Temperature tolerance of the sea cucumber *Holothuria scabra*

Towards a systematic understanding of multi-level temperature effects

A dissertation by

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`Das nachdenkende, betrachtende, forschende Leben ist eigentlich das höchste´

- Alexander von Humboldt

`The oceans are warming´

- IPCC 2007

Summary

The Sea cucumber *Holothuria scabra* is a sluggish, bottom-dwelling marine invertebrate that adds significantly to the release and recycling of nutrients in tropical coastal ecosystems. Besides its fundamental ecological role, the dried body wall of *H. scabra*, known as Trepang or Bêche-de-mer, is ranked among the highest priced global trade commodities from the ocean. The consequential overexploitation of *H. scabra* brought the artificial rearing of this—by now scarce—species in the center of interest, especially in regions of the Global South. Nowadays, *H. scabra* has become a key species in community-based aquaculture, on the level of extensive sea ranching and restocking. Temperature is the key environmental factor that determines biological fitness of *H. scabra* and thus aquaculture production efficiency. Hence, a detailed understanding of temperature effects on *H. scabra* are important in two different ways: 1) to determine the optimal thermal range and delimiting critical temperature levels for best aquaculture practices; 2) to predict the susceptibility of *H. scabra* to future global warming scenarios. This thesis aims to provide first insights into a mechanistic understanding of how temperature affects *H. scabra* at different biological organization levels. This doctoral thesis is a cumulative work where the scientific core output is divided into four chapters, each representing a published or submitted research paper, which are embedded in a general introduction and discussion.

Chapter 1 addresses the thermal acclimation capacity of *H. scabra*. In this study changes in cellular energy allocation (CEA), oxygen consumption rate and energy related enzymes' activity (IDH and LDH) were measured in juvenile *H. scabra,* held at different temperatures: 21, 27 and 33 °C. The results show initial temperature effects on CEA. In contrast, oxygen consumption and metabolic enzyme activities were continuously affected by temperature. Results imply that juvenile *H. scabra* were able to recover from initial disturbances in energy balance. However, the prolonged exposure to 33 °C led to a metabolic shift, indicated by changes in oxygen consumption rate, LDH and IDH. The synergy of CEA and oxygen consumption proved to be a viable indicator to assess acclimation capability in *H. scabra*.

Chapter 2 builds on the same temperature conditions as Chapter 1. This time, however, the analytical focus is the level of the antioxidant- and immune- response of juvenile H. scabra. Immune responses were addressed by studying the activity of phenoloxidase (PO) and prophenoloxidase (ProPO) in the coelomic fluid. Antioxidant defense responses—catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR) enzymatic activities—were measured in the muscle and respiratory tree tissues, whereas oxidative damage was

evaluated by measuring levels of superoxide radicals (ROS), DNA-strand breaks and lipid peroxidation (LPO). Juvenile *H. scabra* increased SOD and PO activities when temperature was elevated, and revealed low levels of ROS and damage in both cold and warm treatments throughout the experiment, confirming the organism's moderate thermal stress. After the short acclimation period (15 and 30 days), the immune and antioxidant responses prevented damage and maintained homeostasis. This multi-biomarker approach highlights its usefulness to monitor stress levels in *H. scabra* aquaculture.

Chapter 3 investigates temperature-induced maximal- and minimal- O_2 consumption in combination with mRNA expression levels of Hsp70, to determine critical temperature limits for *H. scabra*. The results revealed an aerobic performance window (22 – 38 °C) with distinct critical warm- (CT_{max}; >38 °C) and cold- (CT_{min}; <23 °C) temperature limits. The expression levels of Hsp70 were used to determine disruption of functional homeostasis, which only occurred above 38 °C. The characterization of temperature-induced maximal- (≈ maximal metabolic rate—MMR) and minimal- (≈ standard metabolic rate—SMR) O_2 consumption rates, may serve as reliable alternative to precisely determine aerobic scope in sea cucumbers and other slow bottom-dwelling marine species. Moreover, we showcase the important linkage between energy-related physiological markers and molecular defense mechanisms to predict stress effects, such as future climate change.

Chapter 4 compares acclimation capacity between the tropical species *H. scabra* and the temperate sea cucumber *Holothuria forskali* to detect latitudinal differences in thermal tolerance. The results of this study reveal much broader respiratory adjustments in *H. scabra* compared to *H. forskali,* accompanied by clear temperature effects on enzyme activity and energy reserves in the tropical species, while energy turnover in the temperate species remains consistent. These findings indicate enhanced metabolic plasticity in *H. scabra*, at the cost of clearly elevated energy expenditures. Beside energetic burden, however, results indicate better acclimation capacity in the tropical stenotherm species. This study reveals the importance to holistically explore metabolic strategies in conspecifics and congeners to predict the heterogeneous effects of climate change across latitudinal gradients.

In conclusion this thesis shows that a multi-level analytical approach is required to comprehensively understand temperature effects. The present results reveal a remarkable functional capacity of *H. scabra* to sustain acute and long-term exposure to temperature stress, especially at upper maxima levels. Finally, the variety of methods established and tested throughout this thesis provide an extensive repertoire for future studies in any field of stress detection in *H. scabra* and other sea cucumber species.

Zusammenfassung

Die Seegurke *Holothuria scabra* ist ein wirbelloses, benthisches Meerestier, welches sich kriechend fortbewegt. *H. scabra* vergräbt sich täglich im Sediment wobei es die obere Schicht des Sediments auflockert und dabei signifikant zum Kreislauf von Nährstoffen und deren Freisetzung beiträgt. Somit spielt diese Art eine entscheidende ökologische Rolle in tropischen Küstensystemen. Neben der großen ökologischen Relevanz ist diese Art außerdem von großer kommerzieller Bedeutung. Der getrocknete Muskelschlauch des Tieres wird unter dem Synonymen Trepang oder Bêche-de-mer als eins der teuersten Produkte aus dem Meer gehandelt. Der hohe Preis hat bereits zu einer starken Überfischung dieser Art geführt. Dies wiederum hat ein starkes Interesse geweckt, diese Tiere künstlich, in Aquakulturen zu züchten. Die Aufzucht von *H. scabra* ist vor allem in Regionen des Globalen Südens von großem Interesse. Momentan ist *H. scabra* ein Schlüsselkandidat für extensive Aquakulturvorhaben in kleinen, lokalen Bevölkerungsgruppen, mit dem Zwecke der Subsistenzwirtschaft und Aufstockung von wilden *H. scabra* Populationen. Temperatur ist der entscheidende Umweltparameter welcher ausschlaggebend ist für biologische Fitness, und damit auch entscheidend für die Produktionseffizienz von *H. scabra* Aquakulturen. Daher ist ein detailliertes Verständnis über die Auswirkungen von Temperaturstress auf *H. scabra* aus zweierlei Hinsicht sehr relevant: 1) zur Feststellung des optimalen Temperaturfensters sowie von kritischen Temperaturgrenzwerten, um bestmögliche Aquakulturbedingungen zu erzeugen; 2) zur Vorhersage der Suszeptibilität von *H. scabra*, bezüglich der zukünftigen Szenarien des Klimawandels. Diese Doktorarbeit hat das Ziel die Temperatureffekte in *H. scabra* zum ersten Mal mechanistisch, auf verschiedenen biologischen Ebenen zu verstehen und zu beschreiben. Diese Doktorarbeit ist kumulativ, der wissenschaftliche Kern besteht aus vier Kapiteln—einzeln in Fachzeitschriften veröffentlicht oder eingreicht—welche durch eine gesamten Einleitung und Diskussion in Zusammenhang gestellt werden.

