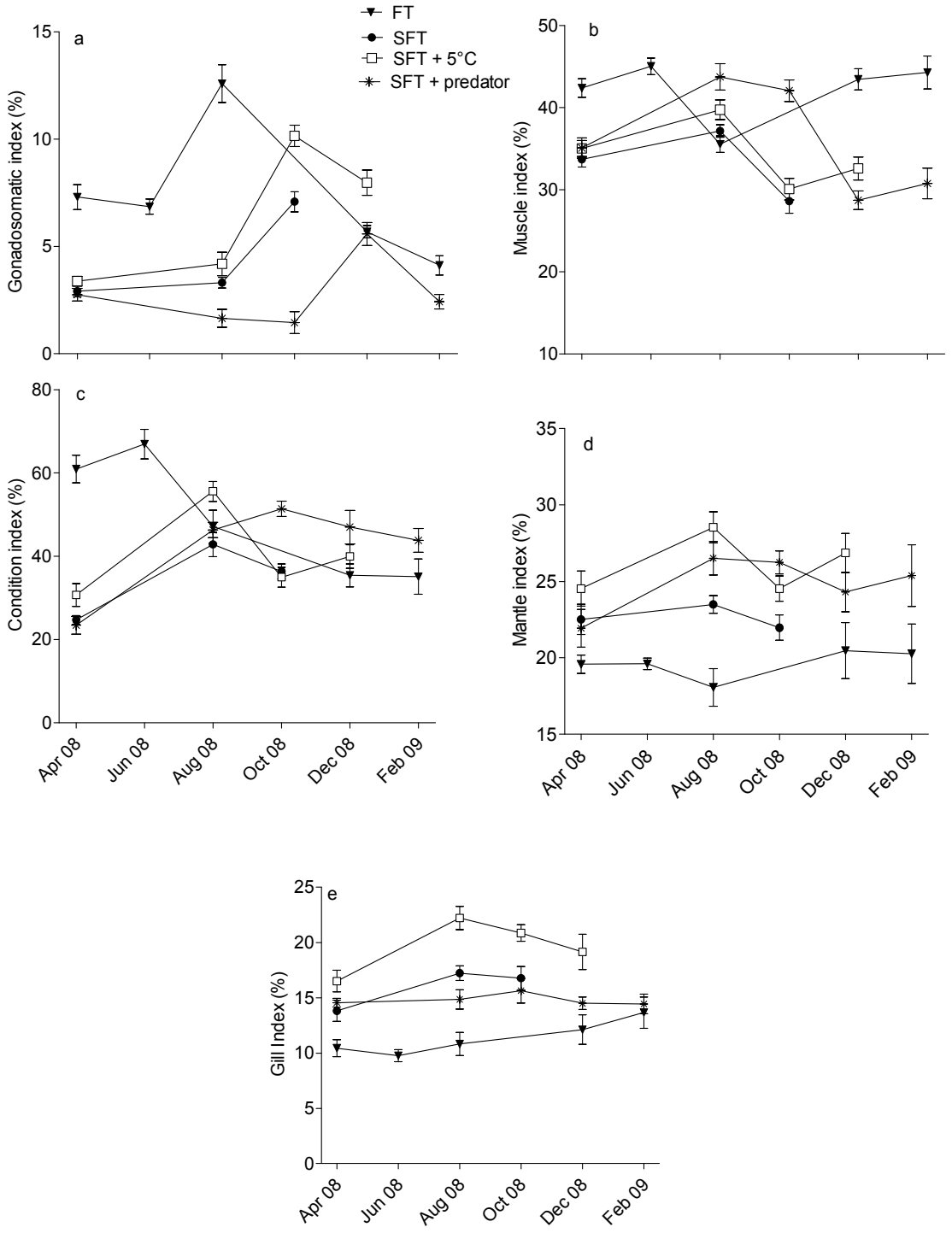


Fig.5: Gonadosomatic a) Muscle b) Condition c) Mantel d) and Gills e) indices of *A. ventricosus* in the different treatments. Values are means ± S.E.M. (N = 8). Details of statistical differences are given in the text. SFT, simulated field temperatures; SFT+5°C, 5°C above SFT; SFT + predator, SFT in presence of predator and FT, field.

GSI in the SFT group (SFT *vs* SFT + predator: t test, $p = 0.02$). Scallops spawned thereafter (personal observation) which caused a decrease in GSI.

Muscle and condition indices followed an inverse pattern to the GSI in all treatments (Fig. 5b and c). Peak muscle and condition indices were higher in FT individuals compared to SFT scallops (muscle index: t test, $p < 0.0001$; condition index: t test, $p < 0.0001$) and occurred in June 2008 in FT and in August 2008 in SFT scallops. In the SFT + predator group, the highest muscle index was observed in August 2008 and condition index in October 2008 and both were higher than peak indices in the SFT group (SFT *vs* SFT + predator: muscle index: t test, $p < 0.0001$, condition index: t test, $p = 0.005$). No differences in peak muscle index between SFT and SFT+ 5°C were found (t test, $p = 0.2$) but the latter group displayed higher maximal condition index (t test, $p < 0.0001$) in August 2008.

In the SFT+5°C group, the mantle index increased significantly between April and August 2008 and decreased in October 2008 (Fig. 6 ANOVA, $p < 0.0001$). The mantle index did not vary with time in SFT, SFT + predator and FT group (SFT: ANOVA, $p = 0.22$; SFT + predator: ANOVA, $p = 0.09$; FT: ANOVA, $p = 0.44$). This index was significantly higher for SFT+5°C and the SFT + predator individuals compared to SFT scallops in August and October 2008 (SFT+5°C vs SFT August 2008: t test, $p < 0.0001$; October 2008: $p = 0.0036$; SFT *vs* SFT + predator August 2008: t test, $p = 0.01$; October 2008: $p = 0.003$). Field scallops tented to have lowest mantle indices compare to SFT, but only significantly different in August 2008 (Fig. 5d).

Differences in gill index were significant when comparing SFT and SFT+5°C scallops in August and October 2008 (t test in August: t test $p = 0.001$; October: t test $p = 0.0045$) and between SFT and FT in April and August 2008 (t test, $p < 0.0001$, Fig. 5e).

DISCUSSION

Growth and survival

In this long-term experiment, we observed increased shell growth (height) in scallops exposed 11 months to 5°C elevated temperatures (SFT+5°C: 22.1°C – 27.5°C) compared to scallops grown at simulated field temperatures (SFT: 17.2°C - 23.0°C). This contrasts results obtained by Sicard et al. (1999), who observed decreased growth in juveniles from the same population acclimated for 55 days to higher temperatures (25°C and 28°C) compared to a control group maintained at lower temperature (19°C and 22°C). This discrepancy arises from comparing short-term with long-term investigations such as the present study, in which higher growth for SFT+5°C scallops occurred only after 7 months of exposure (from June 2008 on). We propose that the higher mortality in the SFT+5°C group from April 2008 on (250 days of age) possibly selected for scallops of better physiological condition and stronger growth in this later period of the long-term experiment.

Within the laboratory treatments, we observed an increase in condition index in SFT and SFT+5°C between April and August 2008. Thus, scallops in both laboratory treatments gained in tissue weight relative to shell weight indicating good general growth conditions. However, this index was higher in survivals of the SFT+5°C scallops. Higher tissue weight

gain was due to higher mantle and gill growth, while muscle and gonad grew less. Higher mantle and gill indices in the SFT+5°C group in August 2008 may afford better oxygen uptake (gill and mantle) and food absorption (gill) at the higher temperature, where oxygen solubility is decreased and scallop metabolism is higher. Indeed, food absorption rates were higher in SFT+5°C animals in April and August 2008 (data not shown).

The higher condition index in field scallops before maturation, the strikingly higher shell growth over the entire study period, and longer survival compared to laboratory SFT animals implies that controlled conditions (e.g. temperature control and normalized microalgal mixture) are not optimal to sustain physiological status. Sicard González (2006) found that oscillating night and day temperatures led to faster growth of the scallop *Nodipecten subnodosus*, and that individuals exposed to thermal fluctuations lived longer in spite of earlier onset of reproduction when compared to animals maintained permanently at an experimentally determined optimum temperature. In our study, the influence of the tides in the field resulted in more pronounced thermal fluctuations compared to the day and night oscillations in the laboratory (Fig. 2). In addition, food quality and composition at the scallop experimental field site (Rancho Bueno in Bahía Magdalena) are more variable in algal species composition (Gárate-Lizárraga et al 2006) compared to the uniform control feeding regime in the laboratory. Under field conditions, scallops preferentially allocated energy into muscle and gonad rather than in mantle and gill growth compared SFT and SFT+5°C scallops. This differential energy investment into tissues in field and laboratory scallops may have important implications for growth, reproduction and survival. Apparently, *ad libitum* feeding with a standardized plankton mixture does not present the same food quality as the natural algal community of the habitat and indicates variability and

diversity to be more important for the scallops' physiological conditions than the food quantity.

Scallops reared in the presence of predators invested significantly more energy into muscle than gonad growth (Fig. 5a and b) and developed heavier shells (Table2). Similarly, blue mussels, *Mytilus edulis*, develop stronger byssal attachments, thicker shells and larger adductor muscles in the presence of starfish and crab predator signals (Reimer and Tedengren 1996; Reimer and Harms-Ringdahl 2001).Thus, predator exposed animals appear to invest more energy into defensive mechanisms, which may delay reproduction

Reproduction

A. ventricosus grown in the field as well as in all laboratory treatments displayed a conservative gametogenic cycle, meaning that gametogenesis is fueled by energy stored in tissues, mainly the adductor muscle (Luna-Gonzalez et al. 2000). Dependence of the gonad energy allocation on muscle mass was confirmed even for the older field animals and corroborates earlier studies of *A. ventricosus* from different sites of Baja California Sur (Felix-Pico 1993). Epp et al. (1988) suggested that mantle tissue may also play a role as energy storage site for gonad development, and mantle mass declines as gonads mature. We observed a slight decrease in mantle index in mature scallops in all treatments which was, however, only significant in SFT+5°C scallops.

Field animals reached maturation in August 2008 concomitantly with a pronounced increase in temperature by 5°C between July and August 2008. In marine poïkilotherm species, temperature plays an important role through modulation of most physiological

processes, including reproduction (Clarke 1987; Lubet 1983; Jalabert 2005). In Bahía Magdalena*, A. ventricosus* is known to reproduce throughout the year with two main spawning peaks in March-April and August-September when water temperatures are 19-23°C and 27-28°C, respectively (Felix-Pico 1993). However, in a laboratory study, Villalaz et al. (1994) found no evidence for a direct effect of water temperature on scallop reproductive condition. Instead, temperature, together with food abundance as a second important parameter, modulates gonad development in mature animals. The authors suggested that temperature could be important as both, initiator of gametogenesis and as a spawning cue. In the laboratory, the delay in maturation in SFT and SFT+5°C could be linked to the more modest increase in temperature by 2°C between July and October 2008, and could further reflect the delay in water temperature change in the laboratory, which was lagged by 1 month compared with FT. Despites higher investments into reproduction of field compared to SFT scallops, (shown by the higher GSI) scallops in the field survived and reproduced again in the second year. In contrast, the SFT and SFT+5°C animals reproduced virtually to death in October 2008, when almost all individuals died after the first spawning.

Exposure to predators under laboratory conditions delayed maturation for 2 months. The effect was not related to temperature, which was the same in the SFT as in the SFT + predator treatment. Scallops lived longer but at the costs of a decrease in reproductive output, deduced from a lower GSI compared to scallops reared without predators. This confirms that predators can modulate prey life history traits such as age and size at maturation, reproductive output and mean lifespan (Abrams 1993, Abrams & Rowe 1996, Stearns 2000, Beckerman et al. 2007). The optimal investment into life history traits will

depend on the environmental conditions that organisms experience during lifetime (Alonso-Alvarez 2006). When *A. ventricosus* scallops are sufficiently protected from predators, and when food and temperature conditions are favourable, investment into growth but also into reproduction is possible. In the present study, field animals were protected in nestier trays against direct predator attack and had optimal and diverse feeding conditions. All parameters together apparently afford higher growth and repeated reproduction in the field group compared to all laboratory treatments. The results corroborate previous studies on energy allocation in *Placopecten magellanicus* (MacDonald and Bayne, 1993) and the freshwater clam (*Anodonta piscinalis*) (Jokela and Mutikainen, 1995). The authors suggest that under favourable conditions, animals invest energy into reproduction without cutting on growth or maintenance. However, under energy limited conditions, energy investments is prioritized for growth and maintenance (*Placopecten magellanicus*) or for reproduction (*Anodonta piscinalis*) depending on the species. Our results suggest that under limited conditions such as in the laboratory, and in presence of a predator, *A. ventricosus* prioritizes energy allocation into muscle growth at the cost of reproduction.

CONCLUSIONS

Environmental factors shape life-history parameters in scallops. Animals experimentally exposed to predators allocate energy to muscle growth and protract spawning. As spawning means a major energetic drainage, late spawning simultaneously extends survival compared to animals kept without predators. In contrast, higher temperatures induce higher growth and enhance investment into gonad production but at the costs of population survival.

Further, standardized conditions, such as *ad libitum* feeding with even a mixed plankton culture, cannot appropriately substitute for the natural food quality in the field, which apparently reduced the physiological fitness of artificially fed animals held under "optimized" laboratory conditions. Thus for optimal scallop culture with high market yield (high muscle index) rearing with fluctuating environmental conditions as well as predators is recommended.

ACKNOWLEDGEMENTS

The authors would like to thank Mario Osuna, Ernesto León and Carmen Abundis for the technical support, the students Max Ostendorf and Belisama Carmona for assistance in the hatchery and animal maintenance and Edouard Kraffe for suggestions on the manuscript. Also we would like to thank the Cooperativa del Puerto de San Carlos, Baja California Sur for the supply of scallops. This research was supported by the DAAD grant D/06/47197 and the SAGARPA- CONACyT project 11947.

CHAPTER 4: MANUSCRIPTS

Manuscripts

Manuscript 1

Guerra, C., Zenteno-Savín, T., Maeda-Martínez, A.N., Abele, D., Philipp, E.E.R. The impact of long-term exposure to elevated temperatures on oxidative stress and scope for growth in the Catarina scallop *Argopecten ventricosus*

The last author and I developed the scientific ideas of this manuscript. I performed sampling of the animals. All analyses were carried out in the laboratory of the second author and were conducted by me. I wrote the manuscript which was improved with the comments of all co-authors.

Status of manuscript: supplemental results of the thesis.

Manuscript 2

Guerra, C., Zenteno-Savín, T., Maeda-Martínez, A.N., Abele, D., Philipp, E.E.R. The effect of predator exposure and reproduction on metabolic and oxidative stress parameters in the Catarina scallop *Argopecten ventricosus*

The last author and I developed the scientific concept of the manuscript. I conducted the samplings and experimental analyses within the laboratory of the second author. I wrote the manuscript in discussion with the last author and received productive feedback of the second and third and the fourth author.

Status of manuscript: to be submittend in the journal: Comparative Biochemistry and Physiology Part A

Manuscript 3

Guerra, C., Zenteno-Savín, T., Maeda-Martínez, A.N., Philipp, E.E.R, Abele, D. Changes in oxidative stress parameters in relation to age, growth and reproduction in the short-lived Catarina scallop *Argopecten ventricosus* reared in its natural environment.

I developed the scientific concept of the manuscript in discussion with the fourth author. I maintained the scallops in the field and conducted the samplings with cooperation of Mario Osuna and Ernesto León. For cleaning of nestier trays, I received help from fishermen in Rancho Bueno. The second author provided the laboratory where I conducted all the measurements and analyzed the data. I wrote the manuscript in discussion with the last author and received productive feedback of the second and third and the fourth author.

Status of the manuscript: Submitted to the journal: Comparative Biochemistry and Physiology Part A.

4.1. Manuscript 1: The impact of long-term exposure to elevated temperatures on oxidative stress and scope for growth in the Catarina scallop *Argopecten ventricosus*

Citlali Guerra, Tania Zenteno-Savín, Alfonso N. Maeda-Martínez, Doris Abele,

Eva E.R.Philipp

ABSTRACT

In ectothermal animals, temperature has a direct influence on physiological functions. An increase in temperature within species tolerance limits can enhance metabolic rates and influence individual energetic balance (scope for growth). At a cellular level, the increased metabolic rates can lead to elevated production of reactive oxygen species accompanied by somatic damage if compensatory mechanisms are not following up. In the present study we investigated changes in the energy available for growth (scope for growth) as well as tissue specific responses in antioxidant defense and oxidative damage within a population of the pacific scallop *Argopecten ventricosus* from Baja California Sur México exposed to long-term (5 and 9 months) elevated but sublethal temperatures. Scallops held at 5°C above simulated field temperature (SFT+5°C) exhibited higher respiration and higher absorption rates after 5 and 9 months of exposure whereas scope for growth was higher only after 9 months exposure. Oxidative damage (lipid peroxidation = TBARS and protein carbonyls) showed controversy results being high after 5 months but lower after 9 months exposure in

mantle and gills. The high levels of damage after 5 months exposure occurred despite the elevated antioxidant capacities (catalase; CAT and superoxide dismutase; SOD) indicating higher oxidative stress in the higher temperature group and that capacities were not sufficient to prevent accumulation of oxidative damage. We suggest that individual *A. ventricosus* posses limited capacity for acclimation to constant elevated temperature. Temperature tolerance may however be achieved by higher mortality rates in the SFT+5°C group, which on the long run, (after 9 months exposure to elevated temperatures), might select for the observed "better quality individuals" that exhibit higher scope for growth and lower levels of oxidative stress.

Keywords: *Argopecten ventricosus*, oxidative stress, scope for growth, temperature

INTRODUCTION

In marine ectotherms, the ambient temperature directly influences the body temperature and thus affects biochemical, cellular and physiological reaction rates (Hochachka and Somero 2002).

Physiological traits such as ingestion-, food absorption-, respiration- and excretion rate may be affected by an overall increase in biochemical and physiological processes, which represents a typical response to elevated temperatures in bivalves (Tremblay et al. 1998 a,d). The integrated analysis of these physiological traits can provide insights into the growth efficiency (scope for growth) and how scope for growth may be disrupted by environmental stress (Widdows and Johnson 1988) such as an increase in environmental temperature. At the cellular level, an increase in metabolic rates can be accompanied by an

enhanced accumulation of cellular oxidative damage. Such damage can occur when reactive oxygen species (ROS) generated by mitochondria during aerobic metabolism oxidise essential cell constituents including membrane lipids, proteins and DNA and may compromise cellular homeostasis and in extreme cases, survival of the individual. In order to counterbalance ROS production and minimize tissue damage, aerobic cells are endowed with an array of protection and repair mechanisms. Some of these protective mechanisms are antioxidant enzymes such as superoxide dismutase (SOD), and catalase (CAT), which neutralize ROS into inactive molecules such water and oxygen (Kregel and Zhang 2006) before they cause damage. When the balance between ROS production and the antioxidant capacity is lost, oxidative damage occurs and damaged products accumulate (Storey 1996; Abele et al. 1998). The accumulation of oxidative by-products, such as lipofuscin and lipid peroxides, after exposure to temperatures above ambient temperature in limpets, protobranch bivalves and mussels illustrates how warming may exacerbate cellular oxidative stress in bivalves (Abele 1998 2001; 2002).

In the scallop *Argopecten ventricosus,* physiological parameters such as ingestion rates and growth (shell height increment) appeared to be optimal at 19-22°C but diminished when scallops are maintained 55 days to higher temperatures (25 and 28°) (Sicard-González et al. 1999). This indicates decrease in physiological homeostasis during exposure to higher temperatures. However, it is still not known how scallops deal with oxidative stress. In the present study, we investigated whether long-term exposure of scallops to constantly higher temperatures of 5°C above the environmental mean but within the species tolerance limits (< 29°), is linked to increased metabolic rates and a parallel loss in cellular homeostasis and induction of oxidative stress. The alternative view is that thermal tolerance varies after a

long-term exposure and scallops undergo acclamatory responses involving long-term compensatory adjustments of metabolic functions that allow them to keep their scope for growth and oxidative physiology independent of temperature.

The long-standing use of the catarina scallop in aquaculture (Maeda-Martìnez et al. 2000; Sicard-Gonzàlez 2006) enabled us to investigate the effect of elevated temperature over a long time-frame within the same cohort of scallops raised in the laboratory.

MATERIAL AND METHODS

Brood stock and larvae culture

Sixty mature adult *A. ventricosus* were purchased from local fishermen in August 2007 in Bahía Magdalena. Animals were transported as described by Maeda-Martinez et al. (2000) to the hatchery station of CIBNOR. Spawning of mature scallops was induced by the thermal shock method following Uriate et al. (2001). Larvae settlement in the tanks occurred naturally. Larvae were maintained at 25 ± 1°C in 1500 L tanks and fed a 1:1 ratio of *Isochrisis galbana* and *Pavlova lutheri* during their first 13 days. On day 14, the diatoms *Chaetoceros calcitrans* and *Chaetoceros gracilis* were added to the food mix to an end concentration of 3:1:2:1, respectively. After 47 days, the scallops were transferred to tanks with a continuous upwelling flow of 5000 L • d^{-1} using the same microalgae species for feeding. The juveniles were reared until they reached 5-7 mm shell height and 3 months of age. From November 2007 on, scallops were maintained in a flow-through system consisting of 8 parallel 70 L-aquaria under constant water flow of 210 L day^{-1}. Sea water from the Bay of La Paz was filtered mechanically through a sand filter (Jacuzzi 225 L,

Little Rock AR USA) and a 1 μm gaft filter before entering the system. The animals were fed *ad-libitum* throughout the experiment using a 1:1 mixture of *Chaetoceros calcitrans* and *Isochrisis galbana* delivered by an automated system containing a mixing tank and a turbidimeter-controlled pump (Hach 1720, Loveland, CO, USA). Cell concentrations were monitored using an electronic cell coulter Cell Counter (Multisizer 3, Beckham, Coulter, Fullerton, CA, USA). Concentrations in the mixing tank varied between 60 000 and 250 000 cells ml^{-1} and between 20 and 50 cells ml^{-1} in the outflow water of each of the 8 aquaria throughout the experimental time, ensuring that scallops were fed *ad libitum*. Salinity was kept at 33-36 ppt., similar to values found in the field site (Sánchez-Montante et al. 2007). Of the 8 aquaria, 4 were held at simulated field temperature (SFT), and 4 aquaria at 5°C above SFT (SFT+5°C) (Fig. 1). We exposed the scallops to simulated monthly field temperature in order to follow a natural cycle of variation that was however, 5°C elevated compared to a control group. Field temperatures were deduced from field measurements in the Rancho Bueno estuary (geographical position: N 24´19´ 17, 3´´ W 111´ 25´ 37, 3´´), located in Bahía Magdalena, Baja California Sur, Mexico where optimal temperatures for *A. ventricosus* growth occurs (Sicard-Gonzàlez et al. 1999). Water temperatures in the SFT setups were adjusted to the average water temperature recorded in the field during the previous month and the SFT+5°C setup accordingly to 5°C higher temperatures. Temperature in each aquaria was controlled using aquarium heaters (Hagen Aquaclear 22952) and recorded at 30 min intervals by temperature loggers (WTA32-5+37, Onset Computer Corp., Bourne MA, USA). Each aquarium contained an initial number of ~ 630 scallops. Scallops sampling was conducted in April and August 2008 i.e. after 5 and 9 months exposure to the respective temperature regime.

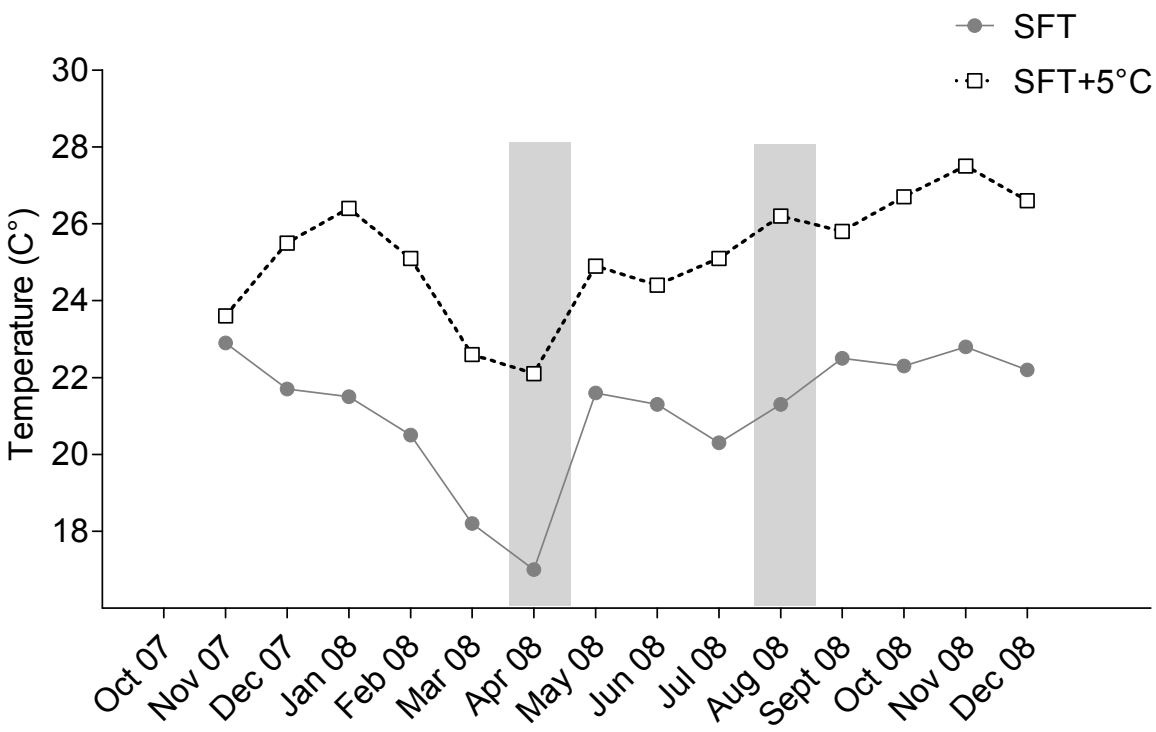


Fig. 1: Mean temperature pattern in the laboratory aquaria. Scallops were reared at simulated field temperatures in the laboratory (SFT) and 5°C above SFT (SFT+5°C). The upper temperature limit of *A. ventricosus* is 29°C (Sicard-González 2006). Grey bars mark the sampling times in April 08 after 5 months exposure and in August 08 after 9 months exposure to the respective temperatures

Scope for growth

The scope for growth (SFG) is essentially an energy balance measurement of an individual, determined from the difference of the energy absorbed from ingested food and the energy expenditure via respiration and excretion. It provides an estimation of the production for somatic growth and is a measurement of the quantitative energy status of the animal (Widdows 1976) (Widdows and Johnson 1988).

The determination of scope for growth (SFG) was assessed after (Ivelev 1945), (Winberg 1960) and (Warren and Davis 1967) and resumed in Sicard (2006):

SFG = AR- (RR + ER)

where SFG is the scope for growth, AR the absorption rate, RR the respiration rate and ER the excretion rate.

The physiological rates (AR, RR, ER) are converted to energy units (Joules h^{-1}) and expressed as Joules g dry $weight^{-1}$ h^{-1}.

Previous to the measurements, all animals were kept at the respective treatment temperature and salinity in 70 L aquaria with filtered seawater for 2 days until defecation ceased. After 2 days, animals were placed in 700 ml chambers connected to a multi channel flow-through system (60 ml min^{-1}). Animals were allowed to acclimate in the chambers for 2 h in filtered seawater at the respective treatment temperature condition. Following acclimation, food absorption (AR), respiration (RR) and excretion rates (ER) were then determined in April 08, and in August 08 for the calculation of scope for growth.

Absorption rate measurement:

After 2 h acclimation period, filtered inflowing seawater was enriched with *Isochrisis galbana* cells to a concentration of 60.000-70.000 cells ml^{-1}.

The absorption rate was calculated as follows:

$AR = IR \times AE$

where IR is the ingestion rate (ingested cells h^{-1}) and AE the absorption efficiency (%). After 3 hours feeding, a sample of 10 ml was taken from the outflow water. Samples were collected from each chamber and the cells were counted using an electronic cell counter (Coulter Cell Counter, Multisizer 3, Beckham, Coulter, Fullerton, CA, USA). Cell concentrations were the mean of 3 counts. Ingestion rates per unit time was calculated using the following equation:

$IR = [(Cb - Ce)] \times F$

where Cb is the cell concentration in blank chamber (cel ml^{-1}), Ce the cell concentration in the experimental chamber (cel ml^{-1}) and F the water flux through the chamber (ml h^{-1}). In order to relate the ingestion rate to the dry tissue weight, the whole tissue of the individual scallops was removed at the end of the experiment and dried for 48 h at 60°C. Ingestion rates were expressed as ingested cells in ml g dry $weight^{-1}$ h^{-1}. Ingestion rates were subsequently converted to energetic units using the energetic equivalent for organic matter (POM) of 23500 J g^{-1} (Widows et al. 1979) and the weight of *Isochrisis galbana* 28.5 pg cel^{-1}(Lora-Vilchis and Doktor 2001).

The absorption efficiency (AE) was measured using the ratio method of (Conover 1996) which represents the efficiency by which organic matter is absorbed from the ingested food. It assumes that an animal can absorb the organic matter but not the inorganic fraction. The Conover method is based on the ration of ash free dry weight: dry weight of food and faeces:

$$AE = (F-E) / (1-E) \times F$$

Where F is the organic content food / dry mass food, E is organic content feces / dry mass feces

The organic content of food and feces is equivalent to their ash free dry mass (AFDM). AFDM and dry mass of *Isochrisis galbana* are 24.7 and 28.5 pg cel^{-1} respectively and were obtained from Lora-Vilchis and Doctor (2001). The AFDM and dry mass of the feces was measured by filtering the chamber-water through pre-combusted glass fiber filters (500°C). Filters were washed with a 3% ammonium solution to eliminate salts and dried at 65°C for 24 h for dry mass determination and combusted at 500°C to obtain AFDM. Since unfiltered microalgae and feces can be mixed in the chamber, the AFDM of algae not absorbed by scallops was calculated and subtracted from total feces AFDM.

Respiration rate measurements

The respiration rate was determined simultaneously to ingestion rates by calculating the difference in oxygen values measured between the in- and out-flowing water in the chambers. In April, the animals were too small to reliably measure respiration, so that 8

replicate measurements of 3-5 equally sized animals were undertaken per treatment. In August, physiological rates were measured individually. Care was taken to eliminate all air bubbles from the system prior to respiration measurements. Oxygen concentration was measured using fiber-optical oxygen optodes of 50 µm diameter (PreSens GmbH, Regensburg Germany) calibrated with air bubbled water (100 % O_2 = 21 kPa) and water saturated with sodium sulfite to deplete oxygen (0 % O_2 = 0 kPa). The oxygen consumption was measured over 5 min in each chamber and three replicate measurements were made per chamber. A blank chamber was run without animals to correct for microbial and microalgal respiration.

