Metabolic and Oxidative Stress

Responses of *Cassiopea* sp. to Environmental Stress

Towards a Better Physiological Understanding

of Jellyfish's Tolerance

A dissertation by

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(Dr. rer. nat.)

Bremen 2017

Universität Bremen

ZMT LEIBNIZ-ZENTRUM für Marine Tropenforschung

A dissertation submitted to the faculty of Biology and Chemistry of the University of Bremen in partial fulfilment of the requirements for the degree of Doctor of Natural Sciences
(Dr. rer. nat.)

This thesis was conducted in the period 2015-2017 at the Leibniz Centre for Tropical Marine Research (ZMT), Bremen. The Project was financed through Leibniz IN-HOUSE project (number 6057). Additional travel funding was received through the Bremen Graduate School of Marine Sciences (GLOMAR).

Gutachter: **Prof. Dr. Martin Zimmer**
Dr. Andreas Kunzmann

Prüfer: **Prof. Dr. Wilhelm Hagen**
Dr. Karin Springer

Weiter Mitglieder de Prüfungsausschusses:
Ernesto Acosta (Doktorand)
Katharina Berg (Bachelor)

Datum des Promotionskolloquiums: 14.12.2017

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Acknowledgements

Firstly and foremost, I'd like to express my sincere gratitude to my advisor **Dr. Andreas Kunzman** for the continuous support of my Ph.D. study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D. study. Thank **TO** You **Andreas.**

Besides my advisor, I would like to thank the rest of my thesis committee: **Prof. Martin Zimmer** and **Dr. Achim Meyer**, Thank You for your insightful comments and encouragement. Thank You for the hard questions which motivated me to widen my research from various perspectives.

My sincere thanks also go to the MAREE and Bio-Lab staff at ZMT. Thank you to the MAREE and Bio-Lab staff at ZMT. Thanks to You Conny, Steffi, Nico and Christian, Sivia and Nina.

My sincere thanks also go to **Dr. Fuad Al-Horani** for his support in the field. Thank You to the staff of the Bio/Chem-lab and Diving Center in MSS for their support in field. Thank You to Everybody in the MSS from the door Entrance to the Red Sea shoreline. Special Thank you to **Eyad** from MSS.

My family in Jordan, no words could be enough to thank you. My **mother** and **father**, both of you have dedicated your life for us. My father, I have never seen a wisdom man like you, hope one day I can adopt your way of thinking. Your concise and precise wisdoms are shaping my life. Thank you my Father.

My Brothers, **Fayeq** and **Naif**, every time the sky darken with heavy clouds, every time the light becomes scarce in my life; you shine my sky with your Crazy Wisdoms-sandwiched Jocks. I am so grateful that I've you in my life. Last but not least, Thanks a lot for everybody who surrounded me everywhere.

امي وأبي: أنتما كالبحر يعطي بلا حدود. في كل حرف من كلامكما حكمة وفي كل فعل لأجلكما أجرٌ عظيم. أخي فائق، أخي نايف: تعجز الكلمات عن وصف ثنائي عليكما. أشكركما جداً.

List of abbreviations

CG	Coral Garden
Chla	Chlorophyll-a
CRR	Cellular Respiration Rates
Cyt-c	Cytochrome C
DIC	Dissolved Inorganic Carbon
ETS	Electron Transport System
GoA	Gulf of Aqaba
GPx	Glutathione Peroxidase
H_2O_2	Hydrogen Peroxide
$HO^{\bullet}$	Hydroxyl Radical
IA	Industrial Area
INT	Iodonitrotetrazolium
KPi,	Potassium Phosphate Buffer
LDH	Lactate Dehydrogenase
LPO	Lipid Peroxidation
MDA	Malondialdehyde
MO_2	Oxygen Consumption
MSS	Marine Science Station
O_2^-	Superoxide Anion
OC	Oxygen Consumption
OML	Oxygen Minimum Layer
PK	Pyruvate Kinase
PLB	Phosphate Loading Berth
ROS	Reactive Oxygen Species
RuBisCO	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase
UV-B	Ultraviolet-B Radiation

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Summary

Jellyfish are commonly seen as robust, noxious and unwelcoming animals. In recent decades, reports concerning jellyfish outbreaks and invasions are increasing worldwide. Regardless of the debate about the underlying drivers of jellyfish blooms, climate change and anthropogenic activities are commonly perceived as the main drivers. There is, however, scarcity of strong evidences to support this perception, because only few manipulative experiments have addressed the physiological responses of jellies to varying environmental stressors. The overall aim of this thesis is to use manipulative experiments and field excursions to test hypotheses about tolerance of jellyfish to stressors associated with climate change and anthropogenic activities for better prediction of their fate in the future.

Due to climate change, extreme weather conditions are becoming more frequent and severe. In chapters 2 and 3, I investigate the metabolic and oxidative responses of the upside-down jellyfish *Cassiopea* sp. (*Cassiopea* hereafter) medusae to sudden changes in seawater temperature (i.e., either rise or drop by 6 °C from the control temperature). Medusae responded in contrasting manners to drop and rise in seawater temperature. While medusae treated at low temperature (20 °C) looked unhealthy and showed signs of decreased physiological performance (i.e., in term of decreased body mass and size) after two weeks, medusae treated at high temperature (32 °C) gained in body mass and size, indicating an enhanced performance for the same period. At the cellular level, medusae treated at low temperature suffered from oxidative stress-induced cellular damage and elevated metabolic demand, while no oxidative stress or signs of increased energy demand were evident in medusae at higher temperature. The overall results of these two chapters suggest that *Cassiopea* medusae are more tolerant to temperature rise than drop. They might benefit from global warming to spread and expand their populations in the future as well.

Coastal systems experience a variety of pollutants, nutrient loading and other burdens associated with anthropogenic activities. In chapter 4, I investigate the anaerobic–potential and oxidative stress responses in *Cassiopea* medusae collected from anthropogenically impacted and protected marine coastal habitats. While medusae from all investigated locations did not show signs of oxidative stress-induced damage (e.g., lipid peroxidation), the medusae from polluted locations had more anaerobic potential (e.g., high PK and LDH activities). While the results of Chlorophyll-a (Chla) contents measurements did not show clear trends in medusae from the studied locations, it seems that medusae Chla content is

more sensitive to water clarity than to pollution status in the studied sites. Overall these results suggest that *Cassiopea* seems to be robust to the level of pollution at the studied sites and they might be anaerobically poised to live and thrive at such habitats.

Noteworthy to mention here is that while the studies conducted in this thesis could work as a framework for future studies aimed for better understanding of jellyfish physiological responses, the thesis findings do not claim ultimate proofs for tolerance or sensitivity in all jellies. However, this thesis, and for the first time, highlights the feasibility and importance of understanding the underlying mechanisms of jellyfish's physiological tolerance/sensitivity to changing environmental conditions. By using the epibenthic jellyfish *Cassiopea* as a representative model for studying tropical jellyfish's ecological roles and responses to environmental stressors, this thesis encourages doing further researches on this jellyfish.

Zusammenfassung

Quallen werden allgemein als robuste, schädliche und unerwünschte Lebewesen betrachtet und in den letzten Jahrzehnten nahmen weltweit Berichte über massenhaftes Auftreten und Invasionen von Quallen zu. Allgemein werden Klimawandel und anthropogene Einflüsse als die Hauptursache angenommen, unabhängig der Debatte über zusätzliche Ursachen von Quallenblüten. Es gibt jedoch einen Mangel an eindeutigen Beweisen zur Unterstützung dieser Beobachtungen, da die physiologischen Reaktionen von Quallen auf verschiedene Umweltstressoren lediglich anhand weniger Studien untersucht wurden. Das übergeordnete Ziel dieser Arbeit ist es, mittels manipulativer Experimente die Hypothesen über die Toleranz von Quallen gegenüber Stressfaktoren im Zusammenhang mit Klimawandel und anthropogenen Aktivitäten zu testen, sowie ihr zukünftiges Schicksal besser vorhersagen zu können.

Durch den Klimawandel werden Extremwetterphänomene häufiger und verheerender. In den Kapiteln 2 und 3 untersuche ich die metabolischen und oxidativen Reaktionen des Medusen-Stadiums der Upside-down Qualle *Cassiopea* sp. (*Cassiopea* hiernach) auf plötzliche Änderungen der Meerwassertemperatur (d.h. Anstieg bzw. Abfall um 6°C von der Kontrolltemperatur). Medusen reagierten auf unterschiedliche Weise auf Erhöhung bzw. Abfall der Wassertemperatur. Während Medusen, die bei niedriger Temperatur (20°C) behandelt wurden, ungesund aussahen und nach zwei Wochen Anzeichen einer verminderten physiologischen Leistung zeigten (verringerte Körpermasse und -größe), gewannen Medusen welche bei höherer Temperatur (32°C) gehalten wurden, an Körpermasse und Körpergröße, was eine verbesserte physiologische Leistung innerhalb des gleichen Zeitraums anzeigte. Auf zellulärer Ebene litten bei niedrigen Temperaturen behandelte Medusen unter oxidativem Stress, induziertem Zellschaden und erhöhtem metabolischen Bedarf. Kein oxidativer Stress oder Anzeichen eines erhöhten Energiebedarfs wurden ersichtlich in Medusen welche mit höherer Temperatur behandelt wurden. Die Gesamtergebnisse dieser beiden Kapitel legen nahe, dass *Cassiopea*-Medusen gegenüber steigenden Temperaturen toleranter sind als gegenüber sinkenden. Sie könnten von der globalen Erwärmung profitieren, um ihre Population auch in Zukunft auszudehnen.

Küstensysteme leiden unter einer Vielzahl von Belastungen (Schadstoff- bzw. Nährstoffbelastung), welche durch anthropogene Aktivitäten verursacht sind. In Kapitel 4 untersuche ich das anaerobe Potential und die oxidativen Stressreaktionen in *Cassiopea*

Medusen, sowohl aus anthropogen beeinflussten als auch geschützten marinen Küstenhabitaten. Während Medusen von allen untersuchten Standorten keine Anzeichen einer durch oxidativen Stress induzierten Schädigung (z.B. Lipidperoxidation) aufwiesen, hatten die Medusen von verunreinigten Orten ein erhöhtes anaerobes Potential (z.B. hohe PK- und LDH-Aktivitäten). Obwohl die Ergebnisse der Chlorophyll-a (Chla)-Gehaltmessungen keine eindeutigen Trends aufzeigten, scheint der Chla-Gehalt der Medusen in Bezug auf die Transparenz des Wassers empfindlicher zu sein als auf den Verschmutzungszustand der untersuchten Gebiete. Insgesamt legen diese Ergebnisse nahe, dass *Cassiopea* robust gegenüber dem Verschmutzungs-Grad der untersuchten Standorte ist und einen anaeroben Stoffwechsel aufweist, um in solchen Lebensräumen zu leben und zu gedeihen.

Erwähnenswert ist hier, dass die in der vorliegenden Arbeit durchgeführten Experimente als Grundlage für zukünftige Studien dienen können, welche auf ein besseres Verständnis der physiologischen Reaktion von Quallen abzielen. Die Ergebnisse dieser Arbeit bringen keine endgültigen Beweise für die Toleranz oder die Empfindlichkeit in Quallen allgemein. In dieser Arbeit wird jedoch zum ersten Mal die Wichtigkeit und auch die Durchführbarkeit gezeigt hinsichtlich des Verständnisses der zugrundeliegenden Mechanismen zur physiologischen Toleranz bzw. Empfindlichkeit von Quallen gegenüber sich verändernden Umweltbedingungen. Durch die Verwendung der epibenthischen Qualle *Cassiopea* als ein repräsentatives Modell zur Untersuchung tropischer Quallen auf ihre ökologische Rolle und ihre Reaktion auf Umweltstressoren ermutigt diese Dissertation zu weiteren Studien dieser Quallenart.

Thesis Outline

This thesis consists of a general introduction (chapter 1), three chapters presenting the core research of the PhD (i.e., chapters 2-4) and a general discussion of the key findings (chapter 5). Each of the three core chapters are intended for publication as an independent research article. Listed below are the abstracts of the articles.

Chapter 2

Cellular respiration, oxygen consumption, and trade-offs of the jellyfish *Cassiopea* sp. in response to temperature change

Samir M. Aljbour, Martin Zimmer, Andreas Kunzmann

This chapter experimentally investigated the physiological responses of *Cassiopea* medusae when confronted with sudden changes in seawater temperature. The aim is to demonstrate how the jellies would respond to an extreme drop/rise in seawater temperature due to extreme weather events. Real-time oxygen consumption (MO_2) and the potential maximal cellular respiration rate (ETS), bell pulsation rate, changes in body mass and size, in addition to morphological observations were used as proxies in this study. Overall these results suggest an enhanced growth in response to global warming, whereas low temperatures may set the limits for successful invasion of *Cassiopea* into colder water bodies. Our results provide a framework for understanding the physiological tolerance of *Cassiopea* under possible future climate changes.

This study was initiated by S.M. Aljbour and A. Kunzmann. All experimental setups and laboratory work was done by S.M. Aljbour. I analyzed the data and wrote the manuscript with input from all authors. This chapter was published in the Journal of Sea Research, Volume 128, October 2017, Pages 92-97.

Chapter 3

Are jellyfish physiologically well adapted to global warming? Surprising oxidative stress and metabolic demand responses in *Cassiopea* sp.

Samir M. Aljbour, Martin Zimmer, Andreas Kunzmann

This chapter experimentally investigated the physiological responses of *Cassiopea* medusae when confronted with sudden changes in seawater temperature. The aim is to demonstrate how the jellies would respond to extreme drop/rise in seawater temperature due to extreme weather events. Superoxide dismutase activity, lipid peroxidation, chlorophyll-a content and cellular respiration in addition to changes in body mass and size in addition to morphological observations were used as proxies in this study. Our findings bring new evidence that an oxidative stress-mediated increased metabolic demand is the main mechanism setting the limits to *Cassiopea's* physiological performance at cold temperature. We conclude that *Cassiopea* populations may flourish and extend their geographical distributions in response to global warming. In the view of global jellyfish blooms at the cost of deteriorating reefs and diminishing fish stocks, our findings are confirming the high competitiveness of jellyfish in future warming coastal ecosystems.

This study was initiated by S.M., Aljbour and A., Kunzmann. All experimental setups and laboratory work was done by S.M., Aljbour. I analyzed the data and wrote the manuscript with input from all authors. This chapter is submitted for publication in the Journal of Sea Research.

Chapter 4

Metabolic responses of the upside-down jellyfish *Cassiopea* sp. to pollution in the Gulf of Aqaba, Jordan

Samir M. Aljbour, Fuad A. Al-Horani, Andreas Kunzmann

This chapter investigated the physiological responses of *Cassiopea* medusae in the field, where the medusae have been collected from polluted and control non-polluted marine coastal locations along the Gulf of Aqaba. The aim is to demonstrate if whether the jellies were anaerobically poised and if they experience oxidative stress or not in response to habitat pollution. The activities of main glycolytic enzymes (i.e., pyruvate kinase and lactate dehydrogenase), in addition to lipid peroxidation and chlorophyll-a content were measured in medusae from different locations. Our findings bring new evidence at the physiological levels supporting the common perception about jellies' robustness to environmental disturbances and metal pollution in this study. Overall these results suggest that *Cassiopea* seems to be robust to the level of pollution at the studied sites and they might be anaerobically poised to live and thrive at such habitats.

This study was initiated by S.M., Aljbour and A., Kunzmann. All experimental setups and laboratory work was done by S.M., Aljbour. I analyzed the data and wrote the manuscript with input from all authors. Fuad A. Al-Horani provided field work support. This chapter is submitted for publication in journal Marine Pollution Bulletin.

Chapter 1

General Introduction

What is *Cassiopea*? Scyphozoan blooms? Oxidative stress, antioxidant system and homeostasis?

Section I. What is *Cassiopea*?

The genus *Cassiopea* (Upside-down jellyfish; Phylum: Cnidaria, Class: Scyphozoa, Order: Rhizostomae, Family: Cassiopeidae, see Fig. 1), is globally distributed in tropical and sub-tropical marine environments, inhabiting mangrove forests, seagrass beds, and coral reefs (Gohar & Eisawy 1960; Holland et al. 2004; Niggl & Wild 2009; Welsh et al. 2009; Stoner et al. 2011, 2014). Alternatively, the jellyfish is sometimes called the mangrove jellyfish or the zooxanthellate jellyfish.

Fig. 1. A *Cassiopea* medusa from the Red Sea.

Most scyphozoans have metagenic life cycle, and *Cassiopea* is no exception: the jellyfish life cycle alternates mainly between two totally different forms, the very tiny sessile polyp form and the conspicuous motile medusoid form (Gohar & Eisawy 1960; Hofman et al. 1996). While in most scyphozoans the medusoid form is entirely pelagic, *Cassiopea* on the other hand has semi-sessile epibenthic medusae (Gohar & Eisawy 1960). While *Cassiopea* medusae retain good ability to swim well, they prefer to set on the sea bottom, pulsating their bells instead of swimming. Like corals do, but unlike most scyphozoan jellyfish, *Cassiopea* exhibits an intimate mutualistic symbiosis with a photosynthetic dinoflagellate endosymbiont (i.e., zooxanthellae, specifically S*ymbiodinium microadriaticum*; Hofmann & Kremer 1981). The jellyfish harbors this endosymbiont during the medusoid and the polyp stage of its life cycle, but not in the embryonic 'planula' and the metamorphic stages of the life cycle (Hofmann & Kremer 1981).

Anatomy, physiology and behavior of jellyfish

Like all cnidarians, jellyfish are among the simplest existing ancient animals. They are diploblastic animals, where the body is made of two main cellular layers (i.e., ecto- and endoderm) and an acellular layer (i.e., mesoglea) in between (Fig. 2). Having only nerve nets and no central nervous system, neurobiologists have been surprised by Nath and his colleague's recent findings that *Cassiopea* shows signs of undergoing sleep-like behavior, a phenomenon thought to be lacking in cnidarians for a long time (Nath et al. 2017). In general, jellyfish are considered watery organisms (>95%) with low carbon (usually <1% of wet mass) content (Lucas et al. 2011). However, they are protein-rich animals, with protein

making up to 50% of the total dry weight (Ding et al. 2011). In the mesoglea, the structural protein (collagen) makes up about 50 % of the total protein content (Khong et al. 2015).

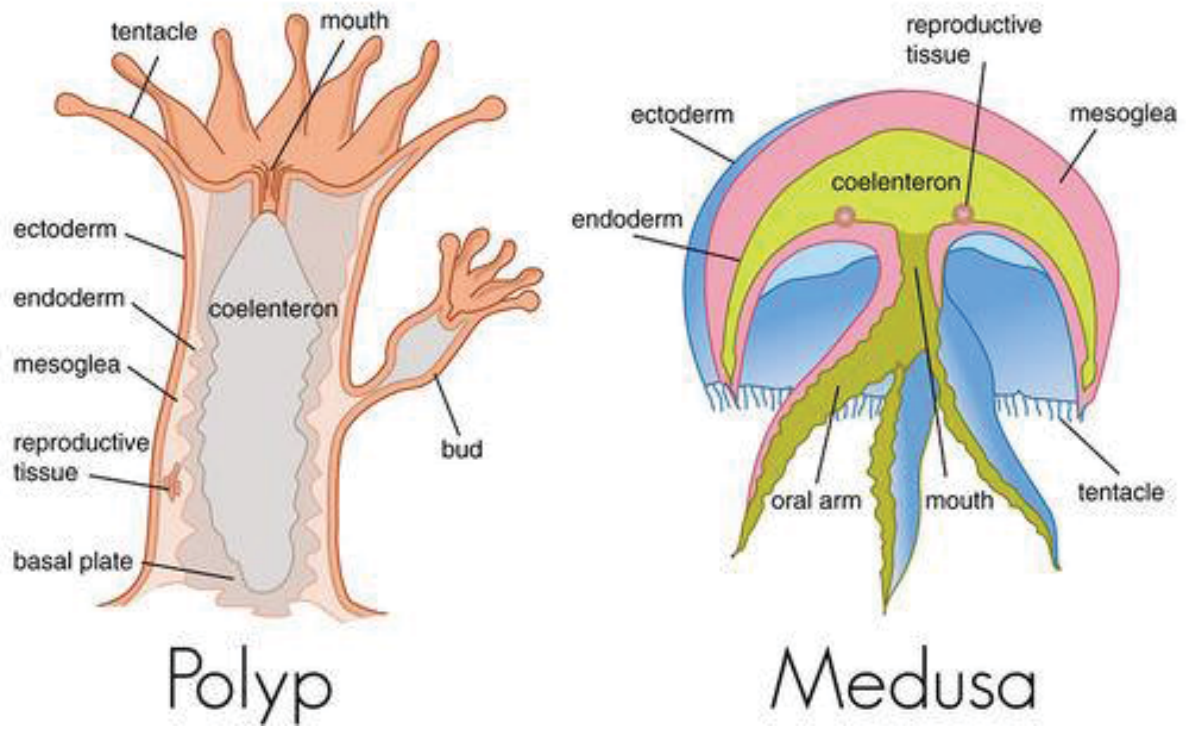

Fig. 2. A representative diagram of a typical scyphozoan life cycle, showing the two main forms. Note the massive amount of mesoglea in the medusoid form. The diagrams is not to scale (i.e., while polyps are measured in mm, medusae of some jellies could reach ca. 2 m in bell diameter). Adapted from https://www.ck12.org website.