Kapitel 1 beschäftigt sich mit der Akklimatisierungskapazität von H. scabra. Diese Studie betrachtet Änderungen in der zellulären Energieverteilung (CEA), Sauerstoffversorgung sowie in der Aktivität von metabolischen Enzymen (LDH und IDH) in juvenilen *H. scabra*, bei unterschiedlichen Temperaturen: 21, 27 und 33 °C. Die Ergebnisse zeigten, das die anfängliche Temperaturänderung (1 °C/Tag) in beide Richtungen, bis die angestrebten Temperaturen (21 und 33 °C) erreicht waren (Tag 0), eine CEA Reaktion auslöste. Im Gegensatz zu dem anfänglichen CEA Ausschlag, waren sowohl Sauerstoffverbrauch als auch

die Aktivität der metabolischen Enzyme über den gesamten Verlauf des Experiments durch die warme und kalte Temperatur beeinflusst. Die Ergebnisse implizieren, das juvenile *H. scabra* in der Lage waren sich von den anfänglichen Temperatureffekten auf die Energiereserven zu erholen. Die Tiere die der warmen Temperatur (33 °C) ausgesetzt waren zeigten jedoch klare Anzeichen einer metabolischen Veränderung, erkennbar durch erhöhten Sauerstoffverbrauch und anpassende Aktivitäten von LDH und IDH. Das Messen von Sauerstoffverbrauch, CEA und metabolischen Enzymen bewies gute Synergien um die Akklimatisierungskapazität von juvenile H. scabra zu bewerten. Überraschenderweise waren die Tiere in der Lage bis 33 °C Energiehomöostase zu bewahren.

Kapitel 2 bezieht sich auf die gleichen Temperaturmanipulationen wie Kapitel 1. Diesesmal liegt der analytische Fokus jedoch auf der Bewertung der Akklimatisierungskapazität auf Basis des Antioxidations- und Immunsystems. Die Antwort des Immunsystems wurde durch Aktivitätsänderungen von Phenoloxidase (PO) und Prophenoloxidase (ProPO) in der Coelomflüssigkeit von juvenilen *H. scabra* gemessen. Die antioxidative Abwehrreaktion—Katalase (CAT), Superoxid-Dismutase (SOD), Glutathione-Reduktase (GR) enzymatische Aktivitäten—wurden in Gewebeproben der Muskulatur und der Wasserlunge gemessen, wobei oxidative Schädigungen durch die Menge von Superoxid-Radikalen (ROS), Brüche in DNA-Strängen und Lipiperoxidation (LPO) quantifiziert wurden. Juvenile H. scabra zeigten erhöhte SOD und PO Aktivität bei wärmerer Temperatur (33 °C), und geringe Mengen an ROS und DNA Schädigungen bei kalten und warmen Temperaturen, was moderaten Stress erkennen lässt. Nach kurzer Akklimatisierungszeit (15 und 30 Tage) waren jedoch keine Immun- und Antioxidationsreaktionen messbar mehr, und Homöostasis war wieder hergestellt. Dieser multiple Biomarkeransatz zeigt eine große Nützlichkeit auf, um Stresslevel in *H. scabra* Aquakulturen zu bewerten.

Kapitel 3 untersucht den durch Temperaturveränderungen ausgelösten maximalen- und minimalen- Sauerstoffverbrauch in Kombination mit mRNA Expremierung des Hitzeschockgens (Hsp70), um Temperaturgrenzwerte von *H. scabra* zu definieren. Die Ergebnisse ergeben eine aerobes Temperaturfenster (22 – 38 °C) für H. scabra anhand dessen kritische warme (CT_{max}; >38 °C) und kalte (CT_{min}; <23 °C) Temperaturen sowie eine theoretische optimal Temperatur (T_{opt}; 30.5 °C) eindeutig festgestellt werden konnten. Die Expremierung von Hsp70 wurde benutzt um den Abbruch von funktioneller Homöostase festzustellen, dies wurde nur oberhalb der kritischen Temperatur von 38 °C festgestellt. Die Charakterisierung von Temperatur erzeugten maximalem- (≈ maximale metabolische Rate—MMR) und minimalem- (≈ standard metabolischen Rate—SMR) Sauerstoffverbrauchs, stellt

eine möglicheweise eine Alternative dar um den aerobe Bereichs auch in Seegurken und anderen schwerfälligen benthischen Arten verlässlich zu messen. Dieses Kapitel demonstriert wie physiologische Parameter und molekulare Abwehrmechanismen nützlich vereint werden können um die Effekte von Stress, wie bevorstehende Klimaänderungen.

Kapitel 4 vergleicht die Akklimatisierungskapazität zwischen der tropischen Seegurke H. scabra und einem Artgenossen aus gemäßigten Breiten (*Holothuria forskali*), um den geographischen Einfluss auf Temperaturtoleranz zu erforschen. Die Ergebnisse dieser Studie zeigen eine sehr viel breitere aerobe Kapazität von *H. scabra* im Vergleich zu *H. forskali*, begleitet von eindeutigen Akklimatisierungseffekten auf der Ebene von Enzymaktivität und Energiereserven in der tropischen Art, wobei die gemäßigte Art keine Reaktion auf diesen Ebenen zeigte. Die Befunde deuten auf eine erhöhte metabolische Plastizität von *H. scabra*, welche allerdings mit einem erhöhtem Energieverbrauch einhergeht. Der Nachteil der höheren Energiebelastung hat jedoch anscheinend die positive Kehrseite einer erhöhten Akklimatisierungskapazität vor allem bei warmen Temperaturextremen. Diese Vergleichsstudie zeigt die Relevanz eines holistischen Verständnisses von metabolischen Strategien in Artgenossen und Artverwandten Tieren von verschiedenen Breitengraden um die heterogenen Effekte des Klimawandels besser vorhersagen zu können.