The rate of oxygen consumption was calculated as follows:

$$RR = (\%\ O2e - \%\ O2b) \times F$$

were % O_2e is the percent oxygen in the experimental chamber and % O_2b is the percent oxygen in the blank chamber and F is the water flux trough the chamber (ml h^{-1}). Percent oxygen was transformed to mmoles of dissolved oxygen in seawater, using known values of oxygen solubility according to Benson and Krause (1984) and converted to mg O_2 by a conversion factor of 33.2 mg $mmol^{-1}$ (Brey 2001). The respiration rate was expressed as µg O_2 g dry weight $^{-1}$ h^{-1} and transformed to energetic units considering that 1 mg consumed O_2 is equivalent to 14.1 Joules (Elliot and Davison 1975).

Excretion rate measurement

Excretion rate was calculated as the difference of nitrogen content in the chambers respective to a blank chamber. Tree 2 ml samples from the outflow water of each chamber were taken in eppendof tubes and stored at -80°C previous to analysis. Ammonium concentration (mg NH4 ml^{-1}) was estimated by the phenol hypochlorid method of (Solorzano 1969) adapted to microplates by Hernández-López and Vargas-Albores (2003).

Excretion rate was measured as follows:

$ER = (ACe - ACb) \times F$

where ACe is the ammonium concentration in the experimental chamber (mg NH_4 ml^{-1}) and ACb is the ammonium concentration in the blank chamber (mg NH_4 ml^{-1}) and F is the water flux trough the chamber (ml h^{-1}). The excretion rate was determined as mg NH_4 g dry $weight^{-1}$ h^{-1} and transformed to energetic units considering that 1 mg NH_4 is equivalent to 7.37 (Logan and Epifanio 1978).

Biochemical analysis

At each sampling time mantle, muscle and gill of additional 8 to 10 scallops from each treatment were dissected, weighed and frozen by immersion in liquid nitrogen for biochemical analysis. Tissues were stored at – 80°C until analysis.

Antioxidant enzyme assays

Activities of catalase (CAT), superoxide dismutase (SOD), were measured with spectrophotometric assays at 25°C in a Beckman DU 640 spectrophotometer.

Enzyme assay conditions were as follows:

CAT and SOD: samples were homogenized by hand with a glass homogenizer on ice in 1:20 (w/v) in 50 mM phosphate buffer (pH 7.5) containing 1mM EDTA and 1 mM PMSF. Homogenates were centrifuged at 15000×g for 15 min at 4°C and the supernatant immediately used for CAT and SOD activity measurements.

Catalase activity (EC 1.11.1.6) was determined after Aebi (1984) by recording the time of H_2O_2 decomposition, resulting in a decrease of absorption from 0.45 to 0.4 at 240 nm (1 unit). Working solution (20 mM H_2O_2, 100 mM phosphate buffer) and sample were mixed in a cuvette and the change in absorbance was recorded every 15 s for 3 min. Enzymatic activity was expressed as units CAT g^{-1} fresh weight. One unit of catalase is defined as the amount of enzyme necessary to reduce 1 µml of H_2O_2 per min. Results are reported as units CAT g^{-1} fresh weight. Total SOD (EC 1.15.1.1) activity was determined by the method of Susuki (2000). The xanthine/xanthine oxidase (X/XO) system was used to generate O_2•- which reacts with nitroblue tetrazolium (NBT). 1.45 ml Working sodium solution (50 mM sodium-carbonate buffer, 0.025 mM NBT, 1mM Xantine, 0.1 mM EDTA), 25 µl XO (0.1 U ml^{-1} in 2 M ammonium sulfate) and 25 µl homogenized sample or blank were mixed in a cuvette and the change in absorbance at 560 nm recorded for 5 min every 30 sec. One unit of SOD is defined as the amount of enzyme necessary to inhibit the reduction of NBT by 50 %. Results are presented as units SOD per g^{-1} fresh weight.

Oxidative damage measurements

Lipid peroxides: Lipid peroxides are known to produce a variety of intermediate substances including malondialdehyde (MDA). MDA reacts with thiobarbituric acid (TBA) under acidic pH and elevated temperatures. Lipid peroxides were measured using the generation of MDA/TBA adducts (thiobarbituric acid reactive substances, TBARS, assay) to quantify MDA formation. TBARS were measured following the method of Persky et al. (2000). Samples were homogenized by hand in a glass homogenizer on ice in a 1:20 (w/v) saline solution (0.9%) at pH 7.0. 0.8 M HCl in 12.5% trichloroacetic acid (TCA) was added to 250 µl of homogenate prior to the addition of 1% TBA. Samples were incubated for 10 min in a 90°C water bath, cooled to room temperature and centrifuged at 1500 × g for 10 min at 4°C. TBARS level in each sample was measured at 535 nm. TBARS concentrations were derived from a standard curve and the values expressed as TBARS nmolar equivalents per g^{-1} fresh weight.

Protein oxidation (carbonyls): The detection of protein carbonyls was carried out after Levine et al. (1990). Carbonyls react with the carbonyl specific reagent 2,4-dinitrophenylhydrazine (DNTP) and, after precipitation with trichloroacetic acid (TCA), carbonyls can be measured spectrophotometrically. Samples were homogenized by hand in a glass homogenizer on ice in 5 % sulfosalisilic acid and centrifuged at 10000 × g for 15 min at 4°C. The supernatant was discarded and the pellet incubated at room temperature for 1 h with 500 µl 10 mM DNTP. Sample blanks contained 500 µl 2 M HCl instead of DNTP. During the incubation, samples were mixed every 15 min. After 1 h, 500 µl 20% TCA were added to samples and blanks to precipitate the protein and centrifuged at 10000 × g at 4°C for 5 min. The protein pellet was washed 3 times with 1 ml ethanol: ethylacetate (1:1),

resuspended in 1 ml 6 mM guanidine hydrochloride and incubated for 30 min at 37°C for 15 min. The samples were centrifuged at 10000 × g at 4°C for 5 min, and the supernatants of samples and blanks measured in quartz cuvettes at 360 nm. The amount of carbonyls was estimated as the difference in absorbance between samples and blank using a molar extinction coefficient of carbonyls $\varepsilon = 22{,}000\ cm^{-1}\ M^{-1}$. Amount of carbonyls was assessed as nmol per mg^{-1} protein measured in the same samples using the Bradford method (Bradford 1976).

Statistics

The effect of tissue size on antioxidant enzyme activities, protein carbonyls and TBARS was tested by power regression of the respective parameter on tissue wet mass. For the effect of size on physiological rates, (clearance, respiration and excretion rate) whole animal dry weight was used. Statistical analyses were performed with GraphPad Prims 5 Software (La Jolla, California, USA). One-way ANOVA with *post-hoc* test was used to test for differences in antioxidant enzyme activity (SOD and CAT), tissue damage (protein carbonyls and TBARS), between different tissues of same treatment and sampling date. Unpaired t test was used to indicate differences within same tissue and treatment but different sampling dates. Prior to any analysis, data were tested for normality and homogeneity of variance and if assumptions were not met, analyzed by non parametric ANOVA (Kruskal-Wallis) or t-test (Mann-Whitney tests).

RESULTS

Scope for growth (SFG)

The individual ingestion rate (ml h^{-1}), oxygen consumption rates (mg O_2 h^{-1}) and excretion rates (mg NH_4 h^{-1}) were shown to depend on tissue size. The size dependency of each physiological rate can be described by the power function:

Clearance rate: $4.53 \times W^{0.87}$ $r^2 = 0.87$ $N = 32$

Respiration rate: $3.4 \times W^{0.82}$ $r^2 = 0.80$ $N = 32$

Excretion rate: $3.7 \times W^{0.4}$ $r^2 = 0.45$ $N = 32$

In order to remove the size effect between both groups (SFT and SFT+5°C), each rate was corrected according to:

$$Rate = Rate'(W_{mean}/W)^b$$

where Rate and Rate´ are corrected and observed values respectively, W is the observed individual tissue wet mass, W_{mean} the mean tissue dry mass of the all animals (1 g) and b the calculated scaling coefficient for each rate.

A common scaling coefficient was calculated for both sampling times (after 5 and after 9 months exposure) as no significant differences were found between the scaling coefficients of each sampling times in any of the rates.

Each physiological rate was then converted to energy equivalents (J h^{-1}) in order to calculate the scope for growth.

The excretion rate was similar between SFT and SFT+5°C treatments and the two time points (April and August) (Table 1) and did not affect SFG. Absorption and respiration rates had a greater influence on SFG and both rates were significantly higher at elevated temperatures at both sampling times (Table 1). In April, energy expenses (respiration rates) ranged 2.4 times higher and energy absorbed by food (absorption rates) 1.4 higher in SFT+5°C compared to SFT scallops (Table 1). The high energy expenses however resulted only in a slightly and insignificant elevated SFG in the SFT+5°C relative to the SFT group after 5 months of high temperature exposure. In August, the energy expenditure by respiration was 1.4 and the absorption rate 1.5 times higher in the SFT+5°C compared to the SFT group (Table1). The somehow lower energy expenditure by respiration in the SFT+5°C in August resulted in a higher SFG in the SFT+5°C compared to the SFT group.

Biochemical analysis

Neither CAT, SOD activity nor TBARS or protein carbonyl concentrations showed significant size dependence in muscle, mantle or gill tissue within the different treatments. Hence, the uncorrected data were used for the analysis of differences between treatments and sampling time.

Antioxidant capacities

In April, scallops exposed to SFT+5°C had higher CAT and SOD activities in mantle muscle and gill tissues compared to SFT group (Kruskal Wallis, $p < 0.05$) (Fig. 2A, B).

Capacities of both antioxidant enzymes increased in mantle and gills of the SFT treatment between April and August, whereas in the SFT+5°C group, CAT and SOD activities in these tissues remained unchanged (CAT gills) or even decreased (CAT mantle and SOD mantle and gills). CAT and SOD capacities in muscle ranged higher in the SFT+5°C group in both sampling times (Mann-Whitney t-test < 0.05).

Oxidative damage markers

Protein carbonyls and TBARS concentrations followed a similar pattern as antioxidant capacities. Hence, the SFT+5°C group had the highest protein carbonyls concentrations in all tissues in April (Kruskal Wallis, $p < 0.05$) (Fig. 2C). TBARS concentrations were also higher but reached significance only in gills (Kruskal Wallis, $p < 0.05$) (Fig. 2D). Between April and August, protein carbonyls and TBARS levels in mantle and gills increased in SFT scallops and decreased in SFT+5°C (Mann-Whitney t-test $p < 0.05$). In muscle, protein carbonyls remained higher in the SFT+5°C group in both sampling dates (Mann-Whitney t-test $p < 0.05$) while no differences in muscle TBARS concentrations between SFT and SFT+5°C neither for April nor for August were found.

Table 1: Absorption rate (AR), respiration rate (RR), excretion rate (ER) and scope for growth (SFG) of *A. ventricosus* expressed as J h^{-1} for a standard animal of 1 g under simulated field temperatures in the laboratory (SFT) and 5°C above SFT. Values are means ± SD. Lower case letters represent significant differences between the two sampling times within the same treatment and capital letters are used for differences between different treatments among animals of the same sampling time (t test $P < 0.05$).

	AR (J^{-1} h^{-1})		**RR (J^{-1} h^{-1})**		**ER (J^{-1} h^{-1})**		**SFG (J^{-1} h^{-1})**	
	SFT	**SFT+5°C**	**SFT**	**SFT+5°C**	**SFT**	**SFT+5°C**	**SFT**	**SFT+5°C**
April	$18.4 \pm 3.4^{a\,A}$	26.2 ± 6.4^{aB}	$10.9 \pm 3.2^{a\,A}$	25.8 ± 5.2^{aB}	0.035 ± 0.002^{aA}	0.032 ± 0.009^{aA}	4.7 ± 1.6^{aA}	5.2 ± 2.6^{aA}
August	41.6 ± 5.3^{bA}	62.5 ± 7.4^{bB}	29.9 ± 3.5^{bA}	40.6 ± 4.2^{bB}	0.032 ± 0.009^{aA}	0.056 ± 0.04^{aA}	13.9 ± 5.2^{bA}	24.7 ± 3.9^{bB}

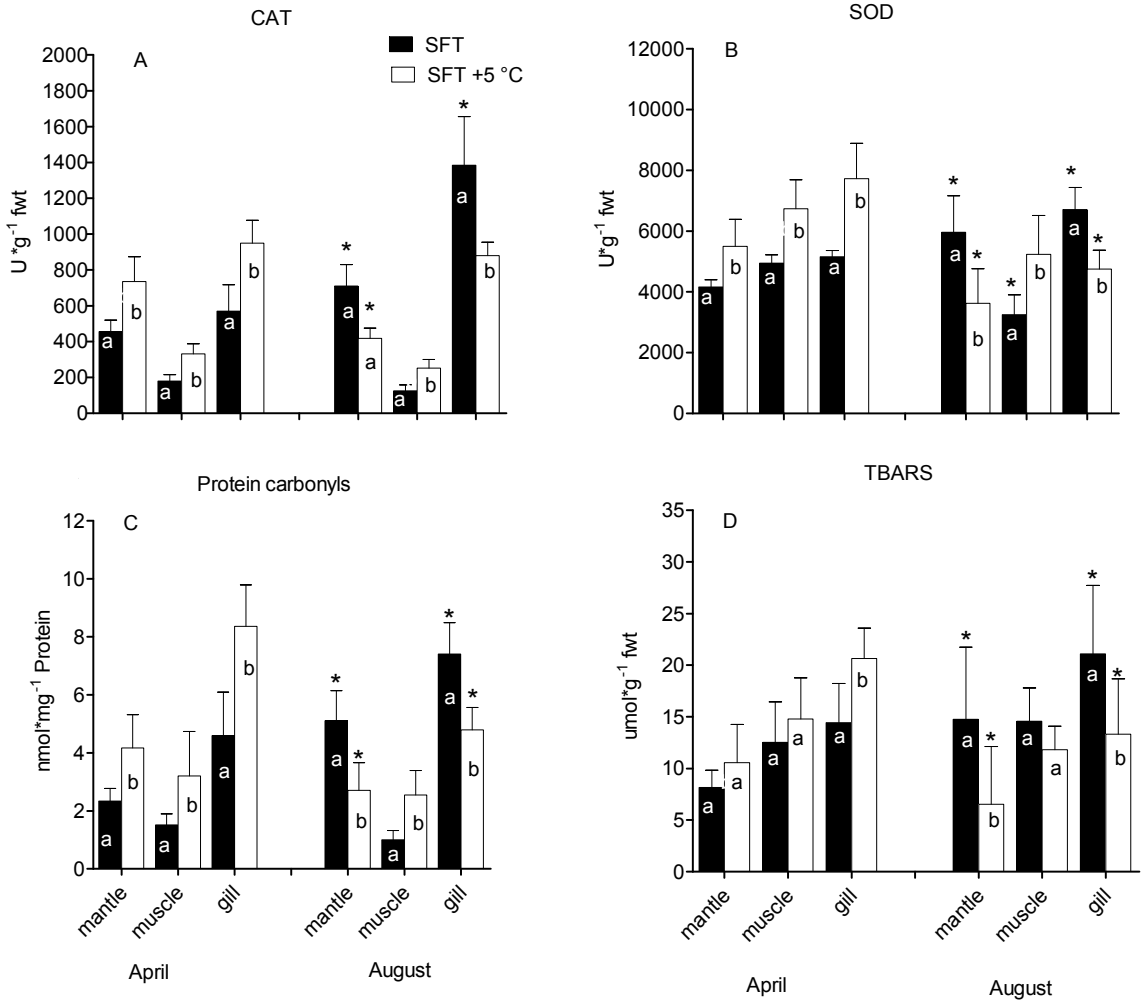


Fig. 2: Enzyme activity of (A) catalase (CAT), (B) superoxide dismutase (SOD) (units g^{-1} fwt), (C) protein carbonyls (nmol mg^{-1} protein) and (D) TBARS content (µmol g^{-1}fwt) in mantle, muscle and gill tissues of *A. ventricosus* in April and August 2008. Scallops were reared at simulated field temperatures in the laboratory (SFT) and 5°C above SFT (SFT+5°C) Values are means ± SD (*N*=8-10). Different letters indicate differences between different treatments within same sampling date and tissue (ANOVA, $p < 0.05$). *Asterisks* (*) indicate differences within same tissue and treatment but between different sampling dates (t test, $p < 0.05$).

DISCUSSION

The exposure of *A. ventricosus* to elevated but subcritical temperatures (SFT+5°C) for 5 months enhanced individual metabolic rates which is reflected by the higher respiration and absorption rates relative to the control group (SFT). According to the rate of living theory (Pearl 1928), increased energetic demands conjunctly increase ROS production and enhance accrual of somatic damage which may compromise future survival in a sense of "living fast, dying young" (Speakman et al. 2002). Indeed, concomitant to the elevated metabolic rates, scallops exhibited higher concentrations of protein carbonyls and TBARS (lipid peroxidation) in gills, mantle and muscle tissues and also higher mortality rates as shown in our previous analysis of growth rates and mortality using the same group of scallops (Guerra et al. 2011). Scallops may compensate the higher ROS production by increasing their antioxidant capacities; however, it seems that the increase in two key antioxidants (SOD and CAT) in the SFT+5°C group failed to counterbalance the damaging effects of ROS.

In *A.ventricosus,* absorption rate was high enough to support growth without utilizing own energy reserves shown by the positive scope for growth after 5 months of warm exposure. However, even with the enhanced absorbtion rates in SFT+5°C scallops, scope for growth was similar compared with the control group (SFT). This may happen when energy expenditure by respiration exceed the energy absorbed by food leading to a lower energy output and thus, lower growth efficiency related to the high respiration rates. It is possible that the high metabolic requirements to enhance cellular maintenance mechanisms such as antioxidants, compromised energy available for growth even if higher antioxidant capacities failed to counterbalance the oxidative damage.

Altogether, it seems that *A. ventricosus* possess a low capacity to adapt to elevated temperatures so that a constant increase by 5°C, even if temperature is kept below the critical temperature for this species, will exacerbate oxidative stress in a variety of tissues. As shown in other invertebrates, high temperatures affect mitochondrial functionality and exacerbate ROS production leading to damage in mitochondrial proteins and lipids (Abele et al. 2002; Heise et al. 2003; Abele and Puntarulo 2004). Together with high-energy expenditure by respiration, this can decrease energy production and affect the individual growth efficiency. The intertwined relation of high susceptibility to oxidative stress and a relative low energy efficiency could finally have contributed to higher mortality rates at elevated temperatures as previously reported (Guerra et al. 2011).

As we found that temperature was directly linked to metabolic rates in *A. ventricosus*, we would have expected that the seasonal temperature elevation of 4°C in the SFT and in the SFT+ 5°C group from April to August 2008 (5 to 9 months exposure) further accelerates energy metabolism and intensifies oxidative damage in both treatments. We also expected to observe a higher impact in the SFT+5°C group. In the SFT scallops, indeed respiration rates as well as protein carbonyls and lipid peroxides increased in gill and mantle tissue despite the enhanced capacities of SOD and CAT in these tissues. It is worth noting however, that time may have conjunctly influence the parameters so that it is not possible to discern whether the changes observed in the SFT group between April and August are due to the increase in temperature, an increase in scallops age or due to a combination of both factors. Interestingly, oxidative damage in the SFT+5°C scallops showed the inverse pattern compared to the SFT group despite a similar increase in temperature. Hence, in August, after 9 months exposure to constant elevated temperatures, SFT+5°C exhibited

lower levels of protein carbonyls and TBARS in mantle and gill in respect to the previous sampling and also in respect to the SFT group. Concomitant, capacities of SOD and CAT were lower in mantle and gill after 9 months exposure indicating that there was a lower need to fight off ROS in these tissues. Further, different as after 5 months exposure, SFT+5°C scallops also exhibited a higher scope for growth compared to SFT scallops. The higher scope for growth was attributed to a relative lower energy expenditure by respiration compared to the previous sampling while keeping high food absorption rates. This corroborates results in the scallop *Argopecten purpuratus* in which respiration rate was the major component that affected scope for growth (González et al. 2002) The somehow improved physiology of the SFT+5 °C scallops after 9 months exposure to the elevated temperatures is difficult to explain. One reason for this improvement may be the significant higher mortalities in the SFT+5°C group compared to SFT scallops between April and August (Guerra et al. 2011) which could have provoke a stronger selection of scallops with better physiological condition, i.e., individuals with less oxidative damage, higher energy efficiency and reduced requirements for antioxidant protection.

From an ecological point of view, *A. ventricosus* might in general have a low capacity to endure long-term elevated temperatures but high mortalities can counterbalance such negative temperature effects by natural selection of less affected individuals; however, selection may result only at cost of population densities.

ACKNOWLEDGEMENTS

The authors would like to thank Mario Osuna, Ernesto León, Carmen Abundis and Stefan Koengistein for the technical support, and for assistance in the hatchery and animal maintenance. We further thank Norma Olguín, Orlando Lugo and Stefanie Meyer for assistance in the laboratory. In addition, we would like to thank the Cooperativa del Puerto de San Carlos, Baja California Sur for the supply of scallops. This research was supported by the DAAD grant D/06/47197 and the SAGARPA- CONACyT project 11947.

4.2. Manuscript 2: The effect of predator exposure and reproduction on metabolic and oxidative stress parameters in the Catarina scallop *Argopecten ventricosus*

Citlali Guerra, Tania Zenteno-Savín, Alfonso N. Maeda-Martínez, Doris Abele,

Eva E.R. Philipp

ABSTRACT

Predation is an ecological factor that is known to impact physiological processes such as metabolic rates, growth and reproduction, and may also influence a prey´s susceptibility to oxidative stress. We investigated how prolonged exposure to predators modulates metabolic rate, tissue specific antioxidant defense and oxidative damage in the short-lived (2 years maximum lifespan) epibenthic swimming scallop *Argopecten ventricosus.* Scallops exposed to predators featured lower standard metabolic rates and lower antioxidant defense levels (superoxide dismutase and catalase), but also lower oxidative damage (protein carbonyls and TBARS = lipid peroxidation) in gills and mantle compared to predator-free individuals. In swimming muscle however, antioxidant levels and oxidative damage were higher in predator-exposed scallops. When predator-exposed scallops were on the verge of spawning, levels of oxidative damage increased in gills and mantle in spite of a parallel

increase in antioxidant defense in both tissues. Pre-spawning, levels of oxidative damage increased also in swimming muscle whereas antioxidant capacities in this tissue decreased. Post-spawned scallops recuperate antioxidant capacities and oxidative damage levels similar to those before maturation. Our results suggest that predator exposure and reproduction modulate antioxidant defense and oxidative damage in a tissue specific manner. While predators induce oxidative damage in scallop's adductor muscle but not in mantle and gill tissues, reproduction increases oxidative damage levels in all somatic tissues. Nevertheless, oxidative damage seemed to be transient as scallops exhibit resilience capacities after spawning.

Keywords: *Argopecten ventricosus*, oxidative stress, predation, reproduction

INTRODUCTION

Sublethal effects of predation risk can markedly shape the morphology and life history of the prey on the individual and population level (Luttbeg and Kerby 2005). Exposure to sea star predators, or even to their chemical signals, induces for example a violent escape response of swimming and jumping movements in scallops (Thomas and Gruffydd 1971; Thompson et al. 1980; Grieshaber et al. 1994; Tremblay et al. 2006; Schmidt et al. 2008). A chronic exposure of animals to the presence of predators in their natural habitat can be anticipated to result in higher basal swimming activity and oxygen turnover. Although there is no strict relationship between the rate of oxygen reduction in mitochondria and the production of reactive oxygen species (ROS) (Buttemer et al. 2010), accelerated ROS production during strenuous exercise was found to increase oxidative stress in fish and

mammals (Ji 1993, 1999; Asami et al. 1998; Aniagu et al. 2006, Power and Jackson 2008 for review). Superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$) can oxidize and damage cellular constituents, such as membrane lipids, proteins and DNA. Antioxidants such as superoxide dismutase (SOD) that catalyses the dismutation of $O_2^{\bullet-}$ to H_2O_2 and catalase (CAT) which catalyses the reaction of H_2O_2 to water and oxygen, mitigate the oxidative damage to some extent (Storey 1996; Abele and Puntarulo 2004). Although escape swimming in scallops is eventually fueled by anaerobic glycolysis, higher ROS production may increase during phases of increased oxygen uptake when recovering from exhaustive swimming (Guderley and Poertner 2010). If ROS are not counterbalanced by cellular maintenance mechanisms, repeated escape swimming and recovery may result in the accumulation of oxidative damage with negative fitness consequences for the prey. Detailed studies that pinpoint the link between predator exposure and oxidative stress in marine ectotherms are however missing. In the only two studies addressing this topic in ectotherms to date, Slos and Stocks (2008) found increased oxidative stress in damselflies (*Enallagma cyathigerum*) after 5 days exposure to predators, which they attributed to the higher metabolic rates and lower investment in antioxidant defense. In a second study with the damselfly *Lestes viridis*, 10 days exposure to predator risk however led to an increase in antioxidative defense mechanisms, that depended on food levels and sex (Slos et al. 2009). So far, it is not really understood whether and by which mechanisms sublethal exposure to predators induces oxidative stress in marine ectotherms.

Increased oxidative stress has also been proposed to be one of the costs arising during reproduction (for review see Constantini 2010). Accumulation of oxidative damage and decrease in antioxidant protection as a cost of reproduction have been demonstrated in

different species such as fruit flies, birds, sheep, and also in bivalves (Wang et al. 2001; Alonso-Alvarez et al. 2006, 2007; Bize et al 2008; Nussey et al. 2009; Soldatov et al. 2008). In bivalves, the initiation of gametogenesis as well as the spawning event is strongly governed by environmental conditions (Vahl 1985; MacDonald and Bayne 1993; Jokela and Mutikainen 1995) with temperature and food availability being the major modulators (Barber and Bayne 1983). As gametogenesis and environmental factors can modulate oxidative stress parameters, it is difficult to disentangle the sole oxidative costs of reproduction.

In the present study, we investigated the tissue specific response of cellular oxidative defense and damage parameters, respiration rate and metabolic enzyme activity to predator presence in *A. ventricosus*. The short lifespan (2 years) (Keen 1971) and the long-standing use of the Catarina scallop in aquaculture enabled us to study the effect of predator exposure over several months within the same population of scallops. The long-term experiment include a reproductive cycle under controlled temperature and feeding conditions that allowed us to disentangle environmental effects from reproductive costs on the different parameters.

MATERIALS AND METHODS

Experimental animals and culture conditions

Argopecten ventricosus larvae were obtained and reared in the hatchery as described in detail in Guerra et al. (2011). Briefly, larvae were obtained from spawn of wild scallops and juveniles reared for 3 months in the hatchery until they reached 5-7 mm shell height.

Thereafter, scallops were maintained in a flow-through system consisting of 8 parallel 70 L-aquaria under constant water flow of 210 L day^{-1} and a salinity of 33-36 ppt. Each aquarium contained an initial number of ~ 630 scallops. Temperature in the aquaria was adjusted each month to natural conditions in the field to mimic seasonal temperature changes (Fig.1). Field temperatures were recorded at the Rancho Bueno estuary (geographical position: ´24°19´17,3´´N, 111°25´37,3´´W) located in Bahía Magdalena, Baja California Sur, Mexico, where optimal temperatures for *A. ventricosus* growth have been reported to occur (Sicard-Gonzàlez et al. 1999). The temperature in each aquaria was controlled using aquarium heaters (Hagen Aquaclear 22952) and recorded at 30 min intervals using temperature loggers (WTA32-5+37, Onset Computer Corp., Bourne MA, USA). The animals were fed *ad-libitum* using a 1:1 mixture of *Chaetoceros calcitrans* and *Isochrysis galbana*. The first sampling was performed at the end of April 2008 at an experimental temperature of 17°C when scallop mean shell height was 24 ± 5.3 mm. After this initial sampling, the blue crab *Callinectes sapidus,* which is a potential predator for *A. ventricosus* in their natural environment (Ciocco and Orensanz 2001), was introduced into the "predator-exposed treatment" i.e. in 4 of the 8 aquaria (one crab per aquarium). The crabs´ pincers were held together with rubber bands to prevent the crabs from eating the scallops. The crabs were fed squid every third night in a separate aquarium. Introducing *C. sapidus* into the aquaria immediately enhanced swimming activity in the scallops (personal observation). The effect prevailed throughout the entire 4 months of experimental period without observable signs of habituation in the scallops. The remaining 4 aquaria were maintained at the same temperature and feeding conditions as in the predator-exposed aquaria but without predators (predator-free treatment). After 4 months (August 2008),

predator-exposed and predator-free scallops were sampled again at an experimental temperature of 21.3°C.

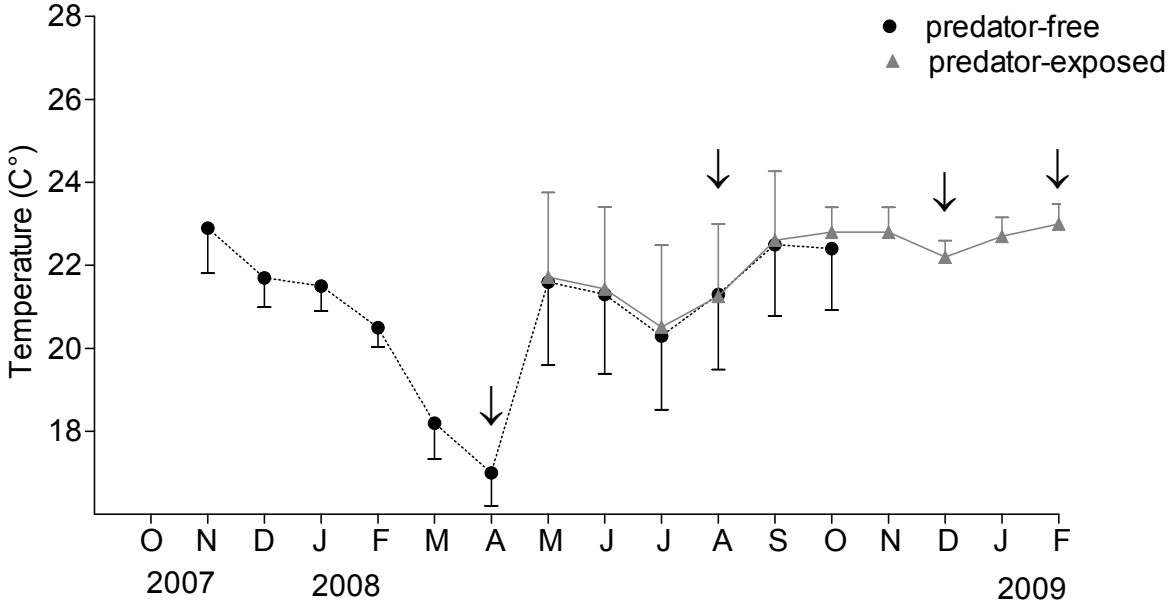


Fig. 1: Temperature pattern regulated in the laboratory. Arrows indicate time-points of sampling.

To investigate changes in oxidative stress during reproduction, it was planned to take samples of immature, pre-spawning and post-spawned individuals of both groups. However, the animals of the predator-free treatment spawned earlier than the predator-exposed group, and all animals in the predator-free group died directly after spawning. In contrast, in the predator-exposed scallops we did not observe increased post-spawned mortality and 8% of the initial population remained alive until the end of the experiment in February 2009. In this group, additional samplings were possible in December 2008 for pre-spawning and in February 2009 in post-spawned scallops. Temperature during this time span was kept at 22-23°C in order to discern differences in oxidative stress parameters related solely to reproduction (Fig. 1).