Why does *Cassiopea* medusa pulsate its bell continuously while sitting upside-down?

Unlike fish, and many other invertebrates, the zooxanthellate jellyfish (*Cassiopea*) is not a vision-dependent predator, however this does not alleviate its reliance on light. In this jellyfish, the light dependency and the unusual upside down position are thought to be due to the fact that they host the light-dependent photosynthetic zooxanthellae endosymbionts in their tissues (Gohar & Eisawy 1960). The upside-down position guarantees a better exposure of the endosymbionts inside their tissues to sunlight, in other words it could be considered as an adaptive mechanism for enhancing photosynthesis. Some authors concluded that *Cassiopea* relies on their bell pulsation for creating water flows that facilitate several functions. For example, food capture, waste removal, swimming, and getting rid of sediment particles falling on the organism (Gohar & Eisawy 1960; Welsh et al. 2009; Hamlet et al. 2012) are among the main apparent functions first established. While it is accepted that seawater flow is generally attributed to enhanced exchange rates of nutrient and dissolved

inorganic carbon (DIC) between seawater and organisms immersed inside it, exchange of oxygen and enhancing photosynthesis rate were considered essential benefits of enhancing flow too (Mass et al. 2010). In photosynthesis, the first major step in carbon fixation, the incorporation of CO_2 into organic molecules, is catalyzed by the enzyme RuBisCO (Ribulose-1,5-bisphosphate carboxylase/oxygenase; Andersson 2008). It is well known that under high oxygen concentrations, the carboxylase activity of RuBisCO is compromised by an opposing oxygenase activity, and as a consequence it becomes less efficient in CO_2 fixation (Andersson 2008). Analogous to *Cassiopea*'s continuous bell pulsation, the common xeniid coral *Heteroxenia fuscescens* pulsate their tentacles. In this coral, Kremien et al. (2013) have shown that the net photosynthesis rate was significantly higher in polyps in the pulsating state compared to the non-pulsating state. Alleviating the problem of reduced RuBisCO affinity to CO_2 under conditions of high oxygen concentrations by pulsation-mediated enhanced O_2 efflux was proposed as the mechanism of the overall enhanced photosynthesis in this xeniid (Kremien et al. 2013). In *Cassiopea* medusae, reducing internal oxygen tension could be an added benefit to the list of aforementioned functions attributed to the continuous bell pulsation in this jellyfish.

What roles do zooxanthellae endosymbionts play in *Cassiopea*?

As already mentioned above, *Cassiopea* medusae and polyps incorporate zooxanthellae into most of their tissues. Beside the traditional role of these endosymbionts in photosynthesis, they play essential roles in the development and physiology of the Upside-down jellyfish, as highlighted in the following brief review. Hofmann & Kremer (1981) have shown that the mixotrophic jellyfish could benefits of 5-10% of net algal photosynthate being translocated to its tissues *in vivo*, mainly in the form of glycerol and glucose. In most scyphozoans, metagenesis (i.e., defined as alternation between sexual and asexual generation; Ceh et al. 2015) is the typical mode of life cycle, which involves pelagic sexually-reproducing medusae and a benthic asexually-reproducing polyp (Hofmann et al. 1996). In the polyps of the scyphozoan *Cassiopea*, the asexual metamorphic process, by which free-swimming disc-shaped ephyrae are produced from the sessile polyps, is called strobilation (Gohar & Eisawy 1960). In scyphozoans, strobilation is induced and controlled by a set of biotic and abiotic factors (Rahat & Adar 1980; Hofmann et al. 1996; Fuchs et al. 2014). Rising seawater temperature and acquisition of zooxanthellae by *Cassiopea*'s polyps were found to be indispensable prerequisites for strobilation (Rahat & Adar 1980; Hofmann et al. 1996). In

fact, while the presence of the photosynthetic endosymbiont could contribute to the ecological success of the holobiont in the oligotrophic tropical marine habitats, it could be a potential source of the cytotoxic reactive oxygen species (ROS) during photosynthesis (Dykens 1984; Shick & Dykens 1985; Aljbour et al. 2017). Therefore, it is a matter of equilibrium between the acquired benefits and the negative impacts of the endosymbiosis that control the level of the endosymbionts inside their tissues.

What roles do *Cassiopea* medusae play in their habitats?

The Upside-down jellyfish could be considered a key organism in many reef habitats (Jantzen et al. 2010; Niggl et al. 2010). For example, in the Gulf of Aqaba (GoA), the jellyfish was found to fuel the reef habitat with their released dissolved and particulate organic matter, through mechanisms such as sloppy feeding, or excretion of fecal materials or mucus (Niggl et al. 2010). Interestingly, the authors have shown that the rate of organic matter release by *Cassiopea* medusae exceeds release rates reported for hermatypic corals by factors of 2 to 15. Furthermore, the continuous bell pulsation could extract the pore water from the sediments underneath the pulsating medusae (Jantzen et al. 2010; see Fig. 3). The extraction process, which is physically based on the pumping action, is generated by the successive contraction-relaxation cycles of the medusa bell, seems to be nonselective. Besides re-suspending the partially buried organic nutrient, dissolution of the associated pollutants (e.g., heavy metals) due to enhanced swirling action is very likely (see Fig. 3).

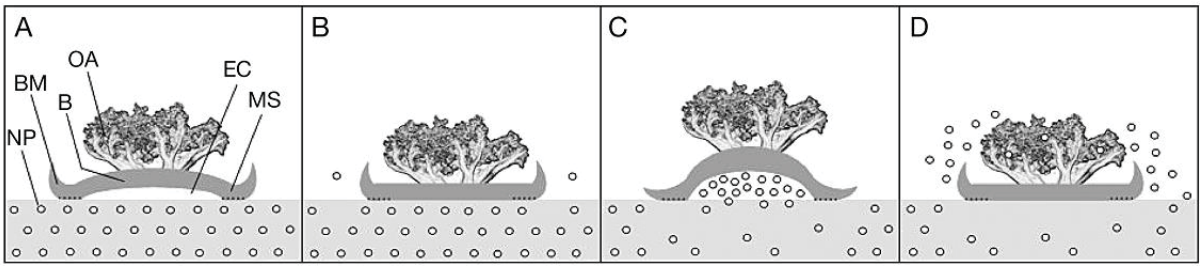

Fig. 3. A scheme of *Cassiopea* medusae bell mediated pumping activity. (A) Freshly settled medusa in relaxed state. (B-D) Represents a contraction-relaxation-contraction cycle; note that the amount of the re-suspended nutrient (or any dissolved solute) in the pore water underneath the medusa could be released with increasing pulsation rate. B: bell; BM: bell margins; EC: exumbrellar cavity; MS: mucus sealed bell seating; NP: nutrient-rich pore water; OA: oral arms (Jantzen et al. 2010).

Are *Cassiopea* an invasive jellyfish?

Generally speaking, invasive species are that subset of alien species (often called 'exotic'or'introduced') which have overall negative impacts in their new habitats (Russell & Blackburn 2017). According to Doty (1961), the introduction of *Cassiopea* into the Pearl Harbor during the Second World War by U.S. Navy vessels (1941-45 period) is the beginning of *Cassiopea's* presence in the Hawaiian Islands. Furthermore, the author confirmed that the jellyfish was restricted to that harbor at least until December 1955, when he left the Island. The author also mentioned that the jellyfish was most likely introduced as scyphistomae on ships or alike, while he excluded the possibility of the medusoid form being the introduced form. In 2010, *Cassiopea andromeda* has been recorded in sizable aggregations for the first time from Marsamxett Harbour in the Maltese Islands (i.e., until then known from the Levantine and Aegean basins in the Mediterranean Sea), in this record the author have suggested "shipping "as the vector of the jellyfish transport (Schembri et al. 2010). After ca. 4 years of this record, Özbek and Oztürk (2015) reported the northernmost location record of the Upside-down jellyfish *Cassiopea andromeda* from the coasts of Turkey (i.e., specifically in the Asin Bay, in the Gulf of Güllük), where the jellyfish was observed for the first time in 2014. In another example, the presence of *Cassiopea andromeda* in the Nayband Bay (i.e., northern part of the Arabian Gulf) has been recorded for the first time in 2014, the jellyfish was found in aggregates (e.g. up to 4-5 jellies per m^2, in the studied lagoon) and therefore had been described by the author as an invasive species (Nabipour et al. 2015). The authors described the jellyfish as being a venomous jellyfish, able to cause envenomation, pain, swellings, rashes, and itching, vomiting and other toxicological effects, depending on sensitivity of the victims to the venom (Nabipour et al. 2015). In spite of the aforementioned reports concerning *Cassiopea's* most recently reached marine habitats and the fact that they are showing increased habitat ranges, describing the jellyfish as invasive and venomous is questionable. Personally, I have not experienced sign of envenomation from the jellyfish *Cassiopea* through three yours working on them.

Section II. Scyphozoan blooms: Are jellyfish going to take over the oceans?

Over the past few decades, jellyfishes were increasingly blooming in many coastal marine systems worldwide (Purcell et al. 2007; Richardson et al. 2009). Global warming due to climate change and increased anthropogenic pressures (e.g., overfishing, eutrophication and habitat alteration) in many coastal systems are hypothesized to be associated (if not drivers) for such intensified blooms (Purcell et al. 2007; Gambill & Peck 2014). However, there is still no solid proof that the current jellyfish blooms phenomena is due to climate change or natural oscillations in jellyfish populations (Condon et al. 2013). Regardless of the current debate and disagreement about the drivers of this phenomenon, jellyfishes seem to be robust and benefit from both global warming and anthropogenic activities to a certain degree. For example, Holst (2012) has shown that increasing seawater temperature had several positive effects on strobilation. The author has found that while the percentage of strobilating polyps of *Aurelia aurita* and *Chrysaora hysoscella* have increased in response to temperature rise, *Cyanea capillata* and *Cyanea lamarckii* polyps' strobilated faster and produced more ephyrae per polyp. In an experimental simulation of the future possible ultraviolet-B (UV-B) radiation levels and temperatures, Klein et al. (2016), have found that strobilation will be facilitated at higher seawater temperature (28 °C) in *Cassiopea*, if the UV-B levels is decreased in the future. However, the authors have found that if seawater temperature rise is paralleled with increase in UV-B levels, the opposite will hold true (i.e., at 28 °C + high UV-B resulted in reduced strobilation). Given that temperature is more homogenous and less attenuated than UV light in seawater (i.e., within few meters depth water temperature changes only slightly, but UV light is highly attenuated), it is more likely that increasing seawater temperatures would enhance *Cassiopea's* reproduction.

Negative impacts of scyphozoan blooms (emphasis on pelagic species):

Jellyfish are well known to have diverse effects on marine life and economy, and in many cases they were associated with envenomation and death of human. Ecologically, most scyphozoans exhibit "boom and bust'' population dynamics, they grow rapidly and die *en masse*, releasing massive amounts of organic matter into the water column following decay of their dead bodies (Pitt et al. 2009; Chelsky et al. 2016). Furthermore, decay of such enormous amounts of tissues introduced in a confined short time could lead to localized hypoxia or anoxia (Pitt et al. 2009; Chelsky et al. 2015, 2016; Qu et al. 2015). Furthermore, decay of dead jellyfish could have significant impacts on microbial plankton (Tinta et al. 2016). The

diet of jellyfishes is highly diverse, they consume zooplankton, small invertebrates and fish larvae, and even fish are on the menu (Breitburg et al. 1997; Mills 2001). By feeding on zooplanktons and fish larvae, jellyfish are able to adversely reduce fish stocks through both competition and predation (Möller 1984; Breitburg et al. 1997; Mills 2001; Gordoa et al. 2013; Robinson et al. 2014). On the other hand, jellyfish are eaten by some fish, sea turtles and others, the so called "medusivores". Kondo et al. (2016) have found evidences for the transmission of some parasitic trematode larvae to their definitive fish hosts through medusivory. Therefore, inducing habitat deterioration (e.g., hypoxia), predation, competition and facilitating parasitism are some negative impacts of jellyfish blooms when they occur.

Economically, the negative impacts on industry, tourism, and fisheries are of higher concerns to the society. For example, the losses in fisheries production due to *Nemopilema nomurai* blooms around China and Japan have caused millions of dollars losses in the past decade (Robinson et al. 2014). By increasing farmed fish gill disorders and mortality, clogging net cages, or inflicting painful stings to field operators, jellyfish blooms severely affected different Mediterranean aquaculture facilities from Tunisia (Sicily Channel) and Spain (Alboran Sea), with severe economic consequences (Bosch-Belmara et al. 2017). Touristic beach closures on the Israeli side of the Mediterranean Sea, because of jellyfish outbreaks, causes 1.8-6.2 million € loss annually (Ghermandi et al. 2015). Blocking power plant cooling system intakes by jellyfish is becoming more common as well (Lynam et al. 2006). The cost and frequency of such blockages are both increasing worldwide, especially in the Chinese and Japanese coastal systems (Uye 2008).

Section III. Homeostasis and physiological responses to a changing environment

Climate change has caused an increase in mean temperature by more than 1.5 °C above pre-industrial levels (IPCC 2017). The changes in temperature, however, are not symmetrical in all oceans and continents (Bozinovic & Pörtner 2015). Urbanization and coastal development are imposing increased and accelerated negative impact on the coastal marine habitat. Eutrophication and pollution are the two main problematic side-effects of coastal system utilization. Water is a good heat conductor, universal solvent, and the main constituent of all living marine organisms. Due to these properties, one can expect that changes in seawater temperature or the dissolved substances would be detected by the organisms that live immersed inside it. In general, all organisms/cells tend to control their internal milieu stable or at least within certain ranges. The American physiologist Walter Cannon has proposed in

1962 the term “homeostasis”, which is defined as “the tendency of an organism/cell to regulate its internal conditions, regardless of the outside changing conditions”. Noteworthy to mention here is that endotherms, ectotherms, sessile and mobile organisms all could differ in how they respond to a given change in environment (Bozinovic & Pörtner 2015).

Generally speaking, organisms try to cope with stressful conditions by different strategies: avoidance, behavioral, molecular or physiological adaptations. For mobile organisms and active swimmers, avoidance would be the first choice. In sessile animals, which are more susceptible to changes in their environment, avoidance is not the choice. In the last animal category, behavioral, physiological or molecular adaptations become more crucial for survival.

Metabolic cost associated with environmental stressors: pollutants and global warming

Anthropogenic inputs of pollutants impose stress upon marine organisms. To cope with such a stress, organisms allocate more energy toward maintaining homeostasis. For example, Oliveira et al. (2017) have shown that the protein and glycogen contents in the mussel *Mytilus galloprovincialis* decreased significantly following acute (96 h) exposure to carbamazepine (i.e., a toxic antiepileptic drug widely encountered in aquatic environment). In contrast, the activity of the mitochondrial electron transport system (ETS; a representative measure of cellular respiration rate) tended to increase in the same treatments. In this mussel, increased cellular respiration and energy reserve catabolism indicated increased overall metabolism (Oliveira et al. 2017). Since organisms have a certain confined amount of energy reserve at any given time, more energy allocated for maintenance and homeostasis means less energy available for growth and reproduction (Smolders et al. 2004). In another example, Baum et al. (2016) have shown that while exposure to diesel resulted in metabolic depression in the coral reef fish *Siganus guttatus*, the metabolic rate increased when the fish were exposed to an anionic surfactant. According to the authors, both responses are signs of stress effects on metabolic rate, where one is associated with increased metabolic demand, while the depression is thought to be attributed to the toxicity of surfactants to the metabolic machinery (Baum et al. 2016). Impairment of antioxidant enzymes in the clam *Ruditapes philippinarum* is attributed to carbamazepine exposure, and thus the ability to cope with oxidative stress (Almeida et al. 2015). In the jellyfish *Cassiopea maremetens*, Rowen et al. (2017) have shown that the photosynthetic efficiency was significantly decreased in the jellyfish in response to diuron and hexazinone (i.e., commonly encountered herbicides in

coastal systems) exposure. Nowadays, heavy metal pollution (e.g. Cadmium ‘‘Cd”) and rising seawater temperature are common stressors in many estuarine and coastal habitats. Bagwe et al. (2015) were able to show that heavy metal and rising seawater temperature could work synergistically to reduce aerobic scope and thermal tolerance in oysters *Crassostrea virginica*. Furthermore, the authors have shown that the oysters showed an early transition to partial anaerobiosis (e.g., indicated by the accumulation of anaerobic end products in their tissues) when exposed to cadmium (Bagwe et al. 2015). The authors concluded that oysters’ exposure to environmentally relevant concentrations of cadmium for long-term could sensitize the organisms to high temperatures during seasonal warming and/or the global climate change in polluted estuaries (Bagwe et al. 2015).

So, impairment of metabolism and induction of oxidative stress are among the main underlying mechanisms involved in pollutants and temperature-mediated stress; especially in benthic organisms living in anthropogenically impacted shallow coastal marine habitats.

Section IV. Reactive oxygen species, oxidative stress and antioxidant systems

The partial reduction of oxygen is known to generate a group of chemically reactive oxygen-containing molecules known as “reactive oxygen species (ROS)”. Whereas low levels of ROS are crucial for normal cellular metabolisms and for fighting pathogens (Wilson et al. 2017), high levels of ROS are very harmful if not lethal in some cases. Development of many diseases (e.g. lung fibrosis and cancer) in mammals is attributed to oxidative stress toxicity (reviewed in Gonzalez-Gonzale et al. 2017). In marine habitats, temperature rise mediated reactive oxygen species (ROS) formation is the main mechanism causing coral bleaching (Lesser et al. 1990). Yang et al. (2017) hypothesized that ROS played crucial roles in animal evolution especially in the Cambrian period. They proposed that the increased cellular production of ROS associated with elevated level of O_2 in that period in combination with the increased mobility and food intake of metazoans, led to enhanced mutation rates that drove evolution through providing new regulatory mechanisms (Yang et al. 2017).

Superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$), among others are collectively called ROS. They are natural byproducts of the normal aerobic metabolism (e.g., superoxide anion is the main byproduct of the NADPH-oxidase enzymes and mitochondrial electron transport system (ETS); reviewed in Wilson et al. 2017). Superoxide anion, the first oxyradical to form during cellular injuries, is capable of initiating the free radical oxidation chain (Fridovich 1975; Richier et al. 2003). Spontaneous dissociation of O_2^- and superoxide dismutase (SOD) transformation of the reactive molecule into H_2O_2 aids the first steps of detoxifying the molecule (Fridovich 1975; Lesser 2006). While H_2O_2 is less reactive than O_2^-, it is more mobile (i.e., uncharged small molecules diffuse freely through biological membranes). Hydroxyl radicals ($HO^\bullet$) are the most reactive of the three molecules, they have incredible potential for biological damage, attacking all biological molecules (Lesser 2006; Regoli & Giuliani 2014). H_2O_2 and iron are initiators of $HO^\bullet$ formation, mainly by Fenton reaction (Fig. 4), where Fe(II) is oxidized by hydrogen peroxide to Fe(III), along with forming a hydroxyl radical and a hydroxide ion. Both O_2^- and some trace metals, (e.g., copper may mimic iron action keeping Fenton and Haber-Weiss reactions ongoing), may participate in $HO^\bullet$ production (Lesser 2006; Regoli & Giuliani 2014).

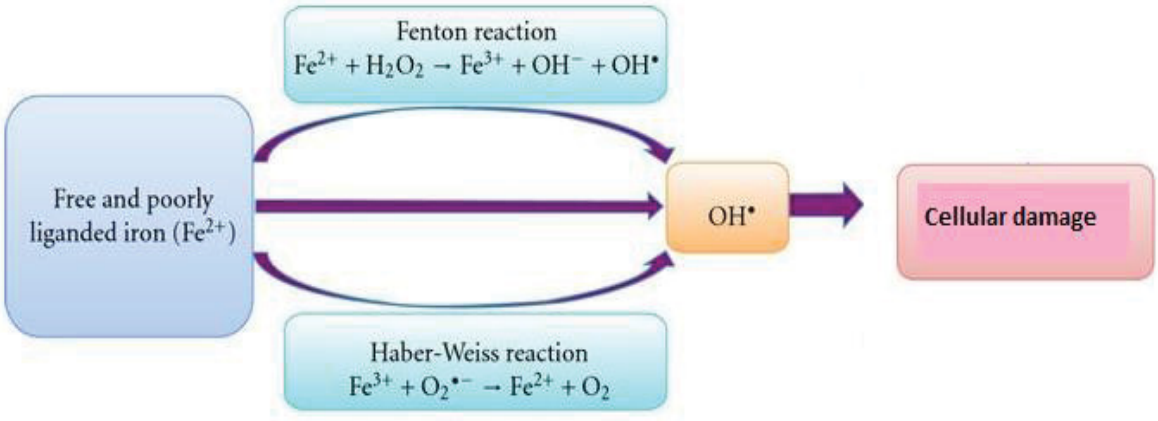

Fig. 4. Fenton and Haber-Weiss reactions mediated ROS formation. Free Iron (Fe^{2+}) reacts trough the Fenton reaction with hydrogen peroxide, leading to the generation of very reactive and damaging hydroxyl radicals ($OH^\bullet$). Superoxide can also react with ferric iron in the Haber-Weiss reaction, leading to the production of Fe^{2+}, which then again affects redox cycling.
Adapted from (http://www.hindawi.com/journals/msi/2011/606807/fig2/)

Most eukaryotic cells are equipped with a repertoire of enzymatic and non-enzymatic antioxidant defense systems. Beside SOD, cells utilize catalase and GSH-Px among other antioxidants for protecting the cells from the toxic effects of ROS (reviewed in Birben et al.