Zusammenfassend zeigt diese Doktorarbeit, das ein analytischer Ansatz auf verschiedenen biologischen Ebenen notwendig ist um die Effekte von Temperaturänderungen umfassend zu verstehen. Mit dieser Herangehensweise wurde eine außergewöhnliche funktionelle Kapazität von *H. scabra*, in Bezug auf langzeit Akklimatisierung und akutem Temperaturstress, festgestellt. Schlussendlich bietet diese Arbeit eine genaue Beschreibung einer Vielzahl von Methoden die ein umfassendes Repertoire für die zukünftige Stressforschung an *H. scabra* und anderen Seegurken darstellen.

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General introduction

The sea cucumber *Holothuria scabra*

The species *Holothuria scabra* (Jaeger, 1833), commonly known as sandfish, is an Aspidochirotid sea cucumber, which has been well studied (rev. by Hamel et al., 2001), mainly due to its importance in artisanal and commercial fisheries in the tropical Indo-Pacific region (Hamel et al., 2001; Purcell et al., 2012). Sandfish are deposit-feeders, inhabiting oligotrophic environments such as sand-flats linked to sea grass and mangroves, or in the vicinity of fringing reefs (Purcell et al., 2012). *Holothuria scabra* exhibit a diurnal burrowing behavior, where the animals dig through the upper sediment layer. This bottom-dwelling lifestyle significantly promotes the productivity of the inhabited ecosystems (rev. by Purcell at al., 2016) through bioturbation (Yamanouti, 1939, 1956; Conand, 1990; Mercier et al., 1999, 2000; Skewes et al., 2000; Purcell, 2004, 2010; Wolkenhauer et al., 2010), which enhances nutrient recycling, mineral dispersion (such as calcium carbonate) (Uthicke and Klumpp, 1998; Uthicke, 1999; Uthicke, 2001 and sea water buffering (Schneider et al., 2011, 2013). Moreover, H. scabra enriches biodiversity by functioning as host for various symbiotic associations (Purcell and Eriksson, 2015) and bridges energy transfer across tropic levels as an important prey species (Dance, 2003, Purcell and Simutoga, 2008, Lavitra et al., 2009, Robinson and Pascal 2012).

Morphology and anatomy

Holothuria scabra has a cylindrical, elongated shape with relatively flat ends. The maximal size of adults varies between 15 – 40 cm. Similarly to the body length, the reported weight can also differ considerably ranging from 500 – 2000 g (Conand, 1989). This indicates distinct weight differences of *H. scabra* across its geographic range, but also reflects weight variation associated to marked changes in coelomic water and amount of sediment in the digestive system (Conand, 1989; Baskar, 1994). The dorsal side is convex and the ventral side is flat (James, 1989). The ellipsoid mouth is located antero-ventrally and surrounded by 20 tentacles. The anus is postero-dorsal, which enables breathing while being buried in the sand. The dorsal surface is rough and gritty with few finely dispersed tube feet. The color of the dorsal surface is variable and can range from dark-yellow to grey-brown and black (Conand 1989,1998; Van den Spiegel et al., 1992; Uthicke and Benzie, 1998). The ventral surface is also rough, with numerous irregularly scattered tube feed for locomotion (Tan Tiu,

1981; Massin, 1999). The ventral color ranges usually from white to cream. Internally, *Holothuria scabra* possesses five bands of muscles, which are characteristic for the five-fold radially symmetry of echinoderms. The muscles span the whole longitudinal body length from the calcite ring (anterior) to the anus (posterior). *Holothuria scabra* rhythmically pumps water for respiration via the cloaca. The freshly entered, oxygenated water is distributed internally through the respiratory tree. The respiratory tree is the main organ associated to gas exchange, it initiates with a main stem from the anterior of the cloaca, from which two main branches spread throughout the entire coelom cavity and surround the organs with finer branches. Minor gas exchange takes also place through the tentacles and the integument (Mary Bai, 1980). The coelom is filled with coelomic fluid, which is circulated internally. Many free cells, known as coelomocytes, are suspended in the coelomic fluid. Coelomocytes play vital roles in nutrition, waste transport and immune activity (Mary Bai, 1980).

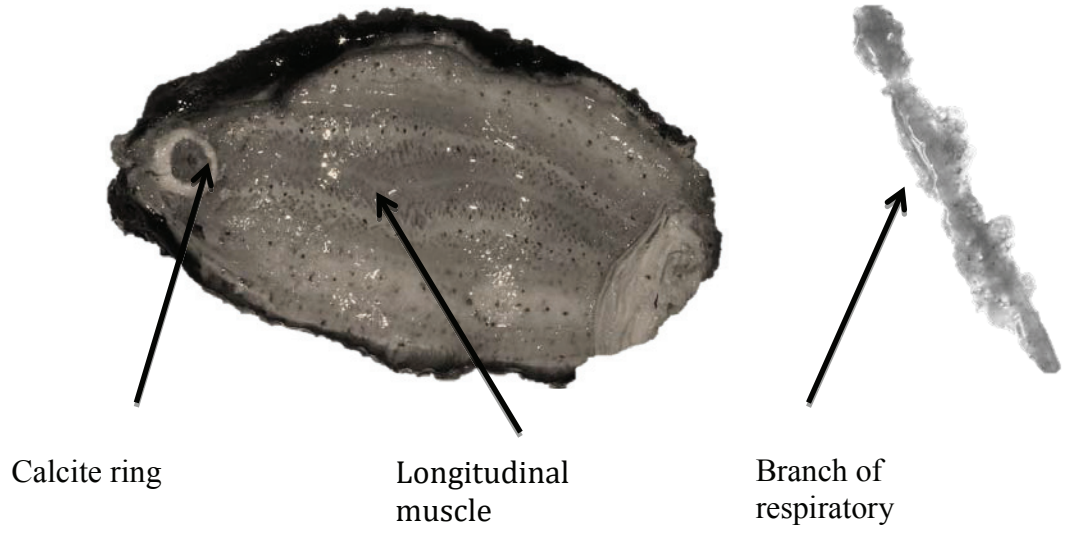


Fig. 1: Sea cucumber (*Holothuria scabra*) anatomy. Dorsal view on opened body wall (left) and separated respiratory tree tissue (right).