During each sampling, two to three scallops were taken from each of the 4 replicate aquaria and dissected into mantle, adductor muscle, gills and gonads. Tissues were weighted and mantle, adductor muscle and gills frozen in liquid nitrogen for biochemical analysis. Further 6-8 animals per aquarium were used for metabolic rate measurements.

Gonad index

The gonad index (GI) was determined on each sampling date and was calculated as follows: (gonad weight/ total shell weight) × 100 (see Lucas and Beninger 1989; Sarkis et al. 2006). A high GI is indicative of mature gonads and a lower GI reflects the onset of gametogenesis or spent gonads.

Enzyme assays

Activities of CAT, SOD, CS and ODH were measured spectrophotometrically at 25°C in a Beckman DU 640 spectrophotometer. Enzyme assay conditions were as follows:

CAT and SOD: Samples were homogenized by hand with a glass homogenizer on ice in 1:20 (w/v) in 50 mM phosphate buffer (pH 7.5) containing 1 mM EDTA and 1 mM PMSF. Homogenates were centrifuged at 15000 × g for 15 min at 4°C and the supernatant immediately used for CAT and SOD activity measurements.

CAT activity (EC 1.11.1.6) was determined after Aebi (1984) by recording the time of H_2O_2 decomposition, resulting in a decrease of absorption from 0.45 to 0.4 at 240nm (1 unit catalase). Working solution (20 mM H_2O_2, 100 mM phosphate buffer) and sample

were mixed in a cuvette and the change in absorbance was recorded every 15 s for 3 min. Enzymatic activity was expressed as units CAT g^{-1} fresh weight. One unit of catalase is defined as the amount of enzyme necessary to reduce 1 µml of H_2O_2 per min. Results are reported as units CAT g^{-1} fresh weight. Total SOD (EC 1.15.1.1) activity was determined by the method of Susuki (2000). The xanthine/xanthine oxidase (X/XO) system was used to generate $O_2^{\bullet-}$ which reacts with nitroblue tetrazolium (NBT). Working solution (50 mM sodium-carbonate buffer, 0.025 mM NBT, 1mM X, 0.1 mM EDTA), XO (0.1 U ml^{-1} in 2 M ammonium sulfate) and 25 µl homogenized sample or blank were mixed in a cuvette and the change in absorbance at 560 nm was recorded for 5 min every 30 sec. One unit of SOD activity is defined as the amount of enzyme necessary to inhibit the reduction of NBT by 50%. Results are presented as units SOD g^{-1} fresh weight.

CS and ODH: Samples were homogenized by hand in a glass homogenizer on ice in a 1:10 (w/v) buffer solution (50 mM imidazol, 1 mM EDTA, 1 mM reduced glutathione). Homogenates were sonicated 4 × 1 5 sec in a Branson Sonifier 450 (output control 4, duty cycle 40 %) at 0°C and centrifuged at 5000 × g for 15 min and 4°C. CS activity was measured after Sidell et al. (1987) by recording the absorbance increase of 0.25 mM 5´,5´-dithio-bis(2-nitro)benzoic acid (DTNB) in 75 mM Tris HCl (pH 8.0), 0.4 mM acetyl CoA and 0.4 mM oxalacetate at 412 nm. Activity was calculated using the extinction coefficient (ε_{412}) of 13.61 mM^{-1} cm^{-1} and expressed as units g^{-1} fresh weight. ODH activity was measured after Ballantyne et al. (1981). The decrease of NADH oxidation by ODH was recorded at 340 nm. 10-25 µl of supernatant were added to a measuring buffer containing 100 mM triethanolamine (pH 7.0), 30 mM pyruvate, and 55 mM arginine and the reaction

followed for 10 min. ODH activity was calculated using ε_{340} = 6.32 mM^{-1} cm^{-1} and expressed as units g^{-1} fresh weight.

Tissue damage

Lipid peroxides (TBARS): Lipid peroxides are known to produce a variety of intermediate substances including malondialdehyde (MDA). MDA reacts with thiobarbituric acid (TBA) under acidic pH and elevated temperatures. Lipid peroxides were measured using the generation of MDA/TBA adducts (thiobarbituric acid reactive substances, TBARS) to quantify MDA formation. TBARS were measured following the method of Persky et al. (2000). Samples were homogenized by hand in a glass homogenizer on ice in a 1:20 (w/v) saline solution (0.9 %) at pH 7.0. 0.8 M HCl in 12.5 % trichloroacetic acid (TCA) was added to 250 µl of homogenate prior to the addition of 1 % TBA. Samples were incubated for 10 min in a 90°C water bath, cooled to room temperature and centrifuged at 1500 × g for 10 min at 4°C. TBARS levels in each sample were measured at 535 nm. TBARS concentrations were derived from a standard curve and the values expressed as TBARS nmolar equivalents g^{-1} fresh weight.

Protein oxidation (carbonyls): The detection of protein carbonyls was carried out after Levine et al. (1990). Carbonyls react with the carbonyl specific reagent 2,4-dinitrophenylhydrazine (DNTP) and, after precipitation with TCA, carbonyls can be measured spectrophotometrically. Samples were homogenized by hand in a glass homogenizer on ice in 5 % sulfosalicylic acid and centrifuged at 10000 × g for 15 min at 4°C. The supernatant was discarded and the pellet incubated at room temperature for 1 h

with 10 mM DNTP. Sample blanks contained 2 M HCl instead of DNTP. During the incubation, samples were mixed every 15 min. After 1 h, 20 % TCA were added to samples and blanks to precipitate the protein and centrifuged at 10000 × g at 4°C for 5 min. The protein pellet was washed 3 times with 1 ml ethanol: ethylacetate (1:1) resuspended in 6 mM guanidine hydrochloride and incubated for 30 min at 37°C for 15 min. The samples were centrifuged at 10000 × g at 4°C for 5 min, and the supernatants of samples and blanks measured in quartz cuvettes at 360 nm. The amount of carbonyls was estimated as the difference in absorbance between samples and blank using a molar extinction coefficient of carbonyls (ε = 22,000 cm^{-1} M^{-1}). Amount of carbonyls was assessed as nmol mg^{-1} protein measured in the same samples using the Bradford method (Bradford 1976).

Measurements of standard and fed metabolic rate (SMR; FMR = SDA, specific dynamic action)

Oxygen consumption of 6-8 individual animals per treatment was measured with 50 µm diameter fiber-optical oxygen optodes (PreSens GmbH, Regensburg Germany) in 700 ml chambers connected to a multi-channel flow-through system (60 ml min^{-1}). In case the animals were too small for reliable respiration measurements, 8 replicate measurements of 3-5 equally sized animals were undertaken per treatment.

Prior to respiration measurements, *A. ventricosus* were maintained without food for 2 days, to eliminate effects of feeding on metabolic rates. Shells were cleaned from epibionts. Before starting the SMR measurements, animals were allowed to acclimate in the chambers for 2 h in filtered seawater at the respective temperature and salinity condition. Experiments

had been previously run to assure that this time was sufficiently long to allow the animals to accommodate to the measuring system. Oxygen optodes were calibrated using air-bubbled water (100 % O_2 = 21 kPa) and water saturated with sodium sulfite to deplete oxygen (0 % O_2 = 0 kPa). Respiration rate was calculated from the difference in oxygen values measured between the in- and outflowing water 3 times over 5 min each. A blank chamber was run without animals to correct for microbial respiration. After SMR measurements, fed metabolic rate (FMR), which includes the energy expenses (oxygen consumption) for burning ingested food, was determined. For this measurement, filtered inflowing seawater was enriched with *Isochrysis galbana* to a concentration of 60000-70000 cells ml^{-1}. Oxygen consumption was measured again 3 h after feeding. After the last measurement, scallops were dissected, and soft tissue dry mass determined after 2 days at 60°C. Percent oxygen was transformed to micromoles of dissolved oxygen in seawater, using known values of oxygen solubility, according to Benson and Krause (1984), and converted to mg O_2. Oxygen consumption of the predator-free and predator-exposed scallops sampled at the same time was expressed as the oxygen consumption per g of tissue dry weight (mg O_2 h^{-1} g^{-1} dw).

The size effect on respiration rates of the predator-exposed scallops sampled at different time points was corrected and expressed for a standard scallop of 1 g dry mass using a power regression:

$$VO_2 = VO_2' \, (W_{mean}/W)^b$$

where VO_2 and VO_2' are corrected and observed values respectively, W is the observed individual tissue wet mass, W_{mean} the mean tissue dry mass of the all animals (1 g) and b

the calculated scaling coefficient for each rate (Sukhotin et al. 2000). The scaling coefficient of b = 0.59 was derived from the log-log linear regression between oxygen consumption and whole animal dry weight ranging from 0.45 to 1.96 g dry mass. In contrast to the respiration rates, the different enzyme activities and oxidative damage parameters were not found to be dependent on body mass and were therefore calculated without correction.

Statistics

Data were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett's test for equal variances) prior to analysis and if necessary LOG-transformed. In the graphs or tables data are displayed as non-logarithmic values. Two-way ANOVA, followed by a Bonferroni post test**,** was used to test for tissue-specific differences in antioxidant and metabolic enzyme activity, oxidative damage parameters, muscle mass, shell size, as well as respiration rates between the predator-exposed and predator-free treatment measured in April and August. One-way ANOVA (Kruskal-Wallis test for non-Gaussian distribution) with Tukey´s post-hoc test (Dunn´s test) was used to test for differences in tissue-specific antioxidant enzyme activity, oxidative damage, metabolic enzymes and respiration rates in immature, pre-spawning and post-spawned scallops. In case of some rare cases when homogeneity of variances could not be achieved by transformation, a t-test with Welch´s correction was performed to test for significant differences between two treatments or time points. Statistical analyses were performed with Graph Pad Prism 5 Software (La Jolla, California, USA) and related software given on

http://www.graphpad.com/quickcalcs/posttest1.cfm for additional Bonferroni post test following two-way ANOVA in case of significant interaction.

RESULTS

Effect of predators on oxidative stress parameters, muscle capacities and metabolic rates

Antioxidant capacities and oxidative damage

After 4 month of treatment, the activity of both antioxidant enzymes, SOD and CAT (Fig. 2A) as well as the concentration of both oxidative damage markers, protein carbonyls and TBARS (Fig. 2B), were higher in gills and mantle tissue of predator-free compared to predator-exposed scallops. The opposite was found in the swimming muscle: predator-free scallops had lower catalase activity and slightly but not significantly lower SOD activity, as well as lower concentrations of protein carbonyls and TBARS compared to predator-exposed scallops. Compared to the first measurement in April, the values of the different parameters were higher in gill and mantle tissue in the predator-free scallops measured in August. In the predator-exposed scallops, values remained unchanged or even decreased in theses tissues with time. Again, the opposite pattern was found for the adductor muscle tissue (Fig. 2A, B).

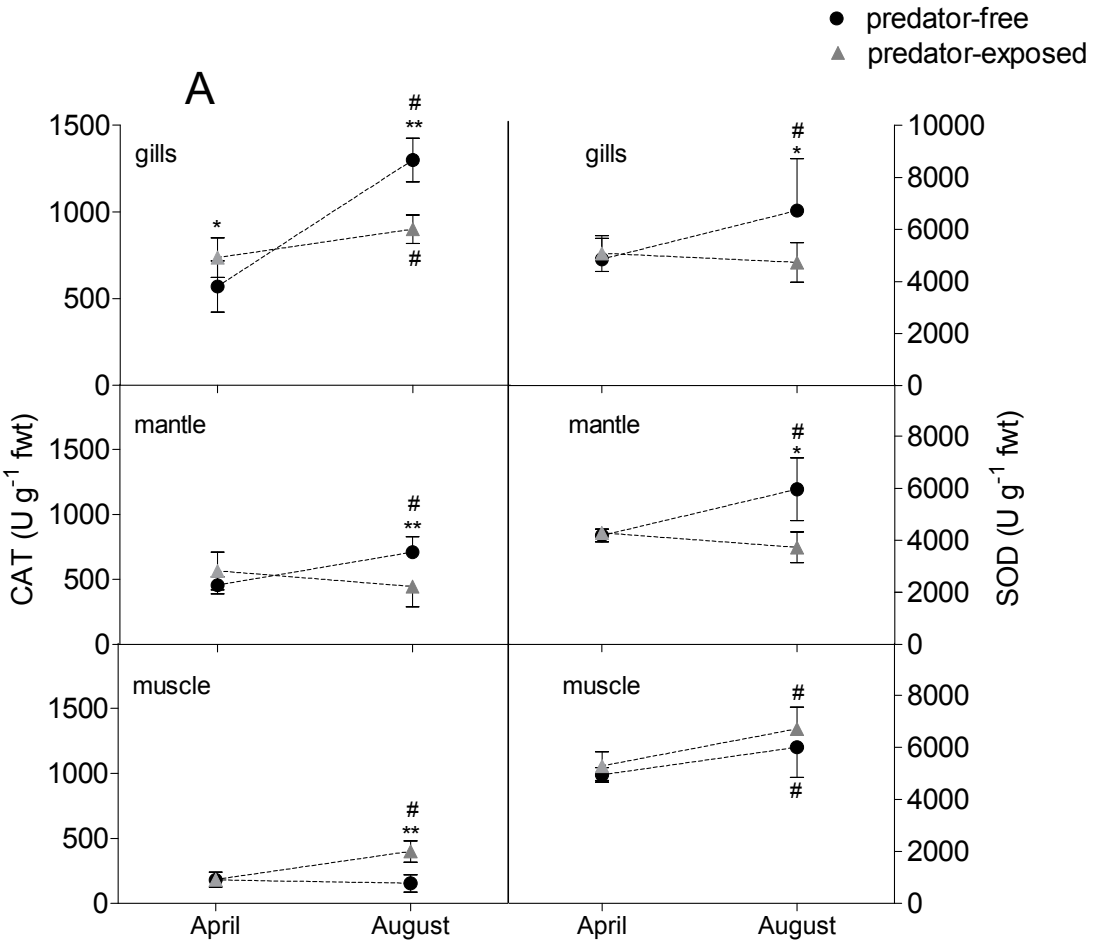


Fig. 2: (A) Enzyme activity of catalase (CAT) and superoxide dismutase (SOD) (units g^{-1} fwt) in gills, mantle, and muscle of *A. ventricosus* kept without predators (predator-free group) and with predators (predator-exposed group) for a time period of 4 months (April-August 2008). Values are means ± SD (N = 6-8 per group). * indicates significant differences between scallops reared without and with predators ($p < 0.001$ ** and $p < 0.05$ *), and # ($p < 0.05$) between the sampling in April and August within the respective treatment.

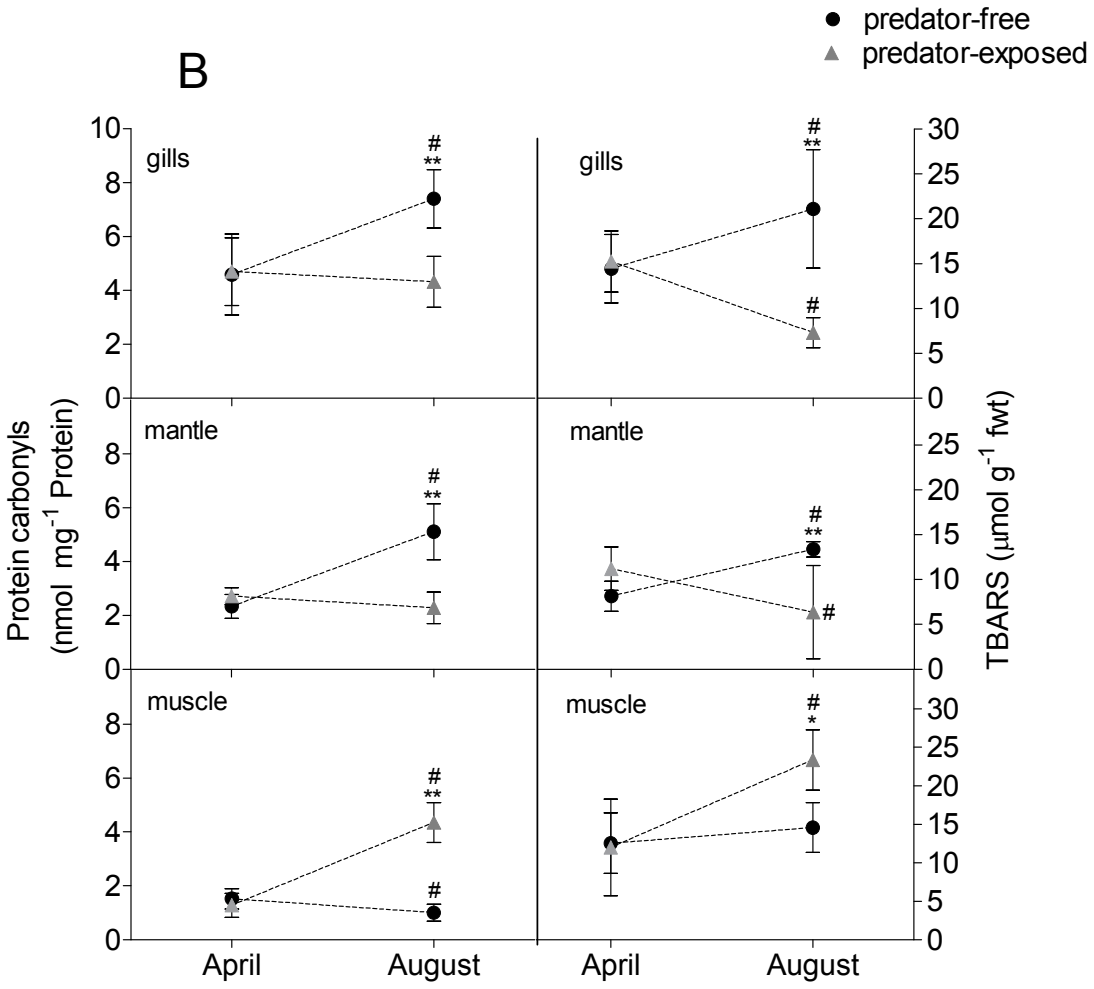


Fig. 2: (B) protein carbonyls (nmol mg^{-1} protein) and lipid peroxidation levels (TBARS, µmol g^{-1}fwt), in gills, mantle, and muscle of *A. ventricosus* kept without predators (predator-free group) and with predators (predator-exposed group) for a time period of 4 months (April-August 2008). Values are means ± SD (N = 6-8 per group). * indicates significant differences between scallops reared without and with predators ($p < 0.001$ ** and $p < 0.05$ *), and # ($p < 0.05$) between the sampling in April and August within the respective treatment.

Muscle mass, metabolic enzymes and metabolic rates

Muscle mass (g^{-1} fresh weight) was higher in the predator-exposed than predator-free scallops following 4 months exposure to predators, but shell sizes (shell height) were the same (Table 1). CS activities as marker for aerobic capacities in muscle were higher in predator-free compared to predator-exposed scallops (Fig. 3A), whereas the opposite was found for ODH activity, a marker of anaerobic capacity (Fig. 3B). From April to August, CS activities increased in predator-free scallops and remained stable in predator-exposed individuals. The opposite was found for ODH where activities increased in predator-exposed scallops and even decreased in predator-free individuals. Standard metabolic rate (SMR) was significantly higher in predator-free compared to 4 month predator-exposed scallops, whereas fed metabolic rate (FMR) was the same in both groups (Fig. 4).

Table 1: Adductor muscle weight (g^{-1} fwt) and shell height (mm) of *A. ventricosus* kept without predators (predator-free group) and with predators (predator-exposed group) for a time period of 4 months (April-August 2008). Values are means ± SD (N = 10-16). ***** indicates significant differences between scallops reared without and with predators ($p < 0.05$). # marks significant differences between the sampling in April and August within the respective treatment ($p < 0.05$).

	April		**August**	
	Predator-free	**Predator-exposed**	**Predator-free**	**Predator-exposed**
Muscle (g^{-1}fwt)	0.36 ± 0.19	0.30 ± 0.11	0.79 ± 0.25$^{\#}$	1.16 ± 0.35*$^{\#}$
Shell height (mm)	24.9 ± 4.45	23.3 ± 2.54	30.1 ± 1.94	30.9 ± 3.0

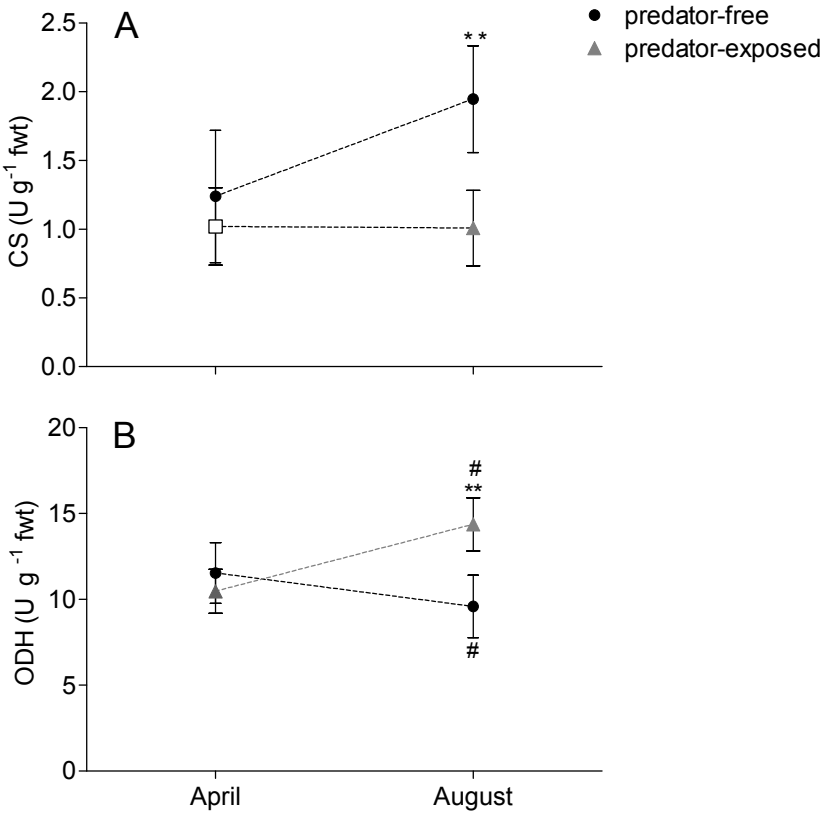


Fig. 3: (A) Citrate synthase (CS) and (B) octopine dehydrogenase (ODH) activities (units g^{-1} fwt) in the adductor muscle of *A. ventricosus* without predators (predator-free group) and with predators (predator-exposed group) for a time period of 4 months (April-August 2008). Values are means ± SD (N = 8-9 per group). * indicates significant differences between scallops reared without and with predators ($p < 0.001$ ** and $p < 0.05$ *), and # ($p < 0.05$) between the sampling in April and August within the respective treatment.

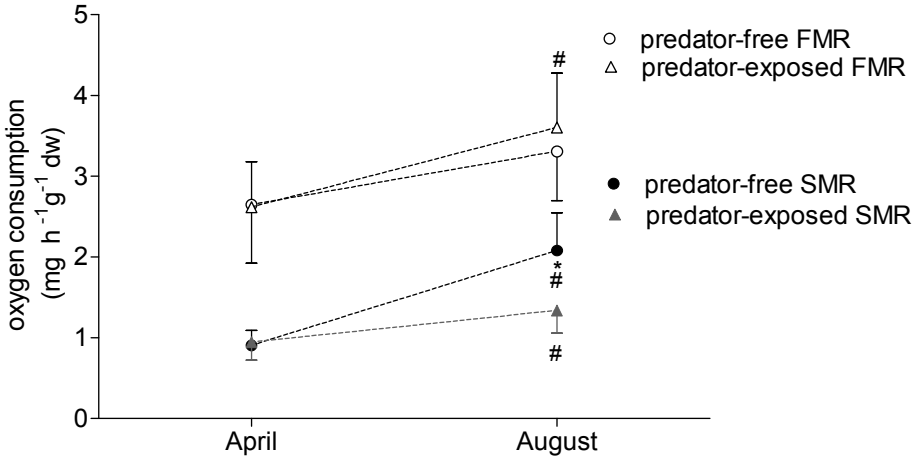


Fig. 4: Weight specific standard metabolic rates (SMR) and fed metabolic rates (FMR) in *A. ventricosus* kept without predators (predator-free group) and with predators (predator-exposed group) for a time period of 4 months (April-August 2008). Values are means ± SD (N = 6 per group). * indicates significant differences between scallops reared without and with predators (Mann-Whitney t-test $p < 0.05$) and # ($p < 0.05$) between the sampling in April and August within the respective treatment.

Effect of reproduction on oxidative stress parameters and metabolic rates

Antioxidant capacities and oxidative damage

The effect of reproduction on antioxidant enzyme activities, cellular damage and metabolic rates could only be investigated in predator-exposed scallops, because the predator-free scallops died after the first spawning before samples could be taken. Mortality did not increase during spawning in the predator-exposed group, which allowed us to sample pre-spawning and post-spawned individuals of this group. In the predator-exposed group, the gonad index peaked in December 2008 (pre-spawning) and was lowest in April (immature), August (immature) 2008 and February 2009 (post-spawned) (Fig.5).

Significant differences in oxidative stress parameters between immature, pre-spawning and post-spawned scallops were observed in all three investigated tissues. In gill and mantle tissue, antioxidant enzymes and oxidative damage markers reached maximum levels when animals were on the verge of spawning (corresponding with the peak in GI) and declined again after the spawning event (Fig. 6A, B, C, D). In contrast, in the adductor muscle activities of both enzymes were lowest in pre-spawning specimens and increased again in post-spawned scallops (Fig. 6A, B). Protein carbonyl levels in muscle tissue did not significantly change throughout the reproductive cycle (Fig. 6C). TBARS levels in muscle however were significantly higher during peak GI, also compared to values in gill and mantle tissue, and decreased in post-spawned scallops to lower values than in immature animals (Fig. 6D).

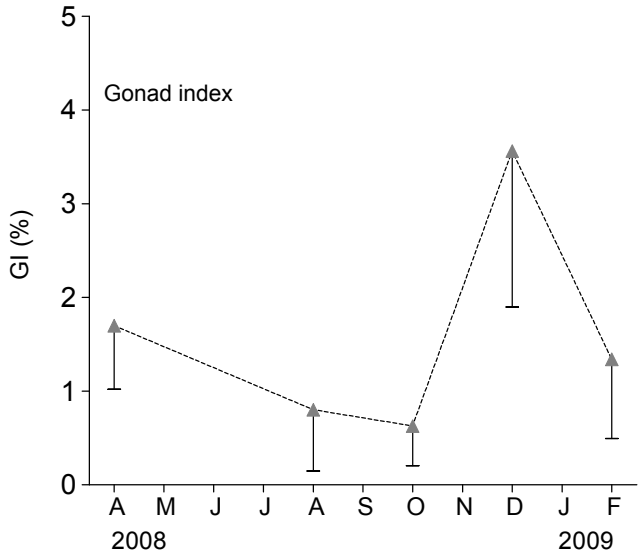


Fig. 5: Gonad- index (GI) of *Argopecten ventricosus* exposed to predator.

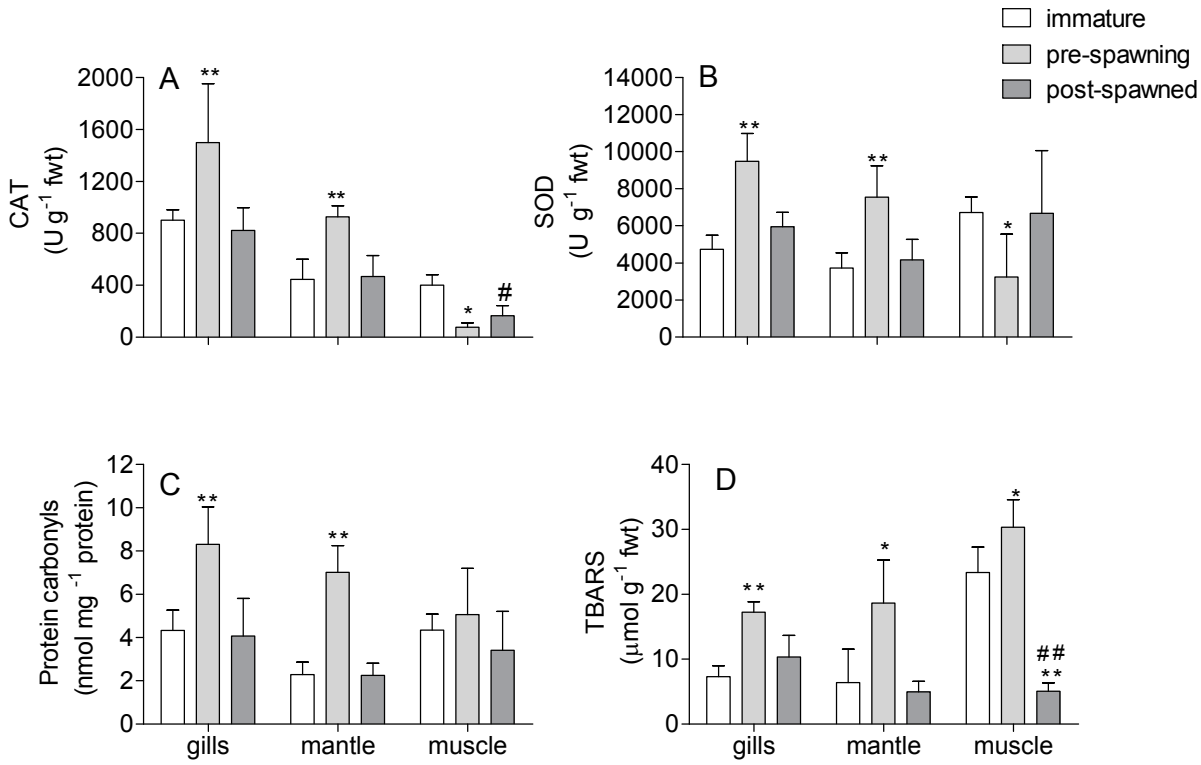


Fig. 6: (A) Catalase (CAT) and (B) superoxide dismutase (SOD) activities (units g^{-1} fwt), (C) protein carbonyl (nmol mg^{-1} protein) and (D) lipid peroxidation levels (TBARS, µmol g^{-1} fwt) in gills, mantle and muscle of predator-exposed *A. ventricosus* scallops. Reproductive stages were deduced from the GSI in Fig 5. Values are means ± SD (*N*= 6-8). * indicate differences between pre-spawning from immature and post-spawned; # indicates differences between immature and post-spawned within each tissue. Differences are set at $p < 0.001$##/** and $p < 0.05$ #/*.

Metabolic enzymes and rates

Respiration rates were temperature independent but influenced by the reproductive state. Lowest SMR and FMR were measured in scallops on the verge of spawning (= pre-spawning) (Fig. 7). After spawning, SMR and FMR returned to pre-maturation levels. Activities of the metabolic enzymes CS and ODH followed the pattern of the respiration

rates with lower activities in pre-spawning animals followed by higher activities again in post-spawned individuals (Fig. 8).