2012). Approximately, 0.3-3% of the electrons transferred through the mitochondrial ETS leak and result in O_2^- (Speakman & Selman 2011). Since all aerobic respiring cells have ETS, SOD is present in all of them as an adaptive mechanism (McCord & Fridovich 1969; Lesser 2006). Under normal metabolic rates, cellular antioxidant systems usually keep pace with ROS production levels, and therefore oxidative stress is avoided. The imbalance between production and detoxification of ROS is defined as oxidative stress, where the cellular redox homeostasis is disturbed (Wilson et al. 2017).

Excess ROS are highly reactive and capable of initiating a chain of cellular macromolecules oxidation: DNA, proteins and membrane lipids are of high susceptibility for such attacks (Lesser 2006). Inhibition of antioxidant enzyme activity and subsequently the onset of oxidative stress were attributed to lipid peroxidation (LPO) and genotoxicity in common carp *Cyprinus carpio,* following exposure to 0.01 mg Hg/L (García-Medina et al. 2017). It is worth to recall that the products of lipid peroxidation (especially of the polyunsaturated fatty acids "PUFA") are extremely dangerous, and due to their high reactivity they are able to induce cellular damage. Furthermore, LPO could be induced by a set of other metals (e.g., Cd, Cu and Pb), which are common in coastal habitats, in this sense LPO is considered one of the main biomarkers for experiencing oxidative stress mediated cellular damages (Knight & Voorhees 1990). Hence, the ability of an organism to fight and keep ROS under control is of critical importance, which could sometimes set the limits between life and death when an organism is confronted with stressful conditions. In *Cassiopea* and other jellyfish, understanding the oxidative responses to both changes in seawater temperature and pollution brings valuable knowledge about their role in the future. These aspects are discussed in chapter 3 and 4.

Research Gaps

The Upside-down jellyfish *Cassiopea* has recently shown clear signs of expanding its population and territory; it invaded the Hawaiian Islands, the Mediterranean Sea and the Arabian Gulf (Doty 1961; Schembri et al. 2010; Nabipour et al. 2015; Özbek & Oztürk 2015). The jellyfish plays key roles in nutrient cycling and food web in coral reefs (Jantzen et al. 2010; Niggl et al. 2010), it is becoming of high interest in aquaculture as food for medusivores in captivity (Pierce 2005). However, *Cassiopea* has been overlooked and almost ignored from studies focusing on the more eye-catching pelagic jellyfish due to their obvious socio-economic negative impacts (Purcell et al. 2007; Richardson et al. 2009; Bosch-Belmara et al. 2017).

Climate change and anthropogenic disturbances have been thought to drive pelagic jellyfish blooms (Purcell et al. 2007; Gambill & Peck 2014); however**,** except for some studies (e.g., Holst 2012; Gambill et al. 2016; Klein et al. 2016; Aljbour et al. 2017), most studies have focused on ecological roles and consequences of pelagic jellyfish blooms only. The epibenthic jellyfish have been proposed to benefit from anthropogenic activities associated with coastal urbanization (Stoner et al. 2011, 2014, and 2016). The authors have found that *Cassiopea* medusae populations in urbanized coastal system were higher in numbers, and their medusae attained bigger sizes and higher zooxanthellae densities in their tissues compared to medusae from the non-urbanized locations. However, no physiological mechanistic cause and effect explanations were provided by the authors.

Thriving in disturbed marine habitats where hypoxia (e.g., due to nutrient overload) and pollutants are common stressors, needs special adaptations at the physiological levels (e.g., aerobic and aerobic metabolism, anti-oxidant system to cope with reactive oxygen species associated with high temperatures and pollutants). In such habitats corals, for example, were and still are deteriorating while jellyfishes seem to thrive. However, studies on physiological adaptations in jellyfish are scarce. Better understanding of physiological responses to environmental disturbances would provide better predictions and insight about their future in marine systems in light of the changing climate and increased anthropogenic disturbances.

Therefore, this thesis tried to partially fill these gaps in current knowledge by studying some physiological responses of the epibenthic jellyfish medusae to changing seawater temperature experimentally, while studying the responses of the jellyfish to anthropogenic disturbance

(i.e., pollution in this study) through field excursions in the Gulf of Aqaba, Red Sea. The overall motivation is that understanding the jellyfish metabolism and oxidative stress response could provide better explanation of the common perception that jellyfish are robust animals.

Aims and specific research questions

This thesis addresses the above mentioned research gaps by raising the following specific questions:

1. Are *Cassiopea* medusae robust to abrupt changes in seawater temperature? And if so, how do they respond to increase/decrease in temperature in terms of:
    a. Aerobic metabolism assessed through cellular respiration rates CRR and oxygen consumption OC
    b. Oxidative responses assessed through superoxide dismutase as antioxidant defensive enzyme and lipid peroxidation as a biomarker of damage associated with experiencing oxidative stress.
2. How is the anaerobic metabolism in medusae is affected in response to pollution (i.e., metal pollution), in this case assessed through the measurement of pyruvate kinase and lactate dehydrogenase activities.
3. Do the current pollutants levels at the Gulf of Aqaba induce oxidative stress in medusae living there, in this case assessed through lipid peroxidation

The following hypotheses were formulated:

1. Increasing seawater temperature increases OC and CRR in *Cassiopea* medusae.
2. *Cassiopea* medusae are robust to both cold and warm temperature
3. No change in anaerobic metabolism is expected in medusae from polluted locations at the Gulf of Aqaba.

The research questions are investigated in a series of experiments with different focus. The experiments were presented in the following manuscripts:

Aljbour et al. 2017. Cellular respiration, oxygen consumption, and trade-offs of the jellyfish *Cassiopea* sp. in response to temperature change. (**Published in *J. Sea Res***). This paper focuses on the OC & CRR responses to short and long-term temperature changes. Acclimatization ability is addressed as well. [Chapter 2]

Aljbour et al. 2017. Are jellyfish physiologically well adapted to global warming? Surprising oxidative stress and metabolic demand responses in *Cassiopea* sp. (**Submitted to *J. Sea Res*.**) This paper focuses on the oxidative stress responses to short and long-term temperature changes. It brings new evidence on jellyfish robustness besides providing a cause-effect mechanistic explanation of CRR responses. [Chapter 3]

Aljbour et al. 2017. Metabolic responses of the upside-down jellyfish *Cassiopea* sp. to pollution in the Gulf of Aqaba, Jordan. (**Submitted to *Mar. Poll. Bull***) This paper focuses on the anaerobic metabolism and oxidative stress responses to current pollutant levels at the Gulf. New evidences on *Cassiopea* robustness to anthropogenic disturbances are brought in this paper as well. [Chapter 4]

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Chapter 2

Thermal Tolerance and Aerobic Metabolism *Cassiopea* Medusae

Does *Cassiopea* like it warm?

This chapter is published as:

Aljbour, S.M., Zimmer, M., Kunzmann, A. 2017. Cellular Respiration, Oxygen Consumption, and Trade-Offs of the Jellyfish *Cassiopea* sp. in Response to Temperature Change. J. Sea Res. 128: 92-97

Abstract: Pelagic jellyfish blooms are increasing worldwide as a potential response to climate-change. However, virtually nothing is known about physiological responses of jellyfish to e.g. sudden changes in water temperature due to extreme weather events. When confronted with a sudden decrease or increase in water temperature by 6 °C, medusae of *Cassiopea* sp. exhibited a strong response in locomotory activity (i.e., bell pulsation increased and decreased by ca. 37 and 46% in hot and cold acute (2 hours) treatments, respectively) relative to control. Although medusae significantly gained in body mass (wet weight) upon chronic (2 weeks) heat treatment, their body size (e.g., bell diameter) did not change over this time interval. In contrast, chronic cold treatment resulted in both significant shrinking (reduced diameter) and mass loss. Measurements of mitochondrial electron transport system (ETS) activities and rate of respiratory oxygen uptake (MO_2) are good estimates of energy consumption and the potential aerobic metabolic rates of an organism. While both acute treatments significantly increased ETS-activities, acclimation over two weeks resulted in a drop in activities to the control levels. Whereas acute heat treatment significantly increased MO_2, chronic exposure resulted in significant MO_2 decrease compared to control; however no changes in MO_2 could be observed in both acute and chronic cold treatments. Overall these results suggest an enhanced growth in response to global warming, whereas low temperatures may set the limits for successful invasion of *Cassiopea* into colder water bodies. Our results provide a framework for understanding the physiological tolerance of *Cassiopea* under possible future climate changes.

Key words: Respiration rate, Aerobic metabolism, Bell pulsation, Jellyfish bloom, Mitochondrial electron transport system (ETS), Global warming.

Introduction

Cassiopea, the upside down jellyfish, is a globally distributed epibenthic scyphozoan in tropical and sub-tropical marine environments, inhabiting mangrove forests, seagrass beds, and coral reefs (Gohar & Eisawy 1960; Holland et al. 2004; Niggl & Wild 2009; Welsh et al. 2009; Stoner et al. 2011, 2014). It has a metagenic life cycle typical of scyphozoans, involving a motile sexually-reproducing medusae and a sessile asexually-reproducing polyp (Hofmann et al. 1996). In *Cassiopea*, the shift from polyp form to medusoid stage (i.e., strobilation) is only induced after they acquire certain species of *Symbiodinium* and when the temperature is above 20 °C (Hofmann et al. 1978; Rahat & Adar 1980). The upside down jellyfish is a key organism in many habitats, fueling pelagic food webs in coral reefs by releasing organic matter (Niggl et al. 2010), and playing an essential role in nutrient cycling (Jantzen et al. 2010). Recently, *Cassiopea maremetens* was found to show rapid uptake and retention of trace metals in their tissues, which has major implications for both biomonitoring and the trophic transfer of pollutants through local ecosystems (Epstein et al. 2016).

Recently, scyphozoans, among other gelatinous cnidarians, are showing increased bloom frequencies worldwide. Global warming and anthropogenic activities (e.g., overfishing, eutrophication, aquaculture, and coastal construction) are thought to be the reason behind the increasing jellyfish blooms (Wei et al. 2015). Climate change has increased the severity and frequency of both hot and cold extreme events where rapid changes in seawater temperature happen. For example, in the shallow seagrass–mangrove flats in Grassy Key in Florida, where *Cassiopea* sp. occurs, the passage of the severe cold front caused a rapid drop in seawater temperature (i.e., to 12-14 °C) in December 1991 (Fitt & Costley 1998). At the same site, the authors noted a variation in temperature of 2 °C per hour following the front passage.

Generally, organisms respond to changes in temperature by increasing or decreasing their metabolic rate in addition to other responses. Metabolic rate is known as the amount of energy used by an animal per unit of time, usually measured as respiration, or oxygen consumption (MO_2). All poikilotherms have certain ranges of temperature where they function optimally, sub-optimally or even cease aerobic metabolism when a critical thermal limit is exceeded (Pörtner & Farrell 2008). Thermal limits for growth and survival arise from oxygen and capacity-limited thermal tolerance (Pörtner & Farrell 2008). When reached, critical thermal limits are normally associated with decreases in aerobic and increases in

anaerobic metabolism (Gambill & Peck 2014). In practice, while MO_2 is the actual rate of respiration (Ishii & Tanaka 2006), the electron transport system (ETS) activities, theoretically, represent the maximum cellular oxygen uptake and the potential respiration that could be supported by the existing enzymatic machinery of an organism undergoing aerobic respiration and the rate of oxygen consumption (Packard 1971; King & Packard 1975).

In spite of the aforementioned importance of *Cassiopea* in their native environment, and that they are considered exotic organisms in many places outside their original natural distributional areas (e.g., in the Gulf of Güllük in Turkey, Özbek & Öztürk 2015), they are not well studied in respect to their role in scyphozoan blooms, except for few studies done by Stoner et al. (2011). The authors showed that *Cassiopea* spp. medusae were more abundant and attained larger sizes in human-impacted systems in The Bahamas, without any further explanations in physiological terms. The lack of research on thermal windows of jellyfish limits our ability to predict how warming might affect (or potentially limit) life cycle and bloom dynamics. Interestingly, to the best of our knowledge, no studies are available on physiological responses of the medusoid stage of the genus *Cassiopea* in response to sudden changes in temperature.

This is the first study on the genus *Cassiopea* that measures the respiratory and metabolic enzymatic efficiency (ETS) responses to sudden temperature anomalies. Since organisms have certain confined amounts of energy available at any given time, increasing energy consumption in maintaining cellular homeostasis will be on the expense of other cellular activities such as growth and reproduction (Gambill & Peck 2014). Therefore, our aim is to investigate responses of ETS and MO_2 to sudden temperature changes (i.e., acute treatments) in *Cassiopea* sp. in order to provide better understanding of their instantaneous performance in response to local extreme weather events as well as to longer term (i.e., chronic long lasting changes) trends in climate change.

Materials and Methods

Experimental organisms:

Cassiopea sp. medusae with a bell diameter of 3.5-5.2 cm and body mass of 3.4-7.1 grams were used in our experiment. Medusae were raised for 3-4 months from ephyrae collected from a strobilating polyp culture kept for years at the MAREE facilities at the Leibniz-Zentrum für Marine Tropenforschung (ZMT), Bremen. The experimental organisms had been

selected to be apparently morphologically healthy (i.e., no signs of pitched bells or lost arms, etc.) and to be of similar size. Prior to the experiment, medusae had been acclimated for two weeks in a single aquarium tank (ca. 120 litre) with a recirculation system (salinity 40.2 ± 0.2, temperature 25.9 ± 0.1 °C; the typical salinity and temperature for *Cassiopea sp*. in the Gulf of Aqaba, Red Sea), 12:12 hours light/dark cycle, and fed twice a week with freshly hatched *Artemia* nauplii.

Experimental design:

Two sets of experimental setups were used to test *Cassiopea's* sp. responses of the selected parameters to thermal (cold/heat, 20 °C and 32 °C, respectively) treatments. Throughout all experiments, a control (salinity 40.2 ± 0.2, temperature 25.9 ± 0.1 °C) treatment was always run in parallel. Whereas the cold treatment (a drop by 6 °C from the control) might mimic the effect of cold front in shallow coastal waters, it might give insight on species translocation by aquaculture activities or ballast water. On the other hand, the heat treatments (an increase by 6 °C from the control) are oriented more towards what might happen in a tidal pool or at longer-term, global warming.

First, in the acute treatment, eight medusae were transferred immediately into pre-cooled or -heated individual 1.0-L glass jars kept in 80-liter water bath set at the same treatment temperature for 2 hours. Medusae (N = 8, for each treatment and a control, respectively) were sampled after two hours of treatment. Second, in the chronic treatment, another 8 medusae were transferred as in acute treatment, but were kept at the same temperature for two weeks.

Henceforth, we use the following terminology: 1- chronic treatment means that the organism have been treated like in acute treatment, but kept for two weeks instead; 2- cold, heat and control always corresponds to 20, 32 and 26 °C, respectively.

Bell diameter and body mass measurements:

Jellyfish bells diameters were measured using scaled glass beakers; diameter was recorded at full relaxation of the bell. Then jellyfish were removed by hand, excess water was removed by shaking and sucking by absorbent tissue without directly letting the tissue touch the jellies tissue, in less than 20 seconds it was weighed in a seawater filled beaker (same water as the incubation aquarium) and mass was measured to the nearest 0.1 gram. The whole diameter

and body mass measurement were done at the beginning of the chronic experiment and at the end of the experiment

MO_2 measurements:

The rates of oxygen consumption were measured in 0.2 µm-filtered seawater aliquots (Sartobran, Germany), using a Fire-sting optical microelectrode (Pyroscience GmbH, Germany). Cylindrical glass chambers with a tightly fitting lid were used as respiratory chambers (closed system), without stirring to avoid animal damage (i.e., stirring was achieved by the animal bell pulsation). Medusae were carefully washed in a bucket filled with filtered seawater to remove other organisms in the mucus and immediately transferred with a glass bowl into the experimental chamber. Decrease in oxygen concentration was logged automatically every 10 seconds to a connected PC for two hours. Slopes of regression lines over time were used to calculate the oxygen consumption rates, and MO_2 values were corrected for background microbial respiration (blanks). Whenever oxygen levels had dropped to 70% saturation, the chambers were opened and refilled with saturated seawater. In all treatments the sensors were calibrated for 0% and 100% air saturation before being used in measurements.

Tissue sampling, enzymatic assays and protein content measurements

Oral arms were cut from the tip to the point where they were attached to the body to avoid interference with other tissues. Samples were immediately kept on dry ice and then at -80 °C until analysis. All oral samples were first dispersed using IKA®-ULTRA-TURRAX dispersers with ice-precooled probes for less than 30 seconds, resulting in crude homogenates, which then were further homogenized in 1.5 mL Eppendorf tubes containing ETS buffer (prepared according to Owens & King 1975), 0.1 M potassium phosphate buffer (KPi) pH 8.5, containing 0.45 mg/ml Poly vinyl pyrolidone (PVP), 22.5 µM $MgSO_4$, and 0.16% Triton X-100), and ca. 500 mg glass bead mixture (0.4 and 1.0 mm diameter) using FastPrep®-24 tissue homogenizer (speed: 4.0 M/S, TN: 12 × 15, time 15 second for each cycle) for two cycles with three minutes cooling on ice between the cycles to avoid excessive heating. Then the homogenates were centrifuged at 5,000 g for 5 minutes at 4 °C, and the supernatant was used for the ETS activity assay directly after centrifugation. All steps were performed on ice where applicable.

ETS activity was measured using the INT reduction assay (Packard 1971; King & Packard 1975; Owens & King 1975). Eighty microliters of the supernatant were added to the reaction mixture (250 µL INT, 167 µL NADH, and 503 µL 0.1 M KPi pH 8.5), which had been incubated at 22 °C in a thermostat for 3 minutes in a disposable plastic cuvette prior to the addition. Then the increase in absorbance was followed spectrophotometrically at 490 nm for 5 minutes. Protein content was determined following the Bradford assay at 595 nm using the Bovine Serum Albumin (BSA) to build the standard curve (Bradford 1976).

Statistical analysis:

Each group has been compared to its control group for differences in the same treatment using a two tailed "Welsh two sample t-test", and 0.05 were set as the significance level. Results of comparing the group means were considered significant if the p-value of the test is ≤ 0.05, and the word 'significant' has this meaning, wherever it appears alone in our text. In all treatments N= 8 unless mentioned elsewhere. T-test statistics are indicated in the test as follow: $t_{(df,N)}$= t-statistic value; where, df= the degrees of freedom for the t-statistic, and N= number of replicates. Correlations between selected variables have been tested using Spearman's rank correlation rho test. The results of the test are presented by indicating the p-value and the association coefficient of the test "rho value".

Results

Acute cold treatment caused a significant ($p < 0.05$ at all times intervals) decrease (by 46%) in bell pulsation rates; whereas heat treatment increased the rates (by 37 %) relative to the control ($p < 0.05$ at all times intervals, Fig. 1).

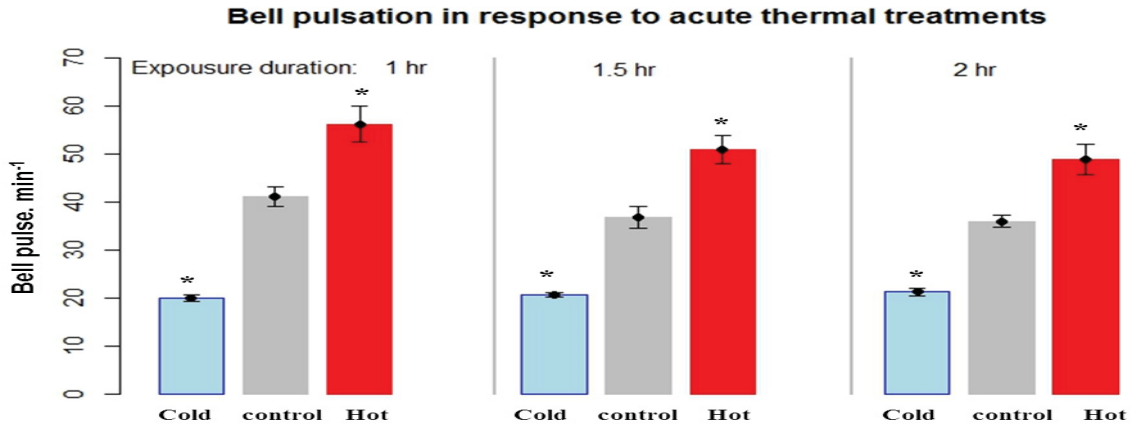

Fig. 1. Effect of acute cold/heat treatment on bell pulsation rate in *Cassiopea medusae*. Bars represent the mean vales ± SE, N= 8. P-value < 0.05 is considered significant and is indicated by an asterisk above bars.