Holothuria scabra mariculture

The dried body wall of *Holothuria scabra,* also known as Trepang or Bêche-de-mer, is a global trade commodity, mainly to serve a luxury market for food and traditional medicine in China (Hamel et al., 2001; Bell et al., 2005). The trade with dried *H. scabra* is ongoing for more than two centuries, however, the demand increased drastically over the last decades as a consequence of a growing middle-income class in China (Slater, 2015). This has led to increased global fishing pressure and over exploitation of this species (see Purcell et al.,

2014) and, accordingly, brought awareness to grow *Holothuria scabra* in mariculture systems (i.e. Hamel et al., 2001; Purcell et al., 2012; 2014). In the early 1980s first Sandfish aquaculture practices were pioneered in India (James, 1996), followed by fast developing hatchery advancements in other regions (Raison, 2008; Mills et al., 2012; Purcell et al., 2012). Currently, *Holothuria scabra* is considered a promising mariculture candidate in the Indo-Pacific region (Battaglene, 1999; Hamel et al., 2001; Bell et al., 2005). Current research focuses on *Holothuria scabra* mariculture opportunities for restocking in combination with sustainable community-based livelihood activities (Eeckhaut et al., 2008; Hair et al., 2012; Purcell et al., 2012; Robinson et al., 2013). Without high investments, the culture of *Holothuria scabra* can create additional income and may serve as high-value protein source, in small fisheries communities that face local overexploitation of marine resources. Moreover, bottom-dwelling sea cucumbers such as Sandfish might be ideal candidates for ecosystem based farming approaches, such as integrated multitrophic aquaculture (IMTA; rev. by Zamora et al., 2016).

After the hatchery phase, juvenile *Holothuria scabra* are generally moved to seawater ponds (Duy, 2012) or to the natural sea environment (Tsiresy et al., 2011; Robinson and Pascal, 2012; Juinio-Menez et al., 2013) for grow out to commercial size. In the sea, extensive culture practices encompass restocking, stock enhancement and sea ranching (Bell et al., 2008), on the semi-intensive production level animals are brought into enclosures, where basic husbandry is applied (Robinson and Pascal, 2009; Rougier et al., 2013). The release of juveniles (Purcell, 2012 recommended 3 – 10g) into grow out facilities is one of the most crucial steps in *Holothuria scabra* aquaculture production. The time subsequent to juvenile planting is associated with the highest mortality rates (Dance et al., 2003; Purcell and Simutoga, 2008). Explanations of juvenile losses in grow out systems include predation, transport stress, salinity fluctuations due to inundation, strong currents, escape, and extreme weather (Purcell, 2004; Robinson and Pascal, 2012). In particular extreme weather could be of growing future concern as in pond as well as in sea-based systems abiotic factors remain largely uncontrolled. Hence, it will be a major challenge to forecast and mitigate the effects of the predicted increased frequencies of extreme heat events and growing severity of the El Niño and La Niña weather phenomenon, in *Holothuria scabra* aquaculture production. For the end of this century Bell et al. (2013) projects a reduction in coastal aquaculture production of 30% in the tropical Pacific, due to climate change. Mass mortalities of *Holothuria scabra* have already been documented on the Philippines, during the last El Niño event, when pond waters varied between temperatures of 34 – 37 °C over prolonged periods

(Gamboa personal communication). It is well studied that water temperature determines feeding rate (Battaglene et al., 1999), scavenging activity, burrowing behavior and growth (Mercier et al. 1999; Purcell and Kirby, 2005; Wolkenhauer, 2008; Lavitra, 2010) in *Holothuria scabra*. In the intensively studied sea cucumber species *Apostichopus japonicas*, similar effects could be directly linked to metabolic changes or temperature sensitivity of feed conversion rates (see An et al., 2007; Dong et al., 2008). It is, therefore, critical to have a holistic and accurate understanding of the temperature effects on metabolism of *H. scabra*, especially at the critical juvenile stage (3 – 10g), to optimize grow out production and to enhance mitigation measures in times of global climate change.

Temperature stress

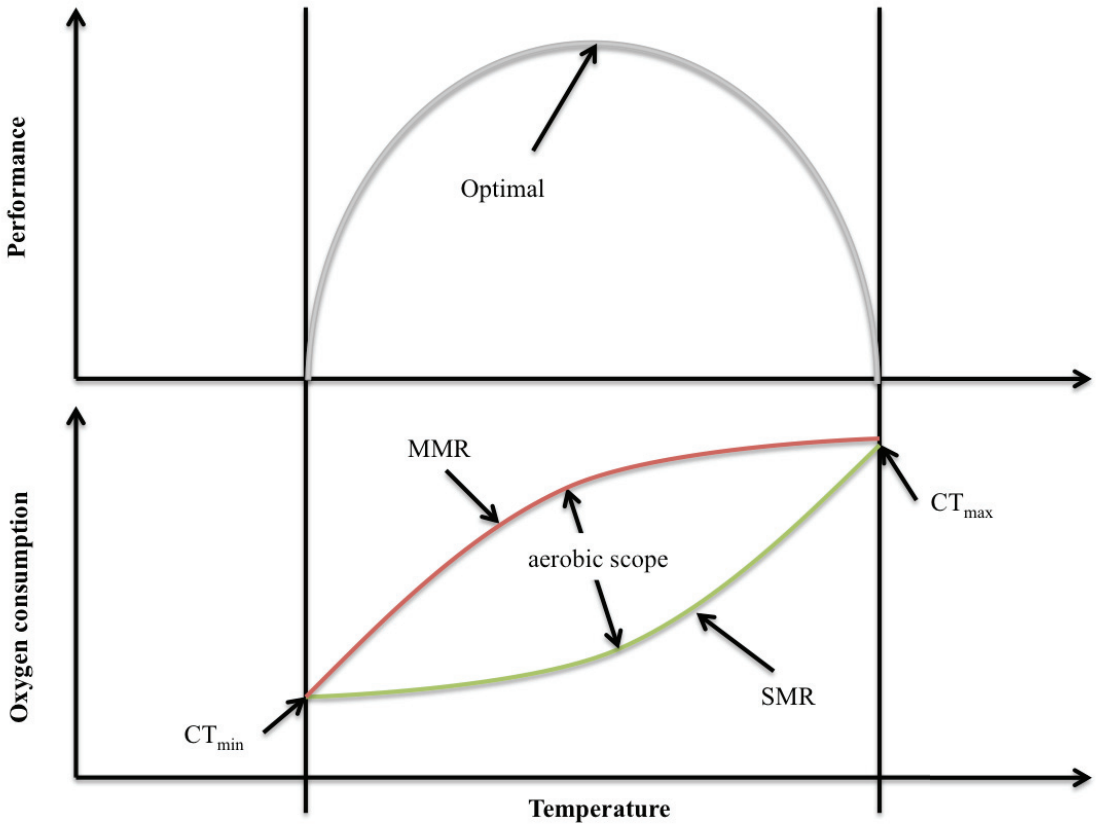
The anthropogenic intervention in the carbon cycle, due to intensive greenhouse gas emission, is among the top three planetary boundaries (Rockström et al., 2009) and a major driver for global climate change. The concentration of greenhouse gases, such as CO_2, in the earth atmosphere are positively linked to air temperature (Jouzel et al., 2007; Lüthi et al., 2008). Consequently, it is inevitable that the current dramatic increase in atmospheric CO_2 levels will lead to reverberate elevations in earth systems temperature. It is predicted that polar- and equatorial- regions will be more affected by global warming than middle-latitudinal areas. For tropical oceans a temperature increase of 2 – 4 °C are projected to occur by the end of this century (IPCC, 2015). This means high acclimation pressure, especially for shallow water marine invertebrates such as *Holothuria scabra*, which are not able to exhibit pole-ward shifts.