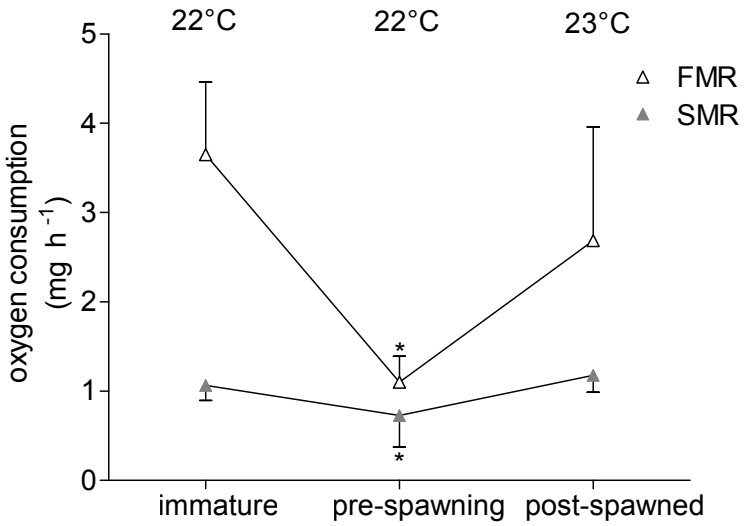


Fig. 7: Standard metabolic rates (SMR) and fed metabolic rates (FMR) of immature, pre-spawning and post-spawned *Argopecten ventricosus* exposed to predators expressed in mg O_2 h^{-1} corrected for a standard scallop of 1 g dry mass. Values are means ± SD (*N*= 6-8). * indicate differences between pre-spawning from immature and post-spawned ($p < 0.05$)

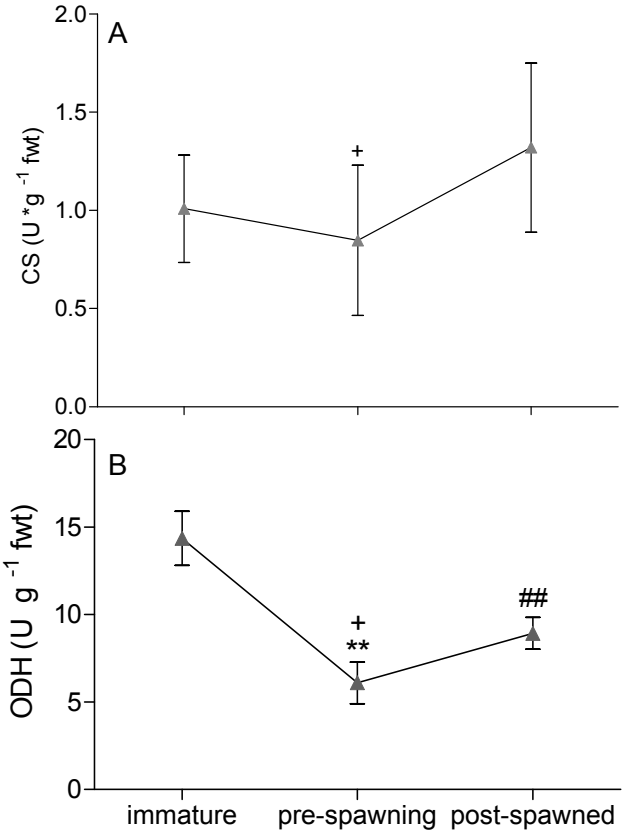


Fig. 8: (A) Citrate synthase (CS) and (B) octopine dehydrogenase (ODH) activities (units g^{-1} fwt) in the adductor muscle of immature, pre-spawning and post-spawned *A. ventricosus* under predator exposure. Values are means ± SD (*N*= 6-9 per group). * indicate differences between pre-spawning from immature; + indicates differences between pre-spawning from post-spawned individuals and # indicate differences between immature and post-spawned. Differences are set at $p < 0.001$++/**/## and $p < 0.05$ +.

DISCUSSION

Effect of predators on oxidative stress parameters, muscle capacities and metabolic rates

The results from our study show that long-term exposure (4 months) of *Argopecten ventricosus* to the predator *Callinectes sapidus* leads especially to changes in growth and biochemical composition of the adductor muscle. Predator-exposed scallops invested more into antioxidant capacities compared to predator-free individuals. Elevated antioxidant activities in predator exposed muscle tissue could however not prevent protein and lipid oxidation. This indicates that predator exposure might have increased burst swimming, causing oxidative stress in the muscle.

Scallops escaping from predators perform intensive valve clapping, brought about by repeated contraction of the swimming muscle (Thomas and Gruffydd 1971, Tremblay et al. 2006). Burst swimming in scallops is fueled by anaerobic metabolism starting with the hydrolysis of arginine phosphate and followed by anaerobic breakdown of glycogen (Bailey, et al., 2003; Chih, et al., 1983). Anaerobic ATP production in the scallop muscle is driven by octopine dehydrogenase (ODH), which catalyses the formation of octopine to restore the electron acceptor NAD^+ needed for glycolysis (Hochachka and Somero 1984). In the present study, predator-exposed scallops had 31 % bigger muscles and 33 % higher ODH levels compared to predator-free scallops, which are indicatives for induced swimming activity in scallops.

Several studies in invertebrates (Magwere et al 2006, Yan et al 2000) and vertebrates (Aniagu et al. 2006, Bejma and Li, 1999, Cooper et al. 2002, Reid 2001) have shown that

vigorous muscle contractions can lead to increased oxidative damage. The results are however not consistent and although strenuous physical activity cause an elevation of metabolic activity, oxidative damage does not necessarily increase in proportion (for review see Constantini 2010). It is difficult to obtain direct confirmation for ROS generation and oxidative damage production in *in-vivo* exercise studies with scallops. Short-term exhaustive exercise in the queen scallop *Aequipecten opercularis* triggered by 3 subsequent experimental sea star "attacks", did not result in significant accumulation of lipid peroxides, but decreased the concentration of reduced glutathione (GSH) (Philipp et al. 2008). This decrease may indicate increased ROS levels in exercising scallop swimming muscle, whereas apparently, only "chronic" exposure to predators, which stimulates repeated escape swimming, overwhelms the muscle´s radical buffering capacities. This may lead to the observed accumulation of oxidative damage products in *A. ventricosus* with 4 times higher protein carbonyls and 2 times higher TBARS concentratiosn compared to predator-free scallops, despite simultaneously induced antioxidant defenses (SOD and CAT). ROS generation during exercise can further "trigger" the up-regulation of antioxidant enzymes (Gomez-Cabrera et al, 2008, Powers and Jackson 2008). This may contribute to explain why predator-exposed scallops had higher CAT and slightly higher SOD activities as compared to predator-free scallops.

The adductor muscle is the most voluminous organ in *A. ventricosus* (~ 50 % of total soft tissue, Guerra et al. 2011). Lower muscle CS activities and thus aerobic capacities can contribute to explain the lower whole animal SMR in predator-exposed compared to the predator-free scallops. The similar FMRs in both groups speak for the capacity to increase oxygen consumption rates to a greater extent in predator exposed than predator-free

scallops, as soon as food becomes available. Thus, SDA dependent increase in oxygen consumption is more conspicuous in predator-exposed specimens with a higher energy allocation to muscle, which metabolizes more food and consumes more oxygen to enhance growth and swimming activity (see Pauly 2010). Given these results, it seems rather the burst swimming activity to cause the higher oxidative damage in the adductor muscle than changes in metabolism, especially as the main respiratory organs, gill and mantle tissue, show even lower oxidative damage compared to predator-free individuals.

Effect of reproduction on oxidative stress parameters and metabolic rates

The energetic and oxidative costs of reproduction have been suggested to represent a constraint in animal´s life history evolution (Dowling and Simmons 2009). To date, these costs have been studied principally in birds, mammals and flies (Wang et al. 2001, Alonso-Alvarez 2006, Bergeron et al. 2011) but the generality by which oxidative stress impacts on reproduction remains to be fully explored across a range of other taxa such as bivalves. Here we show that in *A. ventricosus*, reproduction markedly increases antioxidant defense as well as oxidative damage. In mussels (*Perna perna)* enhanced antioxidant capacities in parallel with an increase in cellular damage (TBARS = lipid peroxidation products) in mature compared to immature individuals where already observed (Wilhelm Filho et al. 2001). However, mussel reproduction in that study coincided with higher summer temperatures, and it was not altogether clear whether higher oxidative stress was mainly due to thermal or reproductive stress, or to a combination of both. In the present study, we aimed to disentangle the effects of temperature and reproduction on oxidative metabolism

by controlling temperatures in a narrow range (between 22-23°C) during maturation. Increased oxidative damage in all investigated tissues in *A. ventricosus* individuals on the verge of spawning can be attributable to the reproductive effort. In gills and mantle, SOD and CAT activities were increased at peak gonad index but failed to counterbalance the pre-spawning oxidative damage in the scallops' respiratory tissues. This suggested that maturation and spawning mainly influence organs directly linked to oxygen uptake (mantle and gills) and food absorption (gills). As respiratory organs, these tissues may be susceptible to an increase in energy demands, such as during the reproductive months (Shumway et al. 1988; Thompson and MacDonald 2006). The oxidative damage in mantle and gill may have accumulated during the process of gonad maturation and highest levels are measured despite the lower metabolic rates close before spawning. The lower metabolic rates (SMR and FMR) in scallops immediately before spawning (pre-spawning = spawning within the next days) compared to immature and post-spawned animals may be attributed to lower energy demand on the verge of spawning when compared to the period of gonad build-up as already found in *Placopecten magellanicus* (Kraffe et al. 2008). In contrast to gill and mantle, antioxidant activities were lower in the adductor muscle during peak GI. During this time of gonad growth, the scallop muscle mass is used as energy reserve for gonad development (Guerra et al. 2011, Barber and Blake 1991; Brokordt et al. 2000a, b). The energy mobilization from adductor muscle towards the gonads conjunctly decreased activity of the metabolic enzymes CS and ODH, which are likely to diminish muscle metabolic capacities and the recuperation after burst swimming. Conjunctly to the decrease in CS and ODH in muscle, an absence of antioxidant induction and increased oxidative damage was observed for *A. ventricosus*.

Interestingly, after spawning, oxidative stress parameters in predator-exposed scallops returned again to the lower levels of immature scallops. Further, no increased post-spawning mortality was observed and scallops reinitiated muscle growth after spawning (see Guerra et al. 2011). This indicates that protein carbonyls and lipid peroxidation products (TBARS) are transient and that they can be efficiently removed or recycled after spawning. Unfortunately, no tissue samples could be taken from the predator-free group, to investigate whether or not this pattern is restricted to the predator-exposed individuals. Assuming that the relation of reproduction and oxidative damage is predator-independent, it still remained unresolved at what extent the observations represents a true biological phenomenon as parameters are measured in a controlled laboratory environment where food and temperature are kept constant. We are conducting a complementary study, using the same cohort of scallops but reared under natural environmental variability in the field and protected from predators. With this complementary study, we aim to disentangle if there is a general trend of reproduction modulating oxidative stress parameters as shown in the present study that is not restricted to the predator-exposed individuals from the laboratory.

CONCLUSIONS

Our study shows that prolonged exposure to the predator *Callinectes sapidus* enhances muscle growth in the scallop *Argopecten ventricosus*, but also causes higher oxidative damage in this tissue. The lower damage in gills and mantle in predator-exposed scallops indicates that predators specifically influence muscle oxidative properties without affecting

the other tissues and supports that the effect is based on enhanced ROS formation during frequent burst swimming periods.

A drastic increase in oxidative damage parameters especially in gills and mantle of scallops on the verge of spawning could be attributed to reproduction and corroborates other studies, which indicate that reproduction directly inflicts somatic damage and overwhelms antioxidant capacities. Oxidative damage is however transient and can be removed from cells in post-spawned scallops indicating that scallops possess repair or removal mechanisms to deal with high oxidative stress following reproduction.

ACKNOWLEDGEMENTS

We thank Mario Osuna, Ernesto León, Carmen Abundis and Stefan Koengistein for technical support and for assistance with the hatchery and the animal maintenance. We further thank Norma Olguín, Orlando Lugo and Stefanie Meyer for excellent technical assistance in the laboratory. We gratefully acknowledge the supply of scallops from Cooperativa del Puerto de San Carlos, Baja California Sur with which we started our culture. This research was supported by the DAAD grant D/06/47197 and the SAGARPA-CONACyT project 11947.

4.3. Manuscript 3: Changes in oxidative stress parameters in relation to age, growth and reproduction in the short-lived Catarina scallop *Argopecten ventricosus* reared in its natural environment

Citlali Guerra, Tania Zenteno-Savín, Alfonso N. Maeda-Martínez, Eva E.R. Philipp,

Doris Abele

ABSTRACT

Increase in oxidative damage and decrease in cellular maintenance is often associated with aging, but, in marine ectotherms, both processes are also strongly influenced by somatic growth, maturation and reproduction. In this study, we used a single cohort of the short-lived catarina scallop *Argopecten ventricosus,* to investigate the effects of somatic growth, reproduction and aging on oxidative damage (protein carbonyls, TBARS and lipofuscin) and cellular maintenance mechanisms (antioxidant activity and apoptosis) in scallops, caged in their natural environment. The concentrations of protein carbonyls and TBARS increased steeply during the early period of fast growth and during reproduction in one-year-old scallops. However, oxidative damage was transient, and apoptotic cell death played a pivotal role in eliminating damage in gill, mantle and muscle tissues of young scallops. Animals were able to reproduce again in the second year, but the reduced intensity of apoptosis impaired subsequent removal of damaged cells. Compared to longer-lived

bivalves, *A. ventricosus* seems more susceptible to oxidative stress with higher tissue-specific protein carbonyl levels. Superoxide dismutase activity and apoptotic cell death intensity were higher in this short-lived scallop than in longer-lived bivalves. The life strategy of this short-lived and intensely predated scallop supports rapid somatic growth and early maturation at young age over cellular maintenance in second year scallops.

INTRODUCTION

Bivalves are useful models to study the influence of environmental variables on life history parameters such as growth, reproduction and longevity. This molluskan class offers a rich diversity of lifestyles and adaptations to specific environmental conditions in which different species have evolved distinct aging strategies (Abele et al. 2009; Philipp and Abele 2010).

The catarina scallop, *Argopecten ventricosus*, is an active swimmer with an energy intensive lifestyle. It is one of the shortest-lived scallops, with a maximum lifespan potential (MLSP) of only 2 years (Keen 1971), characterized by fast growth and high reproductive output (Maeda-Martínez et al. 1993, 1997). Under favorable conditions, catarina scallops attain sexual maturity at an age of 4 months, and mature animals are found throughout the year in the populations around Baja California Sur, México (Cruz et al. 2000; Maeda-Martìnez et al. 2001). High predation pressure, especially in young scallops (Abrams and Rowe 1996; Ciocco and Orensanz 2001), may select for genetic and physiological adaptations that support fast growth, shorten the juvenile period, and allow an early age of maturation that facilitates recruitment before predation strikes in the young

animals. According to the free-radical theory of aging (Rubner 1908; Pearl 1928; Harman 1956), the high metabolic rates necessary to support such an active lifestyle are incompatible with a long lifespan because of an anticipated accumulation of oxidative damage that would compromise cellular functioning (Beckman and Ames 1998). Oxidative damage is caused by reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2,), and the hydroxyl radical ($HO^{\bullet}$), produced mainly within the mitochondria as by-products of aerobic metabolism (Sohal 2002; Kujoth et al. 2005). Cellular protection mechanisms, which include the activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), detoxify ROS before they cause oxidative damage. ROS formation and oxidative damage are, however, not only critical for cellular functioning and homeostasis, but can also play a role in cellular redox signaling and in the induction of apoptosis (Márquez 2007; Terahara and Takahashi 2008, Matés et al. 2008, Eisenberg-Lerner et al. 2009). Apoptosis is a highly regulated cellular self-disintegration program by which damaged cells are eliminated to avoid inflammation and cancerous developments (Edinger and Thompson 2004). This highly conserved mechanism is also present in mollusks (Sokolova 2009; Kiss 2010). Hence, the excessive and progressive accumulation of oxidative damage and loss of cellular integrity in many organisms may not only be linked to an increase in ROS production or a decrease of the antioxidant protection, but also to a progressive decrease of cellular degradation and renewal mechanisms over lifetime (Tomey and Ortega 2000, Philipp and Abele 2010).

Oxidative stress has not only been related to the aging process, but also to periods of intense growth and breeding, and may constitute a potential mechanism explaining life history trade-offs (Costantini 2010). Accumulation of oxidative damage and decrease in

antioxidant protection during periods of intense growth and reproduction have been demonstrated in different species such as fruit flies, birds, sheep, and also in bivalves (Wang et al. 2001; Alonso-Alvarez et al. 2006, 2007; Bize et al. 2008; Nussey et al. 2009; Soldatov et al. 2008). Further, in ectotherms such as bivalves, investment into reproduction, somatic growth and cellular maintenance varies over lifetime and is strongly governed by environmental conditions (Vahl 1985; MacDonald and Bayne 1993; Jokela and Mutikainen 1995) with temperature being one of the major modulators (Barber and Blake 1983).

In the present study, we studied a single cohort of scallops through the early maturation phase and two subsequent spawning events, to identify the effects of aging and reproduction on oxidative stress parameters. Due to its short lifespan, its repeated spawning and its availability in aquaculture in Baja California Sur, México, the catarina scallop *A. ventricosus* allowed us to conduct a high-resolution study of oxidative stress over lifetime. We examined these short-lived scallops for tractable cellular aging, involving oxidative damage to proteins and membrane lipids, and fluorescent age pigment (lipofuscin) accumulation, as well as changes in antioxidant enzyme activities and the capacity to eliminate damaged cells by apoptosis. The fact that scallops invest so heavily into reproduction (Baber and Blake 1991) allowed us to specifically study the effect of reproduction and subsequent recovery on cellular maintenance and oxidative damage in different tissues, and to distinguish the effect of aging from the effect of exhaustive reproduction in field-reared specimens.

MATERIAL AND METHODS

Experimental animals and culture conditions

Argopecten ventricosus larvae were obtained and reared in the hatchery as described in detail in Guerra et al. (2011). Briefly, larvae were obtained from spawn of wild scallops and juveniles were reared for 3 months in the hatchery until they reached 5-7 mm shell height. Thereafter, approximately 10000 scallops were transported to Rancho Bueno estuary, in the vicinity of Bahía Magdalena in October 2007 (geographical position: 24°19´17,3´´N, 111°25´37,3´´W). The field site chosen in the present study represents a beneficial environment for *A. ventricosus* with high chlorophyll concentrations and an optimal temperature range for growth (Acosta-Ruiz and Lara-Lara 1998; Sicard-Gonzàlez et al. 2006). Scallops were kept in fine mesh bags (2 mm) fitted within 20 Nestier trays (55 x 55 x 8 cm) suspended in a long-line system for 2 months until reaching an average size of 24 mm (December 2007). Subsequently, animals were kept without bags in the Nestier trays (10-15 cm water depth). Initial stocking density was set at 500 animals/tray (equivalent to 1700 animals/m^2) for optimal growth (Maeda-Martínez et al. 1997) and adjusted to 150, 90 and 60 animals/tray (495, 297 and 198 animals/m^2) after 187, 337 and 480 days (February, June and December 2008) in order to keep optimal densities for growth.

Scallops were first sampled in April 2008 at an age of 8 months. Further samplings were conducted at 2 month intervals until the last survivals (6 animals) were sampled at an age of 2 years in August 2009. Surface water temperature at the cultivation site was measured during each sampling time with a hand-held thermometer. In addition, a temperature logger (WTA 32-5+37) that recorded water temperature at 60 min intervals was attached to one of

the Nestier trays at a depth of 10-15 cm. The temperature logger was recollected and replaced during field trips at monthly intervals.

Six to twenty animals were retrieved at each sampling time and their shell height (distance from hinge to distal shell margin) was determined to the nearest 0.01 mm using calipers. The animals were then transported 114 km to CIBNOR in La Paz as described by Maeda-Martínez et al. (2000). In the laboratory, animals were cleaned of epibiota and placed in 70 L aquaria with filtered seawater (1 μm). Water temperature was kept at field temperatures measured during collection and held at a salinity of 33-36 ppt. In October 2008, only shell height measurements could be taken as animals died during the transportation.

Gonad and muscle index

The gonad and muscle index (GI and MI) were determined on each sampling date. Both indices were calculated as follows: (gonad or muscle weight/ total shell weight) × 100 (see Lucas and Beninger 1989; Sarkis et al. 2006). A high gonad index is indicative of mature gonads and a lower GI reflects the onset of gametogenesis or spent gonads. At 12 months of age, scallops were close to spawning, with some specimens already spawning during the transport from the field to the laboratory. Spawning animals were excluded from measurements.

Biochemical analysis

One the day after collection, 4-8 scallops were sacrificed and gills, mantle, adductor muscle and gonad tissues dissected. The tissues were weighted and mantle, adductor muscle and gills frozen in liquid nitrogen for biochemical analysis.

Enzyme assays

Catalase and Superoxide-dismutase

CAT and SOD activities were measured spectrophotometrically at 25°C in a Beckman DU 640 spectrophotometer.

Samples were homogenized by hand with a glass homogenizer on ice in 1:20 (w/v) in 50 mM phosphate buffer (pH 7.5) containing 1 mM EDTA and 1 mM PMSF. Homogenates were centrifuged at 15000 × g for 15 min at 4°C and the supernatant immediately used for CAT and SOD activity measurements.

CAT activity (EC 1.11.1.6) was determined after Aebi (1984) by recording the time of H_2O_2 decomposition, resulting in a decrease of absorption from 0.45 to 0.4 at 240 nm (1 unit catalase). Working solution (20 mM H_2O_2, 100 mM phosphate buffer) and sample were mixed in a cuvette and the change in absorbance was recorded every 15 s for 3 min. Enzymatic activity was expressed as units CAT g^{-1} fresh weight. One unit of catalase is defined as the amount of enzyme necessary to reduce 1 μmlo of H_2O_2 per min.

Total SOD (EC 1.15.1.1) activity was determined by the method of (Susuki 2000). The xanthine/xanthine oxidase (X/XO) system was used to generate $O_2^{\bullet-}$ which reacts with

nitroblue tetrazolium (NBT). Working solution (50 mM sodium-carbonate buffer, 0.025 mM NBT, 1mM X, 0.1 mM EDTA), XO (0.1 U mL^{-1} in 2 M ammonium sulfate) and 25 µL homogenized sample or blank were mixed in a cuvette and the change in absorbance at 560 nm was recorded for 5 min every 30 sec. One unit of SOD activity is defined as the amount of enzyme necessary to inhibit the reduction of NBT by 50 %. Results are presented as units SOD g^{-1} fresh weight.

Apoptosis

Apoptosis was assessed via the caspase-3 and -7 activity modified after Liu et al. (2004). Frozen samples of gills, mantle and muscle were ground in liquid nitrogen and homogenized with a glass homogenizer (Nalgene, USA) in lysis buffer (100 mM HEPES, pH 7.5; 5 mM $MgCl_2$ 1 mM EGTA and 1 µg mL^{-1} each leupeptine, pepstatin and aprotinine), with 1:100 (w/v) for gills and mantle and 1:50 (w/v) for muscle. Homogenates were centrifuged for 15 min at 15000 × g and 4°C and apoptosis intensities in supernatants were determined as caspase-3 and -7 activities using a Caspase-Glo 3/7 Assay kit (Promega, Madison, USA). The assay provides a luminogenic caspase-3/7 substrate, diluted in a reagent optimized for caspase activity, luciferase activity and cell lysis. Equal volumes of reagents and supernatant were added to a white-walled 96-well plate and incubated at 25°C for 1 h. The proluminescent substrate was quenched by caspases in the supernatant, where it formed a substrate for luciferase. The resulting luminescence signal is proportional to the amount of caspase activity present in the supernatant. Samples were analyzed using a Multilabel Reader LB 941 TriStar (Berthold Technologies GmbH & Co.

KG, bad Wildbad, Germnay) which gave luminescence readings as relative light units (RLU). Protein concentrations were determined in the supernatant according to Bradford (1976) and apoptosis intensities expressed as RLU mg^{-1} protein.

Tissue damage

Lipid peroxides (TBARS)

Lipid peroxides are known to produce a variety of intermediate substances including malondialdehyde (MDA). MDA reacts with thiobarbituric acid (TBA) under acidic pH and elevated temperatures. Lipid peroxides were measured using the generation of MDA/TBA adducts (thiobarbituric acid reactive substances, TBARS, assay) to quantify MDA formation. TBARS were measured following the method of Persky et al. (2000). Samples were homogenized by hand in a glass homogenizer on ice 1:20 (w/v) in a saline solution (0.9%) at pH 7.0. 250 µL of 6 % HCl (1 M) in 12.5 % trichloroacetic acid (TCA) was added to 250 µL of homogenate prior to the addition of 500 µL of TBA (1%). Samples were incubated for 10 min in a 90°C water bath, cooled to room temperature and centrifuged at 1500 ×g for 10 min at 4°C. TBARS levels in each sample were measured at 535 nm. TBARS concentrations were derived from a standard curve and the values calculated as TBARS nmolar equivalents g^{-1} fresh weight.

Protein carbonyls

The detection of protein carbonyls was carried out after Levine et al. (1990). Carbonyls react with the carbonyl specific reagent 2,4-dinitrophenylhydrazine (DNTP) and, after precipitation with TCA, carbonyls can be measured spectrophotometrically. Samples were homogenized by hand in a glass homogenizer on ice in 5 % sulfosalisilic acid and centrifuged at 10000 × g for 15 min at 4°C. The supernatant was discarded and the pellet incubated at room temperature for 1 h with 10 mM DNTP or 2 M HCl (sample blanks) and mixed every 15 min. After 1 h, 20 % TCA were added to samples and blanks to precipitate the protein and centrifuged at 10000 × g at 4°C for 5 min. The protein pellet was washed 3 times with 1 mL ethanol: ethylacetate (1:1) resuspended in 6 mM guanidine hydrochloride and incubated for 30 min at 37°C for 15 min. The samples were centrifuged at 10000 × g at 4°C for 5 min, and the supernatants of samples and blanks measured in quartz cuvettes at 360 nm. The amount of protein carbonyls was estimated as the difference in absorbance between samples and blank using a molar extinction coefficient of carbonyls (ε = 22,000 cm^{-1} M^{-1}). Amount of protein carbonyls was assessed as nmol mg^{-1} protein measured in the same samples using the Bradford method (Bradford 1976).

Fluorescent age pigment, lipofuscin

Lipofuscin contents were determined by an extraction method modified after Vernet et al. (1988). Frozen gills, mantle and muscle tissues were ground in liquid nitrogen and homogenized (1:20 w/v) in chloroform-methanol solution (2:1 v/v). The homogenate was mixed with 100 mM $MgCl_2$ (1 mL per each 4 mL of chloroform-methanol) solution. After

10 min centrifugation at 2000 × g and 0°C, the chloroform phase was collected and mixed with distilled water (1 mL per 4 mL initial chloroform-methanol). After 10 min at 2000 × g and at 0°C, the chloroform phase was again collected and could be measured in the fluorometer. An emission spectrum was obtained at an excitation wavelength of 350 nm. The fluorescence intensity of each sample was determined at an emission maximum of 536 nm for gills, 434 for mantle and 431 for muscle. According to Hill and Womersley (1991), lipofuscin concentrations were expressed as relative fluorescent intensities (RFI) using 0.1 µg quinine sulphate per mL 1N H_2SO_4.

Standard metabolic rate

Standard metabolic rates (SMR) of 6-8 animals were measured at each sampling event except of August 2009 as not enough animals remained alive to conduct both SMR and biochemical analyses. SMR estimates maintenance requirements of resting, unstressed organisms that are not digesting food and are at a stable temperature within its optimal range (Rolfe and Brown 1997). Oxygen consumption of individual animals was measured with fiber-optical oxygen optodes of 50 µm diameter (PreSens GmbH, Regensburg Germany) in 700 mL chambers connected to a multi-channel flow-through system (60 mL min^{-1}). Prior to respiration measurements, *A. ventricosus* shells were cleaned from epibionts and animals maintained without food for 2 days, to eliminate effects of feeding on metabolic rates. Before starting the SMR measurements, animals were allowed to acclimate in the chambers for 2 h in filtered seawater at the respective temperature and salinity condition. Experiments had been previously run to assure that this time was sufficiently

long to allow the animals to accommodate to the measuring system. Oxygen optodes were calibrated using air-bubbled water (100 % O_2 = 21 kPa) and water saturated with sodium sulfite to deplete oxygen (0 % O_2 = 0 kPa). Respiration rate was calculated from the difference in oxygen values measured between the in- and out-flowing water over 5 minutes. Three replicate measurements were made per individual at each sampling time. A blank chamber was run without animals to correct for microbial respiration.

After the last measurement, scallops were dissected, and soft tissue dry mass determined after 2 days at 60°C. Percent oxygen was transformed to micromoles of dissolved oxygen in seawater, using known values of oxygen solubility according to Benson and Krause (1984) and converted to mg O_2, expressed as the expected rate for a standard scallop (2 g mean dry mass).

Statistics

The effect of tissue size on antioxidant enzyme activities, apoptosis, protein carbonyls, TBARS, lipofuscin and metabolic rates was tested by power regression of the respective parameter on wet tissue mass (antioxidants, apoptosis, protein carbonyls, TBARS lipofuscin) or whole animal dry tissue mass (metabolic rates). One way ANOVA (Kruskal-Wallis test for non-Gaussian distribution) with post-hoc Tukey´s (Dunn´s) was used to analyze the effect of age on tissue-specific antioxidant enzyme activity, oxidative damage, lipofuscin content, apoptosis intensities and metabolic rate. All data were tested for normality and homogeneity of variance prior to analyses.

RESULTS

Field temperature

Monthly field temperature means, minimal and maximal values are shown in Fig. 1. In both years (October 2007-August 2009), water temperature followed a seasonal cycle with decreasing temperatures from October to March, followed by a steady increase between April and September. The greatest monthly temperature variability was recorded in July of both years with temperatures varying over 14°C. The smallest monthly variability was recorded in February 2008 and 2009 with temperatures varying in a range of 4°C during one month.

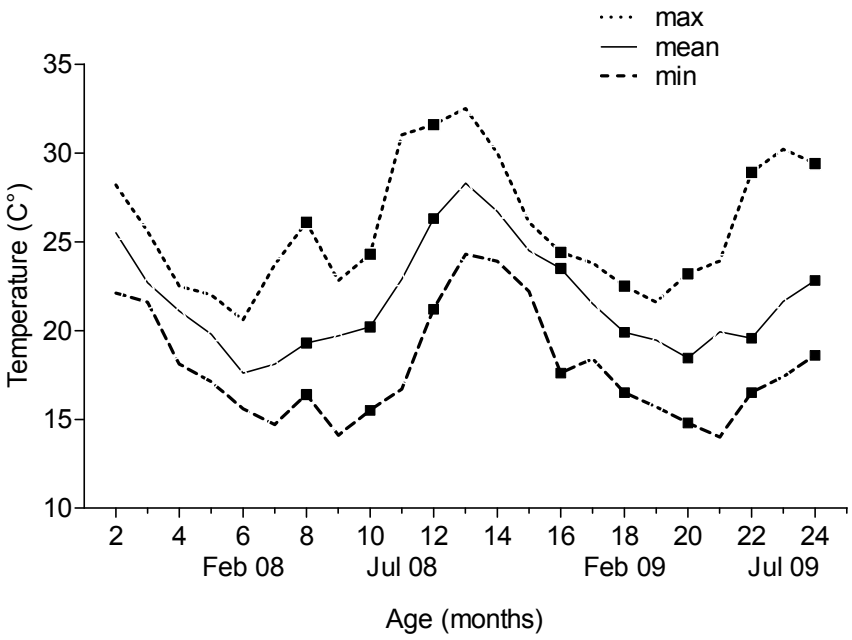


Fig. 1: Water temperature at the field site Rancho Bueno between October 2007 and August 2009 over *A. ventricosus* lifetime (age in months). Each line represents the maximal, the mean and the minimal surface water temperature measured at monthly intervals (water depth: 10-15 cm). The lowest and the highest temperature variability within one month were recorded in February and July, respectively. Squares indicate temperatures at sampling dates.