While both acute cold/heat-treated medusae exhibited increased ETS activities (Cold, $t_{(8, 7)}$= 2.6, $p < 0.05$; heat, $t_{(9, 7)}$= 6.0, $p< 0.0005$; Fig. 2.A), only the acute heat-treated jellyfish had consistently higher oxygen consumption rates (MO_2, $t_{(13, 8)}$= 5.4, $p< 0.0005$; Fig. 2.B). In contrast, acute cold treatment did not induce any changes in MO_2 relative to the control.

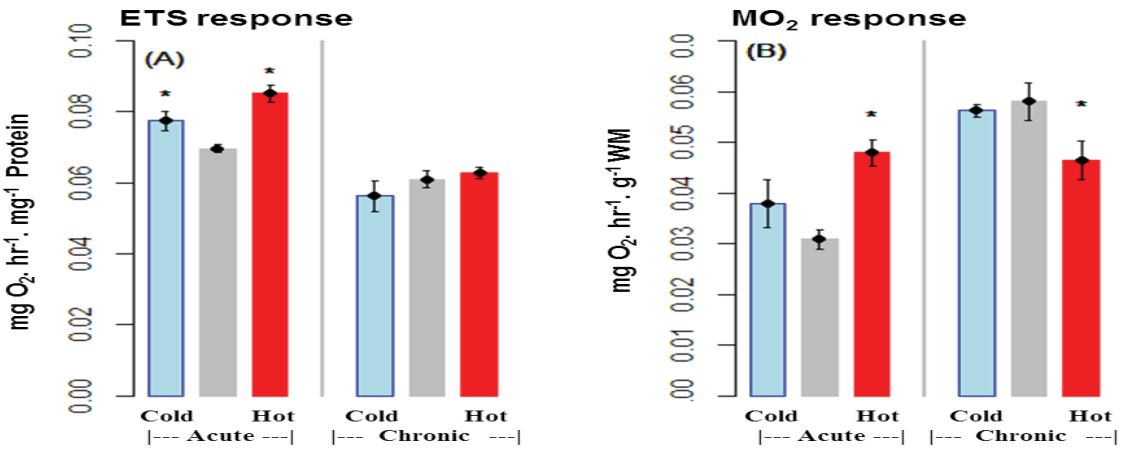

Fig. 2. Effect of acute and chronic cold/heat treatment on respiration rates in *Cassiopea* sp. (a) ETS activity response to acute and chronic cold/heat treatment (2 hours and 2 weeks, respectively). (b) MO2 response to acute and chronic cold/heat treatment; bars represent the mean vales ± SE, N = 7 for acute ETS otherwise N=8. P-value ≤ 0.05 is considered significant and is indicated by an asterisk above bars.

While protein content was increased significantly ($t_{(8, 7)}$= 2.3, p= 0.05) in the acute cold-treated medusae, it has significantly decreased ($t_{(13, 7)}$= -3.1, $p< 0.05$) in the heat-treated ones (Fig. 3.A). Both body wet mass (i.e., final mass – initial mass) and bell diameter (i.e., final diameter – initial diameter) were significantly (bell, $t_{(14, 8)}$= -8.6, $p< 0.000005$; mass, $t_{(10, 8)}$= -8.6, $p< 0.00005$) decreased in the chronic cold treatment in contrast to the increased body mass in chronic heat-treated medusae ($t_{(13, 7)}$= -2.5, $p< 0.05$; Fig. 3.B). Whereas neither cold- nor heat-treatment affected ETS activities after two weeks compared to the control, MO_2 dropped in the chronic heat-treated medusae ($t_{(14, 8)}$= 2.2, $p< 0.05$), but remained unchanged in the cold treatment (Fig. 2.B). Protein content showed the same trend as in the acute cold- and heat-treated organisms, with a significant increase ($t_{(10, 8)}$= 5.0, $p< 0.005$) in the chronic cold-treated medusae and a decrease in chronic heat-treated jellyfish ($t_{(11, 8)}$= 4.3, $p< 0.005$; Fig.3.A, see next page).

It is noteworthy to mention that among the chronic treated medusae, protein content showed a significantly strong negative correlation with medusae body masses (Spearman's rank correlation rho test: $p< 0.00005$, rho= - 0.76; Fig. 4). The results of the Spearman's correlation tests have shown that, in the chronic treated medusae, MO_2 was negatively

correlated with medusae wet body mass (Spearman's rank correlation rho test: $p < 0.0005$, rho= -0.69; Fig. 5)

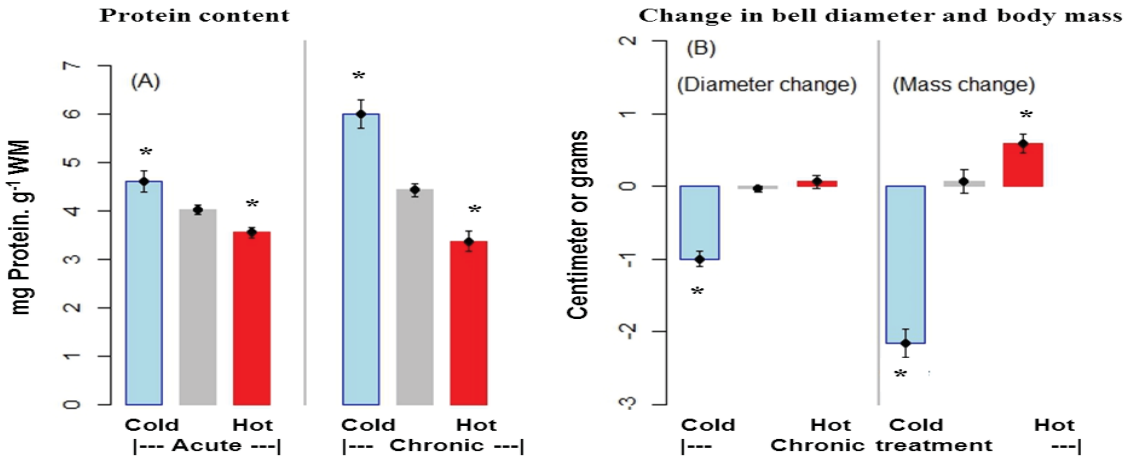

Fig. 3. (a) Effect of acute and chronic cold/heat treatment on total cellular protein content in *Cassiopea* sp. (b) Bell diameter and mass changes in response to chronic cold/heat exposure. bars represent the mean vales ± SE, N= 8 for bell and chronic protein otherwise N=7. P-value < 0.05 is considered significant and is indicated by an asterisk above bars.

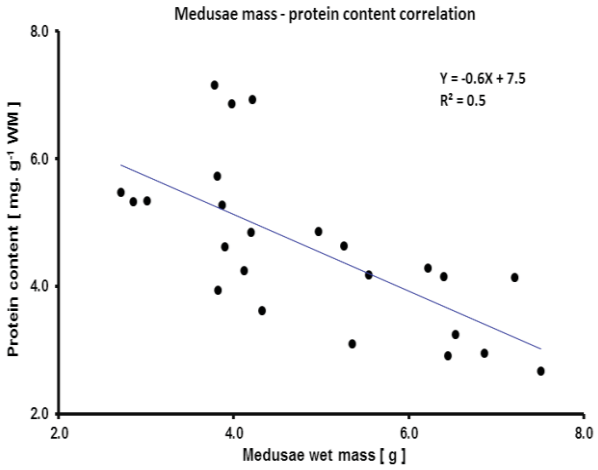

Fig. 4. Relationship between tissue's protein content (oral arms) and wet mass of *Cassiopea's* medusae in the chronically treated medusae.

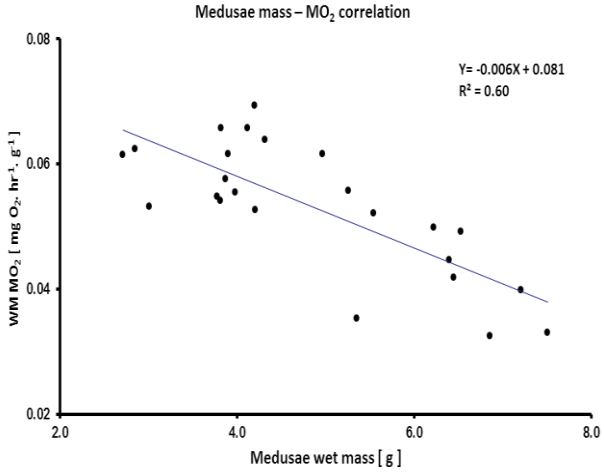

Fig. 5. Relationship between oxygen consumption rate (MO_2) and wet mass in the chronically treated *Cassiopea's* medusae.

Discussion

In this paper we investigated the physiological responses of *Cassiopea sp.* to acute and chronic treatments at cold and hot temperature. We noticed that while the ETS activities showed the same response trend in both acute (i.e., increased) and chronic treatments (i.e., unchanged), pulsation rate, body mass and protein content showed contrasting response trends between cold and heat treatments.

Cassiopea sp. rely on their bell pulsation for creating water currents that facilitate several functions, such as food capture, waste removal (Gohar & Eisawy 1960; Hamlet et al. 2011), swimming, and getting rid of sediment particles falling on the organism (Gohar & Eisawy 1960). They have shown that the pulsation rate is highest at 27 °C and decreases below and above this temperature. In acute heat-treated *Cassiopea* sp., probably, a significant amount of their respiration (MO_2) is spent in energizing the accelerated pulsation rates. In contrast, acute cold-treated jellyfishes showed no changes in the rates of oxygen uptake, possibly because bell pulsation rate was decelerated in these organisms. Thus, the increase in bell pulsation may account for the major energy consumption during acute treatment.

Upon short-term cold exposure, *Cassiopea* sp. decreased its tentacle length, and their ability to transfer captured food to the mouth was disrupted at temperature below 18 °C (Fitt & Costley 1998). In the same study that took place after the passage of a severe cold front through Florida (in December month 1991), numerous medusae had distended tentacles and asymmetric bells, and the population density of medusae was obviously lower than during the previous fall. In another example, a drop in seawater temperature to 14-16 °C, following the passage of an extreme cold front through southern Florida in late January 1977, has caused severe tissue sloughing and loss of normal coloration in the staghorn coral (*Acropora cervicornis*) with ultimate mortality in February of the same year (Davis 1982). Both studies are good observatory examples, revealing the prevalence and impacts of extreme weather events on cnidarians in the studied area. In the present study, about 25% of the chronic cold-treated medusae had an open bell on their aboral side exactly at the center, while some (12%) other medusae had clear (semitransparent) bells (i.e., mostly bleached). As these symptoms were not observed in the heat treatment or the control, we hypothesize that cold stress may initiate endosymbiont loss in *Cassiopea* sp. Moreover, chronic cold-exposed medusae showed decreased body mass, whereas chronic heat-treated jellyfishes exhibited body mass increase, suggesting that they have acclimatized well at 32 °C. These results suggest that chronic cold exposure is more deleterious to *Cassiopea* sp. medusae than the chronic warm exposure.

In contrast to the aforementioned effects of chronic cold treatment (two weeks), the chronic heat-treated organisms looked healthy and acclimatized well after the heat shock, as indicated by their increased body mass and unchanged ETS activity compared to the control. Warming generally increases the overall metabolic rate as long as species-specific thermal limits are

not exceeded. For example, in the polyps of *Aurelia* spp., Gambill & Peck (2014) have found that MO_2 increased in response to warming (e.g. 12 to 15 °C), but the polyps decreased their MO_2 at 20 °C, which suggests that sub-optimally warm temperatures were approached (Gambill & Peck 2014). Surprisingly, the drop of MO_2 in chronic heat-treated medusae coincided with increased body mass, contradicting metabolic depressions. Jellyfishes are characterized by their watery bodies (>95%) and low carbon (usually <1% of wet mass) content of their bodies (Lucas et al. 2011). They grow fast mainly by incorporating large volumes of water into their bodies (termed 'faking giants'; Acuña et al. 2011). This suggests that the observed increase in body mass has diluted the MO_2 signal rather than being metabolically depressed as in *Aurelia* polyps (Gambill & Peck 2014), which are highly different from the medusoid life stage. A supportive finding for this interpretation is the significantly strong negative correlation between medusae body mass and the tissue's protein content and MO_2 in the chronic treated medusae, which again might indicate the watery growth of the medusae (Fig. 4 & 5). Overall, *Cassiopea* sp. seems capable of acclimatizing well at 32 °C without any sign of stress-mediated increase in aerobic metabolism. In contrast, the unaffected metabolism (MO_2 and ETS) in the chronic cold treatment is contradicted by a significant decrease in growth, which suggests that the organisms are metabolically supressed or might be depending more on the anaerobic metabolism, since they showed significant decreases in their body mass.

Interestingly, jellyfishes are protein-rich animals, mainly in the mesoglea, where proteins comprise about 50% to the total dry weight (Ding et al. 2011). Structural proteins (collagen), in turn, make up about 50% of the total protein content (Khong et al. 2015). In *Cassiopea* sp. medusae, while the decreased protein content in acute heat-treated organisms suggests its utilization to fuel the accelerated bell pulsation, the decreased contents upon chronic heat treatment could be explained by the dilution effect of the watery growth. The increased protein content of cold-treated jellyfish could be explained by the concentrating effect of losing body mass (i.e., mainly water). In both cases, this conclusion might be supported by the observed significantly strong negative correlation between medusae body mass and protein content (Fig. 4).

Conclusions

Cassiopea sp. medusae seem to be more tolerant to temperature rise than fall. They seem to acclimate well at 32 °C, gain body mass and reduce the aerobic energy consumption. By

contrast, lowered temperature caused body mass loss, but no apparent change in aerobic metabolism. Water content seems to be rapidly changing in response to both cold and heat exposure. The increased protein content upon cold exposure combined with a decreased body mass, and the opposite scenario upon heat exposure (i.e. decreased protein content and increased body mass) suggest water incorporation rather than real growth in live tissues. Overall these results suggest an enhanced growth of *Cassiopea* sp. medusae in response to global warming, whereas low temperatures may set the limits for successful invasion of *Cassiopea* into colder water bodies. However, it might be beneficial to do a longer time treatment for months and track the whole life cycle to confirm the finding in a bigger scale.

Acknowledgements: This research was supported by Leibniz funds from the Leibniz Gemeinschaft Germany. We are grateful to the staff of MAREE and the ZMT laboratories for support. We thank the reviewers of this manuscript for their constructive comments.

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Chapter 3

Are Jellyfish Physiologically Well Adapted to Global Warming?

Surprising oxidative stress and metabolic demand responses in *Cassiopea* sp.

This chapter is under revision as:

Aljbour, S.M., Zimmer, M., Kunzmann, A. (XXXX). Are Jellyfish Physiologically Well Adapted to Global Warming? Surprising Oxidative Stress and Metabolic Demand Responses in Cassiopea sp. (submitted to the Journal of Sea Research).

Abstract

At present, climatic changes in temperature are hypothesized to be among the main drivers of pelagic jellyfish blooms. Physiological responses of jellies to temperature anomalies, however, are poorly understood. The potentially invasive zooxanthellate benthic jellyfish, *Cassiopea* sp., has been paid little attention. Thus we raised the question: how do extreme weather events (±6 °C from the control 26 °C) affect *Cassiopea's* medusae physiological performance in terms of metabolic demand and oxidative stress response? Therefore, we measured the electron transport system activities (ETS; a proxy of mitochondrial respiration), superoxide dismutase (SOD) activities and lipid peroxidation. In the acute-treated (2 hr) medusae, while cold (20 °C) increased ETS significantly, the heat (32 °C) did not. SOD activities, in contrast remained unchanged in all acute treatments. The chronic (2 wk) cold-treated medusae sustained high ETS-rates, suffered from oxidative stress and body mass loss. In contrast, the heat-treated medusae have not increased their metabolic demand nor any sign of oxidative stress, moreover, they gained body mass. Since chlorophyll-***a*** remained unchanged in all chronic-treated medusae, the cold-induced oxidative stress is more likely due to the increased cellular respiration, not the photosynthesis. Our findings bring new evidence that an oxidative stress-mediated increased metabolic demand is the main mechanism setting the limits to *Cassiopea's* physiological performance at cold temperature. We conclude that *Cassiopea* populations may flourish and extend their geographical distributions in response to global warming. In the view of global jellyfish blooms at the cost of deteriorating reefs and diminishing fish stocks, our findings are confirming the high competitiveness of jellyfish in future warming coastal ecosystems.

Kew words: oxidative stress, mitochondrial respiration, superoxide dismutase, jellyfish bloom, lipid peroxidation, electron transport system.

1. Introduction

In recent years, the pelagic scyphozoan jellyfish blooms are showing increased frequency and extended geographical distribution in costal marine habitats worldwide (Arai 2001; Graham, 2001; Mills 2001; Purcell 2005). Most pelagic scyphozoans proliferate rapidly (Pitt et al. 2009), predate voraciously (Breitburg et al. 1997; Mills 2001) and sting deadly. Jellyfish blooms have negative effects on fish stocks and fisheries (Möller 1984; Breitburg et al. 1997; Mills 2001; Gordoa et al. 2013; Robinson et al. 2014), marine habitats (Pitt et al. 2009; ; Tinta et al. 2012; Chlesky et al. 2015; Qu et al. 2015), tourism (Ghermandi et al. 2015) and industry, resulting in millions of dollars losses annually. The genus *Cassiopea* (also called: the upside-down jellyfish, mangrove jellyfish or sometimes the zooxanthellate jellyfish), is a widely distributed epibenthic scyphozoan jellyfish in tropical and sub-tropical shallow coastal marine habitats (Gohar & Eisawy 1960; Holland et al. 2004; Niggl & Wild 2009; Welsh et al. 2009; Stoner et al. 2011, 2014). Unlike most other scyphozoans, *Cassiopea* is well known to incorporate dinoflagellate endosymbionts (i.e., zooxanthellae; therefore, it is photosynthetically active) into their tissues during the non-embryonic stages of their life cycle (Hofmann & Kremer 1981). Moreover, the zooxanthellate jellyfish is a key organism in many reef habitats playing an essential role in recycling organic matter (Jantzen et al. 2010; Niggl et al. 2010); however, little attention has been paid to its role in jellyfish blooms.

Global warming is thought to be one of the main drivers of jellyfish blooms worldwide (Arai 2001; Mills 2001; Purcell 2005). The seasonal rise in seawater temperature is a critical pre-requisite in the jellyfish life cycle. It is the main physical factor in triggering polyp strobilation (Rahat & Adar 1980; Hofmann et al. 1996). Strobilation, the bottleneck of the asexual reproductive strategy in most jellyfish, produces free swimming medusae from the strobilating sessile polyps. By triggering strobilation, rising seawater temperature was proposed as the main driver of increasing jellyfish populations in the Yellow Sea, China (Wei et al. 2015). Rise in seawater temperature, however, is also one of the main causes of coral reef habitats deterioration, mainly by inducing coral bleaching. Temperature rise-induced reactive oxygen species (ROS) formation is the main mechanism causing coral bleaching (Lesser et al. 1990). Superoxide (O_2^-), one of the first and most reactive ROS produced biologically (Richier et al. 2003), is normally produced in all aerobically respiring cells. Aerobic cellular respiration, the main source of energy for cellular maintenance and growth, involves the biochemical transfer of electrons through the **e**lectron-**t**ransport **s**ystem (ETS) to

oxygen (Relexans, 1996). This system of electrons transfer, the ETS, is the main subcellular site of O_2^- production (Boveris et al. 1972; Boveris & Chance 1973; Fridovich 1975; Nohl 2004), transforming around 0.3-3% of the consumed oxygen into O_2^- (Speakman & Selman 2011). All aerobic respiring organisms have superoxide dismutase (SOD); it converts O_2^- into H_2O_2 and O_2, the initial step in O_2^- detoxification (McCord & Fridovich 1969; Fridovich 1975; Lesser 2006). Under normal metabolic rates, O_2^- levels are kept under control by SOD detoxifying activity, however, under increased levels of energy production (e.g., increased ETS) O_2^- levels could rise to higher levels beyond the SOD detoxifying potential. Excess O_2^- is an extremely reactive molecule; it attacks lipids, proteins, and DNA resulting in severe irreversible cellular damages. Therefore, SOD levels are expected to be directly correlated to O_2^- levels and cellular metabolic rate.