Practically all biological-rates (i.e. metabolic and biochemical activity) increase exponentially with temperature, at least within the normal activity spectrum that ranges from 0 – 40 °C (for most organisms; Brown et al., 2004). Hence, temperature is a central environmental factor with intensive influence on animal life, sloping from low level processes to whole-organism fitness. In contrast to endotherms, which need to allocate their available energy either to heat generation or dissipation, the metabolic rate and level of energy assimilation in ectotherms is directly driven by ambient temperature. Therefore, marine ectotherms, such as sea cucumbers, depend highly on optimal thermal windows, above and below which organism performance decreases (Hochachka and Somero, 2002; Somero, 2010). At optimal performance the fraction of total consumed oxygen that is available for activities beyond resting levels (aerobic scope) peaks, and the surplus energy for

growth, activity and reproduction reaches its maximum. At both sides of this temperature optimum the aerobic performance decreases until species-specific critical –high (CT_{max}) and –low (CT_{min}) temperatures are reached. The oxygen limited thermal tolerance hypothesis (Peck et al., 2002; Pörtner, 2002; Pörtner et al., 2007) determines the thermal performance breadth as consequence of aerobic persistency (Fig.2). This means the thermal window is delimited by an organism capacity to adjust its aerobic metabolism. Consequently, a growing prevalence of an anaerobic performance can be interpreted as stress sign, mainly due to the accumulation of metabolic by-products (i.e. lactate) that become lethal at certain concentrations. At CT_{min} anaerobic energy turnover is initiated due to temperature driven reduced organism activity, encompassing ventilation and respiration. At CT_{max} respiration reaches a peak as oxidative stress (accumulation of O_2-radicals, so-called ROS) correlates positively with oxygen uptake. Hence, at the warm maximum, the burden of the less efficient and time limited anaerobic energy turnover is favored to prevent oxidative damage on DNA, protein and tissue. Because temperature stress becomes increasingly lethal with the accumulation of toxic by-products, exposure time is an important factor. Generally, the temperature spectrum between CT_{max} and CT_{min} is broader when the temperature is changing fast compared to a slow changing rate. It is crucial to understand both stress responses, short- and long- term, to understand and predict species-specific acclimation capacities to future global warming.

Indicators of thermal stress

The first conceptual description of environmental stress was given by Hans Selye (1950), in which he defined stress as ‘the non-specific response of the body to any demand’. Chrousos et al. (1992; 1998) gave a more complex depiction of stress, which is still used today. They described stress as the challenge to sustain an intricate dynamic equilibrium, termed homeostasis. In more detail this defines stress as a behavioral and physiological response due to a given disturbance, underlined by various mechanisms that range from the molecular to the whole organism level, to regain homeostatic conditions (rev. by Schulte, 2014). Thermal stress triggers physiological changes that are initially concentrated on energy production, with changes in oxygen –uptake capacity (respiration) and –assimilation efficiency (mitochondrial function) at the frontline.



Metabolic strategy	Conservation (minimal metabolism)	Compensation (reduced metabolism)	Normal Function	Compensation (increased metabolism)	Conservation (maximal metabolism)
Stress level	Extreme (lethal)	Moderate to high (pejus to pessimum)	No stress	Moderate to high (pejus to pessimum)	Extreme (lethal)
Indicators	Cellular protection through heat shock response (HSR)	Decreasing respiration, switches in energy reserves and enzyme function (LDH, IDH, ETS, SOD CAT, GPX, PO)		Increasing respiration and overall energy demand (LDH, IDH, ETS), growing oxidative stress (ROS, SOD, CAT, GPX, PO)	Cellular protection through heat shock response (HSR)

Fig. 2: (Upper graph) x-axis: Temperature, y-axis: Oxygen consumption and performance. Conceptual depiction of temperature effects on a given performance indicator (bell-shaped curve) with corresponding aerobic scope breadth (below) given as difference between standard metabolic rate (SMR; green line) and maximal metabolic rate (MMR; red line). At optimal temperature (T_{opt}) performance peaks and aerobic scope reaches its maximum. At both sides of the optimal range the scope for aerobic performance declines (pejus to pessimum range) and is delimited by the lower- (CT_{min}) and upper- (CT_{max}) critical temperature where the aerobic scope diminishes. (Bottom) expected metabolic strategy, stress -level and -response are listed for each temperature window.