Somatic growth, shell height, muscle and gonad index

The gonad index (GI) reached peak values in August 2008 and June 2009 at an age of 12 and 22 months (7.24 and 7.69 % GI respectively) (Fig. 2A). Minimum GI values were recorded at 16 and 18 months of scallop age (2.44 and 1.92 % GI respectively) during winter (December 2008 to February 2009). The muscle index showed the inverse pattern to GI (Fig. 2A). Scallops more than doubled their somatic weight between 8 and 12 months of age (0.88 g to 2.1 g dry weight). Thereafter, somatic weight increased continuously but at a lower rate than in the first year (Fig. 2B). Shell height followed the same pattern as somatic weight (Fig. 2B).

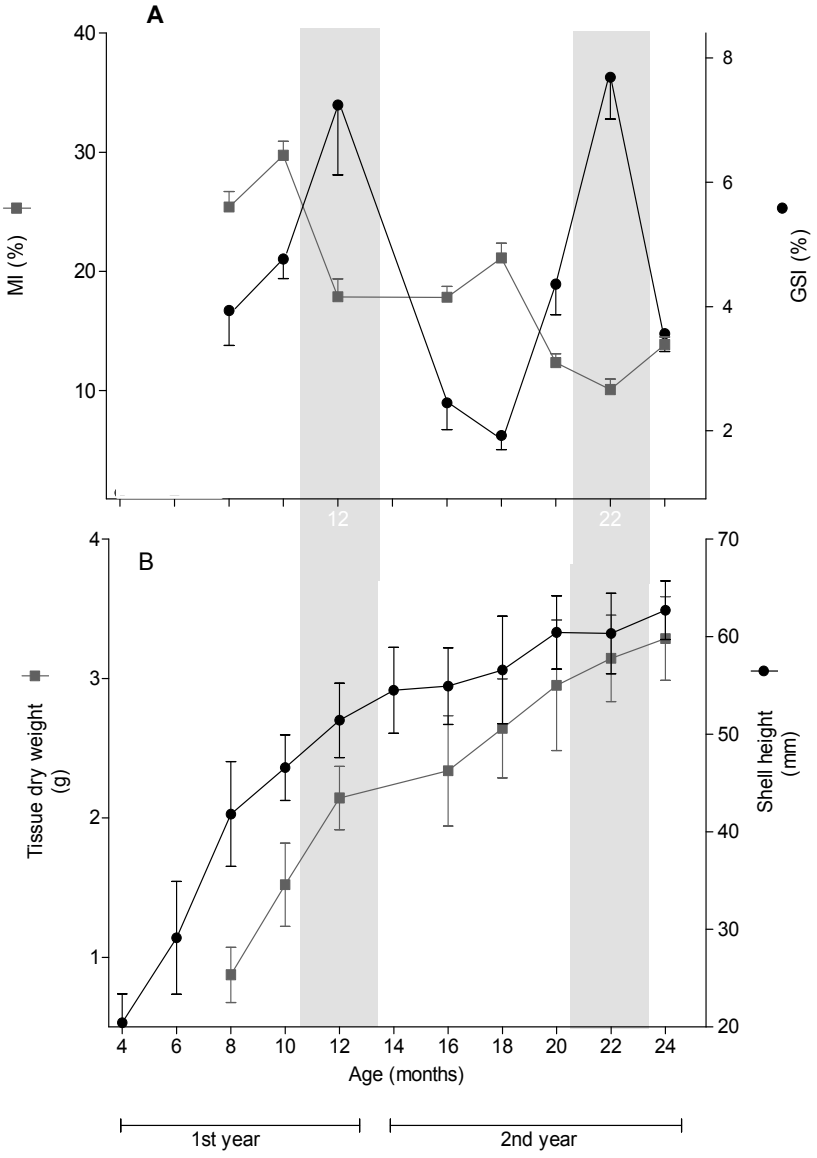


Fig. 2: (A) Muscle (MI) and gonad (GI) index of *A. ventricosus*, calculated as % muscle or gonad weight to empty shell weight.(B) Change in *A. ventricosus* somatic tissue: tissue dry weight without gonad (g dry weight), and scallop size: shell height (mm), over lifetime. Data are presented as means ± SD (tissue dry weigh, MI and GI: N = 6-8; shell height N= 6-20). The slopes of tissue dry weight as well as shell height differed significantly between the first and the second year (tissue dry weight p = 0.03; shell height p = 0.004 ANCOVA). Tissue dry weight 1st year = - 1.099 × age + 0.35 r^2 = 0.45; 2nd year = 0.32 × age + 0.13 r^2 = 0.54. Shell height 1st year = 19.27 × age + 2.5 r^2 = 0.51; 2nd year = 37.51 × age + 1.47 r^2 = 0.44. Grey underlay highlight maturation periods.

Enzyme activities

Antioxidant enzymes

Figs. 3A, B and 4A, B show CAT and SOD enzyme activity in gill, mantle and muscle tissues of field reared *A. ventricosus*. CAT activity was higher in gills, followed by mantle and lowest in muscle (ANOVA $p < 0.0001$), whereas SOD activity did not differ between tissues. CAT activity decreased significantly with size (tissue wet mass) in all tissues. The size dependence can be described as a power functions for each tissue:

CAT (gills): $2.73 \times W^{-0.42}$ $r^2 = 0.2$ $N = 56$

CAT (mantle): $2.74 \times W^{-0.64}$ $r^2 = 0.2$ $N = 59$

CAT (muscle): $2.02 \times W^{-0.54}$ $r^2 = 0.14$ $N = 59$

In order to remove the size effect, CAT activity was corrected according to:

$$CAT = CAT'(W_{mean}/W)^b \qquad \text{Eq. (1)}$$

where CAT and CAT′ are corrected and observed values respectively, W is the observed tissue wet mass, W_{mean} the mean tissue wet mass of the whole data calculated for a standardized animal with mean tissue weight of 1.4 g for gills, 2.5 g for mantle and 4.4 g for muscle, and b the calculated scaling coefficient for each tissue (gills = -0.42; mantle = -0.64 and muscle -0.54).

Standardized CAT activity in gills and mantle was highest at 12 months of age when animals were close to spawning, which was significant only in gills (Kruskal-Wallis $p < 0.0001$; Dunn′s $p < 0.05$). In gills, mantle and muscle, CAT activity reached the lowest

values in old scallops when compared to the youngest animals (Kruskal-Wallis $p < 0.0001$; Dunn´s $p < 0.05$, Fig. 3A, B). SOD activity in gills and mantle did not follow a clear pattern with increasing scallop size or age. Only in muscle, SOD activity was significantly lower in 24 compared to 8 month old scallops (Kruskal-Wallis $p < 0.0001$; Dunn´s $p = 0.05$, Fig. 4A, B).

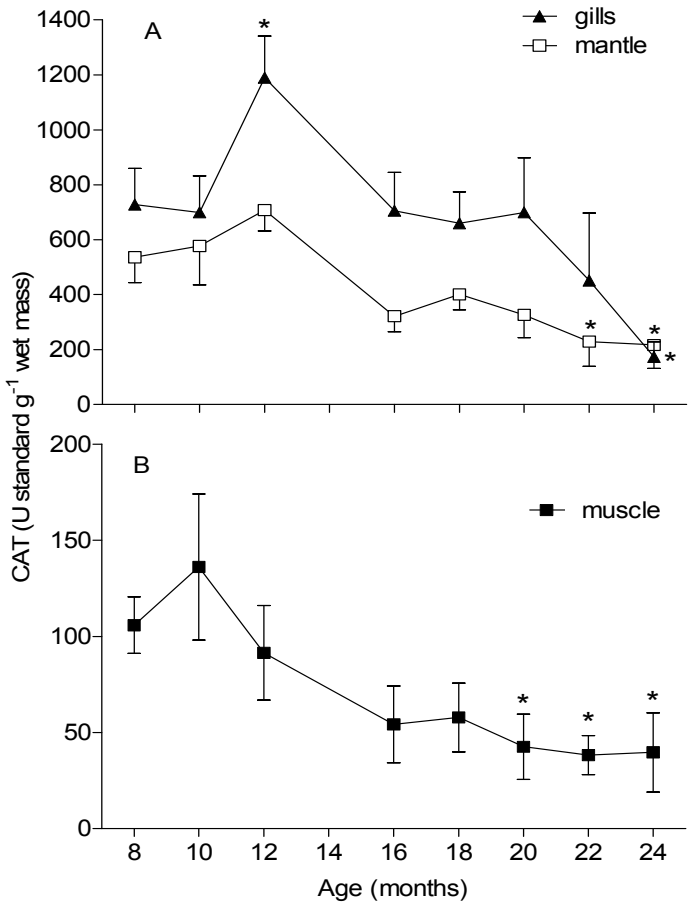


Fig. 3: Catalase (CAT) activity in (A) gills and mantle and (B) muscle of *A. ventricosus* over lifetime. CAT activity decreased significantly with size (tissue wet mass) and therefore values was standardized to the average mean tissue fresh weight of 1.4 g for gills, 2.5 g for mantle and 4.4g for muscle using a power function (see text). Data are presented as means ± SD (N = 4-8). * Significantly different from 8 months old scallops (Kruskal-Wallis $p < 0.0001$; Dunn´s $p < 0.05$)

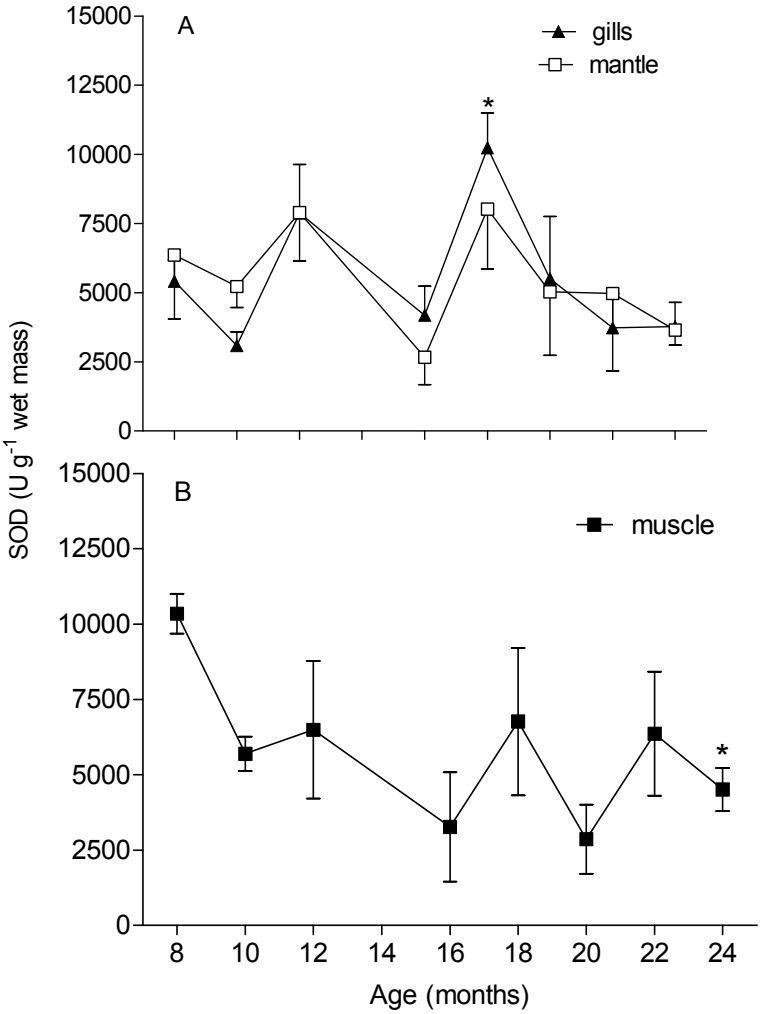


Fig. 4: Superoxide dismutase (SOD) activity in (A) gills and mantle and (B) muscle of *A. ventricosus* over lifetime. Data are presented as means ± SD (N = 4-8). * Significantly different from 8 months old scallops (Kruskal-Wallis $p < 0.0001$; Dunn´s $p < 0.05$).

Apoptosis

Intensities of apoptotic cell death measured as caspase 3 and 7 activity in gill, mantle and muscle tissues of scallops are shown in Fig. 5A, B. Relative intensities were within the same range in all tissues. Highest intensities were measured at 12 months of age in all tissues. Thereafter, apoptotic intensities decreased markedly until 16 months of age and then remained low throughout the rest of the lifetime (Kruskal-Wallis $p < 0.0001$; Dunn´s, $p < 0.05$).

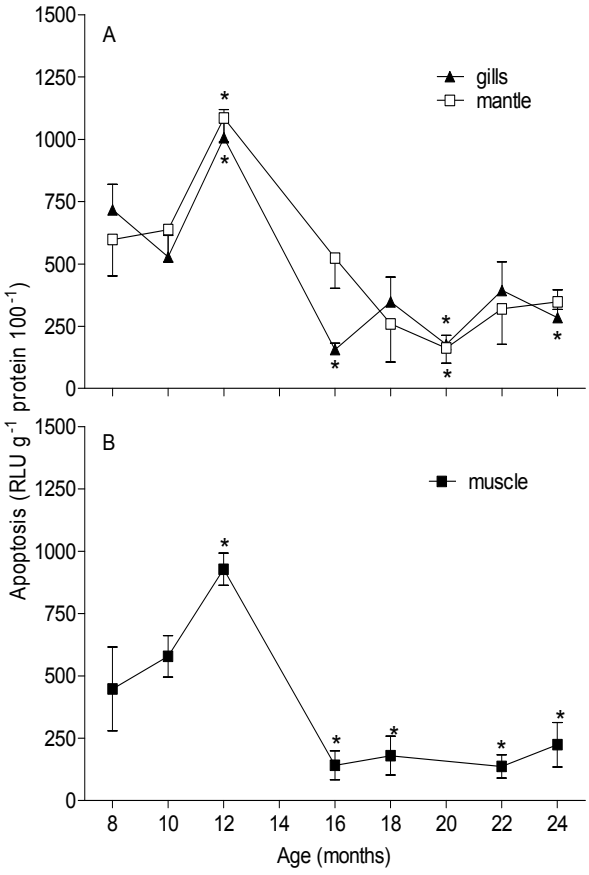


Fig. 5: Apoptosis intensities in (A) gills and mantle and (B) muscle of *A. ventricosus* over lifetime. Data are presented as means ± SD (N = 4-7). * Significantly different from 8 months old scallops (Kruskal-Wallis $p < 0.001$; Dunn´s $p < 0.05$).

Tissue oxidative damage

TBARS, protein carbonyls and lipofuscin

Figs. 6A, B and 7A, B display the concentrations of TBARS and protein carbonyls in different tissues of *A. ventricosus*. TBARS concentrations were similar in all tissues whereas protein carbonyl values were higher in gills than in mantle and muscle (ANOVA $p < 0.001$). Only in muscle, TBARS concentration decreased with increasing size. This relation can be described by the power function:

TBARS (muscle): $1.12 \times W^{-0.59}$ $r^2 = 0.11$ $N = 56$

Size correction was performed in the same way as described for CAT activities (Eq. 1) using the scaling coefficient of -0.59 and the mean muscle wet mass (4.4 g). Only in muscle, standardized TBARS concentrations were slightly increased in 12 month old scallops. In all three tissues, TBARS concentration decreased at 16 months of age and levels remained lower in the second compared to the first year (Kruskal-Wallis $p < 0.001$; Dunn´s < 0.05) (Fig. 6A, B). Protein carbonyls in gills, mantle and muscle peaked at 12 months of age (Kruskal-Wallis $p < 0.0001$; Dunn´s $p < 0.05$). Only in gills, protein carbonyls increased again during the second year, but levels were not significantly different between the youngest and the oldest scallops (Fig. 7A, B).

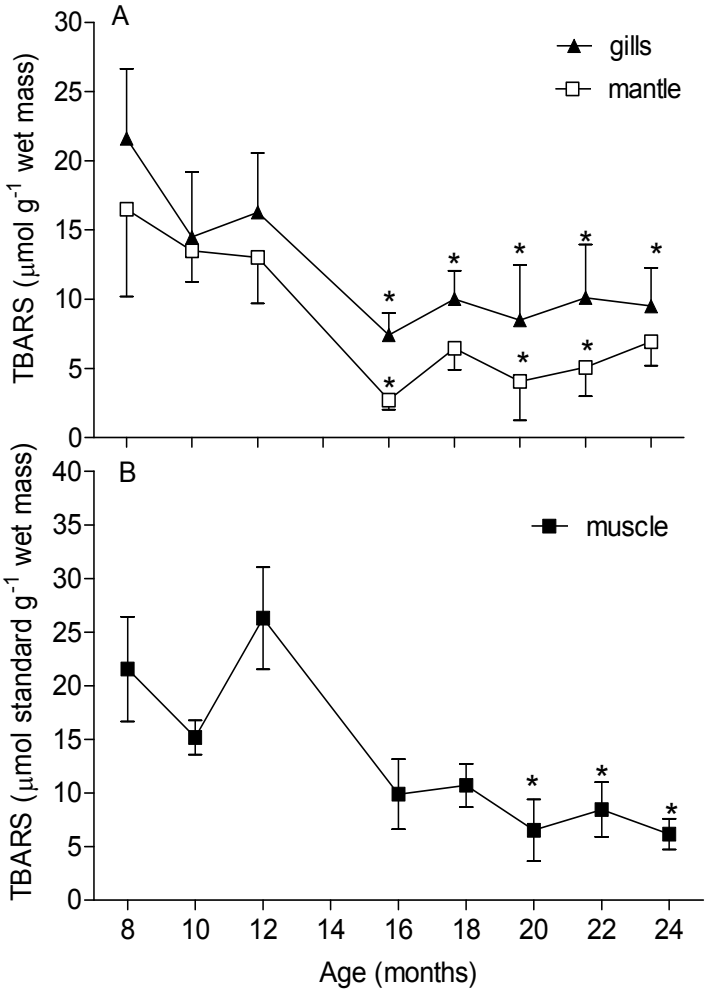


Fig. 6: Lipid peroxide (TBARS) concentration in (A) gills and mantle and (B) muscle of *A. ventricosus* over lifetime. TBARS concentration in gills and mantle are expressed as µmol g^{-1} wet mass while concentration in muscle was standardized to a mean tissue fresh weight of 4.4 g (see text). Data are presented as means ± SD (N = 4-7). * Significantly different from 8 months old scallops (Kruskal-Wallis $p < 0.001$; Dunn´s $p < 0.05$).

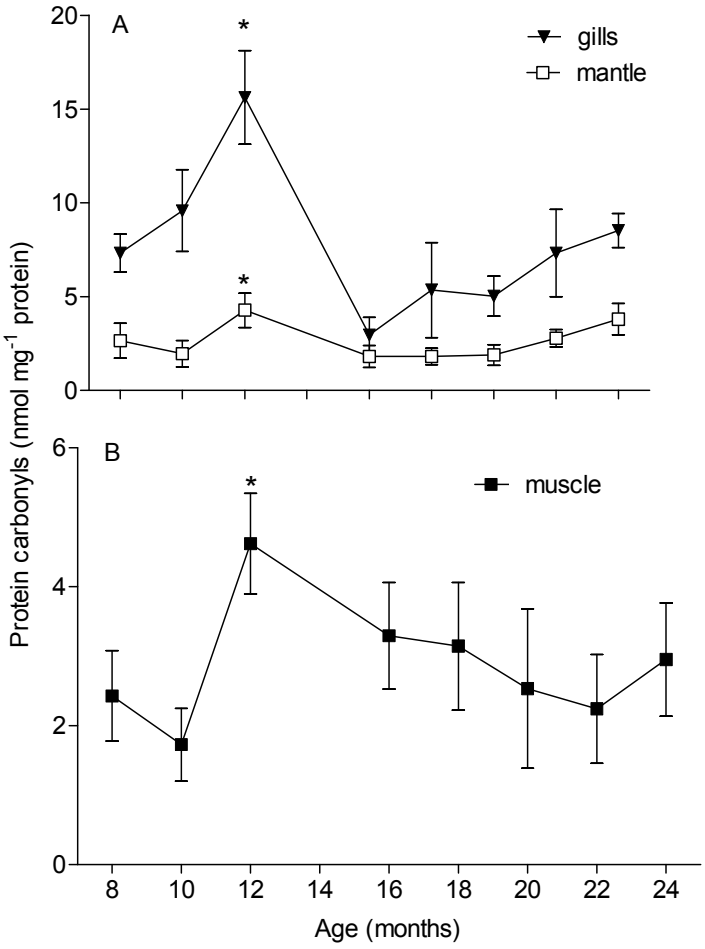


Fig. 7: Protein carbonyl concentrations in (A) gills and mantle and (B) muscle of *A. ventricosus* over lifetime. Data are presented as means ± SD (N = 4-8). * Significantly different from 8 months old scallops (Kruskal-Wallis $p < 0.0001$; Dunn´s $p < 0.05$).

Lipofuscin

Lipofuscin content was highest in gills with lower concentration in mantle and muscle (ANOVA $p < 0.0001$) (Fig. 8A, B). During the last two months of lifetime, lipofuscin concentrations increased pronouncedly in all tissues. In gills and mantle, values measured

in 24 month old scallops were significantly higher than during the first measurement at 8 months of age (Kruskal- Wallis $p < 0.005$, Dunn´s $p < 0.05$).

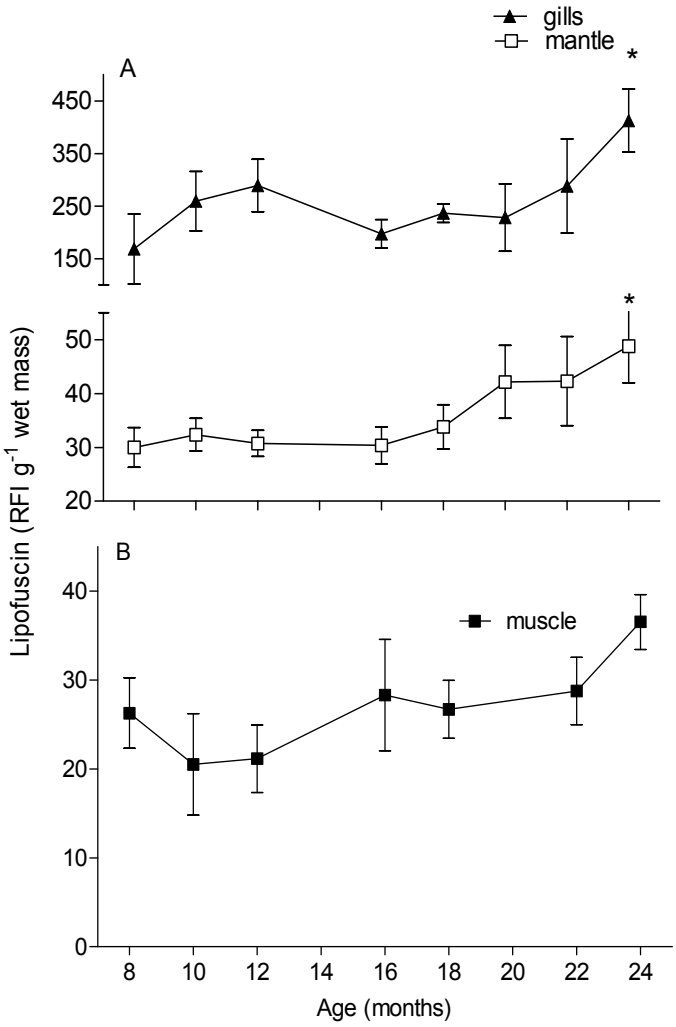


Fig. 8: Lipofuscin content in (A) gills and mantle and (B) muscle of *A. ventricosus* over lifetime. Data are presented as means ± SD (N = 4-6). * Significantly different from 8 months old scallops (Kruskal-Wallis $p < 0.005$; Dunn´s $p < 0.05$).

Standard metabolic rates

Whole animal respiration $v_{s.}$ size increased with the power function:

$VO_2 = 0.1 \times W^{0.68}$ $r^2 = 0.5$ $N = 54$

Oxygen consumption was standardized according to Eq. (1) to mean scallop dry mass (2 g) using the scaling coefficient (0.68), in order to exclude the effect of size. Standardized oxygen consumption was higher in the youngest (8 months) compared to old scallops (22 and 24 months) (Kruskal-Wallis < 0.05; Dunn´s $p < 0.05$, Fig. 9A). In order to see whether there is a relationship between metabolic rate and seasonal water temperature, size corrected metabolic rates were plotted against mean monthly field temperatures at which the oxygen consumption measurements were conducted (Fig. 9B) and no temperature dependence of metabolic rates observed. In contrast, reproductive state seems to influence oxygen consumption to a greater extent. Metabolic rates were higher during the periods of gonad-build up than in scallops that were close to spawn or in post-spawned animals (Fig. 9B).

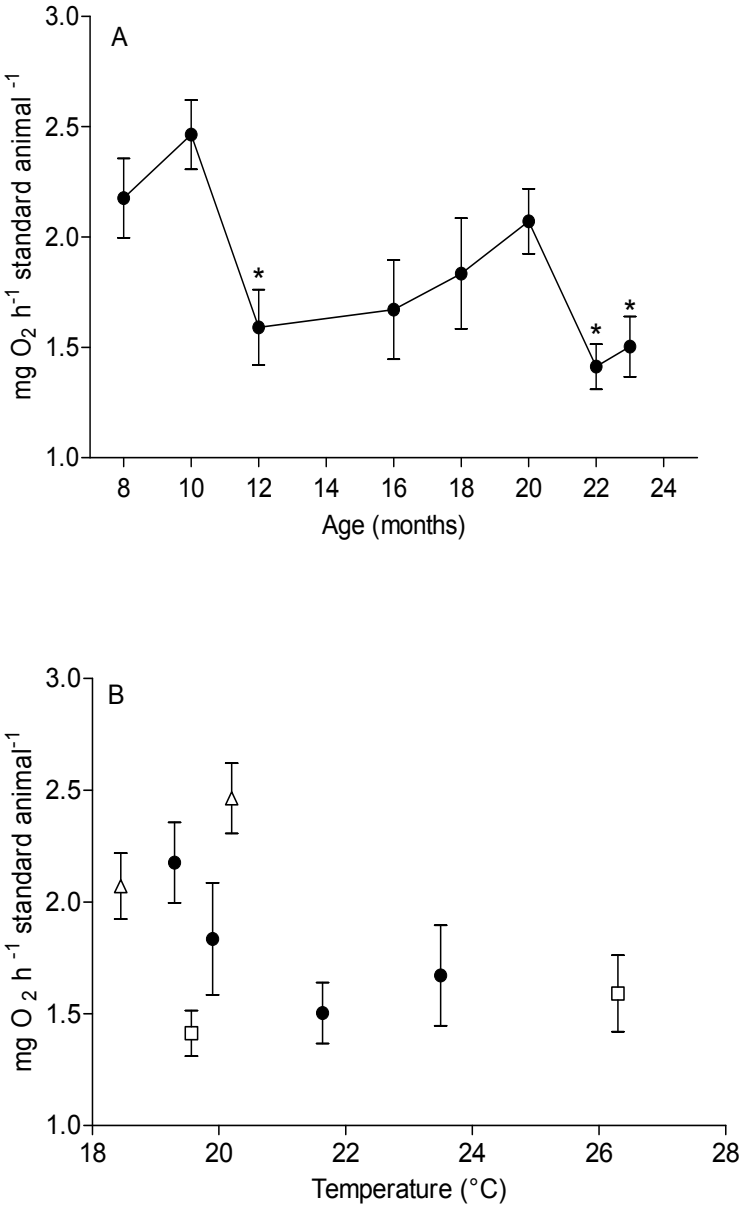


Fig. 9: Respiration rates (mg O_2 h $^{-1}$ standard animal^{-1}) of *A. ventricosus* (A) over lifetime and (B) at mean field temperatures at the respective sampling event. Open triangles indicate oxygen consumption in 10 and 20 months old scallops, during the period of gonad build up. Open rectangles indicate oxygen consumption in 12 and 22 months old scallops, that were close to spawn. Circles indicate immature or post-spawned animals (8, 16, 18, 24 months of age). Oxygen consumption rates were standardized to a mean tissue weight of 2 g dry weight using a power functions (see text). Values are presented as means ± SD (*N*= 6-8). * Significantly different from 8 months old scallops (Kruskal-Wallis $p < 0.05$; Dunn´s $p < 0.05$).

DISCUSSION

Reproduction and the susceptibility to oxidative stress in *A. ventricosus*

Protein carbonyl concentrations in gills, mantle and muscle, and TBARS levels in muscle increased steeply during the first reproductive event at 12 months of age and decreased again following spawning. This dramatic increase in oxidative damage in mature scallops coincided with the peak in apoptotic intensity in gills, mantle and muscle. The high levels of oxidative damage as well as the steep increase in apoptosis intensities illustrate the high costs of reproduction and the need to remove damaged and potentially hazardous cells in first year animals. The link between apoptosis and oxidative damage is not surprising, as ROS and related oxidative damage products are ascribed an important role in initiating apoptotic pathways (Ushida 2007, Mátes 2008). A specific function of apoptosis lies in the regulation of germline development; especially with respect to the elimination of residual or damaged cells in testis and ovaries after gametogenesis. This was already demonstrated for teleost fish (Callard et al. 1993; Wood and Van der Kraak 2001; Terrones et al. 2004). Interestingly, our data for *A. ventricosus* indicate apoptotic mechanisms to be enhanced in gills and mantle, tissues not directly involved in reproduction. As suggested by Soldatov et al. (2008) in a study of blue mussels, respiratory and filtering organs support increased respiration linked to gametogenesis, which may enhance ROS production and oxidative damage in these tissues. In *A. ventricosus*, elevated oxygen consumption indicates enhanced energy demand as scallops begin to grow gonads which decrease again shortly before spawning. Protein carbonyl concentrations in mantle and gills increased during this time of intensive growth and gonad development in spite of the induction of antioxidant

enzymes, especially in the gills. Gills seem especially affected by enhanced oxygen uptake, which raised protein carbonyl levels to 4-times higher values than in mantle and muscle.

Absence of antioxidant enzyme induction and oxidative damage marker accumulation in muscle of mature scallops may relate to the function of this tissue during the reproductive cycle. During maturation, scallop muscle mass is used as energy reserve for gonad development (Barber and Blake 1991; Guderley and Pörtner 2010), which explains the inverse relationship of gonad and muscle index in *A. ventricosus* (Fig. 2A). In a laboratory-reared subgroup from the same scallop population, we showed that the energy mobilization from adductor muscle towards the gonads conjunctly decreases activity of the metabolic enzymes citrate synthase and octopine dehydrogenase (Guerra et al. in prep.) which is likely to diminish the capacity for escape swimming as shown for other scallops (Brokordt et al. 2000 a, b). This decrease seems to be accompanied by an absence of antioxidant induction and increased oxidative damage as observed for *A. ventricosus*.