In addition to the aforementioned importance of *Cassiopea* in their native environments, the zooxanthellate jellyfish is considered invasive (Holland et al. 2004) and exotic (Özbek & Öztürk 2015) in many coastal marine habitats, including the Hawaiian Islands and the Mediterranean Sea (Holland et al. 2004; Özbek & Öztürk 2015). The reason behind this successful habitat extension, however, is still unclear. Stoner et al. (2011, 2016), have recently shown that *Cassiopea* medusae were more abundant and attained larger sizes in human-impacted marine coastal habitats in The Bahamas, without any further mechanistic explanations in physiological terms. The lack of research on thermal responses of jellyfish limits our ability to predict how warming might affect (or potentially drive) the bloom dynamics. Interestingly, to the best of our knowledge, no studies are available on physiological responses of the medusoid *Cassiopea* in response to sudden changes in temperature.

This is the first study on *Cassiopea sp.* that investigates the subcellular physiological responses (e.g., in term of ETS, SOD and lipid peroxidation) to sudden changes in temperature. Since organisms have certain confined amounts of energy available at any given time, increasing energy consumption in maintaining cellular homeostasis will be at the expense of other cellular activities such as growth and reproduction (Gambill & Peck 2014). Therefore, we asked the question: How do temperature changes affect *Cassiopea's* energy consumption and oxidative stress responses? In order to answer this question, we have measured the aerobic mitochondrial respiration (ETS) and the oxidative stress biomarkers (SOD and lipid peroxidation). This study brings new evidence for a better understanding of

the instantaneous physiological performance of *Cassiopea* in response to local extreme weather events, as well as to longer term (i.e., chronic long lasting changes) trends in climate change.

2. Materials and methods

2.1- Experimental organisms and experimental design

Forty eight *Cassiopea sp.* medusae with a bell diameter of 3.5-5.2 cm and body mass of 3.4-7.1 grams were used in our experiment. Medusae were raised for 3-4 months from ephyrae collected from a strobilating polyp culture kept for years at the MAREE facilities at the Leibniz-Zentrum für Marine Tropenforschung (ZMT), Bremen. The experimental organisms had been selected to be apparently morphologically healthy (i.e., no signs of pitched bells or lost arms, etc.) and to be of similar size. Prior to the experiment, medusae had been acclimated for two weeks in a single aquarium tank (ca. 120 litres) with a recirculation system (salinity 40.2 ± 0.2, temperature 25.9 ± 0.1 °C; the typical salinity and temperature for *Cassiopea sp*. in the Gulf of Aqaba, Red Sea), 12:12 hours light/dark cycle, and fed twice a week with freshly hatched *Artemia* nauplii.

Two sets of experimental setups were used to test *Cassiopea's* sp. responses of the selected parameters to thermal (cold/heat, 20 °C and 32 °C, respectively) treatments. Throughout all experiments, a control (salinity 40.2 ± 0.2, temperature 25.9 ± 0.1 °C) treatment was always run in parallel. Whereas the cold treatment (a drop by 6 °C from the control) might mimic the effect of cold front in shallow coastal waters, it might give insight on species translocation by aquaculture activities or ballast water. On the other hand, the heat treatments (an increase by 6 °C from the control) are oriented more towards what might happen in a tidal pool or at longer-term, global warming.

First, in the acute (= temperature shock in this text) treatment, eight medusae were transferred immediately into pre-cooled or -heated individual 1.0-L glass jars kept in 80-liter water bath set at the same treatment temperature for 2 hours. Medusae (N = 8, for each treatment and a control, respectively) were sampled after two hours of treatment. Second, in the chronic treatment, another 8 medusae were transferred as in acute treatment, but were kept at the same temperature for two weeks.

Henceforth, we use the following terminology: 1- chronic treatment means that the organism have been treated like in acute treatment, but kept for two weeks instead; 2- cold, heat and control always corresponds to 20, 32 and 26 °C, respectively.

2.2 Bell diameter and body mass measurements

The jellyfishes' bells diameters were measured using scaled glass beakers; diameter was recorded at full relaxation of the bell. Then jellyfish were removed by hand, excess water was removed by shaking and sucking by absorbent tissue without directly letting the tissue touch the jellies tissues, and then the jellies were immediately weighed in a seawater filled beaker (same water as the incubation aquarium) and mass was measured to the nearest 0.1 gram. The whole diameter and body mass measurement were done at the beginning of the chronic experiment and at the end of the experiment

2.3 Tissue sampling and homogenization

Individual oral arms were cut from the distal tip to the base. Arms base means the point where they arise from the ring-shaped tissue where all oral arms are normally fused. Cutting the arms this way ensure easier and reproducible cutting procedure which avoid interference with other tissues associated with oral complex base. Oral arm samples were immediately put in pre-weighed plastic microtubes (e.g., 3-5 oral arms per tube) and snap frozen at -80 °C. Tissue homogenization in preparation for the biochemical analysis was done as follows: the frozen oral arm masses were measured (i.e., by mass difference while the tissues were within the tubes), semi-thawed on ice (i.e. the tissues were still frozen, but easier to get it out of the microtubes by shaking). The semi-thawed tissue were transferred to clean empty ice-cooled small glass tubes (ca. 3-5 mL volume capacity) and then dispersed using IKA®-ULTRA-TURRAX dispersers with ice-precooled probes for less than 30 seconds, the resulting thick dispersed tissue will be called " crude homogenate" hereafter. The resulting crude homogenates were aliquoted by pipetting into three pre-weighed, pre-cold plastic microtubes and immediately snap frozen at -80 °C. In the next days after crude homogenate preparation for each experiment, the ETS and SOD, Chla, protein and MDA content were measured as described in detail below. For both ETS and SOD activity assays the following common homogenization practice was used, keeping in mind the different homogenization buffers used for each enzyme. First step, in the same frozen tubes containing the crude homogenates assigned for ETS $^{\text{or SOD}}$ we added ice-cold ETS $^{\textbf{or SOD}}$ homogenization buffer (chemicals constituents is described in appendix A) equal to 3 times the mass of the crude homogenate

(e.g., 0.2 g tissue in 0.6 mL buffer); then ca. 500 mg glass beads mixture (0.4 and 1.0 mm diameter) were added to each tube. Then the tissues were further homogenized using FastPrep®-24 tissue homogenizer for two cycles (speed: 4.0 M/S, TN: 12 ×15, 15 second) followed by 3 minutes cooling in ice after each cycle to avoid excessive heating. Second step: immediately after the cooling time followed the second FastPrep homogenization, the homogenates were centrifuged at 5,000 g (or 12,000 rpm in the case of SOD) for 5 minutes at 4 °C, and then the supernatants were pipetted into new microtubes and assayed for the specified enzymes. Therefore, when we use the word (supernatant) we refer to the supernatant just described above.

3.3 Enzymatic assays and protein content measurements

3.3.1 ETS activity was assayed using the common INT (Iodonitrotetrazolium) reduction assay (Packard 1971; Owens & King 1975). In this assay, the rate of INT reduction in the presence of the nonionic detergent Triton X-100 is used as a measure of the electron transport activity and as an index of oxygen consumption rate. Briefly, 503 µL of 0.1 M $KP_{i,}$ pH 8.5, 250 µL of 8 mM INT, and 167 µL of 7.2 mM NADH were added sequentially to a disposable plastic cuvette, stirred gently and incubated at 22°C for 3 minutes in a dry block thermostat (see appendix A for the chemicals descriptions). And then 80 µL of the sample supernatant assigned for ETS measurement (see sec 2.3) were added to start the reaction, and the increase in absorbance over time, due to INT (yellowish) reduction to INT-formazan (reddish colour), was followed at 490 nm for 5 min and time intervals of 10 sec. The slope of the change in absorbance over the middle 3 minutes of the recording recoding period was used in calculating ETS activity after correction for the blank (i.e., treated exactly the same but with 80 µL autoclaved distilled H_2O instead of the sample's supernatant). The results were calculated based on the corrected slopes and presented in mg O_2. hr^{-1}. g^1 WM (see Eqn. 1 & 2).

$$\text{ETS } [\text{U.g}^{-1}\text{WM}] = \frac{\Delta\text{A/min}}{\varepsilon \text{.d}} \times \frac{\text{V.cuvette (µL)}}{\text{V.assayed (µL)}} \times \frac{\text{V.homo buffer(µL)}}{\text{Sample mass(g)}}, \qquad \text{(Eqn. 1)}$$

where**,**

ΔA/min: is the change in absorbance over the measurment time (i.e., the slope). ϵ : is the molar absorptivity or the extinction coefficient of INT-Formazan (i.e., 15.9 $mM^{-1}cm^{-1}$). *d*: is the path length of the light through the cuvette (i.e., usually 1.0 cm unless mentioned). *V.homo buffer*: is the volume of the buffered used in homogenization. *V.sample*: 80 µL in paper.

ETS activity may be expressed in term of oxygen consumption rate as follow:

$$\text{ETS } [\mu\text{mol } O_2.\text{min}^{-1}.\text{g}^{-1}\text{WM}] = \frac{1}{2} \text{ ETS } [\text{U.g}^{-1}\text{WM}]; \text{ Cammen et al. (1990).} \quad \text{(Eqn. 2)}$$

2.3.2-SOD activity was assayed by the competitive inhibition assay based on McCord and Fridovich (1969); and further modifications by Beyer and Fridovich (1987) and Vandewalle and Petersen (1987). In this assay, cytochrome C (cyt-c) reduction by O_2^- generated by the xanthine-xanthine oxidase coupled system is competitively inhibited by the cellular SOD activity. At the defined conditions in this assay, one unit of SOD causes a 50% inhibition in the rate of cyt-c reduction in this system. Briefly, 830 µL of SOD-AB solution, 100 µL of 0.1 mM Cyt-c, 10 µL of 5 mM xanthine, and 10 µL (ca. 1.8 mU) of xanthine oxidase (XO) were added sequentially to a disposable plastic cuvette, stirred gently and incubated at 22 °C in for 3 minutes in a dry block thermostat (see appendix A for the chemicals descriptions). Noteworthy to mention is that the volumes and concentrations of Cyt-c and XO were chosen so that the reduction of Cyt-c causes an increase in absorbance at 550 nm (ΔA_{550}) by 0.025 ± 0.005 per minute. And then 50 µL of the sample supernatant assigned for SOD activity measurement (see sec 2.3) were added to start the competitive inhibition reaction which was followed at 550 nm for 3 minutes and every 10 sec using PerkinElmer-Lambda 35 photometer. The SOD activity was presented in $\text{U.g}^{-1}\text{WM}$.

2.3.3-MDA content was determined using the common method known as **t**hio**b**arbituric**a**cid (TBA)-**r**eactive **s**ubstances (TBARS) based principally on the protocol of Uchiyama and Mihra (1978) with slight modifications. Briefly, the following reagents were added sequentially: 1% H_3PO_4, 0.6% TBA (i.e., freshly prepared in water), and phosphate buffer saline (PBS) pH=7.3 to the crude homogenate in 3:1:1:0.6 ratio for H_3PO_4: TBA: PBS: tissue mass in mg, respectively. Immediately, the reaction mixture was vortexed and incubated at 90°C for 45 minutes. The reaction was stopped by incubation in ice after the 45 minutes incubation, centrifuged twice at 10,000 g for 5 minutes to get clearer supernatant. The supernatant absorbance spectrum (400-700 nm) was measured in triplicates using TECAN-infinite M200 PRO photometer. We have calculated the MDA content using the third derivative approach and MDA standards prepared using the same reagents used in the assay. Results were presented in $\text{nmol.g}^{-1}\text{WM}$.

2.3.4-Chl***a*** content was determined as follows: 96% ethanol was added to the crude homogenate (in the following homogenate (g): ethanol (mL) ratio1:15; for example 0.1 g tissue/homogenate will receive 1.5 mL ethanol), vortexed and immediately incubated in dark

at 4 °C for 24 hours. Supernatants were cleared by centrifugation at 5000g for 10 minutes and immediately the absorbance at 750 and 665 nm were read using a PerkinElmer-Lambda 35 photometer. Chl***a*** contents were calculated using the HELCOM COMBIN formula.

2.4- Statistical analysis: Each group has been compared to its control group for differences in the same treatment using "Welsh two sample t-test", two tailed and 0.05 were set as the significance level. Results of comparison of the groups means were considered significant if the p-value of the test is ≤ 0.05, and the word 'significant' has this meaning, wherever it appears alone in our text. In all treatmens N= 8, unless mentioned elsewhere.

Results

Loss/Gain of body mass and size and ETS activity

At the end of the experiment period (2 weeks), the chronic cold-treated (20 °C) medusae were significantly smaller compared to the first day of the treatment. Both the mean body mass and bell diameter were significantly decreased (p-value < 0.0001 for both parameters, Fig. 1). Chronic heat-treated (32 °C) medusae, in contrast, gained in body mass (p value < 0.05) with no significant changes in bell diameter (p-value > 0.35) even though they looked bigger in the aquarium (Fig. 1). Mitochondrial respiration (ETS), on the other hand had shown a totally contrasting results. In the acute-treated (2 hr) medusae, the cold resulted in highly significant (Welsh two samples t-test, p-value < 0.001) increase in ETS activity by 34% compared to the control, while the heat did not induce any changes. On the other hand, in the chronic (2 wk) treatment, while the cold treated-medusae sustained an elevated ETS (p-value = 0.01) activities, the heat-treated medusae showed significant decrease in ETS (p-value < 0.05), with 22% decrease compared to the control (Fig. 2).

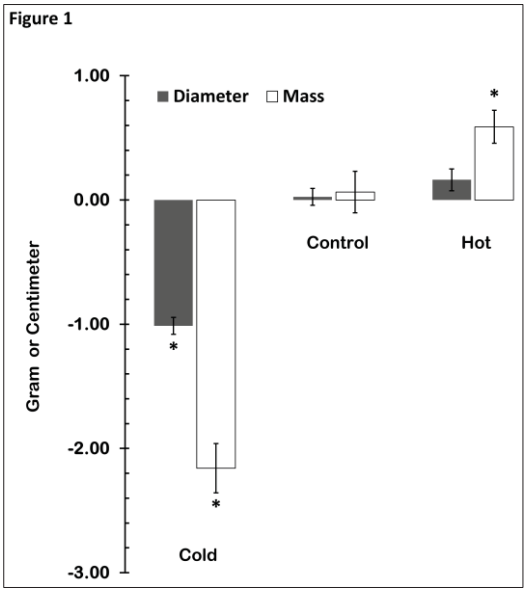

Fig. 1. Changes in bell diameter and mass of *Cassiopea* sp. medusae in response to chronic cold/heat treatment. Bars represent the mean change in bell diameter or medusae mass ± SE, n = 8 in both control and cold treatments; n=7 in hot treatment. Welch two sample t-test, and p-value < 0.05 is considered significant and is indicated by an asterisk above bars.

Figure 2

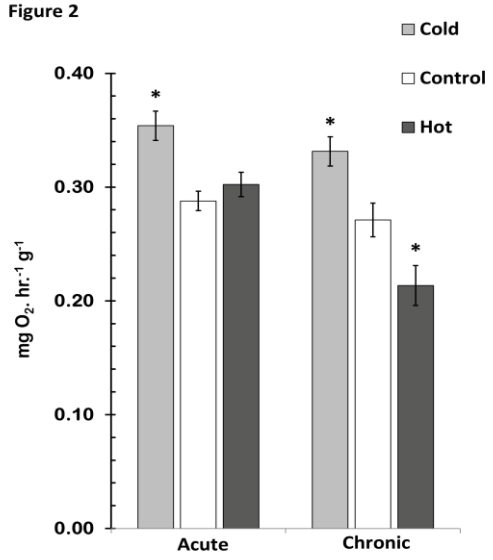

Fig. 2. Acute (left) and chronic (right) ETS responses to cold/heat treatments of *Cassiopea* sp. medusae. Bars represent the mean ETS activity per gram wet mass ± SE, n = 8. Two tailed Welch Two Sample t-test, and p-value < 0.05 is considered significant and is indicated by an asterisk above bars.

Figure 3

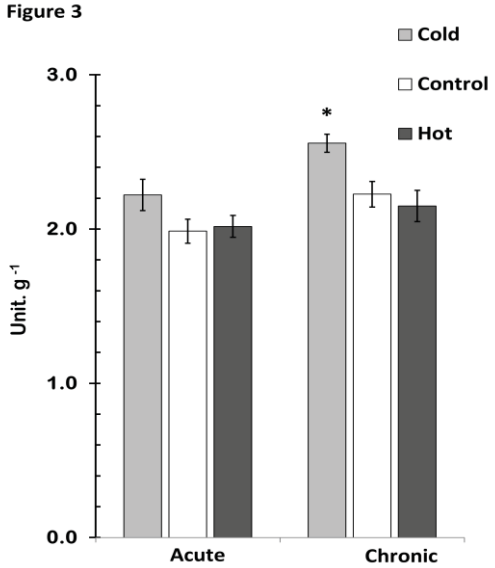

Fig. 3. Acute (left) and chronic (right) SOD responses to cold/heat treatments of *Cassiopea* sp. medusae. Bars represent the mean SOD activity per gram wet mass ± SE, n = 8. Two tailed Welch Two Sample t-test, and p-value < 0.05 is considered significant and is indicated by an asterisk above bars.

3.2- Superoxide dismutase activity and lipid peroxidation (MDA content)

SOD activities did not change significantly in all acute treatments. In the acute cold treated-medusae, however, it is worth to mention that the SOD was increased by 13% compared to the control (p-value = 0.06; Fig. 3). On the other hand, in the chronic (2 wk) treatment, while the cold treated-medusae showed a highly significant increase (by 18% compared to control, p-value < 0.01) in SOD activity and MDA content (increased by 60% compared to control, p-value < 0.001), in the chronic heat-treated medusae both oxidative stress biomarkers were not changed (Fig. 3 & 4; next page).

3.3- Chl*a* concentration response and its correlation to SOD activity

In both chronic cold/heat treated-medusae, Chl*a* concentrations did not show any significant changes from the control (Fig. 5; next page). The results of the Pearson's product-moment correlation test, have shown that the SOD activities displayed a direct positive correlation to Chl*a* concentrations in both control and heat treated groups (p-value < 0.05), but not in the chronically cold-treated medusae.

Figure 4

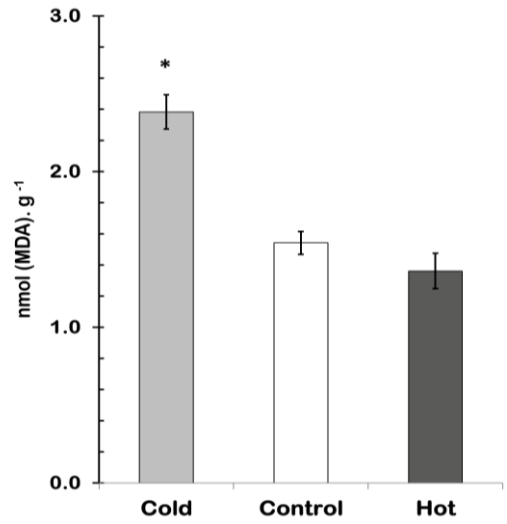

Fig. 4. Chronic lipid peroxidation (in terms of [MDA]) responses to cold/heat treatments of *Cassiopea* sp. medusae. Bars represent the mean [MDA] per gram wet mass ± SE, n = 8. Two tailed Welch Two Sample t-test, and p-value < 0.05 is considered significant and is indicated by an asterisk above bars.

Figure 5

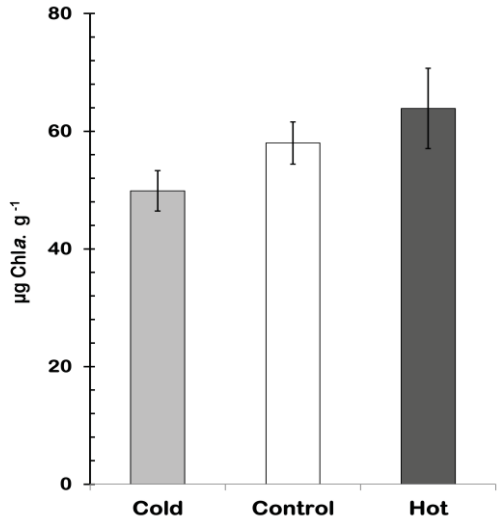

Fig. 5. Chronic Chla responses to cold/heat treatments of *Cassiopea* sp. medusae. Bars represent the mean [Chla] per gram wet mass ± SE, n = 8. Two tailed Welch Two Sample t-test, and p-value < 0.05 is considered significant and is indicated by an asterisk above bars.

4. Discussion

In this study, while jellyfish medusae have shown minor responses to short term temperature changes, the long-term treatments have induced significantly contrasting responses at the physiological and subcellular levels. Whereas they suffered oxidative stress and reduced physiological performance in response to long-term cold treatment, the long-term heat treated medusae have not shown signs of oxidative stress damage or reduced physiological performance. In our discussion, the major focus will be on the response to the long-term treatments (i.e., chronic treatments) where we draw the major conclusions.