The respiration rate of the whole organism represents the energy expenditure for momentary basal maintenance functions, also known as standard metabolic rate (SMR). The energy demand for basal maintenance plus normal activity, such as scavenging, is termed routine metabolic rate (RMR). The fully stretched respiration capacity (i.e. due to maximal swimming speed or stress) is defined as maximal metabolic rate (MMR). The difference between SMR and MMR (aerobic scope) represent a useful measure to define organism fitness (Verberk et al., 2015) (Fig.2) and is often used to define stress levels. On the biochemical tier the activity of the electron transport system (ETS), located in the mitochondria, is an indirect measure to quantify respiration. Next to the actual oxygen uptake of the whole organism, the ETS activity provides a measure of the tissue specific maximal oxygen uptake potential. While changes in respiration through ventilation represent immediate adjustments, modifications in ETS activity are usually lacking behind and indicate longer-term alterations (days to weeks) of the energy metabolic system. Changes in key metabolic enzymes activity can provide useful information about the prevalent energy turnover. The enzyme lactate dehydrogenase (LDH) catalyzes the reduction of pyruvate, which makes it a key player in anaerobic energy cycling. Iso-citrate dehydrogenase (IDH) on the other hand, catalyzes the reduction of iso-citrate, which is a crucial step in the aerobic citrate cycle. Relative changes of both enzymes are important indicators on how efficiently the available energy (aerobic scope) is used and when time-limited anaerobic metabolism becomes critical. Thermal stress can also cause switches in the utilization of energy reserves. Signs of stress can either be a reduction of total energy reserves (carbohydrates, lipids and proteins) in relation to energy consumption (ETS), also known as cellular energy allocation (CEA; De Coen and Janssen, 1997; 2003), or a switch from a carbohydrate and lipid dominated to a protein-fueled catabolism (Bayne, 1973). When metabolic demand outpaces the oxidation of food and energy reserves (i.e. at critically high temperature), animals are approaching the boundary of oxidative stress. Although efficient, the respiration of oxygen is a `radical life giver´ (Abele, 2002) as its partial reduction leads to the formation of toxic bi-products, termed reactive oxygen species (ROS) (Halliwell and Gutteridge, 1985). The most common ROS are the radical species superoxide anion ($O_2^{\bullet -}$); 1-electron reduction) and hydroxyl ($OH^{\bullet}$; 3-electron reduction), and the non-radical species H_2O_2 (2-electron reduction). Whereby, the most potent radical ($OH^{\bullet}$) forms through the reaction between $O_2^{\bullet -}$ and H_2O_2 (Livingstone, 2003). Free ROS can be taken up by radical scavengers (i.e. reduced glutathione (GSH), carotenoids and vitamin C, E, A), or can be converted to less-reactive products through antioxidant enzymes (Hermes-Lima et al., 1998; Livingstone, 2003). The

enzymatic defense chain converts $O_2^{\bullet -}$ to H_2O_2, through superoxide dismutase (SOD), which is then further detoxified by catalase (CAT) and glutathione peroxidase (GPX). Under normal conditions there is a balance between pro-oxidant products and the detoxification by the antioxidant system. Under stressful conditions, however, the balance can be disturbed towards an accumulation of ROS, which causes enhanced activity of antioxidant enzymes and ultimately lead to DNA damage and tissue degradation (Sokolova, 2003), such as lipid peroxidation (LPO). Prolonged oxidative stress with its associated damaging effects on macromolecules and alterations of critical cellular processes (rev. by Kültz, 2005) can have deleterious consequences on organism health and fitness (Livingstone, 2003). Therefore, a non-centralized response of the immune system such as elevated activity of phenoloxidase (PO), which is known as general reaction to wound healing, can also serve as indirect measure of oxidative stress. Under acute stress, when the damage of cellular components by ROS is ongoing, the heat shock response (HSR) provides the last line of defense. The HSR involves the release of last energy reserves, to synthesise a highly conserved group of molecular chaperons, known as heat shock proteins (Hsps). These chaperons are critical to sustain protein homeostasis by refolding denatured proteins and through breakdown and removal of irreparable proteins (rev. by Tomanek, 2008). The increased activity of a number of Hsps has been associated with decreasing aerobic activity (Anestis et al., 2008).

The heterogeneous effects of global warming

The adaptation to distinct thermal environments has led to different acclimation capacities in various taxa as well as in congeners of one species. The `temperature variability hypothesis´ predicts that animals from temperate zones (eurytherms) are able to acclimatize over a wide temperature range, while tropical and polar animals (stenotherms) have a much more narrow temperature spectrum, to which they can successfully acclimate (Janzen, 1967; Addo-Bediako et al., 2000; Sunday et al., 2011). The striving for efficient energy use can be seen as driving force for thermal specialization (Pörtner, 2006a). The large scope for phenotypic plasticity, which is inherent for eurytherms is costly. Functions that cause additional energy expenditure include temperature-driven gene regulation, broad variety of enzymatic iso-forms that cover a wide thermal range, thermal adjustments of mitochondria and compensatory regulations of the respiratory system (Pörtner, 2006b; Pörtner, 2010; Somero et al., 2011). This means that under stable conditions stenotherms can outcompete eurytherms, due to their lower energetic maintenance costs (Angilletta, 2009). However, it is assumed that

the narrow thermal window breadth of specialized stenotherms leads to a steeper slope of the metabolic curve and, thus, to higher metabolic costs, for a temperature change of the same magnitude, compared to eurytherms (Verberk et al., 2015). Hence, it is expected that the adaptation to relatively smaller temperature variations at lower latitudes and in polar-regions make organisms in these areas more susceptible to thermal fluctuations (Tewksbury et al., 2008). This means that global climate change will have heterogeneous effects on different species fitness, reproduction and survival (Deutsch et al., 2008; Somero, 2010). A comprehensive understanding of mechanisms underlying thermal tolerance levels and acclimation performance is required to determine species-specific vulnerabilities to climate change. In this context, studies between individuals of one species, across its distribution breadth, and congeners that inhabit contrasting thermal environments are highly relevant to predict population shifts on local and global scales.

Gaps of knowledge

Despite *Holothuria scabra's* aquaculture relevance and considerable advances in hatchery production of this species, a detailed understanding of the thermal tolerance from the metabolic perspective is lacking. Such data, though, will be imperative in the face of a changing climate and expected increased frequencies of heat events. Mass mortalities associated to over heating have been documented, yet the mechanisms underlying fatal stress levels remain elusive. Moreover, potential connections between temperature stress and disease outbreaks due to impaired immune performance remain unsolved. On the other hand the characterization of positive temperature effects on growth and performance of *H. scabra*, through moderate temperature changes within the coping range, might be a lucrative yet untapped research field. While thermal conditions close to the upper limit might be preferable for optimal growth, reduced temperatures, with means to slow-down the metabolism, could be favorable during transport. Until now, the effects of temperature on *H. scabra* have only been measured on the level of behavior (i.e. burrowing cycle and scavenging activity) and growth. In all conscience, there are no reports on optimum and critical temperatures on the basis of metabolic rate and cellular stress levels available. It can be expected that there is no one optimal temperature that fits for all life stages, thermal preference and temperature for optimal growth need to be evaluated for each age and size class. These questions need to be solved to exploit this endangered species in a more sustainable way and to predict the effects of future global warming.