Reproduction has a temperature independent effect on metabolic rate and oxidative stress parameters

In the first year, gonad maturation occurred during a period of rapid and pronounced rise in ambient temperature. We were therefore not sure, whether or not some of the oxidative stress effects observed during this reproductive period was due also to thermal stress (Viarengo et al. 1991, Power and Sheehan 1996, Wilhelm Filho et al. 2001). Aerobic metabolic rates were, however, not exclusively, or to a major extent, dependent on temperature in our scallop culture, which may be due to the scallops being kept in and even

above their thermal optimum range (16-25°C, Sicard-González et al. 1999), where temperature has only little (optimum) or even a negative (above optimum) effect on respiration (Poertner et al 2002). In fact, a major increase in oxygen consumption in 8 to 10 months old scallops occurred before temperatures began to rise steeply, to cover costs of gonad growth, as earlier reported for *Argopecten irradians* (Bricelij 1987). In this short-lived (~ 2 years) bay scallop a significant increase in oxygen uptake was associated with gamete production.

Trade-off between reproduction, cellular maintenance and aging in *A. ventricosus*

A. ventricosus reproduces and grows fast in the first year of lifetime (Maeda-Martínez et al. 1997). Afterwards, somatic growth in *A. ventricosus* is reduced as more energy is allocated to gonad development (Villalaz 1994). In this study, somatic growth diminished after the first year, while investment into gonad development remained similar in the first and second year of scallop lifetime (GI of 7.69 % in the second compared to 7.24 % in the first year). Assessment of GI is, however, only a rough approximation of the reproductive effort, and larval quality and quantity need to be measured in both years to assess whether the second reproductive effort is equally successful as the first one.

Diminished somatic growth in the second year was moreover connected to reduced somatic maintenance (decrease in apoptotic intensity and CAT activity). Specifically the decrease in apoptotic cell removal may relate to a prolonged prevalence of defective cells and cell components that can exacerbate oxidative damage. Oxidized proteins and lipids can build cross linkages, forming lipofuscin that can hardly be degraded and, consequently,

accumulates in the cell (Terman and Brunk 2004). Enhanced accumulation of age pigment granula in the cells can be interpreted as stocking of garbage that compromise cellular flux processes and physiological functioning, including mitochondrial energy production (Kurz 2007, 2008). Indeed, lipofuscin increased steeply within the last two months of scallop lifetime and was the only stress/age marker that was finally higher in older compared to young scallops. Contrary, protein carbonyls and lipid peroxides remained low even following maturation in the second year. Both markers, thus, respond to acute oxidative stress occurring during enhanced metabolic activity as already found in fish (Kammer et al. 2011), and slower metabolism and presumably lower ROS production in the second year may explain the lower levels of oxidative damage at the second half of scallop lifetime. The fact that lipofuscin content increased in the late survivors reflects reduced investments into cellular waste removal in old specimens, and indicate that the lack of cellular renewal may be actually more important for damage accumulation than an increase of prooxidative processes at the end of life (see also Yin and Chen 2005).

Levels of antioxidant activity and oxidative damage differ between short-lived *A. ventricosus* and longer-lived bivalves

The oxidative stress theory of aging predicts that the differences in the rate of aging among species are attributable to the differences in oxidative damage accrual (Harman 1956, Perez et al. 2009). Hence, one of our hypotheses was that the active lifestyle characterized by the early onset of reproduction, fast growth, and swimming activity of *A. ventricosus* results in a short lifespan, caused by fast accumulation of oxidative damage. Indeed, *A. ventricosus*

exhibit higher absolute protein carbonyl concentrations in mantle and gills when compared to longer-lived bivalve species, which however, cannot be attributed to a low investment into cellular defense mechanisms as, at least, apoptosis intensities and SOD activity, are also higher in *A. ventricosus* (Table 1). High SOD activities in mantle and gills correspond to the higher metabolic rates of *A. ventricosus* compared to the longer-lived bivalves (Philipp et al. 2005, 2006 Begum et al. 2009). SOD detoxifies superoxide ($O_2\bullet^-$), the first ROS product formed in mitochondria, and the activities of this enzyme are known to be strongly correlated to species metabolic rates (Abele and Puntarulo 2004; Abele et al. 2007). Nevertheless, high SOD activities do apparently not compensate the resulting oxidative damage and, indeed, elevated activity of only one antioxidant enzyme is not sufficient to prevent oxidative damage accumulation (Costantini and Verhulst 2009, Horak and Cohen 2010). In contrast to SOD, CAT activity in *A. ventricosus* is in the same range compared to the longer lived (~ 8 years) and also actively swimming scallop *A. opercularis* but 6 times lower than in the long-lived (~ 400 years) quahog *A. islandica* (see Table 1). Further, CAT but not SOD activity declines steeply in older bivalves (Sukhotin et al. 2002; Philipp et al. 2005; Ivanina et al. 2008; Abele et al. 2008) indicating that the decreased protection against cellular H_2O_2 formation may be a major cause for age related oxidative stress.

The accumulation rate of the irreparable damage that remains after all repairing and removal mechanisms have acted, may be more essential to disentangle real differences in aging rate between species. Whereas *A. ventricosus* control lipofuscin levels in young animals and accumulate this age pigment only in the oldest scallops, in the longest-lived bivalve known so far, the ocean quahog *Arctica islandica,* lipofuscin concentrations

increased between young (5-11 years) and older (110-192 years) animals (Strahl et al. 2007), but reached a steady-state within the older (110-192) age class. It is worth noting that no older specimens between 200 and 400y are available and is still questionable if lipofuscin maintain the seady state levels also until the end of the quahog´s lifespan. The ocean quahog is characterized by constancy of cellular maintenance mechanisms (antioxidant activity and apoptosis rates in all tissues except for the heart) up to at least 200y of lifetime, and it keeps the accumulation of protein carbonyls low also following sexual maturation (Abele et al. 2008; Strahl and Abele 2010). This contrasts the life strategy in short lived *A. ventricosus* that supports cellular maintenance, reproduction and fitness during the first year at the costs of fast damage accumulation (steep increase in lipofuscin) in last survivors. *A. ventricosus* is therefore a good example for the evolutionary theory of aging, which predicts that efficient somatic maintenance conserves organismal fitness only as long as there is another reasonable chance to survive and reproduce in the wild (Kirkwood and Austad, 2000). Ridgway et al. (2010) already described the inverse relationship between developmental schedules (time to maturity and growth rate) and longevity in different bivalves. In *A. ventricosus*, high predation pressure in the field apparently supports selection of early fitness at the cost of rapid aging after the second reproductive event.

Table 1:. Comparison of aging parameters between bivalve species with different lifestyles and maximum lifespan.

	A.ventricosus	*A. opercularis*	*A. islandica*	*M. arenaria*
Maximum lifespan	~2 years[1]	~ 10 years[2]	~ 350years[3]	~ 13 [4]
Lifestyle	swimming	swimming	burrowing	burrowing
Earliest age at sexual maturity	4 months [5]	1 year [6]	7 years [7]	1 year [8]
Investigated age range	8 months - 2years	1-5 years	7-192 years	2-8 years
Location	Baja California Sur (Pacific Coast)	Isle of Man (Irish Sea)	Iceland (Atlantic Ocean)	The Netherlands
SOD				
mantle	5681.1 ± 1674.3	379 ± 169 [9]	658.2 ± 364.2 [10]	956.3 ± 194.2 [12]
gills	5720.1 ± 1380.5	/	810 ± 406.5[10]	/
CAT				
mantle	393.4 ± 121.6	384 ± 69[9]	2330.5 ± 452.4[10]	136.5 ± 44.1[12]
gills	622.7 ± 158.5	/	3526.2 ± 740.3[10]	/
Protein carbonyls				
mantle	2.4 ± 0.7	1.0 ± 0.1[9]	0.58 ± 0.46 [11]	1.1 ± 0.3[12]
gills	7.7 ± 1.6	/	1.14 ± 0.54 [11]	/
Apoptosis				
mantle	491.5 ± 292.5	209.9 ± 126.4 [10]	73.7 ± 51.8[10]	/
gills	451± 289.6	570.5 ± 236.7[10]	326.3 ± 146.1 [10]	/
muscle	383.1 ± 270.9	487.9 ± 571.6[10]	47.8 ± 39.2[10]	/

[1)] Keen 1971, [2)] Ansell et al. 1991, [3)] Wanamaker et al. 2008, [4)] Strasser 1999, [5)] Cruz et al. 2000, [6)] Aravindakshan 1955, [7)] Thorarinsdottir and Steingrimsson 2000, [8)] Coe and Turner 1938, [9)] Philipp et al. 2006, [10)] Strahl and Abele 2010 (age range for apoptosis: 7-148 years, age range for CAT and SOD: 29-141), [11)] Strahl et al. 2007 (age range for protein carbonyls: 110- 192years), [12)] Philipp et al. 2005.

CONCLUSIONS

In conclusion, we have shown that oxidative damage does not increase steeply and continuously throughout the short lifespan of the scallop *A ventricosus*. The short-term markers of oxidative damage, protein carbonyls and TBARS, increase transiently in response to enhanced growth rates and reproduction events in the first and to a lesser extent in the second year of life. Antioxidant capacity did not fully counterbalance ROS formation, and oxidative damage accumulation in mantle muscle and gill tissues during gonad maturation in the first year, which may however be necessary to trigger the removal of damaged cells through programmed cell death. The decrease in apoptosis intensities and in CAT activity during the second year of life indicate aging in second year scallops and may be causal for the observed rapid accumulation of the undegradable fluorescent age pigment, lipofuscin, in the last survivors. Altogether, this seems to be part of the life strategy of *A. ventricosus* favoring rapid growth and early maturation at young age over cellular maintenance and longevity in old scallops.

ACKNOWLEDGEMENTS

We thank Mario Osuna, Ernesto León, Carmen Abundis and Stefan Koengistein for technical support, assistance with the hatchery and animal maintenance. We further thank Norma Olguín, Orlando Lugo and Stefanie Meyer for excellent technical assistance in the laboratory. We gratefully acknowledge the supply of scallops from Cooperativa del Puerto de San Carlos, Baja California Sur with which we started our culture. This research was supported by the DAAD grant D/06/47197 and the SAGARPA- CONACyT project 11947.

CHAPTER 5: GENERAL DISCUSSION

General discussion

Within this synoptic discussion, I will highlight the central findings of publication 1 and of the manuscripts 1-3. In the first part of the discussion, I focus on the link between oxidative stress and aging in *A. ventricosus*. Then I concentrate in the specific extrinsic (temperature and predation) and intrinsic (reproduction) factors that influence individual life history traits and population lifespan and discuss if oxidative stress plays an underlying modulatory role. In the last part, I discuss the limitations of long-term laboratory studies and highlight the importance of conducting field experiments within an ecological meaningful context.

5.1. Is oxidative stress a driver of aging in *Argopecten ventricosus*?

The oxidative stress theory of aging has been one of the dominant mechanistic theories explaining how multicellular organisms age and why they die (Speakman and Selman 2011). Despite substantial correlative evidence among antioxidant capacities, oxidative damage accrual and aging, the role of oxidative stress as a determinant of lifespan is still a matter of debate (Buffenstein et al 2008).

The high absolute levels of protein carbonyls conjunctly to a high metabolic rate and the short lifespan of *A. ventricosus* compared to other bivalve species (manuscript 3) in part support the oxidative stress theory of aging. However, the fine-tune analysis of oxidative stress throughout the lifespan of *A. ventricosus* reveal that young (1 < year) *A. ventricosus* scallops acquire more TBARS (lipid peroxidation products) and similar protein carbonyls in gill, mantle and muscle tissues compared to the old individuals (> 1 year) (manuscript 3). Moreover, the increase in the antioxidant capacities did not appear to prevent the

accumulation of damage in this species weather if oxidative stress is induced by thermal stress, swimming activity or reproduction (manuscript 1, 2 and 3). In the contrary, levels of antioxidants tend to be rather positively related with levels of oxidative damage independent of environmental factors or reproductive strage. These results are in conflict with the oxidative stress theory that predicts that the steep increase in oxidative damage accrual is linked to a precipitated loss of antioxidant capacities in short lived species. The fact that antioxidant capacities and oxidative damage may react in an orchestrated manner as observed for *A. ventricosus* is in line with recent findings that proposed that it is certainly a selective advantage of organisms to counterbalance ROS and to protect cells against oxidative stress by inducing antioxidant capacities. However, a complete neutralization of ROS is not desirable (Buffenstein 2008; Horak and Cohen 2010; Pamplona and Costantini 2011). This is because ROS and certain amounts of oxidative damage are necessary to induce a stress response in the cells, which ultimately activates other processes that are also primordial to maintain cellular homeostasis. Contrary to the traditional view that cells of longer-lived species are better protected by high antioxidant capacities, the higher levels of SOD in *A. ventricosus* when compared to longer-lived species (manuscript 3) may be related to relative higher respiration rates, compared to respiration rates of 59 bivalve species (Figure 5.1) and the need to dismutate superoxide radicals to H_2O_2.

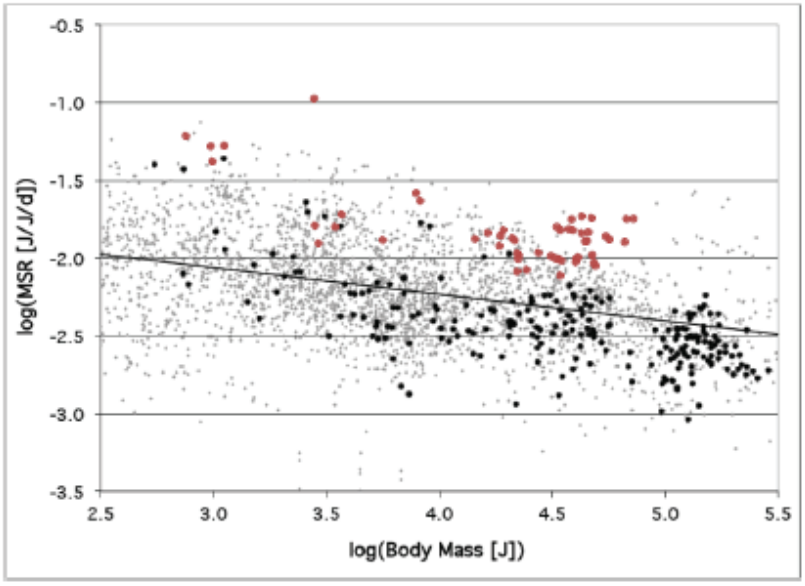


Figure 5.1: Temperature adjusted (10°C) mass specific respiration rates (MSR J/J/d) versus body mass M (J) modified after Begum et al. 2009. Black dots represent MSR from the longest-lived bivalve (*Arctica Islandica* record lifespan ~400 y *N* = 234). Grey dots represent MSR from 58 bivalve species (*N*= 3583) and red dots MSR of *A. ventricosus* reared in the field over the study period October 2007-August 2009 (*N* = 54). Respiration rates in *A. ventricosus* were measured at a temperature around 20°C (manuscript 3). We used a Q_{10} of 2.06 to estimate our data at 10°C. To get the Q_{10}, respiration rates of a standard bivalve was calculated at 10°C and at 20°C using linear regression of log (MSR) on log (M) over the data of the 59 bivalve species represented by the grey and black dots and the straight line. The parameters of the linear regression are described in Brey (2001). Body mass was converted to Joules (J) (1 mg dry weight = 22.3 Joules, Brey 2001) and respiration rates to Joules per day (J/d) (1 mg O_2 = 14.1, Joules Elliot and Davison 1975).

It was previously reported that H_2O_2 can initiate a variety of signaling cascades and it is the most important signaling radical (Foman et al. 2010). The generation of H_2O_2 by SOD, an enzyme that is known by its fast catalytic velocities (10^9 M^{-1} s^{-1} Forman et al. 2010), as well as the subsequent enzymatic degradation by catalase, and gluthation peroxidase makes this radical a suitable second messenger. The decrease of catalase in the older individuals did not directly lead to an increase in levels of oxidized proteins or lipids but the decrease of

this antioxidant could be an indicator of a deregulation in signaling pathways. In fact, recent studies suggest that the disruption of signaling processes in the cell is more likely to serve as a pacemaker of the aging process (Buffenstein 2008; Metcalfe and Alonso-Alvarez 2010). In mammals, transcription factors such as NF_kB and NF_k2 are known to be regulated by their fine tuning oxidation that allow them to bind in DNA and coordinate a variety of "stress" genes that finally induce antioxidant responses, marshal inflammatory responses, and resistance to infections and diseases (Martindale and Holbrook 2002; Lane 2003; Safdar et al. 2011). Furthermore, the oxidation of special lipids such as the mitochondrial lipid cardiolipin triggers programmed cell death (apoptosis) (Mcmillin and Dowhan 2002). In A. *ventricosus*, apoptosis clearly appeared to be an elemental cell maintenance mechanism for elimination of excessive oxidative damage (manuscript 3). Apoptosis intensities in mantle, muscle and gills are comparable to intensities found in the short lived scallop *A. opercularis* (recorded lifespan ~ 8 years), which in that species, are combined with high cell proliferation rates (Strahl and Abele 2010). High rates of cellular removal and renewal in scallops that have a higher scope of activity and high lifetime metabolic rates may be more important than antioxidant protection in order to conserve cellular homeostasis compared with sessile and less active bivalve species that exhibit lower turnover rates (manuscript 3, Strahl and Abele 2010). According to Yin and Chen (2005), it is the remaining oxidation products after all repairing and removal mechanisms have acted, e.g the non-degradable cross-linkages such as lipofuscin and non-enzymatic glycation, that may strongly contribute to a vicious cycle that increasingly interferes with cellular functioning (lysosomal autodigestive capacity) and affect cellular homeostasis (Terman and Brunk 2004; Jung 2010). Even if protein carbonyls and TBARS concentrations ranged at higher or similar levels in young compared to old *A. ventricosus* individuals, reduced

apoptotic intensities during the second year of scallops´ lifetime, lowered their removal capacity indicating that old individuals have a lower tolerance threshold for "cleaning" oxidative damage. Indeed, lipofuscin was the only marker that accumulated steeply in the last survivors (at 20-24 months of age). It is worth noting that lipofuscin increased only just at the end of scallop lifetime so that only when the very oldest (24 months of age) individuals are compared to the youngest (8 months of age) individuals, a significant difference in tissue specific lipofscuin concentration can be detected (manuscript 3). This reflects the fact that *A. ventricosus* scallops succeed to keep waste accumulation at a steady state level over the most part of their life despites their short lifespan and active lifestyle and support surprising findings of the short-lived scallop, *Aequipecten opercularis*. Indeed, *A. operularis* exhibits a marginal decrease in mitochondrial functions (assessed as low and constant mitochondrial H_2O_2 generation rates) with age (Philipp et al. 2006). The author suggested that high mitochondrial functions allow the queen scallop to "keep as fit as possible and evade extrinsic mortality from predators as long as possible" (Philipp 2006). In that study however, no queen scallops older than 5 years could be obtained because of stock over-exploitation in the Irish sea. Hence, it remained unknown if mitochondrial function is affected in the very oldest individuals (Philipp 2006). In contrast, *A. ventricosus* scallops could have been raised until the end of the record lifespan known for this species (~ 2 years) in a predator-free environment. Under these conditions, it was possible to demonstrate that in this species, but maybe also in other scallop species, only if last survivors of a population are included an increase in lipofuscin becomes evident. This situation is often not given in field studies due to the impracticability to sample the oldest specimens and to discern physiological parameters across all age classes (Abele et al. 2009).

In light of the results obtained for *A. ventricosus* as model species, I conclude that oxidative damage does not accumulate inexorably regardless of time of life. In the young scallops, damage is transient and should be eliminated through processes such as apoptosis before the undegradable cross-links (lipofuscin) start to build and accumulate. Thus, I suggest that for the process of aging, it is less important to determine whether animals have low or high levels of oxidative damage but rather how animals deal with these damage products and the implications that certain accumulation has for the whole individual. In accordance with this idea, old (> 1 year) *A. ventricosus* scallops do not accumulate more oxidative damage products but they rather fail to remove these oxidized products allowing formation and accumulation of lipofuscin within the cells. Lipofuscin represents only one of the results of aging but the disruption of signaling pathways by destabilization of redox processes during aging may lead to other negative effects on homeostasis. Even if it would be too anticipated to make a direct link between the increase of lipofuscin and scallops´ lifespan, lipofuscin certainly contributes to loss of cellular redox homeostasis and is an indicator of aging.

As *Argopecten ventricosus* showed to have a great flexibility in how it deals with oxidative challenges over time in its natural environment, the question arising is how extrinsic (temperature, predation) and intrinsic (reproduction) factors modulate tissue specific oxidative stress patterns and to which extent this is linked with individual survival. In order to answer these questions, experiments were conducted under controlled laboratory conditions where changes can be attributed to the specific independent variables: temperature, predation and reproduction.

5.2. Temperature, metabolic rate and the rate of living

Argopecten ventricosus, and ectotherms in general, cannot regulate their body temperature in accordance with surrounding water temperature. As a result, *A. ventricosus* increased the metabolic rates conjunctly with the exposure to constantly elevated but sublethal temperatures, so that respiration and food absorption rates ranged over 20-50 % higher in warm exposed individuals compared to the control group (manuscript 1). Concomitantly to the higher respiration rates, warm exposed scallops exhibited also higher mortality rates (Publication 1). This notion can be incorporated into the rate of living theory (Pearl 1928) in which is proposed that the duration of life varies inversely to the rate of metabolic rates in the sense of "living fast dying young" (Speakman et al. 2002).

The experimental design in this thesis circumvents one major critic of previous tests of the theory, specifically the failure to simultaneously determine metabolic rate conjunctly with lifespan within individuals of the same species and under the same environmental conditions (Speakman et al. 2002). To achieve this aim, metabolic rates were experimentally increased and impact on lifespan was assessed in different experimental groups. Our initial prediction was that an acceleration of the rate of living would inevitably increase the oxidative damage accrual compromising cellular homeostasis in a great extent leading to the enhanced mortalities (Pearl 1928; Harman 1956). Indeed, the high damage accrual measured in mantle, muscle and gill tissues after 5 months exposure may have contributed to the higher mortalities observed in warm exposed scallops. According to Salmon et al. (2010), oxidative stress has little impact in modulating the aging process and determining lifespan under natural and favorable conditions. However, under stressful and unfavorable conditions, as in our temperature experiment, the ability to resist oxidative

stress might be reduced which compromise cellular homeostasis and individual survival to a greater extent.

Even if there is a conspicuous link, it would be incorrect to conclude that the elevated oxidative damage found in scallops after 5 months exposure is attributed to the elevated metabolic rates per se. In situations where oxygen is available, energy supply and demand is balanced and mitochondria are well coupled, oxidative phosphorylation proceeds more rapidly at higher metabolic rates. This is accompanied by a rapid fall of the membrane potential and even a reduction of superoxide formation and no increase in ROS induced oxidative damage relative to the oxygen consumption (Buettemer et al. 2010). Higher metabolic rates may go hand in hand with excessive ROS production if oxygen solubility is decreased; a situation that can happen at elevated temperatures where oxygen shortage may induce hypoxic conditions and exacerbate oxidative stress (Abele et al. 1998; Poertner et al. 2002; Abele and Puntarulo 2004). In the present study, a lack of oxygen is less possible explanation as scallops exposed to elevated temperatures where constantly aerated and exhibited a higher mantle and gill index compared to the control group, which may afford better oxygenation of tissues (publication 1). Increase in ROS production may occur if the higher respiration rates overwhelm the kinetic capacities of mitochondrial enzymes and promote excessive ROS generation leading to dysfunctional mitochondria and the excessive oxidative damage. Antioxidant capacities in mantle, muscle and gill tissues increased as response to the accelerated cellular metabolism at elevated temperatures however, antioxidant capacities obviously failed to counterbalance excessive ROS-production.

Another consequence of thermal stress in *A. ventricosus* is the lower energy allocation for growth (scope for growth) relative to the oxygen consumed. The relative low scope for

growth in warm exposed scallops is related to the higher energy expenses by respiration rates in relation to the energy absorbed by food (preliminary chapter). Enhanced expression of energy costing antioxidants may have reduced energy investments for growth under thermal stress. During exposure to higher temperatures, the costs associated with cellular maintenance and growth requires temperature compensation of metabolic rate-limiting enzymes to support the flux of electrons through the respiratory chain of mitochondria and supply the energy demands (Lesser and Kruse 2004). Lesser and Kruse (2004) showed that mussels (*Modiolus modiolus*), enhance concentration of the mitochondrial rate-limiting enzymes citrate synthase, and activity of cytochrome *c* oxidase while keeping the same level of antioxidant protection during a decrease in temperature. This was suggested as a strategy to prevent excessive ROS production and to allow mussels to keep a close to steady-state of energy production (ATP) independent of temperature variation.

The dysfunctional mitochondria and the lack of compensatory capacities of rate limiting metabolic enzymes could be the missing link between the higher respiration rates and the higher oxidative damage in warm exposed *A. ventricosus* scallops. In fact, in Arctic and Antarctic bivalves the increase in ROS induced oxidative damage is related to damaged and uncoupled components of the mitochondrial respiratory chain (Abele et al. 1998b, 2002, Heise et al. 2003). For A. *ventricosus*, the functional state of mitochondria, as well as the evaluation of metabolic rate-limiting enzymes still has to be investigated.

Even if mortality rates were higher at elevated temperature, a small proportion (2.6 %) of the initial population exposed to higher temperatures, lived longer compared to the control group (publication 1). Interestingly, after 9 months of warm exposure, *A. ventricosus* exhibited lower levels of protein carbonyls and lipid peroxides in gill and mantle tissues in

respect to the control group despites the continuous elevation of temperature (Manuscript 1). An explanation is that higher mortality rates measured at elevated temperatures selected strongly for individuals with better physiological condition so that the older age classes include “better quality” individuals i.e individuals with less oxidative damage formation or scallops that indeed could compensate the elevated temperatures. Hence, during the lifelong exposure to elevated temperatures two periods could be distinguished: The first period, which compose the time until 5 months exposure, where susceptible individuals are in abundance and a second period after 9 months exposure where all susceptible individuals already died. In this case, high mortalities at elevated temperatures counterbalanced such temperature effect by natural selection of the least susceptible specimens. This would not only explain the inconsistent levels of oxidative damage, but also the higher condition index and the higher scope for growth that was measured after 9 months but not after 5 months exposure in respect to the control group (publication 1 and manuscript 1).

In conclusion, a long-term exposure to higher but sublethal temperatures can potentially select for more stress tolerant animals with better physiological condition, lower oxidative damage and higher scope for growth. These factors can contribute to the longer lifespan of some individual within a population of *A. ventricosus* scallops. The longer lifespan comes however at the costs of a decrease in population densities due to higher mortality rates in “low quality” or more susceptible animals. From an ecological point of view, mortality rates induced by thermal stress could lead to considerable consequences for population dynamics. In fact, *A. ventricosus* stocks in the field show irregular pulses of high abundance followed by periods of scarcity or collapse (Maeda-Martínez et al. 1993) which are typical for short living species and are classified as “spasmodic stocks” (Orensanz et al.

2006). The variations are partially linked to drastic temperature fluctuations for example by increasing temperatures during El Niño events. Such a drastic increase in temperature in 1991 as found in Bahía Concepción, in the Gulf of California, México for example, led to catastrophic mortalities of *A. ventricosus* in this zone (Maeda-Martínez et al. 1993). Together with the intense fisheries, this resulted in the collapse of the Bahía Concepción population between 1991-1993 (Félix-Pico et al. 1997).

5.3. The link between predation, reproduction and survival

The precocious lifestyle of *A. ventricosus* in its natural environment is thought to be linked to the high predation pressure attributed mainly by fishes (Ballistidae, Tetradontidae), gastropods (*Muricanthus*), and crabs (*Callinectes, Cronius*) (Ciocco and Orensanz 2001). Predation is suggested to be an important factor that determines the fast growth rate and the early onset of reproduction, which however cannot be combined with a long lifespan. Although this assumption would explain why this scallop is so short-lived, I clearly found the opposite pattern, means that scallops that are reared in presence of a potential predator *Callinectes sapidus* exhibit lower mortality rates and a longer lifespan compared to scallops that are reared under the same conditions but in a predator-free environment (publication 1). Hence, I suggest that in *A. ventricosus* scallops, higher rates of predation selected for higher performance and a longer intrinsic lifespan as already posit in critical studies by Williams and Day (2003) and Reznick et al. (1990, 2001, 2004). Indeed, scallops that were exposed to a predator exhibited enhanced swimming performance reflected by the bigger adductor muscles and higher ODH levels compared to predator-free scallops (Manuscript 2). The enhanced swimming behavior in scallops is powered by muscle contractions, which

are fueled mainly by the breakdown of glycogen to octopine that is catalyzed by ODH (Bailey 2003). Moreover predator-exposed scallops exhibited thicker shells without compromising shell growth (shell height increment) compared to the predator-free group (publication 1). At the cellular level, the lower content of protein carbonyls and lipid peroxides in mantle and gill tissues in predator-exposed scallops speaks for better cellular maintenance. Only in faster growing and more active muscle tissues of the predator-exposed scallops, oxidative damage appeared to be elevated. However, the higher oxidative damage in muscle did not compromise individual performance (swimming capacity) or survival, which suggests that consequences of oxidative damage for the whole organism have a tissue-specific basis. In the laboratory, where individuals were exposed to predators but could not be eaten because the crab´s pincers were held together, the improvements in physiological performance and cellular maintenance in gills and mantle can translate into longer survival rates (see also Reznick 2004).

In *A. ventricosus* the physiological improvement could be sustained by diversion of resources away from reproduction (deduced by the lower gonadosomatic index GSI). Indeed predator-exposed scallops delayed spawning by 2 months compared to predator-free individuals and also reached a 25% lower peak gonadosomatic index (Publication 1). Hence, by definition, scallops were not really fitter when reared with predators because reproductive effort, an important parameter that defines fitness, was decreased. Due to the high energetic cost of reproduction, the observed delay in spawning and decrease in reproductive effort may have attributed to the longer lifespan in the predator-exposed group in addition to the improvement in physiological performance and tissue specific cellular maintenance.

Altogether, the results point out that if reproduction effort decreases because of an enhanced ability to resist predators, the increase in lifespan will come only at the cost of reproduction and recruitment. *A. ventricosus* scallops exposed to predators may reproduce for longer time because they live longer but would reproduce less frequently and with lower gamete output. As a corollary, any extra investment in durability would not be beneficial for this species because this implicates a reduction in future population density.

Lifespan is a by-product of a complex life history that evolved under particular environmental conditions. An increase in both analyzed environmental factors: temperature and predator exposure may prolong population lifespan in *A. ventricosus*, which may however lead to negative implications for population dynamics in this short living species as it may decrease reproduction output (predation) or population densities (temperature) and affect future recruitment.