In recent decades and due to the climatic changes, rapid fluctuations in seawater temperature became more extreme and frequent. Such temperature fluctuations could have deleterious effects on marine ectotherms. For example, in response to cold shock (10 °C below the control, 1 h), the zebrafish (*Danio rerio*) has increased SOD activity in their brain tissue by 60% (Tseng et al. 2011). Since SOD is one of the main antioxidant enzymes, the observed results indicate elevated levels of intracellular reactive oxygen species (ROS) formation in the fish. In this study, in cold shocked (20 °C, 2h) *Cassiopea* medusae, where they were very calm and shown reduced mobility in term of reduced bell pulsation, the 13% increase in SOD activities could partially explain the elevated cellular respiration (i.e., in term of ETS activity,

Fig. 2 & 3). In other words, this could mean that the slight increase in SOD does account for more energy allocated for ROS detoxification. While the normal trend in marine organisms is that organisms increase SOD activity upon thermal shocks; Wang et al. (2008) have found that SOD activities were decreased significantly in the sea cucumber, *Apostichopus japonicus* shocked at 32 °C for 1 hour. In heat shocked (32 °C, 2h) medusae, the unchanged activities of both SOD and ETS enzymes might indicate that this temperature, 32 °C, is not stressful at the cellular levels even though the medusae have shown a moderately increased bell pulsation activity. One might conclude that the jellyfish with an overall low metabolic rate do not show immediate reaction to short-term treatments.

In contrast to the minor responses of *Cassiopea* medusae to temperature shocks (i.e., acute treatments), the chronic treated medusae showed significantly contrasting responses in term of both enzymatic and morphological aspects.

In aerobic cellular respiration and photosynthesis, O_2^- is a common intermediate of molecular oxygen reduction (Fridovich 1975). Furthermore they are the main source of O_2^- formation in all normally functioning cells. In this discussion, recall that SOD activities, which is the first line of defensive line against O_2^- toxic reducing power, are usually considered in direct correlation to O_2^- exposure levels (e.g., associated with cellular respiration or photosynthesis levels; McCord & Fridovich, 1969). In chronic cold treated medusae (20 °C, 2 wk), while the increased superoxide dismutase activity could obviously indicate an elevated ROS formation, the very significant increase in lipid peroxidation clearly indicates that the antioxidatitive response failed to keep ROS under control (Fig. 3 & 4). Lipid peroxidation (LPO) is commonly used as biomarker of cellular damage associated with experiencing oxidative stress within tissues (Ayala et al. 2014). For example, it was used in the polychaete worm *Diopatra neapolitana*, through the measurement of tissues' MDA contents, as a good indicator of metal-induced oxidative stress cellular damage (Freitas et al. 2012). Given that photosynthesis is one source of O_2^-, and therefore is expected to be positively correlated with SOD levels, we have measured Chla (i.e., an estimated proxy for photosynthesis and oxygen production) in *Cassiopea*. Our results confirmed the association between Chla and SOD activities. Both parameters were positively correlated in the control medusae. This correlation has already been established before in some symbiotic cnidarians including *Cassiopea* (Dykens 1984; Shick & Dykens 1985), where they have found that SOD levels were directly correlated to the Chl*a* content. We found that SOD activities are positively correlated to the Chl*a* content in medusae from the control treatment (Pearson correlation test, p-value <

0.01), but not in cold treated medusae. The lack of SOD-Chl*a* correlation in chronic cold-treated medusae could mean that cellular respiration, rather than photosynthesis, is the main source of ROS and the subsequently increased SOD and LPO in this treatment. The unchanged Chl*a* content in all treatments confirms this conclusion as well (Fig. 5). Morphologically, the very significant loss (>40%; Fig. 1) in body size and mass of the chronic cold-treated medusae, in addition to other signs of being unhealthy (e.g., open bells, overall shrinkage), indicates deteriorated physiological performance. On the effects of cold exposure on medusae in the field, Fitt and Costley (1998) have observed that *Cassiopea* populations were decreased after the passage of a severe cold front through Florida. Furthermore, many medusae had distended tentacles and asymmetric bells. They have also found that *Cassiopea* polyps' tentacles were decreased and showed disturbed feeding ability upon short-term cold treatment (≤ 18 °C). Although we were not investigating the feeding behaviour in our experiment, we have observed that in the chronic cold treatment most of the fed *Artemia* were found in clumps of mucus filled with undigested, immobilized *Artemia* beside the medusae on the next day after feeding. These clumps clearly indicate a disturbed feeding ability since these observations were not observed in the control or heat treatments. In conclusion, the decreased body mass and increased cellular respiration in this treatment might be explained by two reasons, the reduced feeding ability and the cost of maintenance (i.e., more energy to maintain cellular homeostasis).

Global warming and rising seawater temperature is generally accepted as the main cause for coral reef deterioration, namely coral bleaching. Oxidative stress mediated cytotoxicity is the hypothesized mechanism of endosymbiont loss (Lesser 1997; Downs et al. 2002). According to Lesser (1997), increasing seawater temperature by only a few degrees is enough to cause a severe oxidative stress in corals. In *A. japonicas*, oxidative stress was associated with death when the organisms were kept at 32 °C for 16 hours; this was indicated by the rapid change in catalase and SOD activities in the coelomic fluid (Wang et al. 2008). In jellyfish, however, the story of warming could be quite different. For example, the increased *Aurelia aurita* population in the Seto Inland Sea of Japan was thought to be driven mainly by overfishing and increase in seawater temperature (i.e., associated with global warming; Uye & Ueta 2004). In the present study, the unchanged SOD and LPO activity in the heat-treated (32 °C, 2 wk) *Cassiopea* medusae indicates their ability to avoid oxidative stress toxicity; it could also mean that they did not experience it at all. Furthermore, showing no signs of increased cellular respiration, gaining body mass and the unchanged Chla suggest that *Cassiopea* were

not stressed from the incubation temperature, if not to say they are performing better at 32 °C.

Jellyfishes are well known to tolerate different environmental unfavorable conditions. For example, the moon jellyfish *Aurelia aurita* increased its feeding rate, while the Spanish mackerel fish *Scomberomorus niphonius* decreased its feeding rate under experimentally induced hypoxic conditions (Shoji et al. 2005). Therefore, in marine hypoxic zones, which expected to increase in magnitude and spatial distribution due to global warming and anthropogenic activities in coastal zones, the voracious jellyfish might displace other hypoxia less tolerant fish species, which might affect and change the food web structure in the affected areas (Breitburg at al. 1994; Shoji et al. 2005). In the present study, our findings add new molecular evidences on the robustness of jellyfishes to increasing seawater temperatures. Given that both hypoxia and rising seawater temperature are increasing in magnitude and spatial distribution in many coastal zones worldwide, our findings suggest that the jellyfish *Cassiopea* might be more invasive and show more potential to increase their population, and maybe form blooms in the future.

5. Conclusions

Based on our findings, we conclude that the medusae of the jellyfish *Cassiopea* are more sensitive to cold (20 °C) than warm temperature (32 °C). They seem to benefit from the expected increase in seawater temperature associated with global warming, by increasing their population size and invading new warmer coastal marine habitats. Oxidative stress, however, seems to be the main mechanism limiting *Cassiopea's* medusae ability to invade colder marine habitats. Since this is the first study on *Cassiopea sp.* to investigate its physiological responses to climate change induced temperature changes, we encourage more research on this organism with longer experimental periods and different stress factors.

7. Acknowledgements

This work was supported by Leibniz funds from the Leibniz Gemeinschaft Germany [grant number ZMT-PB-3100/6057]. We would like to specially thank Stefanie Meyer, from the Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar- und Meeresforschung (AWI), and Constanze von Waldthausen, from ZMT, for their initial help in the lab. We acknowledge the MAREE staff at ZMT for their help in aquaria setup. Many thanks go directly to Dr. Achim Meyer for his fruitful discussions on the manuscript.

7. Appendix A: Brief descriptions on the preparation of some chemicals used in this paper

A.1- ETS chemicals

1- ETS assay buffer (ETS-AB): mix 0.1 M K_2HPO_4 with 0.1 M KH_2PO_4 to obtain potassium phosphate buffer (KPi) solution with pH 8.5 then keep it in the refrigerator, could be used as stock to prepare other reagents. 2- ETS homogenization buffer (ETS-HB); Dissolve the following chemicals: 0.45 g polyvinyl pyrolidone (PVP), 1.6 mL Triton X-10, and 2.7 mg $MgSO_4$ in 1.0 L final volume of 0.1 M $KP_{i,}$ pH 8.5 (prepared according to Gopalan et al. 1996).

ETS substrates solutions: 1- Prepare 7.2 mM NADH in ETS-A containing 0.2% Triton X-100 (e.g., dissolve 25.5 mg NADH and 10.0 µL Triton-X100 in 5.0 mL KPi). Prepared fresh, stored on ice and exposure to direct intense light was avoided. 2- Prepare 8 mM INT (Iodonitrotetrazolium chloride) in 0.1 M KPi, pH 8.5 (e.g., dissolve 0.041g INT in 10 mL KPi). Prepared fresh, stored on ice and the exposure to direct intense light was avoided.

A.2- SOD chemicals

1- SOD homogenization buffer (SOD-HB): mix 0.1 M K_2HPO_4 with 0.1 M KH_2PO_4 to obtain KPi solution with pH 7.4. 2- SOD assay buffer (SOD-AB): prepare 0.05 M KPi solution with 0.1 mM EDTA and adjust pH to 7.68 then keep it in the refrigerator.

SOD substrates solutions: 1- Prepare 0.1 mM Cyt-c in Milli-Q H_2O. 2- Prepare 5 mM xanthine solution by dissolving 3.04 mg xanthine in 4 mL of 0.1 M NaOH. 3- Prepare 1.8 mU of xanthine oxidase (XO) in SOD-AB.

Some reagents molecular masses (g. mol^{-1}) and source of purchase:

NADH (709.41) from Merck, INT (505.7) from Sigma-Aldrich, xanthine (152.1) from Sigma-Aldrich. Cyt-c, XO, NaOH, EDTA, K_2HPO_4, KH_2PO_4, $MgSO_4$, PVP and all other chemicals mentioned in this paper were the product of Sigma-Aldrich.

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Chapter 4

Anaerobic Metabolism and Oxidative Stress Responses

Metabolic performance of *Cassiopea* in response to anthropogenic stressors

This chapter is under revision as:

Samir M. Aljbour, Fuad A. Al-Horani , Andreas Kunzmann. (XXXX). Metabolic Responses of the Upside-Down Jellyfish *Cassiopea sp.* to Pollution in the Gulf of Aqaba, Jordan. (submitted to the journal Marine Pollution Pollutin).

Abstract:

The physiological responses of jellies to pollution are virtually overlooked. We measured two glycolytic enzymes (PK, LDH) activities, lipid peroxidation (LPO), protein and chlorophyll-a content in *Cassiopea* sp., from polluted and control locations along the Gulf of Aqaba, Jordan. In medusae from polluted locations, both PK and LDH activities were significantly higher compared to controls, suggesting increased reliance on anaerobic metabolism. PK and LDH were positively correlated in *Cassiopea* from all locations. While medusae from polluted locations have shown no signs of oxidative stress damage, protein content was significantly lower, which might suggest the utilization of protein to sustain the increased maintenance energy demand, as also indicated by the increased anaerobic metabolism. Overall these results suggest that *Cassiopea* seems to be robust to the level of pollution at the studied sites and they might be anaerobically poised to live and thrive at such habitats.

Kew words: anaerobic metabolism, lipid peroxidation, oxidative stress, sedimentation rate, pyruvate kinase, lactate dehydrogenase.

Introduction:

Scyphozoan jellyfishes, in contrast to the more sensitive corals, are generally robust, noxious, short-lived animals, and tolerant to a wide range of environmental conditions and pollutants. At present, many pelagic jellyfishes are increasing massively worldwide forming noxious blooms (Arai 2001; Graham 2001; Mills 2001; Purcell 2005). Jellyfishes grow rapidly and die *en masse*, releasing massive amounts of nutrients into the water column following decomposition of their dead bodies (Pitt et al. 2009). They can change fish community structure and reduce fish stocks, since they compete voraciously with fishes for the same planktonic prey; furthermore they are able to predate on fish larvae themselves (Breitburg et al. 1997; Mills 2001). Economically, the negative impacts of jellyfish blooms on fisheries and tourism are of higher concern for society. For example, while in the past decade *Nemopilema nomurai* blooms caused millions of dollars loss in fisheries around China and Japan (Robinson et al. 2014), other jellyfish invasions in the Mediterranean Sea caused 1.8-6.2 million € annually due to beach closures (Ghermandi et al. 2015). The scyphozoan jellyfish *Cassiopea*, sometimes called "upside-down jellyfish or mangrove jellyfish", leads an epibenthic life style and unlike most other scyphozoan, it incorporates zooxanthellae into their tissues during all non-embryonic stages of their life cycle (Hofmann & Kremer 1981). *Cassiopea* spp., are widely distributed in tropical and subtropical shallow coastal marine habitats (Gohar & Eisawy 1960; Holland et al. 2004; Niggl & Wild 2009; Welsh et al. 2009; Stoner et al. 2011, 2014). In some reef habitats, *Cassiopea* is a key organism with a leading essential role in nutrient recycling (Jantzen et al. 2010; Niggl et al. 2010); however, little attention has been paid to its role in jellyfish blooms.

Global warming and anthropogenic activities (e.g. eutrophication and coastal constructions) are proposed as the main drivers of jellyfish blooms (Arai 2001; Mills 2001; Purcell 2005). Because of their proximity to main cities, coastal systems are usually subjected to a wide array of pollutant inputs from effluents associated with industrial, agricultural and domestic activities. Heavy metals are of major concern among these pollutants. In *Cassiopea* spp., it has been shown recently that while some metals (e.g. Cu, Mn, Cd, Zn) have been accumulated up to 200 fold of the ambient seawater concentrations, other metals (e.g. lithium) were actively regulated within the tissues, others (e.g., Ca, Mg and Sr) reflected the ambient environment (Templeman & Kingsford 2010, 2012). In mollusks, crustaceans and other marine invertebrates, the ability to regulate the intracellular metal concentrations and

accumulation of excess metals in nontoxic forms attributes to their tolerance to high tissue concentrations of metals (Rainbow 2002). Particularly important in this context is the fact that "toxicity is related to a threshold concentration of metabolically available metal and not to total accumulated metal concentration" (Rainbow 2002).

Oxidative stress, a result of reactive oxygen species (ROS) production, which leads to cellular damage, could be induced by metal exposure of the organisms (Zhang et al. 2010). Lipid peroxidation (LPO) could be induced by ROS formed due to exposure to transition metals (e.g. as Cd, Cu and Pb), and is one of the main signs of experiencing oxidative stress mediated cellular damage (Knight & Voorhees 1990). In another mechanism of toxicity, cadmium for example is known to inhibit the mitochondrial electron transport system (ETS) enzymes, which results in more ROS and less ATP synthesis (Livingstone 2001). Furthermore, even low concentrations of Cd might result in uncoupled mitochondrial respiration and less efficient energy production (Jacobs et al. 1956). In decapod crustaceans, heavy metal exposure was associated with decreased oxygen consumption (MO_2) rates too (Barbieri 2009; Barbieri et al. 2013). On the other hand, oyster exposure to heavy metal (i.e., Cd) suppressed the anaerobic metabolism (Ivanina et al. 2010). Therefore, stimulating oxidative stress, impairment of aerobic/anaerobic metabolism and mitochondrial functions, which result in cellular and organismal energy imbalance, are common mechanisms of metal toxicity.

Stress represents an extra sink of stored energy for maintenance of cellular homeostasis; moreover, this re-allocation of cellular energy reserves is at the expense of other cellular activities such as growth and reproduction (Gambill & Peck 2014). Under normal conditions, animals sustain their energy demand through aerobic metabolism; however, under stressful conditions when energy demands are exaggerated, the anaerobic energy metabolism becomes essential.

In animal physiology, the onset of anaerobiosis is a well-known sign of elevated energy demand beyond aerobic potential. At this stage, aerobic scope declines severely, transition to anaerobic metabolisms becomes essential for sustaining cellular energy demands (Pörtner 2002). Transition to anaerobiosis in oyster, was found to be a sensitive biomarker of energetic stress induced by temperature and Cd exposure (Bagwe et al. 2015). Pyruvate kinase (PK) and lactate dehydrogenase (LDH) are two universal glycolytic rate controlling enzymes in most animals. While PK, one of the main rate controlling enzymes in glycolysis, sits at the

crossroad directing the fate of glucose carbon to biosynthesis or being used for glycolytic energy production (Mazurek et al. 2002), LDH, the main enzyme sitting at the crossroad between aerobic and anaerobic metabolisms, is well known to be increased under conditions of increased cellular energy demand. Moreover, LDH correlates well with anaerobic capacities and therefore is commonly used as a proxy of anaerobiosis (Hochachka et al. 1983).

In the Gulf of Aqaba, Jordan, *Cassiopea* sp., is a key epibenthic organisms in reef habitats playing roles in food webs by fueling the coral reefs with their released organic matter (Niggl et al. 2010). Furthermore, Niggl and Wild (2009), have observed their increased occurrence over years. Field and experimental studies on *Cassiopea* sp., from the Great Barrier Reef have shown that the jellyfish is able to bioconcentrate metals in its tissues well above the ambient seawater concentrations (Templeman & Kingsford 2010, 2012; Epstein et al. 2016). Moreover, it is considered invasive (Holland et al. 2004) and exotic (Özbek & Öztürk 2015) in many coastal marine habitats, including the Hawaiian Islands and the Mediterranean Sea (Holland et al. 2004; Özbek & Öztürk 2015). The reason behind this successful habitat extension, however, is still unclear. Stoner et al. (2011, 2016), have recently shown that *Cassiopea* medusae were more abundant and attained larger sizes in human-impacted marine coastal habitats in The Bahamas, without any further mechanistic explanations in physiological terms. Aljbour et al. (2017) could show in a laboratory study that *Cassiopea* sp., seems to acclimate well at 32 °C, gain body mass and reduce the aerobic energy consumption. The authors have drawn their conclusion based on cellular respiration (ETS) and oxygen consumption (MO_2) measurements, they concluded that *Cassiopea* medusa are more tolerant to heat than cold temperatures. The lack of research on physiological responses of jellyfish to pollution limits our ability to explain the observed association of jellyfish with anthropogenic activities. Interestingly, and to the best of our knowledge, no studies are available on physiological responses of the medusoid *Cassiopea* to pollution and climatic change induced disturbances except for the aforementioned paper of Aljbour et al. (2017).

This is the first study on *Cassiopea* that investigates the subcellular physiological responses (e.g., in term of cellular glycolytic (PK, LDH) potential and oxidative stress damage in term of LPO) to pollution. Since organisms have certain confined amounts of energy available at any given time, increasing energy consumption in maintaining cellular homeostasis will be at the expense of other cellular activities such as growth and reproduction (Gambill & Peck

2014). Therefore, we asked the questions: How does pollution status of benthic habitat affect *Cassiopea's* glycolytic potential and anaerobic metabolism? Do *Cassiopea* medusae experience oxidative stress induced damage under the defined conditions of pollution in the selected site? In order to answer these questions, we have measured the activities of two main glycolytic enzymes (PK and LDH), malondialdehyde content (MDA, an oxidative stress biomarker used as a proxy to asses LPO), and both protein and chlorophyll-a (Chla) content in the medusae of the upside down jellyfish *Cassiopea* sp., collected from four environmentally different locations along the coastal line of the Gulf of Aqaba, Jordan. This study brings new evidence for a better understanding of the physiological performance of *Cassiopea* in response to the general pollutant status in coastal systems, which helps in explaining the general association between jellyfish and anthropogenic activities.

2. Materials and Methods

2.1 Study areas

The Gulf of Aqaba (GoA) is a narrow (2-25 km wide), deep (max. depth 1,820 m) and long (180 Km) most northern extension of the Red Sea. It is surrounded by dry desert mountains, no riverine inputs and only a negligible runoff. The GoA is the only access of Jordan to the sea. The whole Jordanian coastal system access to the GoA is 26 Km long and is heavily exploited for both industrial and touristic business. In this study we chose four locations along the Jordanian coastal line to sample the *Cassiopea* medusa. While the Phosphate Loading Berth (PLB) and the Industrial Area (IA) are locally well known polluted locations, the Marine Science Station (MSS) and the Coral Garden (CG) are considered to be marine reserved areas (Fig. 1; next page). Our choice of the polluted locations, PLB and IA, was based on literature review and *in situ* observation acquired from prior knowledge. While both PLB and IA are known to be polluted, especially with the heavy metal cadmium (Al-Najjar et al. 2011; Al-Rousan et al. 2016), they differ mainly in the sedimentation rate, which is very high in PLB compared to all other location along the Jordanian coastline (Badran & Al Zibdah 2005; Al-Rousan et al. 2016). Compared to the other locations, the PLB has the highest metal pollution loading index (PLI), followed by IA (Al-Najjar et al. 2011). PLB has the finest sediments grain size, dominated by clay resulting from the deposition of large amounts of phosphate powder during the shipment processes (Badran & Al Zibdah 2005; Al-Najjar et al. 2011), while the MSS, CG and IA have sandy texture resulting from the washing out of the fine sediment particles in this area (Al-Najjar et al. 2011). In conclusion and for the

purpose of this study we refer hereafter to PLB and IA as polluted locations and we mean that they are anthropogenically impacted, while MSS and CG are considered marine nature reserves, representative of protected areas, less affected by anthropogenic activities.