Objectives

The principal goal of this thesis is a holistic depiction of temperature effects on the ecologically and economically relevant sea cucumber species *Holothuria scabra*. The first objective was to establish a selection of suitable biomarkers encompassing molecular, biochemical and physiological methods, which enable thermal stress detection at different biological organization levels. The second objective was to identify patterns of tissue specific stress levels that characterize acute thermal shock and long-term thermal acclimation. The third goal was to compare thermal acclimation patterns identified for *H. scabra* with acclamation characteristics in a temperate congener species *Holothuria forskali*. Temperature is a critical environmental variable in *H. scabra* aquaculture that directly determines animal health and production output. Moreover, as global warming is expected to outpace the thermal adaptation capacity for most species (Hofmann and Todgham, 2010), the capability of *H. scabra* to defend multilevel homeostasis through acclimatory adjustments can be considered as critical ability to buffer future warming events. Hence, this thesis strives to answer the following three research questions.

1. What are the underlying mechanisms of long-term thermal acclimation on the level of respiratory physiology, energy metabolism, cellular oxidative stress and immune activity in juvenile *Holothuria scabra*?

2. What is the temperature-induced aerobic performance breadth of juvenile *Holothuria scabra*? At which critical cold- and warm- temperature limits occurs functional disruption of homeostasis, due to metabolic regression?

3. Is the tropical species *Holothuria scabra* facing a higher metabolic challenge as response to thermal acclimation than the temperate congener species *Holothuria forskali*? What are potential implications for future ocean-warming?

Throughout this study we used respiratory tree and longitudinal muscle as target tissue for all analyses. The suitability of both tissues will be compared.

Chapter and publication outline

This general introduction aims to address relevant background information that are important for the reader to understand the overarching context of this study. In the following four chapters the core research work of this doctorate thesis is presented. The subsequent final part provides the overall discussion, including conclusion on final outcomes and future perspectives.

Chapter 1: This chapter focuses on the metabolic capacity of juvenile *Holothuria scabra* to cope with mild cold- and warm- temperature stress, over an extended period of 30 days. Lab-based temperature manipulation experiments were conducted in which the animals acclimated to constant temperature conditions (6 °C above and below optimal conditions). In this study the analytical scope encompassed whole organism and cellular oxygen uptake, key metabolic enzymes activity and status of energy reserves.

Chapter 2: This chapter is based on the same experiment as described in chapter 1. In this part, though, the analytical focus is on changes of cellular oxidative stress levels and immune function in juvenile *Holothuria scabra,* as response to the thermal-acclimation stress (6 °C above and below optimal conditions).

Chapter 3: The focus of this chapter is the characterization of critical high- (CT_{max}) and low- (CT_{min}) temperature limits, due to acute thermal stress, for juvenile *H. scabra*. Test animals were exposed to fast temperature up- (+2 °C per hour) and down- (-2 °C per hour) regulations until the trajectory of temperature driven respiration rate was interrupted. Next to respiration performance this chapter focuses on the cellular heat shock response, through the gene expression of heat shock proteins.

Chapter 4: This part compares the findings on *Holothuria scabra's* metabolic acclimation capacity with the outcome of an identical experiment that was implemented with the congener species *Holothuria forskali*. The aim of this chapter is to compare metabolic strategies between a tropical (*H. scabra*) and a temperate (*H. forskali*) congener and discuss the results in the context of climate change susceptibility.

List of publications

Publication 1)

Kühnhold, H., Kamyab, E., Novais, S., Indriana, L., Kunzmann, A., Slater, M., Lemos, M., 2017. Thermal Stress Effects on Energy Resource Allocation and Oxygen Consumption Rate in the Juvenile Sea Cucumber *Holothuria scabra*. Aquaculture 467: 109–17.

This study was initiated and implemented by H. Kühnhold. Experiments were setup in collaboration with M. Slater. Laboratory analyses were conducted in the laboratory facilities of S. Novais and M. Lemos. Experimental maintenance and lab-work were strongly supported by E. Kamyab, who worked as masters student under the supervision of H. Kühnhold. Data analyses and manuscript writing was done by H. Kühnhold with revisions from all co-authors.

Publication 2)

Kühnhold, H., Kamyab, E., Novais, S., Alves, L., Indriana, L., Kunzmann, A., Slater, M., Lemos, M., 2017. Effects of thermal stress on the immune and oxidative stress responses of juvenile sea cucumber *Holothuria scabra*. J Comp. Physiol. B 187(1): 51-61.

The experimental setup and work procedures were identical to publication 1. This time a bigger share of the data interpretations and manuscript writing was done by E. Kamyab (both authors share first-authorship).

Publication 3)

Kühnhold, H., Steinmann, N., Huang, Y., Indriana, L., Meyer, A., Kunzmann, A. (XXXX) Respiration and Hsp70 induction can characterize thermal tolerance in marine ectothermic species. Submitted to Journal of Experimental Biology.

This work was initiated and implemented by H. Kühnhold. Respiration data were independently collected from H. Kühnhold during field work in Indonesia. Lab protocols for the gene expression analyses were established with the help of bachelor student N. Steinmann

and the master student Y. Huang under the supervision of A. Meyer and H. Kühnhold. Data analyses and manuscript writing were conducted by H. Kühnhold with revisions from all co-authors.

Publication 4)

Kühnhold, H., Novais, S., Alves, L., Indriana, L., Kamyab, E., Lemos, M., Slater, M., Kunzmann, A. (XXXX) Acclimation capability inferred by metabolic performance in two sea cucumber species from different latitudes. Submitted to the journal Global Change Biology.

This study required the same infrastructure as used for publication one. The majority of the experimental planning, lab-work, data analyses and manuscript writing were independently initiated and conducted by H. Kühnhold, with minor support and revisions from all co-authors.