5.4. The cost of reproduction

The cost of reproduction is a fundamental selective force that shapes life history trajectories in organisms (Stearns 1992). Scallops in general are known to invest so heavily into gametogenesis and spawning, that reproduction represents a major stress (Barber and Blake 1991). In *A. ventricosus*, gonadal maturation is fueled by energy diverted from other tissues principally from the adductor muscle (publication 1 and manuscript 3). The energy mobilization from adductor muscle towards the gonad reduces muscle metabolic capacities (manuscript 2) which have been shown to compromise the escape response and recovery after burst swimming in other scallops (Brokordt et al. 2000 a,b). Moreover, after

spawning, *A. ventricosus* scallops exhibit a steep decrease in physiological condition (deduced by the condition index) (publication 1). Hence, after gonadal maturation and spawning, individuals are more vulnerable to mortality and more susceptible to predation (Blake and Moyer 1991, Brokordt et al. 2000 a, b). In *A. ventricosus*, high investments into reproduction are not only paid in form of energy allocation to gonad and energy drainage after spawning but are also connected to the peak increase in protein carbonyls and lipid peroxides in scallops´ muscle, mantle and gill tissues. Mantle and gill tissues are not directly involved in gametogenesis but maintain high metabolic rates to support energy demands at this period. Gametogenesis in the field occurred at the period of a steep increase in water temperature (manuscript 3) so that the effects of temperature could mask the resulting oxidative damage in muscle, mantle and gill tissues. However, under controlled temperature conditions in the laboratory, oxidative damage reflected a temperature-independent correlation with gametogenesis indicating that in *A. ventricosus*, mainly maturation modulates oxidative damage formation (manuscript 2).

The reproductive strategy in short lived bivalves such as *A. ventricosus* differs from the strategy of longer-lived species that do not exhibit peak reproductive periods and rather budget energy investments in low but persistent gametogenesis (Strahl and Abele. 2010). A study by Cardoso et al. (2009) showed that a long-lived clam can use gonads not only for reproduction but also for energy storage while a short-lived clam spawns completely releasing all the energy in form of gametes. These findings points towards the fact that longer-lived bivalves benefit from minimizing the cost for current reproduction and may prevent the peak increase in oxidative damage and the necessity to enhance energy-costing mechanisms such as apoptosis to remove the damage (see Fig. 5.2).

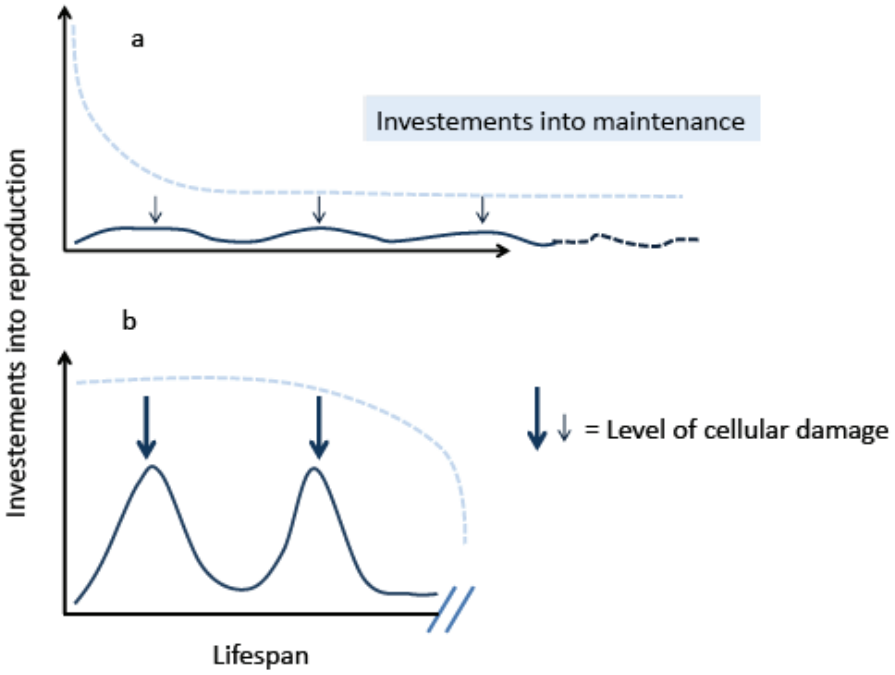


Figure 5.2: A simplified illustration of the relationship among reproductive investments, oxidative costs and cellular maintenance in long (a), compared to short lived species (b).

It is certainly true, that broadcast spawning in scallops may compromise survival. However, it is questionable if the peak increase in oxidative damage is involved. In fact, apoptosis is triggered parallel to oxidative damage in mantle, muscle and gill tissues that afford the elimination of damaged cells (manuscript 3).

Interestingly, the oxidative cost of reproduction is not constant throughout *A. ventricosus´* lifetime and depends on the age class that is studied. In *A. ventricosus*, the steep increase in metabolic rates accompanied by the transient accumulation of protein carbonyls and TBARS related to gametogenesis are only observed during the first year of scallop's lifetime. A diminished reproductive effort does not explain these differences, as the gonad index was similar in both consecutive years (manuscript 3). This is different to some mammals and birds where the relationship between litter size and oxidative damage (or

susceptibility to oxidative stress) seems to be age-independent (Bize et al. 2008, Bergeron et al. 2011). It is possible that in *A. ventricosus,* the prevention in oxidative damage is related to the lower metabolic rates and growth in the second year, which could be also linekd to the diminished gamete production and larvae quality that is not obvious by measuring only the gonad index (Román et al. 2001).

5.5. The controversy between laboratory and field conducted experiments

In the present study, all scallops were hatched at the same time from the same spawning event so that all individuals had the same chronological age, and experienced the same breeding conditions in the hatchery until an age of 3 months. Compared to the control group that remained in the laboratory at simulated field temperatures (SFT group), the group that was subsequently reared and maintained in the field, reached 1.5 times higher shell size after 1 year, 3 times better physiological condition (deduced by the condition index) and invested 2 times more energy into reproduction (deduced by the GSI). Moreover, 50 % of the initial stock survived to the second year and scallops reproduced again. Contrary, the SFT group reared in the laboratory literally reproduced to death after the first spawning event at the end of their first year when all animals died directly after spawning. Only when predators were introduced, post-spawning mortality did not occur which may be due to changes in energy investments such as reproduction. These results indicate that standardized laboratory conditions are not optimal and cannot appropriately

substitute for the natural conditions in the field so that long-term laboratory maintenance can be anticipated to reduce physiological condition in scallops.

The fast growth observed in the field scallops occured mainly within the first 2 months after release in the field while the spat that remained in the laboratory continued to grow at a constant but lower rate. Bivalves can exhibit sudden compensatory growth when environmental conditions become favorable which allow them to reach their optimal physiological size at a given age (Broekhuisen 1994). Consequently, field animals were 2-times bigger than scallops in the laboratory at the respective time of reproduction and probably were more robust and less susceptible to energetic drainage after spawning. Hence, even if the reproductive effort was higher (shown by a 20 % steeper increase in GSI), field scallops kept a 20 % higher condition index before spawning and even though post-spawning mortality also occured in the field, it did not compromise the survival of the whole population, as for the laboratory cohort. This reflects that the environmental conditions experienced during the early stages have a substantial impact on future life history trajectories such as reproductive output and survival. In scallops, gametogenesis is a genetically controlled process, which depends on the interactions between exogenous and endogenous factors (Barber and Blake 1991). However, a minimum age and size has to be reached before the beginning of gametogenesis (Barber and Blake 1991). Hence, the fast growth can be a selective advantage in order to attain early sexual maturity in this scallop, which is primordial to ensure recruitment in the field before falling prey to predators.

From these results, I conclude that standardized conditions in the laboratory e.g *ad libitum* feeding with a mixed and “appropriate” microalgal culture for scallop growth, cannot substitute the natural conditions in the field. It was already shown in mussels that after

acclimation to laboratory conditions, scope for growth decrease markedly caused by a decrease in food ingestion rates, which authors describe as "not surprising due to the obvious differences between field and laboratory conditions in terms of food quantity and quality" (Mubiana and Blust 2007). Similar, *A. ventricosus* also has a marked lower scope for growth and ingestion rates in laboratory vs. field read animals (Table 5.1). Additionally to the lack of food variability, the less variable temperatures may not be necessarily more favorable (Publication 1). This was already shown in a comparative study with lion spawn scallop (*Nudipecten subnodosus*) (Sicard-González et al. 2006) where oscillating day and night temperatures led to a faster growth an longer survival rates in spite of their earlier onset of reproduction when compared to scallops maintained at an experimentally determined constant optimal temperature.

Table 5.1: Absorption rate (AR), and scope for growth (SFG) of *A. ventricosus* expressed as J h^{-1} for a standard animal of 1 g under simulated field temperatures in the laboratory (SFT) as well as in the field. Values are means ± SD. Lower case letters represent significant differences between treatments. (t-test $P < 0.05$).

	AR (J h^{-1})		**SFG (J h^{-1})**	
	April 2008	August 2008	April 2008	August 2008
SFT	18.4 ± 3.4[a]	41.6 ± 5.3 [a]	4.7 ± 1.6 [a]	13.9 ± 5.2 [a]
Field	27.41± 4.9 [b]	61.7 ± 6.4[b]	16.3 ± 6.2[b]	28.3± 5.4[b]

Laboratory studies offer a variety of advantages. For example, it is easier to control biotic and abiotic parameters in such as way that findings are more likely to be due to the independent variable. Moreover, they are easy to replicate due to the exact conditions being

controlled. However, keeping animals in the laboratory may influence physiological processes so that it's hard to know which results might be generalized and which might be particular to the laboratory environment (see review Cohen et al. 2010). For example, under the energy-limited conditions from the laboratory, predators may have stronger effect in modulating the trade-offs between somatic growth, reproduction and survival as under field conditions.

I support the idea that is an increasing appeal for conducting experiments in the field (Nussey 2009; Costanini 2010, Mecalfe and Alonso-Alvarez 2010; Bergeron 2011) mainly in studies that have the approach to understand physiological processes within a more meaningful ecological context.

CHAPTER 6: CONCLUSIONS AND PERSPECTIVES

Conclusions and perspectives

This study provides a comprehensive picture on cellular oxidative processes throughout the life stages of the short living scallop *Argopecten ventricosus*. The aim was to obtain a better understanding of extrinsic and intrinsic factors that can contribute in shaping the life history of an ectothermic marine species and to examine the role of oxidative stress in modulating aging and longevity.

Despite substantial evidence that suggest that reduced reactive oxygen radical formation, low oxidative damage accrual and increased antioxidant capacities are general characteristics of some long lived bivalves (reviews by Abele 2009 and Bodnar 2009), the data obtained for *A. ventricosus* speak against a simple link between antioxidant capacities, oxidative damage accrual, and life span. The strikingly higher enzymatic capacities of SOD in *A. ventricosus* when compared to longer-lived species indicate that antioxidant capacities are not always related with a long lifespan. In *A. ventricosus*, high SOD activities may certainly counterbalance superoxide radical formation, but it appeared that oxidative damage is rather efficiently eliminated by high apoptotic activity. To go further in these findings, it will be important to investigate the way scallops deal with oxidative damage by measuring DNA integrity, protein stability as well as other repairing mechanisms and systems that degrade oxidative damage such as proteolytic activity. Moreover, it would be promising to focus in the roles of redox signaling pathways. In mammals, it has been shown that the disruption of redox signaling pathways (e.g. reduction/oxidation of transcriptional factors) are linked to a plethora of negative physiological consequences ranging from metabolic disturbances to modulation of “stress genes” expression which may

play a pivotal role in the aging process (Buffenstein 2008; Leloup et al. 2011). Bivalves have a low ontogenetic complexity when compared to mammals so that the interplays between signaling pathways, genetic triggering and physiological responses might be less complex, might adjust more rapidly and might depend strongly on environmental changes.

The possibility to rear *A. ventricosus* scallops in its natural environment until the end of species record lifespan allowed us to address that despites scallops short lifespan, oxidative damage did not increase steeply with aging. Scallops accumulate the undegradable oxidized products (lipofuscin) just at the end of their lifetime. This finding brings into question if other bivalve species, that show rather constant levels of damage accrual in different age classes, would also show a decline in cellular homeostasis just at the end of their lives or is this pattern only found in scallops? To date comparative data of "old" individuals of different bivalve species are missing in order to have a comprehensive picture of changes in physiological and cellular functions throughout the later lifespan of short but also of long lived species.

The fact that protein carbonyls and TBARS ranged highest in pre-spawning animals and lowest in post-spawned individuals within the first year, suggest that these cell damage markers are transient and modulated by life history traits such as reproduction and growth. The fine tune analysis over two consecutive years and reproduction periods allowed discerning that the oxidative stress of reproduction is lower in the second compared to the first year. The question is: Do the prevention of peak oxidative damage during the second reproductive year come at the expense of a decrease in fecundity? Changes in larval quality and quantity with age are important determinants that give a more detailed picture about the fecundity and fitness of individuals (Sukhotin and Flyanchinskaya 2009). A deeper analysis

of larvae quality and quantity in both consecutive years would give insights into understanding changes in fitness and reproductive senescence. Within this context, it would be also promising to compare oxidative damage accrual in relation to fecundity over lifetime in other bivalve species in order to compare life history strategies depending on species and lifestyles.

Under thermal stress in the laboratory (temperature elevation), respiration rates, oxidative damage accrual (protein carbonyls and TBARS) and mortality rates were elevated. *A. ventricosus* could be an interesting model for the question of how scallops "handle" temperature changes in their environment. In this aspect, it would be relevant to examine mitochondrial functionalities of a scallop species that is adapted to a temperate environment but which can also experienced fast and important temperature fluctuations. It would be also promising to investigate conjunctly if scallops exposed to the high temperatures also failed to repair or remove the damage, which would corroborate more precisely to a failure in cellular homeostasis and to higher mortalities. Interestingly, the high mortalities in the warm exposed group seemed to select for scallops of better physiological condition opening the question if high, but sub-lethal temperatures, may serve as a selection mechanism for individuals under cultivation.

The exposure to predators revealed that phenotypic plasticity in *A. ventricosus* scallops permits allocation of energy to swimming muscle and development of thicker shells to evade predators. Reallocation of energy to muscle growth, however constrained reproduction as indicated by the deferment and the lower investment into gametogenesis. As spawning means a major energetic drainage, the lower reproductive effort may have prevented post-spawned mortalities. One question that arises is if the observed trade-offs

are restricted to the laboratory conditions. It was evident that the standardized conditions in the laboratory could not appropriately substitute the natural environment in the field, which was reflected by the lower growth, condition index and reproduction effort in laboratory-reared scallops. Under the energy limited conditions from the laboratory, predators may have exerted a stronger effect in modulating the trade-offs between muscle growth, reproduction and survival. The question is: Would an increase an increase in physiological condition (swimming performance/shell thickness) also constrain reproduction under the favorable field conditions? Or are the trade-offs found in the laboratory only valid under conditions of limited resource availability/quality? Even if very ambitious, it is necessary to conduct experiments in the field in order to define the biological relevance of physiological and cellular processes within a more meaningful ecological context.

REFERENCES

Abele D., Brey T., Philipp E. (2009) Bivalve models of aging and the determination of molluscan lifespan. *Experimental Gerontology* **44**, 307-315

Abele D., Burlando B., Viarengo A., Pörtner H-O. (1998) Exposure to elevated temperatures and hydrogen peroxide elicits oxidative stress and antioxidant response in the Antarctic intertidal limpet *Nacella concinna*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **120**, 425-435

Abele D., Tesch C., Wencke P., Pörtner H-O. (2001) How Does Oxidative Stress Relate to Thermal Tolerance in the Antarctic Bivalve *Yoldia eightsi*? *Antarctic Science* **13**, 111-118

Abele D., Heise K., Pörtner H-O., Puntarulo S. (2002) Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. *Journal of Experimental Biology* **205**, 1831-1841

Abele D., Puntarulo S. (2004) Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* **138**, 405-415

Abele D., Philipp E., Gonzalez P., Puntarulo S. (2007) Marine invertebrate mitochondria and oxidative stress. *Frontiers in Bioscience* **12**, 933-946

Abele D., Strahl J., Brey T., Philipp E. (2008). Imperceptible senescence: Aging in the ocean quahog *Arctica islandica. Free Radical Research* **42**, 474-480.

Aebi H. (1984) Catalase in vitro. *Methods in Enzymology* **105**, 121-126

Abrams P.A. (1993) Does increased mortality favor the evolution of more rapid senescence? *Evolution* **47**, 877-887

Abrams P.A., Rowe L. (1996) The effects of predation on the age and size of maturity on prey. *Evolution* **50**,1052-1061

Acosta-Ruiz M.J., Lara-Lara J.R. (1998) Resultados fisico-químicos en un estudio de variación diurnal en el área central de Bahía Magdalena, B.C.S. *Ciencias Marinas* **5**, 37-46

Alonso-Alvarez C., Bertrand S., Devevey G., Prost J., Faivre B., Sorci G. (2004) Increase susceptibility to oxidative stress as a proxiamte cost of reproduction. *Ecology letters* **7**, 363-368

Alonso-Alvarez C., Bertrand S., Devevey G., Prost J., Faivre B., Chastel O., Sorci G. (2006) An experimental manipulation of life-history trajectories and resistance to oxidative stress. *Evolution* **60**, 1913-1924

Andreyev A.Y., Kushnareva Y.E., Starkov A.A. (2005) Mitochondrial metabolism of reactive oxygen species *Biochemistry* **70**, 201-214

Aniagu S.O., Day N., Chipman J.K., Taylor E.W., Butler P.J., Winter M.J. (2006) Does exhaustive excercise result in oxidative stress and associated DNA damage in the Chub (*Leuciscus cephalus)*?. *Environmental and Molecular Mutagenesis* **47**, 616-623

Ansell A.D., Dao, J.C., Mason, J. (1991). Three european scallops: *Pecten maximus*, *Chlamys* (*Aequipecten*) *opercularis* and *Chlamys varia*. In: *Scallops: Biology,*

Ecology and Aquaculture (ed. by S.E Shumway), pp.715-751. Elsvier, Amsterdam, The Netherlands

Aravindakshan I. (1955) *Studies on the biology of the queen scallop Chlamys opercularis (L.).* Doctoral Thesis, University of Liverpool, United Kingdom 230 pp

Asami S., Hirano T., Yamaguchi Y., Tsurudome Y., Itoh H., Kasai H. (1998) Effects of the forced and spontaneous exercise on 8-hydroxydeoxy-guanosine levels in rat organs. Biochem. *Biophysical Research Communications* **243**, 678-682

Ballantyne J.S., Hochachka P.W., Mommsen T.P. (1981) Studies on the metabolism of the migratory squid *Loligo opalescens*: enzymes of tissues and heart mitochondria. *Marine Biology Letters* **2**, 75-85

Bailey D.M., Peck L.S., Bock C., Pörtner H-O. (2003) High-energy phosphate metabolism during exercise and recovery in temperate and Antarctic scallops: an in vivo 31P-NMR study. *Physiological and Biochemical Zoology* **76**, 622-633

Barber B.J. and Blake N.J. (1983). Growth and reproduction of the bay scallop *Argopecten irradians* (Lamark) at its southern distrubution limits. Journal of Experimental *Marine Biology and Ecology* **66**, 247-256

Barber B.J., Blake N.J. (1991) Reproductive physiology. In: *Scallops: Biology, Ecology and Aquaculture* (ed. by S. E. Schumway), pp. 377-428. Elsvier, Amsterdam, The Netherlands

Beaulieu M., Reichert S., LeMaho Y., Ancel A., Criscuolo A. (2010) Oxidative status and telomere lenght in a long-lived bird facing a costly reproductive event. *Functional Ecology* doi:10.111/j.1365-2435.2010.01825.x

Beckerman A.P., Wieski K., Baird D.J. (2007) Behavioural versus physiological mediation of life history under predation risk. *Oecologia* **152**, 335-343

Begum S., Basova L., Strahl J., Sukhotin A., Heilmayer O., Philipp E., Brey T., Abele D. (2009). A metabolic model for the ocean quahog *Arctica islandica* – Effects of animal mass and age, temperature, salinity and geography on respiration rate. *Journal of Shellfish Research* **28**, 533-539.

Benson B.B., Krause D.J. (1984) The concentration and isotopic fractionation of oxygen dissolved in freshwater and seawater in equilibrium with the atmosphere. *Limnology and Oceanography* **29**, 620-632

Bergeron P., Careau V., Humphires M.M., Reale D., Speakman J.R., Garant D. (2011) The energetic and oxidative costs of reproduction in a free-ranging rodent. *Functional Ecology* **25**, 1063-1071

Berlett B.S., Stadtman E.R. (1997) Protein oxidation in aging, disease, and oxidative stress. *Journal of Biological Chemistry* **272**, 20313-20316

Bize P., Devevey G., Monaghan P., Doligez B., Christe P. (2008) Fecundity and survival in relation to resistance to oxidative stress in a free-living bird. *Ecology* **89**, 2584-2593

Blake N.J., Moyer M.A. (1991) The calico scallop *Argopecten gibbus*, fishery of Cape Canaveral, Florida. In: *Scallops: Biology, Ecology and Aquaculture* (ed. by S.E. Shumway), pp.899-912. Elsvier, Amsterdam, The Netherlands

Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. **72**, 248-254

Brey T. (2001) Population dynamics in Benthic Invertebrates. A Virtual Handbook. Version 01.2. http:// www.awi-

bremerhaven.de/Benthic/Ecosystem/FoodWb/Handbook/main.html Alfred-Wegener Institute for Polar and Marine Research, Germany

Bricelj M., Epp V., Malouf J., Robert E. (1987). Comparative physiology of young and old cohorts of bay scallop *Argopecten irradians irradians* (Lamarck): mortality, growth, and oxygen consumption. *Journal of Experimental Marine Biology and Ecology* **112**, 73-91.

Broekhuizen N., Gurney W.S.C., Jones A., Bryant A.D. (1994) Modelling compensatory growth. *Functional Ecology* **8**, 770-782

Brokordt K.B., Himmelman J.H., Nusetti O.A., Guderley H.E. (2000b) Reproductive investement reduces recuperation from exhaustive escape activity in the tropical scallop *Euvola ziczac*. *Marine Biology* **137**, 857-865

Brokordt K.B., Himmelmann J.H., Guderley H.E. (2000a) Effects of reproduction on escape response and muscle capacities in the scallop *Chlamys islandica* (Müller 1776). *Journal of Experimental Marine Biology and Ecology* **251**, 205-225

Brown M.R., Jeffrey S.W., Volkman J.K., Dunstan C.K. (1997) Nutritional properties of microalgae for mariculture. Aquaculture **151**, 315-331

Buettemer W., Abele D., Costantini D. (2010) From bivalves to birds: oxidative stress and longevity. *Functional Ecology* **24**, 971-983

Buffenstein R., Edrey Y.H., Yang T. (2008) The oxidative stress theory of aging:embattled or invincible? Insights from non-traditional model organisms. *Age* **30**, 99-109

Callard G., Jorgensen, J.C., Redding, M. (1993). Biochemical analysis of programmed cell death during premeiotic stages of spermatogenesis *in vivo* and *in vitro*. *Developmental Genetics* **18**, 140-147

Camus L., Gulliksen B., Depledge M., Jones M.B. (2005) Polar bivalves are characterized by high antioxidant defences. *Polar Research* **24**, 111-118

Cardoso J.F.M.F., Witte J.I.J., van der Veer H.W. (2009) Differential reproductive strategies of two bivalves in the Dutch Wadden Sea. Estuarine, Coastal and Shelf *Science* **84**, 37-44

Cheung S.G., Lam S., Gao Q.F., Mak K.K., Shin P.K.S. (2004) Induced anti-predator responses of the green mussel, *Perna viridis* (L.), on exposure to the predatory gastropod, *Thais clavigera* Kuester, and the swimming crab, *Thalamita danae* Stimpson. *Marine Biology* **44**, 675-684

Chih C.P., Ellington W.R. 1983. Energy metabolism during contractile activity and environmental hypoxia in the phasic adductor muscle of the bay scallop *Argopecten iridans concentricus*. *Physiological Zoology* **56**, 623-631

Ciocco N.F., Orensanz J.M. (2001) Depredación. In: *Los Moluscos Pectínidos de Iberoamerica: Ciencia y Acuicultura* (ed. by A.N. Maeda-Martínez), pp. 267-284. Noriega Editores, Mexico City, Mexico

Clarke A. (1987) Temperature, latitude and reproductive effort. *Marine Ecology Progress Series* **38**, 89-99

Clarke A., Fraser K.P.P. (2004) Why does temperature scale with temperature?. *Functional Ecology* **18**, 243-251

Coe W.R., Turner, H.J. (1938). Development of the gonads and gamets of the soft shell clam (*Mya arenaria*) *Journal of Morphology* **62**, 91-111

Cohen A.A., de Magalhaes J.P., Gohil K. (2010) Ecological, biomedical and epidemiological approaches to understand oxidative balance and ageing: what they can teach each other. *Functional Ecology* **24**, 997-1006

Conover R.J. (1996) Assimilation of organic matter by zooplankton. Limnology and *Oceanography* **11**, 338-345

Cooper C.E., Vollaard N.B.J., Choueiri T., Wilson M.T. (2002) Exercise, free radicals and oxidative stress. *Biochemical Society Transactions* **30**, 280-284

Costantini D., Verhulst S. (2009). Does high antioxidant capacity indicate low oxidative stress? *Functional Ecology* **23**, 506-509

Costantini D. (2010) Redox physiology in animal functions: The struggle of living in an oxidant environment. *Current Zoology* **56**: 687-702

Costantini D., Rowe M., Butler M.W., McGraw K. (2010) From molecules to living systems: historical and contemporary issues in oxidative stress and antioxidant ecology. *Functional Ecology* **24**, 950-959

Crowl T.A., Covich A.P. (1990) Predator-induced life history shifts in a fresh water snail *Science* **247**, 949-951

Cruz P., Ibarra A.M. (1997) Larval growth and survival of two catarina scallop (*Argopecten circularis*, Swobery, 1985) populations and their reciprocal crosses. Journal of *Experimental Marine Biology and Ecology* **212**, 95-110

Cruz P., Ramirez J.L., Garcia G.A., Ibarra A.M. (1998) Genetic differences between two populations of catarina scallop (*Argopecten ventricosus*) for adaptations for growth and survival in a stressful environment. *Aquaculture* **166**, 321-335

Cruz P., Rodriguez-Jaramillo C., Ibarra A.M. (2000) Environment and population origin effects on first sexual maturity of Catarina scallop, *Argopecten ventricosus* (Sowerby II, 1842). *Journal of Shellfish Research* **19**, 89-93

Delgado G.A., Glazer R.A., Steward N.J. (2002) Predator-induced behavioral and morphological plasticity in the tropical marine gastropod *Strombus gigas. Biological Bulletin* **203**: 112-120

De Magalhaes J.P., Church G.M. (2006) Cells discover fire: Employing reactive oxygen species in development and consequences for aging. *Experimental Gerontology* **41**, 1-10

Dowling D.K., Simmons L.W. (2009) Reactive oxygen species as universal constraints in life-history evolution. *Proceedings of the Royal Society B* **276**, 1737-1745

Edinger A.L., Thompson C.B. (2004) Death by design: apoptosis, necrosis and autophagy. *Current Opinion in Cell Biology* **16**, 663-669

Eisenberg-Lerner A., Bialik S., Simon H-U., Kimchi A. (2009) Life and death partners: apoptosis, autophagy and the cross-talks between them. *Cell Death and Differentiation* **16**, 1-10

Elliot J.M., Davison W. (1975) Energy equivalents of oxygen consumption in animal energetics. *Oecologia* **19**, 195-201

Epp J., Bricelj M., Malouf R.E. (1988) Seasonal partitioning and utilization of energy reserves in two age classes of the bay scallop *Argopecten irradians irradians* (Lamark). *Journal of Experimental Marine Biology and Ecology* **121**, 113-136

Etim L., Brey T., Arntz W. (1997) Quatification of of the sinusoidal trajectory in tissue mass and condition index of a bivalve (*Egeria radiata*) of the Cross River, Nigeria. *Journal of Molluscan Studies* **63**, 101-108

Felix-Pico E.E., Tripp-Quezada A., Castro.Ortíz G., Serrano-Casillas G., González-Ramírez P.G., Villalejo-Fuerte M., Palomares-García R., García-Dominguez F.A., Mazón-Suástegui M., Bojorquéz-Verástica G., López-García G. (1997)

Repopulation and culture of the Pacific calico scallops in Bahía Concepción, Baja California Sur, México. *Aquaculture International* **5**, 551-563

Felix-Pico E.F. (1993) Estudio biológico de la almeja Catarina *Argopecten circularis* (Sowerby 1835) en Bahía Magdalena B.C.S, México. Master Thesis Centro Interdisciplinario de Ciencias Marinas (CICIMAR), Baja California Sur, México. 77 pp

Forman H.J., Maiorino M., Ursini F. (2010) Signaling functions of reactive oxygen species. *Biochemsirty* **49**, 835-842

Gárarte-Lizárraga I., Band-Schmidt C.J. S, Verdugo-Díaz G., Muñetón-Gómez M.S, Félix-Pico E.F (2007) Dinoflagelados (Dinophycea) del sistema Lagunar Magdalena-Almejas. In: *Estudios Biológicos en Bahía Magdalena* (ed. by R. Fuentes, R. Rodríguez, J. Gutierrez-Gómez, R. García Palomares), pp. 145-175. Instituto Politécnico Nacional, México

González M.L., López D.A., Pérez M.C., Castro J.M. (2002) Effect of temperature on the scope for growth in juvenile sallops *Argopecten purpuratus* (Lamark, 1819). *Aquaculture International* **10**, 339-349

Grieshaber M.K., Hardewig I., Kreutzer U., Poertner, H-O. (1994) Physiological and metabolic responses to hypoxia in invertebrates. Reviews of Physiology, *Biochemistry and Pharmacology* **125**: 44-118

Guderley H., Poertner H-O. (2010) Metabolic power budgeting and adaptive strategies in zoology: Examples from scallops and fish. *Canadian Journal of Zoology* **88**, 753-763

Guderley H., Rojas F.M., Nusetti O.A. (1995) Metabolic specialization of mitochondria from scallop phasic muscles. *Marine Biology* **122**, 409-416

Guerra, C. Maeda-Martínez A.N., Hernandez-Llamas A., Sicard-González M.T., Koenigstein S., Abele D., Philipp E.E.R. (2011) The influence of temperature and presence of predators on growth, survival and energy allocation for reproduction in the pacific scallop *Argopecten ventricosus*. *Aquaculture Research* doi:10.1111/j.1365-2109.2011.02885.x

Gomez-Cabrera M.C, Domenech E., Viña J. (2008) Exercise is an antioxidant: Upregulation of antioxidant genes by training. *Free Radical Biology and Medicine* **44**, 126-131

Harman D. (1956) Aging: a theory based on free radical and radiation biology. *Journal of Gerontology* **11**, 298-300

Hernandez-Llamas A., Magallon-Barajas F.J., Lechuga-Devez C.H., Bustillos-Guzman J.J., Lopez-Cortes D. (1995) Growth potential of wild juvenile *Penaeus stylirostris* in earthen ponds receiving chemical and organic fertilizers, and pelleted feed. *Aquaculture Engineering* **14**, 317-330

Hernandez-Llamas A. and Ratkowsky D.A. (2004) Growth of fishes, crustaceans and molluscs: estimation of the von Bertalanffy, Logistic, Gompertz and Richards curves and a new growth model. *Marine Ecology Progress Series* **282**, 237-244

Hernández-López L., Vargas-Albores F. (2003) A microplate technique to quantify nutrients (NO_2^-, NH_4^- and PO_4^{-3}) in seawater. *Aquaculture Research* **34**, 1201-1204

Heise K., Puntarulo S., Pörtner H-O., Abele D. (2003) Production of reactive oxygen species by isolated mitochondria of the Antarctic bivalve *Laternula elliptica* (King and Broderip) under heat stress. *Comparative Biochemistry and Physiology Part B*: *Biochemistry and Molecular Biology* **134**, 79-90

Hill K.T., Womersley C. (1991). Critical aspects of fluorescent age-pigment methologies: modification for accurate analysis and age assessments in aquatic organisms. *Marine Biology* **109**, 1-11.