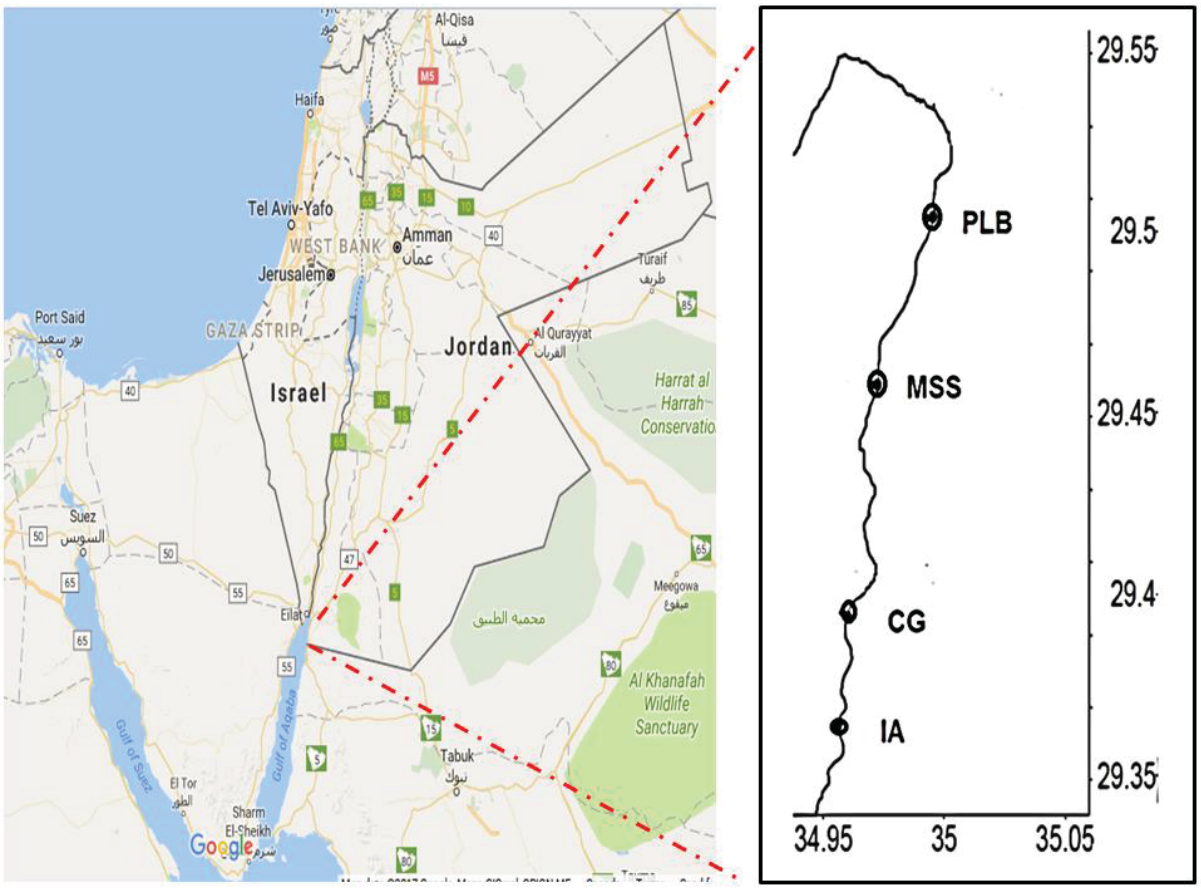

Fig. 1. Sampling locations along the Jordanian coastal line of the Gulf of Aqaba, Jordan. PLB= Phosphate Loading Berth, MSS= Marine Science Station, CG= Coral Garden, IA= Industrial Area. Adapted from Google maps.

2.2 Experimental organisms, tissue sampling and preparation procedures

Thirty two *Cassiopea* sp., medusae with average bell diameter of 5.8 ± 1.6 cm, were collected by scuba diving in May 2016 from the 4 selected locations mentioned above. Individual medusae were collected in plastic bags (ca. 3.5 litres water capacity), tied and then kept immersed in ca. 100 L tank filled with seawater on board until being dissected in the laboratory at the MSS within one hour from collection. Immediately upon arrival to the laboratory, medusae's bells diameters were measured using scaled glass beaker, diameter was recorded to the nearest 0.1 cm at full relaxation of the bell. Individual oral arms were then cut from the distal tip to the base. Arms base means the point where they arise from the ring-shaped tissue where all oral arms are normally fused. Cutting the arms this way ensure easier

and reproducible cutting procedure which avoid interference with other tissues associated with the oral complex base. Oral arm samples were immediately put in pre-weighed plastic microtubes (e.g., 2-4 oral arms per tube) and snap frozen at -80 °C. Tissue homogenization in preparation for the biochemical analysis was done as follow: the frozen oral arms were semi-thawed on ice (i.e., so the tissue are still frozen but easier to get it out of the microtubes by shaking), transferred to a clean empty ice-cooled small glass beaker (ca. 3-5 mL volume capacity) and then dispersed using IKA®-ULTRA-TURRAX dispersers with ice-precooled probes for less than 30 seconds. The resulting thick dispersed tissue will be called "crude homogenate" hereafter. The crude homogenates were aliquoted by pipetting (i.e., using cut-tip tips) into pre-weighed, pre-cold plastic microtubes and immediately snap frozen at -80 °C. Noteworthy to mention here is that we have made sure that the medusae had no pitched bells, cut tentacles or other signs of unhealthy conditions.

2.3 The measurements of PK and LDH activities's and protein content

The crude homogenates were further homogenized using FastPrep tissue homogenizer in 3 volumes (e.g., 0.2 g is homogenized in 0.6 mL) ice-cold homogenization buffer (75 mM Tris-HCl, 1 mM EDTA, and pH 7.5) for 15 seconds and immediately incubated in ice for ca. 3 minutes for cooling. Homogenates were immediately centrifuged at 5000 rpm for 10 minutes at 2 °C. From each sample, the supernatant was pipetted into three aliquots and immediately frozen at -80 °C, to be used for PK and LDH assays and protein content measurement. All measurements were done in less than one week of homogenization. Hereafter, when we say "PK, LDH or protein aliquot" we mean the frozen supernatant just mentioned above. Noteworthy to mention here is that the incubation temperature (i.e., 24 °C) in the following sections represents the average seawater temperature at a depth of 14-19 m in the sampling locations, given that the temperature between locations varied only by 0.2 °C.

2.3.1 PK activity was assayed spectrophotometrically following Hickey and Clements (2003), with minor modifications, by recording the rate of decrease in absorbance due to NADH (ϵ= 6.31 $mM^{-1}cm^{-1}$) oxidation to NAD^+ combined with pyruvate conversion to lactate. Briefly, in this assay, samples supernatants (110 µL) were added to the reaction mixture in 1.0 mL plastic cuvette (containing: 25 µL of 10 mM NADH, 20 µL of 25 mM PEP (phosphoenolpyruvate), 2 µL (ca. 5.5 U) of LDH enzyme solution and 823 µL of the PK assay buffer (containing: 60 mM Tris-HCl, 60 mM KCl and 6 mM $MgSO_4$ x $7H_2O$; pH 7.6). The mixture was stirred, incubated at 24 °C in a thermostat for 5 minutes in disposable plastic

cuvettes, then the reaction was started by adding 20 µL of 50 mM ADP (**a**denosine 5-**dip**hosphate), and the decrease in absorbance was followed at 340 nm for 5 minutes using a PerkinElmer-Lambda 35 photometer, Germany. Results were presented in $\mu U.mg^{-1}$ Protein**.**

2.3.2 LDH activity was assayed spectrophotometrically following Lushchak et al. (1998, 2001), with minor modifications, by recording the rate of decrease in absorbance due to NADH (ϵ= 6.31 $mM^{-1}cm^{-1}$) oxidation to NAD^+ combined with pyruvate conversion to lactate. Briefly, in this assay, samples supernatants (70 µL) were added to the reaction mixture in 1.0 mL plastic cuvette (containing: 500 µL LDH assay buffer (i.e., 0.1 M Tris-HCl, pH 6.8), 15 µL of 10 mM NADH, and 400 µL dH_2O). The mixture were stirred, incubated at 24 °C in a thermostat for 5 minutes in disposable plastic cuvettes, then the reaction was started by adding 15 µL of 53 mM sodium pyruvate, and the decrease in absorbance was followed at 340 nm for 5 minutes using a PerkinElmer-Lambda 35 photometer. Results were presented in $\mu U.mg^{-1}$ Protein.

2.3.3 Protein content was determined following the common Bradford assay at 595 nm using Bovine Serum Albumin (BSA) to build the standard curve (Bradford 1976). Results were presented in mg^{-1} Protein. g^{-1} WM.

2.4 MDA content was determined using the common method known as thiobarbituricacid (TBA)-reactive substances (TBARS) based principally on the protocol of Uchiyama and Mihra (1978) with slight modifications. Briefly, the following reagents were added sequentially: 1% H_3PO_4, 0.6% TBA (i.e., freshly prepared in ultrapure distilled water), and phosphate buffer saline (PBS) pH= 7.3 to the crude homogenate in 3:1:1:0.6 ratio for H_3PO_4 (mL): TBA (mL): PBS (mL): homogenate (g), respectively. For example, 0.6 g homogenate will receive 3.0 ml H_3PO_4, 1.0 ml TBA and 1.0 ml PBS. The reaction mixture was immediately vortexed and incubated at 90°C for 45 minutes a dry block thermostat. The reaction was stopped by incubation in ice after the 45 minutes incubation, centrifuged twice at 10,000g for 5 minutes to get clearer supernatant. The supernatant absorbance spectrum (400-700nm) was measured in triplicates using TECAN-infinite M200 PRO photometer. We have calculated the MDA content using the third derivative approach and MDA standards prepared in using the same reagents used in the assay. Results were presented in $nmol.g^{-1}$ WM of tissue.

2.5 Chl***a*** content was determined as follows: 96% ethanol was added to the crude homogenate (in the following homogenate (g): ethanol (mL) ratio1:15; for example 0.1 g tissue/homogenate will receive 1.5 mL ethanol), vortexed and immediately incubated in dark at 4°C for 24 hours. Supernatants were cleared by centrifugation at 5000g for 10 minutes and immediately the absorbance at 750 and 665 nm were read using a PerkinElmer-Lambda 35 photometer. Chla contents were calculated using the HELCOM COMBIN formula

2.6 Statistical analysis: Testing statistical significance differences were done with the non-parametric Wilcoxon rank sum test with continuity correction and Kruskal-Wallis rank sum test to test the statistical differences between groups from different locations. Spearman's rank correlation rho was used to test the correlation between two variables. The p-value of $\leq$ 0.05 is used as the border line of statistical significance to reject the null hypothesis for each test, and the word "significant" is used only if the p-value of the test is ≤ 0.05. All statistical tests were performed with the help of the free software R-Studio (R i386 3.4.0).

Results

The activity of the two main glycolytic enzymes (PK and LDH) assayed in this study were found to be significantly different among the 4 studied sites, showing a general trend of being higher in medusae from polluted areas (PLB and IA), compared to controls (MSS and CG, Kruskal-Wallis test, $p<0.05$ for both enzymes, Fig. 2.A and B). However, the PLB medusae PK activities were not significantly different from either MSS or CG in pairwise comparison (Fig. 2.A). In contrast, the LDH activities were ca. 0.6-2 fold higher in both polluted locations compared to the controls (Wilcoxon tests, $p<0.05$ at all inter-locations comparisons, Fig. 2.B).

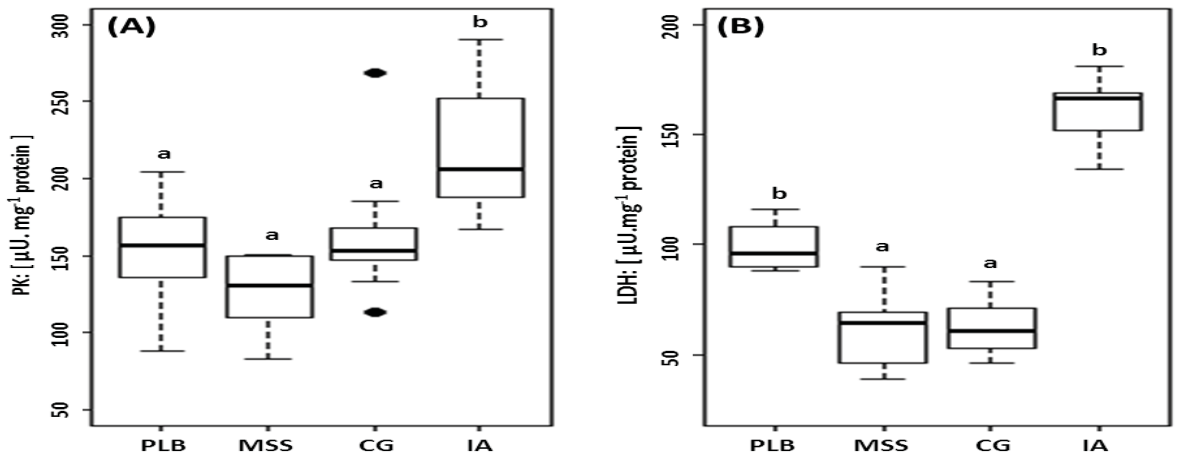

Fig. 2. PK and LDH activities in *Cassiopea* medusae (A = PK, B = LDH). Letters above boxplots indicate statistically significant differences between locations (i.e., different letter means significant difference). Wilcoxon tests, $p<0.05$ set as borderline for statistical significance. Black circles represent outlier values.

Interestingly, we have found that the PK activities were positively correlated with the LDH activities in medusae from all locations combined (Spearman's tests, $p<0.001$ and rho= +0.59; Fig. 3). Furthermore, the pairwise comparison of (PK/LDH) ratio in each individual polluted location with the control locations showed that the ratio in both polluted locations are significantly lower compared to the controls (Wilcoxon tests, $p<0.05$ for all polluted-control pairwise comparison, Fig. 4.B).

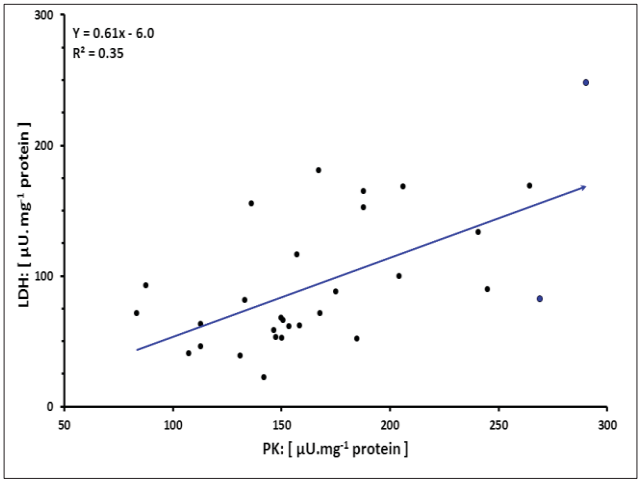

Fig. 3. Correlation of PK and LDH activities. Spearman's test, $p<0.001$.

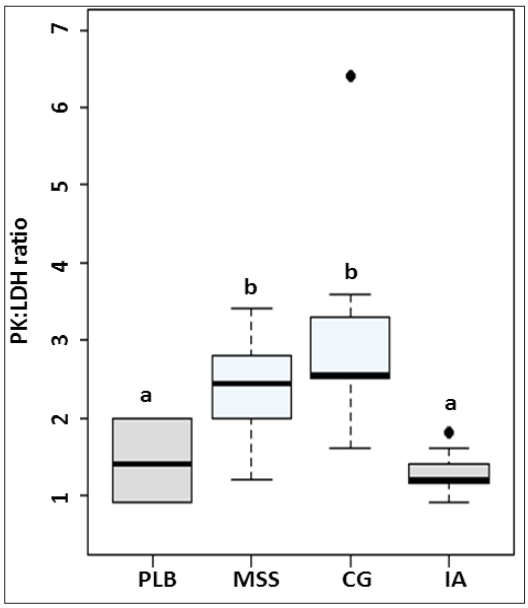

Fig. 4. PK/LDH ratios. Letters above boxplots indicate statistically significant differences between locations (i.e., different letter means significant difference). Wilcoxon tests, $p<0.05$ set as borderline for statistical significance. Black circles represent outlier values.

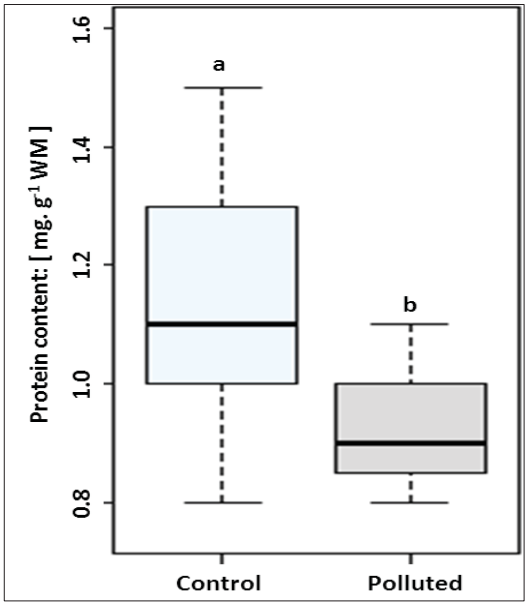

Fig. 5. Cassiopea's medusae protein content in polluted *vs*. control locations. Letters above boxplots indicate statistically significant differences between locations (i.e., different letter means significant difference). Wilcoxon tests, $p<0.05$ set as borderline for statistical significance.

Protein contents were found to be significantly lower in medusae from polluted locations compared to medusae from control locations (Wilcoxon test, $p<0.005$; Fig. 5). While Chla content values were significantly (Wilcoxon test, $p<0.05$; Fig. 6) lowest in PLB medusae compared to all other stations (i.e., MSS, CG or IA), the values were significantly the highest in IA medusae compared to all other sites (Wilcoxon test, $p<0.05$, Fig. 6). In contrast to the

glycolytic enzymes, which have shown contrasting responses in anthropogenically impacted areas compared to the control ones, MDA contents have shown no significant differences in polluted locations compared to the control (IA+PLB *vs*. MSS only in this case, Wilcoxon test, p>0.90; Fig. 7.A).

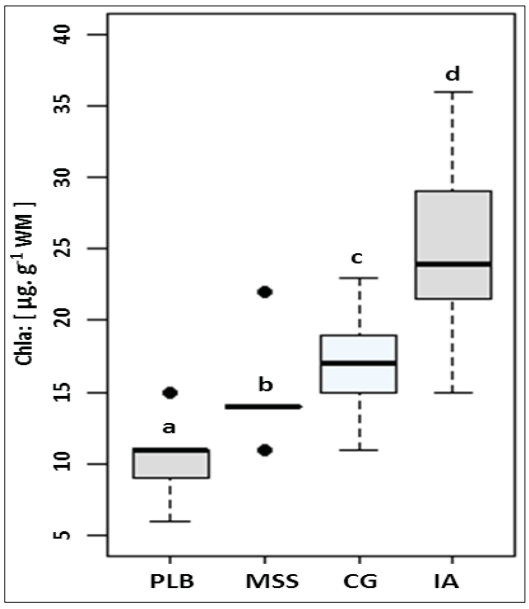

Fig. 6. *Cassiopea's* Chla content in sampling locations. Letters above boxplots indicate statistically significant differences between locations (i.e., different letter means significant difference). Wilcoxon tests, p<0.05 set as borderline for statistical significance. Black circles represent outlier values. PLB= Phosphate Loading Berth, MSS= Marine Science Station, CG= Coral Garden, IA= Industrial Area.

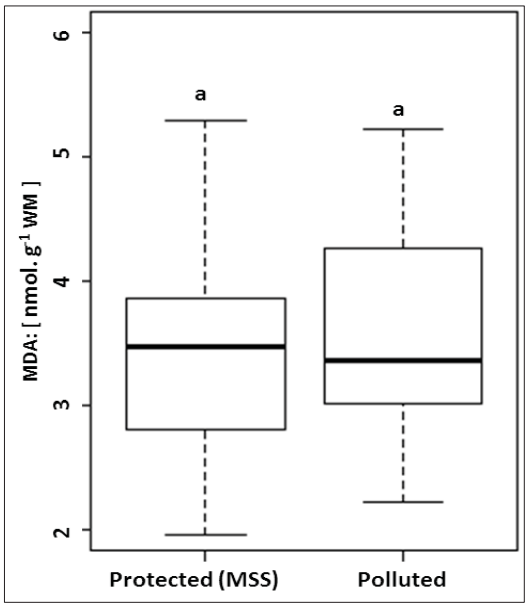

Fig. 7. *Cassiopea's* medusae MDA content in polluted *vs*. control locations. Letters above boxplots indicate statistically significant differences between locations (i.e., different letter means significant difference). Wilcoxon tests, p<0.05 set as borderline for statistical significance.

Discussion

In this study, while metabolic enzymes, protein content and Chl*a* contents have shown contrasting trends in polluted compared to control locations; no signs of oxidative stress damage (in term of LPO) were detected in all locations.

In mollusks, PK activities were found to show a significant increase in response to cadmium treatment (Dailianis & Kaloyianni 2004). In both studied locations, PLB and IA, cadmium concentrations are elevated compared to the control locations (Al-Najjar et al. 2011; Al-Rousan et al. 2016). We have found that PK activities are significantly higher in polluted compared to the control locations (Fig. 2.A). Pyruvate kinase (PK), which controls the fate of glucose carbon toward biosynthesis or glycolytic energy production, is one of the main rate

controlling enzymes in glycolysis (Mazurek et al. 2002). Therefore, an increase in PK activities could result in more carbon being directed toward glycolysis, in other words accelerating glycolysis. Moreover, accelerated glycolysis reflects the increased demand for energy under stress (Dailianis & Kaloyianni 2004). In the absence of molecular oxygen (O_2), the Krebs cycle shuts down and NADH concentrations build up, which exerts negative feedback inhibitory actions on glycolysis. However, the reoxidation of NADH to NAD^+ in the process of lactate formation through the activity of lactate dehydrogenase frees glycolysis from the inhibitory control of NADH, allowing glycolysis to proceed further. Lactate dehydrogenase (LDH) activity is well known to be increased under conditions of increased cellular energy demand. Moreover, LDH correlates well with anaerobic capacities and therefore it is commonly used as a proxy of anaerobiosis (Hochachka et al. 1983). In the green mussel *Perna viridis*, Purushothaman and Rajendran (2010) have found that the LDH activity in the adductor muscles was significantly elevated in response to copper treatment. In our study, the significantly higher activities of LDH in polluted sites compared to both control sites suggest an increased reliance on anaerobic metabolism for energy supply in medusa from these locations (Fig. 2.B).

Interestingly, we found a significant strong positive correlation between the PK and LDH activities in medusae from all locations combined (Spearman's tests, $p<0.05$; Fig. 3). Since both PK and LDH activities were increased in parallel in polluted areas, this confirms the synergistic action of both enzymes in pushing the anaerobic metabolism further to meet the energy requirement of medusa in these locations.

Keeping in mind the fact that pyruvate kinase (PK) could participate in aerobic as well as the anaerobic metabolism; we used the approach of PK/LDH activity ratio analysis to obtain a relative index of aerobic versus anaerobic glycolysis capacity (Hochachka 1980). Applying this approach in our study reveals that *Cassiopea* medusae from polluted locations are more dependent on anaerobic glycolysis than the medusae from control locations. This is clearly indicated by the significantly lower PK/LDH activity ratios in medusae from polluted compared to control locations (Fig. 4). As mentioned earlier, the significantly higher LDH activities in polluted locations confirm our conclusion too.

Stoner et al. (2016) have shown that *Cassiopea* medusae from heavily human impacted areas in the Abaco Island, The Bahamas, attained higher zooxanthellae densities. In light of their findings, the higher values of Chla in IA (i.e., polluted location) suggest that *Cassiopea's*

photosynthetic ability was not negatively impacted by the present pollution status at the IA location (Fig. 6). The PLB site, the second polluted location in our study, is similarly polluted compared to the IA industrial area (Al-Najjar et al. 2011, Al-Rousan et al. 2016) except for the sedimentation rate, which is comparably higher at PLB (Badran and Al Zibdah 2005; Al-Rousan et al. 2016). In corals, it is known that the sedimentation has negative effects on coral photophysiology (Philipp & Fabricius 2003). In this location, PLB, which showed the lowest Chla content values compared to the other locations, might be explained by the negative effect of the higher sedimentation rate on the photosynthetic ability (Fig. 6).

Lipid peroxidation (LPO) is one of the ultimate signs of damage associated with experiencing oxidative stress within tissues. Polyunsaturated fatty acids (PUFA) are essential components of cellular membranes that control cellular functions and maintain their structural integrity. However, PUFA are preferentially attacked by ROS resulting in LPO and consequently the loss of their biological functioning (Ayala et al. 2014). It is well known that membrane lipid peroxidation could be stimulated by transition metals such as Cd, Cu, Hg, Ni, Pb, and Fe (Knight & Voorhees 1990). In the polychaete worm *Diopatra neapolitana*, MDA content, a proxy of LPO, was correlated to the retention of soluble forms of metals in their tissues (Freitas et al. 2012). In this worm, the authors have shown that LPO is a good indicator of experiencing oxidative damages due to metal-induced oxidative stress. Field and experimental studies on *Cassiopea* sp. from the Great Barrier Reef have shown that the jellyfish are able to bioconcentrate metals in their tissues well above the ambient seawater concentrations (Templeman & Kingsford 2010, 2012; Epstein et al. 2016). Interestingly, while some metals (e.g., Cu, Mn, Cd, Zn) have been accumulated up to 200 fold the ambient seawater concentrations; other metals (e.g. lithium) were actively regulated within the tissues, others (e.g., Ca, Mg and Sr) reflected the ambient environment (Templeman & Kingsford 2010, 2012). In *Cassiopea* sp., from the Gulf of Aqaba, Jantzen et al. (2010) have shown that the medusae were capable of remobilizing pore water nutrients from the sediment as a feeding strategy. However, it is obvious that the remobilization process is not selective and re-dissolving the buried metals is very likely. In our study, the absence of any significant difference in the level of LPO, in term of MDA tissue content, between polluted and control locations suggests that the jellyfish medusae have not experienced oxidative stress damage at the current pollutant concentrations at the polluted locations (Fig. 7). However, it cannot be ruled out that they may have elevated ROS in their tissues. In general, heavy metals have detrimental impacts on the invertebrates' physiology, such as reduced survival and impaired

fertilization success (Brown et al. 2004); however, this is not always the case. Heavy metals bioaccumulation in itself does not always mean the occurrence of detrimental effects (Wood et al. 2012). For example, in hydroids it was found that the sub lethal doses of heavy metal (i.e. copper in this case) has stimulated the reproductive growth, a stress response phenomenon termed hormesis (Stebbing 2002). In human-impacted coastal systems (assumed to be polluted) in the Bahamas, *Cassiopea* medusae were more abundant and attained larger sizes compared to the uninhabited areas on Abaco Island, Bahamas (Stoner et al. 2011). In fact, in light of our findings in combination with preceding discussion, *Cassiopea* medusae seem to show signs of being robust to many environmental disturbances.

In the polychaete worm *Hediste diversicolor*, under stressful conditions of metal treatment (i.e., Hg for 28 days), the increased energy demand significantly reduced their energy reserve, protein and glycogen content (Freitas et al. 2017). In this study, the authors suggested that both protein and glycogen served as major sources of energy to fuel defense mechanisms against the metal-induced oxidative stress. In a field study, lower protein content was correlated with higher metal accumulation and oxidative stress levels in the same polychaete (Freitas et al. 2012). The significantly low protein contents of *Cassiopea* medusae tissues in our study were not correlated to oxidative stress induced damage (e.g. lipid peroxidation); however, it cannot be ruled out the probability of using this energy reserve to fuel the anti-oxidative defensive mechanisms to keep ROS levels under control. In fact, this might suggest that medusae were able to keep oxidative stress under control.

Conclusions

In *Cassiopea* medusae, the increased PK and LDH activities, combined with decreased PK/LDH activities in medusae from the anthropogenically impacted locations suggest clearly their reliance on anaerobic metabolism. While LPO is a common cellular damage sign induced by metal pollution in most invertebrates, the jellyfish seems to be able to keep ROS levels under control and this is indicted by the unchanged LPO activity in their tissues. The increased anaerobic metabolism and the ability to avoid LPO suggest that the medusae from polluted locations are using anaerobiosis to sustain the increased metabolic demand. The significant decrease in protein contents in polluted location suggests its use as source of energy to meet the energy demands needed for detoxification of pollutants induced toxicity. All in all, *Cassiopea* seems to be robust to the level of pollution at the studied sites and they might anaerobically be poised to live and thrive at such habitats. This could mean, that with

assumed increasing anthropogenic activity and increased pollution, the jellyfish populations might increase as well and potentially form large aggregates, if not harmful blooms.

Acknowledgements

We would like to especially thank the MSS diving staff for their help in the field, namely Eyad Azgool, Morsi Abuabdalh and Tareq Alsalman. We are grateful to the staff of MSS and ZMT laboratories for support. Special thanks go to Dr. Achim Meyer for his fruitful comments. Funding: This work was supported by Leibniz funds from the Leibniz Gemeinschaft Germany [grant number ZMT-PB-3100/6057].

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Chapter 5

General Discussion

A discussion of the main findings and future research outlooks

Thesis synthesis

Jellyfish blooms, regardless of the debate about their underlying drivers, are noxious, directly interfere with human health, business activities and impart many socio-economic effects (Purcell et al. 2007; Richardson et al. 2009; Bosch-Belmara et al. 2017). Despite the increased common perception that jellyfish are generally robust invertebrates, and the facts that they are a key component of marine nutrient cycling and their populations are increasing globally in response to global warming and anthropogenic activities (Purcell et al. 2007; Pitt et al. 2009; Richardson et al. 2009; Chelsky et al. 2016; Tinta et al. 2016); only few studies have empirically examined the effects of such environmental stressors on jellyfishes.

Our understanding of how jellyfish might respond to climatic change stressors is limited by studies on polyps, where some studies have demonstrated positive effects of rising seawater temperature on asexual reproduction (Holst 2012; Gambill et al. 2016; Klein et al. 2016). Indeed, problems are attributed to the conspicuous medusae not the polyps; therefore, studying the medusoid stage might be of higher importance, because successful reproduction does not always mean successful growth and survival of offspring. By choosing the upside-down jelly fish *Cassiopea* as a model organism for tropical marine jellyfish in this thesis, manipulative experiments and field excursions were used to investigate the influences of selected environmental stressors on jellyfish medusae. The thesis findings demonstrated that *Cassiopeia* medusae showed high tolerance and ability to acclimatize well at higher temperature, while they were sensitive to cold temperature (chapter 2). The higher sensitivity at lower temperature was attributed to oxidative stress-mediated damage and increased metabolic demands (chapter 3). *Cassiopea* medusae were unexpectedly tolerant to anthropogenic impacts, where they were anaerobically poised, however, did not experience oxidative stress (chapter 4).

Metabolic performance, oxidative homeostasis and tradeoffs in *Cassiopea*

The metabolic status of an organism can determine its performance under both normal and stressful conditions, which ultimately affects the survival and functions of the organism (Sokolova et al. 2012; Lesser 2013). Since all organisms have only a certain confined amount of available energy at any given time, regulation of energy allocation (expenditure) to different functions is fundamental to the organism's fitness (Sokolova et al. 2012). Rising seawater temperature and metal pollutants were found to cause an energy imbalance in

marine invertebrates (Jacobs et al. 1956; Knight & Voorhees 1990; Livingstone 2001; Barbieri 2009; Ivanina et al. 2010; Barbieri et al. 2013; Bagwe et al. 2015). The use of the physiological ecology approach, a bottom-up approach to understand the interaction of an organism with its environment (Leser 2013), enriches our knowledge on how to explain organismal responses to environmental stress.

In light of the aforementioned discussion, chapter 2 & 3 bring new explanations for some of the upside-down jellyfish responses to environmental stress. In December 1991, Fitt & Costley (1998) noticed that the *Cassiopea* medusae population in Grassy Key was unhealthy (i.e. numerous medusae had distended oral arms and asymmetric bells) and less in number following the passage of a cold front (which caused rapid drops in temperature down to 12-14 °C) through Florida. In this thesis, the losses in body mass and size of medusae exposed to a sudden drop (from 26 to 20 °C) in seawater temperature, paralleled with experiencing oxidative stress and increased metabolic demand, suggest that the medusae are sensitive to cold temperature. Furthermore, medusae showed decreased feeding ability, therefore, it is likely that the increased energy consumption (due to oxidative stress) and decreased energy acquisition (due to reduced feeding ability of medusae) are the underlying mechanisms of medusae cold sensitivity.

The recent massive bleaching of corals in the Northern Great Barrier Reef following the 2015/16 El Niño events was attributed to the increased seawater temperature up to 32 °C in large patches of the reef (Wolanski et al. 2017). According to Lesser et al. (1990), rising seawater temperature-mediated reactive oxygen species (ROS) formation is the main mechanism for coral bleaching (i.e., the breakdown of the algal-cnidarian symbiosis). Unlike corals, the zooxanthellate jellyfish *Cassiopea* has not shown signs of bleaching when incubated at 32 °C for two weeks (chapter 3). Furthermore, the medusae gained in body mass, did not experience oxidative stress, and did not show signs of increased metabolic demands (i.e., both oxygen uptake rates (MO_2) and cellular respiration in term of the activity of electron transport system (ETS) enzymes were not increased). These findings clearly indicate that the medusae were able to allocate more energy towards growth (assessed in term of body mass gain in this thesis). Overall these results suggest an enhanced growth in response to global warming, whereas low temperatures may set the limits for successful invasion of *Cassiopea* into colder water bodies**.**

Oxidative stress and anaerobic metabolism: a sink and a source of energy under stress

The use of anaerobic metabolism for energy production, development of mechanisms (e.g., large gill surface area and high hemocyanin affinity for O_2, and shorter diffusion distances from the water to the blood) for effective removal of oxygen from seawater, and reduction in metabolic rates are the main physiological adaptations in animals living in oceanic oxygen minimum layers (OML; Childress & Seibel 1998). Whereas OML are characterized by permanent low temperature and O_2 levels, seasonal hypoxia occurs in many coastal shallow habitats as well (Childress & Seibel 1998; Purcell et al. 2001). While some jellyfish live in OML, peak production of many coastal jellyfish species coincides with the summertime seasonal hypoxia in some coastal systems (Purcell et al. 2001).

Despite of the aforementioned reports about the outstanding ability of some jellyfish species to thrive in many oxygen deficient environments, the physiological mechanisms that permit these organisms to live in hypoxia remain unknown. While the possibility that *Cassiopea* medusa have experienced environmental hypoxia in the studied location, functional hypoxia could not be ruled out however. Functional hypoxia resulting from insufficient O_2 supply to the Krebs cycle could be attributed to a variety of environmental stressors such as elevated temperature and metal pollution (Livingstone 2001; Pörtner 2002; Barbieri 2009; Bagwe et al.2015). In *Cassiopea* medusae collected from the anthropogenically impacted locations along the Gulf of Aqaba (GoA), the elevated activities of the glycolytic enzymes (PK and LDH) might indicate the onset of anaerobiosis. In oyster, Bagwe et al. (2015) have used the onset of anaerobic metabolisms as an informative and sensitive biomarker of energetic stress induced by temperature and Cadmium (Cd) exposure. Given that the aforementioned locations show transition metals (e.g., Cd & Pb), and analog to oysters, the studied medusae might have suffered from energetic stress due to metal pollution.

Experiencing energetic stress in marine invertebrates exposed to transition metals could be attributed to experiencing oxidative stress due to metal exposure (Knight and Voorhees 1990; Freitas et al. 2012). While the exposure of the polychaete worm *Diopatra neapolitana* to transition metals resulted in the buildup of high levels of the lipid peroxidation (LPO) end product malondialdehyde (MDA), an oxidative stress biomarker used as a proxy to asses membrane LPO (Freitas et al. 2012), *Cassiopea* medusae from polluted location in the GoA did not show any signs of experiencing oxidative stress-induced cellular damage in term of LPO. In animal physiology, it is well known that in the absence of molecular oxygen (O_2),

the Krebs cycle shuts down and NADH concentrations build up, which exerts negative feedback inhibitory actions on glycolysis. It is well known that LDH, a major anaerobic enzyme, could allow glycolysis to proceed again by replenishing it with an oxidized form of the NADH (i.e., NAD^+) in an oxygen-independent mechanism.

In light of the preceding discussion, anaerobic metabolism serves in providing an additional and alternative route of energy production to meet the exaggerated cost of maintenance and homeostasis (Pörtner 2002). Unlike higher invertebrates, jellyfish have no gills or hemocyanin to improve oxygen uptake from seawater, and their bodies are characterized by a massive amount of an acellular mesoglea, sandwiched between two very thin layers (i.e., epi- and endoderm). Thuesen et al. (2005) have shown that some scyphozoan medusae are able to maintain oxygen consumption (i.e., oxyregulator) down to below 10% air saturation. The authors have demonstrated that the massive amount of the mesoglea in medusae bodies acts as an oxygen reserve. The oxygen trapped in the mesoglea has been called as "intragel oxygen" by the authors. While the intragel oxygen was depleted after two hours from *Aurelia labiata* when moved experimentally into anoxic conditions, it recovered to 70% of normoxic oxygen saturation after 2.5 hours, when moved to normoxic seawater (Thuesen et al. 2005). In the context of the above discussion and our findings in chapter 4, which demonstrated that *Cassiopea* medusae are likely to be anaerobically poised and less susceptible to oxidative stress-induced damages, the jellyfish seems to be robust to the levels of pollutants at the studied location. On a larger scale, our results bring new physiological evidences to explain the common perception that 'jellyfish are hypoxia tolerant'; however, it is important to highlight here that the full explanation needs further research.

Future Research Outlooks

Breakdown of the cnidarian-algal symbiosis in corals is associated with decreased fitness of both organisms. It has been shown that the loss of coral endosymbionts is attributed to the enhanced production of ROS in corals under thermal and light stress (Lesser et al. 1990; Downs et al. 2002). This thesis, especially the findings of both chapter 3 & 4, suggests a stable endosymbiosis in *Cassiopea* medusae compared to corals, however, this thesis was not oriented towards photophysiology studies (i.e., only Chla content was measured). Therefore, experiments addressing more precisely other estimates of *Cassiopea's* photophysiology are needed. For example, such experiments could address zooxanthellae count and the rate of photosynthesis at different levels of the stressors.

Most studies addressing the ecological impacts of jellyfish on their environment have focused on pelagic jellyfish such as *Aurelia, Cyanea* and *Mnemiopsis*. Induction of hypoxia and anoxia (e.g., attributed to O_2 depletion in oxidation of the massive amount of organic matter released from the decaying jellyfish's dead bodies), and changing the benthic and planktonic microbial communities are some of the jellyfish post-bloom complications (Breitburg et al. 1997; Mills 2001; Pitt et al. 2009; Qu et al. 2015; Chelsky et al. 2016; Tinta et al. 2016). The role of *Cassiopea* in jellyfish blooms however, is not well studied. Importantly, the upside-down jellyfish *Cassiopea* is different from most pelagic jellyfish in two aspects: they have zooxanthellae (i.e., they produce O_2) and they are benthic long-lived jellies (i.e., ca. up to 1-2 years, while most pelagic medusae live < 6 months). Therefore, it sounds promising to dedicate more research to the potential roles of this zooxanthellate jellyfish in shallow marine habitats.

Interestingly, jellyfish are protein-rich animals, where protein comprises > 50-60% of the total organic matter (Pitt et al. 2009; Ding et al. 2011). Collagen, a medically important structural protein, accounts for approximately 50% of the total protein content (Khong et al. 2015). Furthermore, jellyfish have low lipid (ca. 22%) and carbohydrate (ca. 7%) contents (Pitt et al. 2009; Khong et al. 2015). Having good protein quality and low calories, edible jellyfish are an attractive source of nutritive ingredients. In this sense, given that *Cassiopea* is easy and cheap to culture, and relatively safe to handle, it is worthwhile to study the possibility for using this interesting animal as a source for food and maybe a potential bio-filter in aquarium setups.

Overall Conclusions

This thesis examined the responses of jellyfish to stressors that occur on local and global scales. Rapid changes in temperature and metal pollution are widely distributed and their limitations and impacts on the marine environment are increasing steadily. Importantly, the results of this thesis provide a framework for understanding the physiological tolerance of *Cassiopea* and other jellyfish under possible future climate changes and anthropogenic activities. The findings suggest that *Cassiopea* would benefit from global warming-induced rise in seawater temperature and might spread their populations into new coastal systems. Tolerance to anthropogenic impacts and high water temperature add new evidence to the physiological robustness of jellyfish. Metabolic responses and oxidative stress are good indicators to understand physiological responses of jellyfish to stressors. Overall, more

jellyfish physiology is needed for understanding their ecological roles at present and in the future.

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Declaration on the contribution of the candidate to the multi-author articles/manuscripts which are included as chapters in the submitted doctoral thesis

Chapter (2)

Contribution of the candidate in % of the total workload (up to 100%) for each of the following categories:

Experimental concept and design:	ca.100%
Experimental work and/or acquisition of (experimental) data:	ca.100%
Data analysis and interpretation:	ca. 95%
Preparation of figures and tables:	ca.100%
Drafting of the manuscript:	ca. 80%

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Chapter (4)

Contribution of the candidate in % of the total workload (up to 100%) for each of the following categories:

Experimental concept and design:	ca.100%
Experimental work and/or acquisition of (experimental) data:	ca.100%
Data analysis and interpretation:	ca. 95%
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Date: 15.11.2017

Signatur:

Ort, Datum: Bremen, 15.11.2017

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