Hochachka P.W., Somero G.N. (2002) In: *Biochemical Adaptation: Mechanism and Process in Physiological Evolution*. Oxford University Press, New York, pp. 480

Hochachka P.W, Somero G.N (1984) Biochemical adaptations. Princeton University Press, Princeton, New Jersey pp 537

Horak P., Cohen A.A. (2010) How to measure oxidative stress in an ecological context:methodological and statistical issues. *Functional Ecology* **24**, 960-970

Hoverman J., Auld J., Relyea R. (2005) Putting prey back together again: integrating predator-induced behavior, morphology, and life history. *Oecologia* **144**, 481-491

Ivanina A.V., Skolova I.N., Sukhotin A.A. (2008). Oxidative stress and expression of chaperones in aging mollusks. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **150**, 53-61

Ivelev V.S. (1945) Transformation of energy by aquatic animals. *Integrative Reviews of Hydrobiology and Hydrography* **38**, 449-458

Jalabert B. (2005) Particularities of reproduction and oogenesis in teleost fish compared to mammals. *Reproduction Nutrition Development* **45**, 261-279

Ji L.L. (1993) Antioxidant enzyme response to exercise and aging. *Medical Science in Sports Exercise* **25**, 225-231

Ji L.L. (1999) Antioxidants and oxidative stress in exercise. *Proceedings of the Society of Experimental Biology and Medicine* **222**, 283-292

Jobling M. (1981) The influences of feeding on the metabolic rate of fishes: a short review. *Journal of Fish Biology* **18**, 385-400

Jokela J., Mutikainen P. (1995) Phenotypic plasticity and priority rules for energy allocation in a freshwater clam: a field experiment. Oecologia 104, 122-132

Jung T., Hoen A., Grune T. (2010) Lipofuscin: Detection and quantification by microscopic techniques. *Advanced Protocols in Oxidative Stress II* **594**, 173-193

Kammer A.R., Orzcewska J.I., O´Brien K.M. (2011). Oxidative stress is transient and tissue specific during cold acclimation of threespine stickleback. *Journal of Experimental Biology* **214**, 1248-1256

Keen A.M. (1971) Sea shells of tropical west America. Standford, California, U.S.A: pp 208

Kraffe E., Tremblay R., Belvin S., LeCoz J., Matry J., Guderley H. (2008) Effect of reproduction on escape responses, metabolic rates and muscle mitochondrial properties in the scallop *Placopecten magellanicus*. *Marine Biology* **156**, 25-38

Kim S.Y., Velando A., Sorci G., Alonso-Alvarez C. (2009) Genetic correlation between resistance to oxidative stress and reproductive life span in a bird species. *Evolution* **64**, 852-857

Kirkwood T.B. (2005) Understanding the odd science of aging. *Cell* **120**, 437-447

Kirkwood T.B., Austad S.N. (2000) Why do we age? *Nature* **408**, 233-238

Kiss, T. (2010) Apoptosis and its functional significance in molluscs. *Apoptosis* **15**, 313-321

Kregel K.C., Zhang H.J. (2006) An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. *American Journal of Physiolgical Regulations and Integrated Comparative Physiology* **292**, 18-36

Kujoth G.C., Hion, A., Pugh T.D., Someya S., Panzer K., Wohlgemuth S.E., Hofer T., Seo A.Y., Sullivan R., Jobling W.A., Morrow J.D., Remmen H.V., Sedivy J.M., Yamasoba T., Tanokura M., Weindruch R., Leeuwenburgh C., Prolla T.A. (2005) Mitochondrial DNA Mutations, oxidative stress, and apoptosis in mammalian aging. *Science* **309**, 481-484

Kurz T., Terman A., Brunk U.T. (2007) Autophagy, ageing and apoptois: The role of oxidative stress and lysosomal iron. *Archives in Biochemistry and Biophysics* **462**, 220-230

Kurz T., Terman, A., Gustavsson, B., Brunk, U.T. (2008). Lysosomes in iron metabolism, ageing and apoptosis. *Histochemical and Cellular Biology* **129**, 389-406

Lafrance M., Cliche G., Haugum G.A., Guderley H. (2003) Comparison of cultured and wild sea scallop *Placopecten magellanicus*, using behavioral responses and morphometric and biochemical indices. *Marine Ecology Progress Series* **250**, 183-195

Leloup C., Casteilla L., Carriere A., Galinier A., Benani A., Carneiro L., Penicaud L. (2010) Balancing mitochondrial redox signaling: A key point in metabolic regulation. Antioxidants and Redox Signaling **14**, 519-530

Lane N. (2003) A unifying view of ageing and disease: the double-agent theory. *Journal of Theoretical Biology* **225**, 531-540

Lapointe J., Hekimi S. (2010) When a theory of aging ages bady. *Cellular and Molecular Life Sciences* **67**, 1-8

Leonard G.H., Bertness M.D., Yund P.O. (1999) Crab predation, waterborne cues, and inducible defenses in the blue mussel, *Mytilus edulis. Ecology* **80**, 1-14

Lesser M.P., Kruse V.A. (2004) Seasonal temperature compensation in the horse mussel, *Modiolus modiolus*: metabolic enzymes, oxidative stress and heat shock proteins. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* **137**, 495-504

Levine R.L., Garland D., Oliver C.N., Amici A., Climent I., Lenz A.G., Ahn B.W., Shaltiel S., Stadtman E.R. (1990) Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology* **186**, 464-478

Liu D., Li C., Chen Y., Brunett C., Liu X.Y., Downs S., Collins R.D., Hawiger J. (2004) Nuclear import of proinflammatory transcription factors is requiered for massive liver apoptosis induced by bacterial lipopolysaccharide. *Journal of Biology and Chemistry* **279**, 48434-48442

Livingstone D.R., De Zwaan D., Thomposon R.J. (1981) Aerobic metabolism, oxygen production and phosphoarginine as sources of energy in the phasic and catch adductor muscles of the giant scallop *Placopecten magellanicus* during swimming and the subsequent recovery period. *Comparative Biochemisrty and Physiology Part B: Biochemsirty and Molecular Biology* **708**, 35-44

Lodeiros C., Maeda-Martìnez A.N., Freites L., Uribe E., Luch-Cota D., Sicard M.T. (2001) Ecophysiology of scallops from Iberoamerica. In: *Los Moluscos Pectínidos de Iberoamerica: Ciencia y Acuicultura* (ed. by A.N.Maeda-Martínez), pp. 77-88. Noriega Editores, Mexico City, Mexico.

Logan D.I., Epifanio C.E. (1978) Laboratory energy balance for the larvae and juveniles of the American lobster *Humarus americanus*. *Marine Biology* **47**, 381-389

Lora-Vilchis C., Doktor N. (2001) Evaluation of seven algal diets for spat of the pacific scallop *Argopecten ventricosus*. *Journal of the World Aquaculture Society* **32**, 228-235

Lubet P. (1983) Experimental studies on the action of temperature on the reproductive activity of the mussel (*Mytilus edulis* L., Mollusca, Lamellibranchia) *Journal of Molluscan Studies* **12A**, 100-105

Lucas A., Beninger P.G. (1985). The use of physiological condition in marine bivalve aquaculture. *Aquacultur*e **44**, 187-200

Luch-Belda D., Luch-Cota D.B., Luch-Cota S.E. (2003a) Interannual variability impacts on the Calofornia current large marine ecosystems. In : *Large Marine Ecosystems of the World: Trends in Exploitation, Protection and Research* (ed. by G. Hempel and K. Sherman), pp. 195-226. Elsvier, Amstedam, The Netherlands

Luna-Gonzalez A., Caceres-Martinez C., Zuñiga-Pacheco C., Lopez-Lopez S., Ceballos-Vazquez P. (2000) Reproductive cycle of *Argopecten ventricosus* (Sowerby 1842) (Bivalvia: pectinidae) in the Rada del Puerto de Pichilingüe B.C.S México and its relation to temperature, salinity and food. *Journal of Shellfish Research* **12**, 107-112

Luttbeg B., Kerby J.L. (2005) Are scared prey as good as dead? Trends in Ecology and Evolution 20, 416-418

MacDonald B.A., Bayne B.L. (1993) Food availability and resource allocation in senescent *Placopecten magellanicus*: Evidence From Field Populations. *Functional Ecology* **7**, 40-46

Maeda-Martìnez A.N., Reynoso-Granados T., Monsalvo-Spencer P., Sicard-Gonzàlez M.T., Mazón-Suástegui J.M., Hernandez O., Segovia E., Morales R .(1997) Suspension culture of catarina scallop *Argopecten ventricosus* (=circularis) (Sowerby II, 1842), in Bahia Magdalena, México, at different densities. *Aquaculture* **158**, 235-246

Maeda-Martìnez A.N., Reynoso-Granados T., Solís-Martín F., Leija-Tristán A., Aurioles-Gamboa D., Luch-Cota D., Salinas-Zavala C., Ormart-Castro P., Félix-Pico E. (1993) A model to explain the formation of catarina scallop (*Argopecetn circularis*) beds in Magdalena Bay, Mexico. *Aquaculture Fisheries Management* **24**, 323-339

Maeda-Martìnez A.N., Sicard M.T., Reynoso-Granados T. (2000) A shipment method for scallop seed. *Journal of Shellfish Research* **19**, 765-770

Maeda-Martìnez A.N., Carvahlo L., Luch-Cota S.E., Luch-Cota D.B. (2001) Las poblaciones de almeja catarina *Argopecten ventricosus* en el centro de actividad biológica de Bahía Magdalena, México. In: *Centros de Actiniad Biológica del Pacífico Mexicano* (ed. by D.Luch-Belda, J. Elorduy-Garay, S. Luch-Cota and G. Ponce-Díaz), pp. 220-228. CIBNOR Ediciones, La Paz, B.C.S., México.

Magwere T., Pamplona R., Miwa S., Martinez-Dias P., Portero-Otin O.M., Brand A.R., Partridge L. (2006) Flight activiy, mortality rates and lipoxidative damage in *Drosophila*. *Journal of Gerontology* **61**,136-145

Martindale JL, Holbrook N (2002) Cellular response to oxidative stress:signaling for suicide and survival. *Journal of Cellular Physiology* **192**: 1-15

Márquez A., Treviño-Villa S., Guéraud F. (2007) The LEC rat: a useful model for studying liver carcinogenesis related to oxidative stress and inflammation. *Redox Report* **12**, 35-39

Matés J.M., Segura J.A., Alonso F.J., Marquéz J. (2008). Intracellular redox status and oxidative stress: implications for cell proliferation, apoptosis, and carcinogenesis. *Archives in Toxicology* **82**, 273-299

McCord J.M., Fridovich I. (1969) Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). *The Journal of Biological Chemistry* **244**, 6049-6055

McGraw K., Cohen A.A., Costanini D., Horak P. (2010) The ecological significance of antioxidants and oxidative stress: a marriage between mechanistic and functional perspectives. *Functional Ecology* **24**, 947-949

McMillin J.B., Dowhan W. (2002) Cardiolipin and apoptosis. Biochimica et Biophysica Acta (BBA) *Molecular and Cell Biology of Lipids* **1585**, 97-107

Metcalfe N.B., Alonso-Alvarez C. (2010) Oxidative stress as a life history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. *Functional Ecology* **24**, 984-996

Monaghan P., Metcalfe N.B., Torres R. (2009) Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecology letters* **12**, 75-92

Motulsky H. (1995) Intuitive Biostatistics pp 272-276. Oxford University Press, Inc

Mubiana V.K., Blust R. (2007) Effects of temperature on scope for growth and accumulation of Cd, Co, Cu and Pb by the marine bivalve *Mytilus edulis*. *Marine Environmental Research* **63**, 219-235

Muller F. (2000) The nature and mechanism of superoxide production by the electro transport chain; its relevance to aging. *Journal of the American Aging Association* **23**, 227-253

Mullineaux L.S., Mills S.W., Goldman E. (1998) Recruitment variation during a pilot colonization study of hydrothermal vents (9°50´N, East Pacific Rise). *Deep Sea Research Part II: Topical Studies in Oceanography* **45**, 441-464

Nussey D.H., Pemberton J.M., Pilkington J.G., Blount J.D. (2009) Life History correlated of oxidative damage in a free-living mammal population. *Functional Ecology* **23**, 809-817

Orensanz J.M., Parma A.M., Turk T., Valero J. (2006) Dynamics, assessment and management of exploited natural populations. In: *Scallops: Biology, Ecology and Aquaculture* (ed by S.E. Shumway), pp. 765-897. Elsvier, Amsterdam, The Netherlands

Orzack S. (2001) How and why do aging and life span evolve? In: *Life span: Evolutionary, Ecological, and Demographic perspectives.* (ed. by J.R. Carey and S. Tuljapurkar), pp. 19-39. Population and Development Review, New York.

Osuna-García M., Hernandez-Llamas A., Mazón-Suástegui, J.M. (2008) Production dynamics of the giant lions-pawn scallop *Nodipecten subnodosus* cultivated off-bottom. *Aquaculture* **274**, 260-267

Pamplona R., Costanini D. (2011) Molecular and structural antioxidant defense against oxidative stress in animals. *American Journal of Physiology and Integrative Comparative Physiology* **301**, 843-863

Pauly D. (2010) Five Easy Pieces: The Impact of Fisheries on the World´s Marine Ecosystems. first ed.Island Press, Island.

Pearl R. (1928) The rate of living. Alfred Knopf, New York

Peña J. (2001) Taxonomía, morphología, distribución y hábitat de los pectínidos iberoamericanos. In*: Los moluscos pectínidos de Iberoamérica: Ciencia y Acuicultura* (ed. by A.N.Maeda-Martínez), pp 1-23. Noriega Editores

Pérez V., Bokov A., Remmen H.V., Mele J., Ran Q., Ikeno Y., Richardson A. (2009) Is the oxidative stress theory of aging dead? *Biochimica et Biophysica Acta* **1790**, 1005-1014

Perez V.I., Buffenstein R., Masamsetti V., Leonard S., Salmon A., Mele J., Andziak B., Yang T., Ederly Y., Friguet B., Ward W., Richardson A., Chaudhuri A. (2009) Protein stabiliy and resistance to oxidative stress are determinants of longevity in the longest-living rodent, the naked mole-rat. *Proceedings of the National Academy of Sciences* **106**, 3059-3064

Persky A.M., Green P.S., Stubley L., Hollew C.O., Zaulyanov L., Brazaeua G.A., Simpkins J.W. (2000) Protective effect of estrogens against oxidative damage to heart and skeletal muscle *in vivo* and *in vitro*. *Proceedings of te Society for Experimental Biolology and Medicine* **223**, 59-66

Peter M.E. (2011) Programmed cell death: Apoptosis meets necrosis. *Nature* **471**, 310-312

Philipp E., Brey T., Poertner H-O., Abele D. (2005a) Chronological and physiological ageing in a polar and a temperate mud clam. *Mechanisms of Ageing and Development* **126**, 589-609

Philipp E., Poertner H-O., Abele, D. (2005b) Mitochondrial ageing of a polar and a temperate mud clam. *Mechanisms of Ageing and Development* **126**, 610-619

Philipp E., Heilmayer O., Brey T., Abele D., Poertner H-O. (2006) Physiological ageing in a polar and a temperate swimming scallop. *Marine Ecology Progress Series* **307**, 187-198

Philipp E.E.R., Abele D. (2010) Masters of longevity:Lessons from long-lived bivales - A mini-review. *Gerontology* **56**, 55-65

Philipp E., Schmidt M., Gsottbauer C., Saenger A.M., Abele D. (2008) Size-and-age-dependent changes in adductor muscle swimming physiology of the scallop *Aequipecten opercularis*. *Journal of Experimental Biology* **211**, 2492-2501

Poertner H-O. (2002) Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* **132**, 739-761

Powell E.N., Cummins H. (1985) Are molluscan maximum life spans determined by long-term cycles in benthic communities? *Oecologia (Historical Archive)* **67**, 177-182

Powers S.K, Jackson M.J. Exercise-induced oxidative stress: Cellular mechanisms and impact on muscle force production. *Physiological Reviews* **88**, 1243-1276

Practicó D, (2002) Lipid peroxidation and the aging process. *Science and Aging Knowledge Environment* **50**, 1-5

Ratkowsky D.A. (1986) Statistical properties of alternative parameterizations of the von Bertalanffy growth curve. *Canadian Journal of Fisheries and Aquatic Sciences* **43**, 742-747

Rawlings T.A. (1994) Effect of elevated predation risk on the metabolic rate and spawning intensity of a rocky shore marine gastropod. *Journal of Experimental Marine Biology and Ecology* **181**, 67-79

Regoli F., Nigro M., Bompadre S., Winston G.W. (2000) Total oxidant scavenging capacity (TOSC) of microsomal and cytosolic fractions from Antarctic, Arctic and Mediterranean scallops: differentiation between three potent oxidants. Aquatic *Toxicology* **49**, 13-25

Reid M.B. (2001) Plasticity in skeletal, cardiac, and smooth muscle. Invited review: Redox modulation of skeletal muscle contractions:what we know and what we don´t. *Journal of Applied Physiology* **90**, 724-731

Reimer O., Harms-Ringdahl S. (2001) Predator-inducible changes in blue mussels from the predator-free Baltic Sea. *Marine Biology* **139**, 959-965

Reimer O, Tedengren M. (1996) Phenotypical improvement of morphological defenses in the mussel *Mytilus edulis* induced by exposure to the predator *Asterias rubens*. *Oikos* **75**, 383-390

Reznick D., Bryga H., Endler J.A. (1990) Experimentally induced life history evolution in a natural population. *Nature* **346**, 357-359

Reznick D., Buckwalter G., Groff J., Elder D. (2001) The evolution of senescence in natural populations of guppies (*Poecilia reticulata*): a comparative approach. *Experimental Gerontology* **36**, 791-812

Reznick D.N., Bryant M.J., Roff D., Ghalambor C.K., Ghalambor D.E. (2004) Effect of extrinsic mortality on the evolution of senescence in guppies. *Nature* **431**, 1095-1099

Ridgway I.D., Richardson C.A., Austad S.N. (2010). Maximum shell size, growth rate and maturation age correlate with longevity in bivalve mollusks. *Jornal of Gerontology* **66A**, 183-190

Rolfe D.F.S., Brown G.C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiological Reviews* **77**, 731-758

Román G, Martínez G, García O, Freites L (2001) Reproducción. In: *Los Moluscos Pectínidos de Iberoamerica: Ciencia y Acuicultura* (ed. by A.N.Maeda-Martínez), pp.27-59. Noriega Editores, Mexico.

Rubner, M. (1908). Das Problem der Lebensdauer und seine Beziehungen zum Wachstum und Ernaehrung. Muenchen, Germany, Oldenburg.

Safdar A., deBeer J., Tarnopolsky M.A. (2011) Dysfunctional Nrf2 and Keap1 redox signaling in skeletal muscle of the sedentary old. *Free Radical Biology and Medicine* **49**, 1487-1493

Salmon A.B., Richardson A., Pérez V. (2010) Update of the oxidative stress theory of aging: Does oxidative stess play a role in aging or healthy aging? *Free Radical Biology and Medicine* **48**, 3-28

Sánchez-Montante O., Zaitsev O., Saldivar-Reyes M. (2007) Condiciones hidrofísicas en el sistema lagunar Bahía Magdalena-Alemjas In: *Estudios Biológicos en Bahía Magdalena* (ed. by R. Fuentes Rodríguez, J. Gutierrez-Gómez, R. García Palomares), pp. 1-29. Instituto Politécnico Nacional, Mexico City, Mexico

Sarkis S., Couturier C., Cogwell A. (2006) Reproduction and spawning in calico scallops, *Argopecten gibbus*, from Bermuda. *Journal of Shellfish Research* **25**, 503-508

Sarkis S., Lovelatti A. (2007) Installation and operation of a modular bivalve hatchery. FAO Fisheries Technical Paper No. 492, pp 173, Rome, FAO.

Shamsudin L. (1992) Lipid and fatty acid composition of microalgae used in Malaysian aquaculture as lived food for the early stage of penaeid larvae. *Journal of Applied Phycology* **4**, 371-378

Schmidt M., Philipp E., Abele D. (2008) Age dependent change of escape response from predator attack in the Queen scallop *Aequipecten opercularis*. *Marine Biology Research* **4**, 422-450

Sicard-Gonzàlez M.T. (2006) Efecto de la oscilación térmica en la fisiología de la Mano de León (*Nodipecten Subnodosus*, Sowery 1835) Doctoral thesis in biological sciences. Universidad Autónoma de Nuevo León, México. Facultad de Ciencias Biológicas: 204 pp

Sicard-González M.T., Maeda-Martínez A.N., Ormart P., Carvahlo L. (1999) Optimum temperature for growth in the Catarina scallop (*Argopecten ventricosus* Sowerby II, 1842). *Journal of Shellfish Research* **18**, 385-392

Sidell B.D., Driedzic W.R., Stowe D.B., Johnston I.A. (1987) Biochemical correlations of power development and metabolic fuel preference in fish hearts. *Physiological Zoology* **60**, 221-232

Slos S., Stoks R. (2008) Predation risk induces stress proteins and reduces antioxidant defense. *Functional Ecology* **22**, 637-642

Slos S., De Meester L., Stoks R. (2009) Food level and sex shape predator-induced physiological stress: immune defense and antioxidant defense. *Oekologia*. **161**, 461-467

Sohal R.S. (2002). Role of oxidative stress and protein oxidation in the aging process. *Free Radical Biology and Medicine* **33**, 37-44

Sokolova I.M. (2009). Apoptosis in molluscan immune defense. *Informational Systems Journal* **6**, 49-58

Soldatov A.A., Gostyukhina O.L., Golovina I.V. (2008) State of antioxidant enzyme complex in tissues of the black sea mollusc *Mytilus galloprovincialis* under natural oxdative stress. *Journal of Evolutionary Biochemistry and Physiology* **44**, 175-182

Solorzano L. (1969) Determination of ammonia in natural waters by the phenolhypochlorite method. *Limnology and Oceanography* **14**, 799-800

Sorbal P., Widdows J. (1997) Effect of elevated temperatures on the scope for growth and resistance to air exposure of the clam *Ruditapes decussatus* (L.), from southern Portugal. *Scientia Marina* **61**, 163-171

Speakman J.R., Selman C. (2011) The free-radical damage theory: Accumulating evidence against a simple link of oxidative stress to ageing and lifespan. *Bioessays* **33**, 255-259

Speakman J.R., Selman C., Mclaren J.S., Harper E.J. (2002) Living fast, dying when? The link between aging and energetics. *American Society of Nutritional Sciences* **132**, 1583-1597

Stearns S.C. (1992) The evolution of life histories. Oxford University Press, Oxford

Steller D.L and Cáceres-Martínez, C. (2009) Coralline algal rhodoliths enhance larval settlement and early growth of the Pacific calico scallop *Argopecten ventricosus*. *Marine Ecology Progress Series* **396**, 49-60

Storey K.B. (1996) Oxidative stress: animal adaptations in nature. Brazilian Journal of *Medical and Biological Research* **29**, 1715-1733

Strahl J., Abele D. (2010) Cell turnover in tissues of the long-lived ocean quahog *Arctica islandica* and the short-lived scallop *Aequipecten opercularis*. *Marine Biology* **157**, 1283-1293

Strahl J., Philipp E., Brey T., Broeg K., Abele A. (2007). Physiological aging in the islandic population of the ocean quahog *Arctica islandica*. *Aquatic Biology* **1**, 77-83

Strasser, M. (1999). *Mya Arenaria*- an ancient invader of the North Sea coast. *Helgoländer Meeresuntersuchungen* **52**, 309-324

Sukhotin A.A., Abele D., Poertner H-O. (2002) Growth, metabolism and lipid peroxidation in *Mytilus edulis*: age and size effects. *Marine Ecology Progress Series* **226**, 223-234

Sukhotin A.A., Flyachinskaya L.P. (2009) Aging reduces reproductive success in mussels *Mytilus edulis*. *Mechanisms of Ageing and Development* **130**, 754-761

Susuki K. (2000) Measurement of Mn-SOD and Cu, Zn-SOD. In: *Experimental protocols for reactive nitrogen and oxygen species* (ed. by N. Taniguchi and J. Gutteridge), pp. 91-95. Oxford University Press. United Kingdom

Terahara K., Takahashi K.G. (2008). Mechanisms and immunological roles of apoptosis in molluscs. *Current Pharmaceutical Design* **14**, 131-137

Terman A., Brunk U.T. (2004) Lipofuscin. The international Journal of Biochemistry and *Cell Biology* **36**, 1400-1404

Terrones J., Vidal S., Cal R., Martínez P., Piferrer F. (2004). Apoptosis during gametogenesis in diploid and triploid turbot (*Scophthalmus maximus*). *Fish Physiology and Biochemistry* **28**, 369-370

Thomas G.E., Gruffydd L.D. (1971) The types of escape reactions elicited in the scallop *Pecten maximus* by selected sea-star species. *Marine Biology* **10**, 87-93

Thompson R.J., Livingstone D.R., De Zwaan A. (1980) Physiological and biochemical aspects of the valve snap and valve closure responses in the giant scallop *Placopecten magellanicus*. *Journal of Comparative Physiology Part B: Biochemistyr and Molecular Biology* **137**, 97-104

Thompson RJ, MacDonald BA (2006) Physiologic integations and energy partitioning In: Scallops: Biology, Ecology and Aquaculture (ed. by S.E Shumway, and G.J Parsons), pp. 493-522. Elsvier, Amsterdam, The Netherlands

Thorarinsdottir, G.G., Steingrimsson, S.A. (2000). Size and age at sexual maturity and sex ratio in ocean quahog *Arctica islandica* (Linneaus, 1767), of north-west Iceland. *Journal of Shellfish Research* **15**, 729-733

Tremblay R., Myrand B., Guderley H. (1998a) Thermal sensitivity of organismal and mitochondrial oxygen consumption in relation to susceptibility of blue mussels, *Mytilus edulis* (L.), to summer mortality. *Journal of Shellfish Research* **17**, 141-152

Tremblay R., Myrand B., Sevigny J.M., Blier P., Guderley H. (1998d) Bioenergetic and genetic parameters in relation to susceptibility of blue mussels, *Mytilus edulis* (L.) to summer mortality. *Journal of Experimental Marine Biology and Ecology* **221**, 27-58

Tomey A.V., Ortega Y. (2000) Relación del estrés oxidativo con el catabolismo de proteinas. *Revista Cubana de Investigación Biomedica* **19**, 206-212

Uriate I., Guilherme R., Abarca A. (2001) Producción de juveniles de pectínidos iberoamericanos bajo condiciones controladas. In: *Los Moluscos Pectínidos de Iberoamerica: Ciencia y Acuicultura* (ed. by A.N.Maeda-Martínez), pp. 147-171. Noriega Editores, México

Ushida K. (2007) Lipid peroxidation and redox-sensitive signaling pathways. *Current Atherosclerosis Reports* **9**, 216-221

Vahl O. (1985). Size-specific reproductive effort in *Chlamys islandica*: reproductive senility or stabilizing selection? In: *Proceedings of the 19th European Marine Biology Symposium* (ed. P. E Gibbs), pp. 521-527. United Kingdom, Plymouth, Devon

Vernet M., Hunter J.R., Vetter R.D. (1988) Accumulation of age-pigments (lipofuscin) in two cold-water fishes. *Fishery Bulletin* **86**, 401-407

Viarengo A., Canesi L., Pertica M., Livingstone D.R. (1991). Seasonal variation in the antioxidant defense system and lipid peroxidation of the digestive gland of mussels. *Comparative Biochemisrty and Physiology Part B: Biochemisrty and Molecular Biology* **100**, 189-190

Villalaz G.J.R. (1994) Laboratory study of food concentration and temperature effect on the reproductive cycle of *Argopecten ventricosus*. *Journal of Shellfish Research* **13**, 513-519

Waller T.R. (1991) Evolutionary relationships among commercial scallops (Mollusca: Bivalvia: Pectinidae). *In: Scallops: Biology, Ecology and Aquaculture* (ed. by S.E. Shumway), pp 1-72. Elsevier, Amsterdam, The Netherlands

Wanamaker A.D., Heinemeier J., Scource J.D., Richardson C.A., Butler P.G., Eiriksson J., Knudsen K.L. (2008) Very long-lived mollusk confirm 17th Century ad-tephra based radiocarbon reservoir ages for North Icelandic shelf waters. *Radiocarbon* **50**: 399-412

Wang Y., Salmon A.B., Harshman L.G. (2001). A cost of reproduction: oxidative stress susceptibility is associated with increased egg production in *Drosophila melanogaster. Experimental Gerontology* **36**, 1349-1359

Warren C.E., Davis G.E. (1967) Laboratory studies on the feeding, bioenergetics and growth of fish. In: *The biological basis of fresh water fish production*. (ed. by S.Gerking), pp. 175-214. Blackwell Scientific Publication, United Kingdom

Widdows J. (1976) Physiological Adaptation of *Mytilus edulis* to Cyclic Temperatures. *Journal of Comparative Physiology* **105**, 115-128

Widdows J., Johnson D. (1988) Physiological energetics of *Mytilus edulis:* Scope for Growth*. Marine Ecology Progress Series* **46**, 113-121

Wiersma P., Selman C., Speakman J.R., Verhulst S. (2004) Birds sacrifice oxidative protection for reproduction. *Proceedings of the Royal Society B: Biological Sciences* **271**, 360-363

Wilhelm-Filho W., Tribes T., Gáspari C., Claudio F.D., Torres M.A., Magalhaes A.R.M. (2001) Seasonal changes in antioxidant defense of the digestive gland of the brown mussel (*Perna perna*) *Aquaculture* **203**, 149-158

Williams P.D., Day T. (2003) Antagonistic pleiotropy, mortality source interactions, and the evolutionary theory of senescence. *Evolution* **57**, 1478-1488

Winberg G.G .(1960) Rate of metabolism and food requirements of fishes. *Fish Research* **194**, 1-253

Wood A.W., Van der Kraak G.J. (2001). Apoptosis and ovarian function: novel perspectives from teleosts. *Biology of Reproduction* **64**, 264-271

Yan L.J, Sohal R.S (2000) Prevention of flight activity prolonges the life span of the housfly, *Musca domestica*, and attenuates the age associated oxidative damage to specific mitochondrial proteins. *Free Radical Biology and Medicine* **29**, 1143

Yin D., Chen K. (2005) The essential mechanism of aging: irreparable damage accumulation of biochemical side-reactions. *Experimental Gerontology* **40**, 455-465

Ziguanov V., Miguel E.S., Neves R.J., Longa A., Fernandez C., Amaro R., Beletsky V., Popkovitch E., Kaliuzhin S., Johnson T. (2000) Life span varaition of the fresh water pearl: a model species for testing longevity mechanisms in animals. *AMBIO: A Journal of the Human Environment* **29**, 102-105