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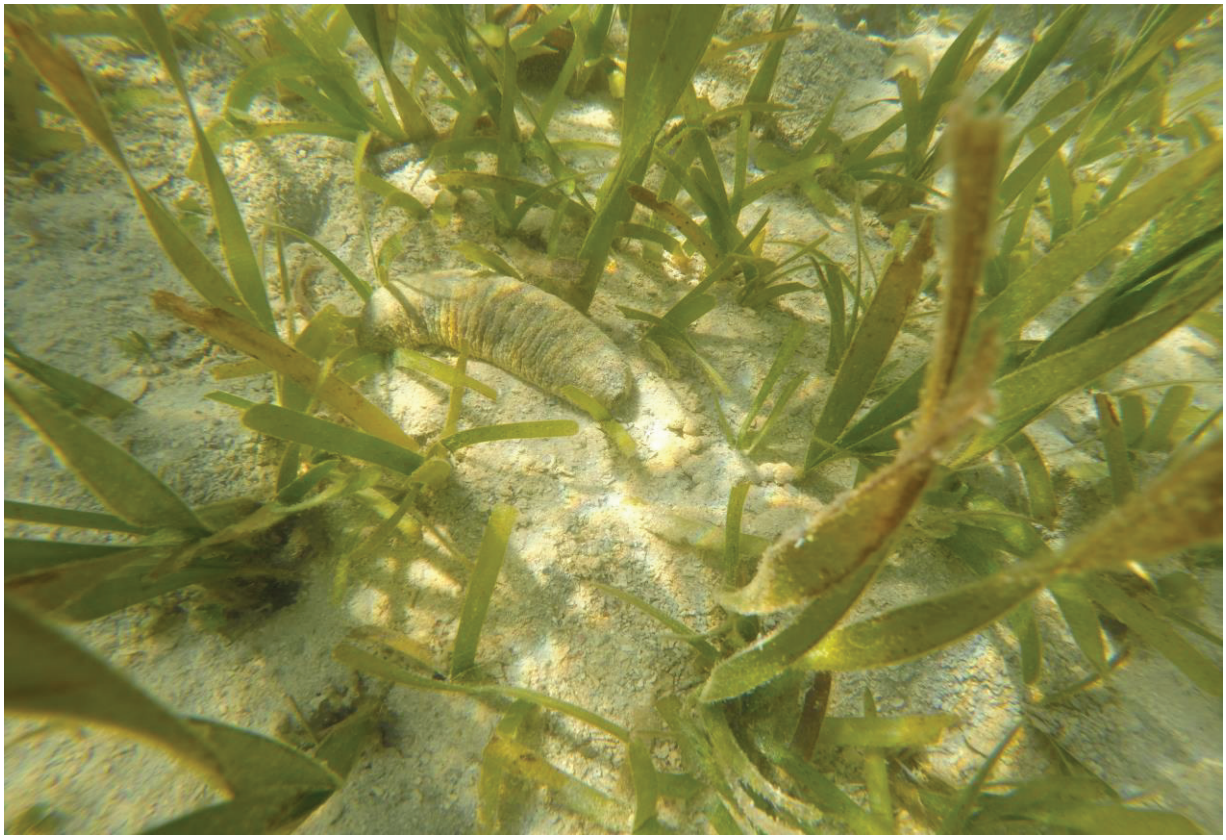
Chapter 1:
Thermal acclimation effects
on metabolic system components



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Thermal stress effects on energy resource allocation and oxygen consumption rate in the juvenile sea cucumber, *Holothuria scabra* (Jaeger, 1833)

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Abstract

Water temperature is a key factor in aquaculture production of the commercially valuable sea cucumber *Holothuria scabra*. Knowledge is scarce about actual energetic costs that can be associated with internal acclimatization processes as a response to thermal extremes. In the present study changes in cellular energy allocation, oxygen consumption rate and energy related enzymes' activity (IDH and LDH) were measured in juvenile *H. scabra,* held at different temperatures: 21, 27 and 33 °C. The results showed that the steady temperature change (1 °C/day) to both temperature treatments, until reaching the testing temperatures (day 0), clearly affected cellular energy consumption and available energy reserves, measured in the respiratory tree and muscle tissue, respectively. However, 15 and 30 days after acclimation, the initial differences in cellular energy allocation between treatments decreased. In contrast to the variations measured in cellular energy allocation, oxygen consumption was highest at 33 °C and lowest at 21 °C at all three measurement times. Moreover, a significant positive correlation between oxygen consumption rate and temperature was detected at day 15 and day 30. Likewise, a shift from anaerobic to aerobic energy metabolism, indicated by changes in LDH and IDH activities, was observed in the animals from the warm temperature treatment. Results imply that juvenile *H. scabra* were able to recover from initial disturbances in energy balance, caused by the incremental temperature change of ± 6 °C. Over the experimental period of 30 days, elevated temperature did however, lead to a metabolic shift and more efficient energy turnover, indicated by changes in oxygen consumption rate, LDH and IDH. The synergy of cellular energy allocation and oxygen consumption proved to be a viable indicator to assess the capability of sea cucumbers like *H.*

scabra to cope with extreme temperature conditions. Surprisingly, juvenile *H. scabra* were able to sustain their energy balance and oxygen consumption rate within the homeostatic range, even at 33 °C. Thus, we assume that rearing temperatures of 33 °C might be possible, which could improve aquaculture production of *H. scabra*. However, further research is required to understand the mechanisms and effects of acclimation under aquaculture conditions.

***Keywords**: Rearing temperature, cellular energy allocation, metabolic enzymes, energy balance, respiration, oxygen consumption rate*

Introduction

Global demand for sea cucumbers, sold as a luxury food product, has led to signs of over-exploitation reported in at least 24 countries (Purcell et al., 2014). Consequently, the relevance of sea cucumber aquaculture has increased considerably over the last few decades (Lovatelli et al., 2004). While aquaculture production contributed only less than 1 % of the total production in 2002, it had already increased to 25% in 2011 (Eriksson and Clarke, 2015). This significant growth can be primarily attributed to the increased aquaculture production of *Apostichopus japonicus* (94% in 2011), mostly in China (Eriksson and Clarke, 2015). Driven by this development, interest in establishing aquaculture practices for tropical species has also increased. The sea cucumber *Holothuria scabra* is among the highest priced species and is considered a promising aquaculture candidate in the tropics (Hamel et al., 2001; Eriksson and Clarke, 2015). Prior to the grow-out phase in outdoor ponds, juvenile *H. scabra* are cultured in indoor tank systems, where optimal environmental conditions are critical for growth and survival. Water temperature is a key parameter, affecting virtually all physiological, as well as, intercellular mechanisms in ectothermic animals (Somero, 2010). Therefore, best aquaculture management for sea cucumbers requires the detection of thermal stress at an early, sub-lethal stage. To date, however, to our knowledge, information about reliable condition indicators for thermal stress in sea cucumbers is scarce.

Previous studies on *A. japonicus*, clearly demonstrate a strong effect of ambient water temperature on growth and feeding (Yuan et al., 2007, 2009, 2013; Yang et al., 2005), metabolic performance (An et al., 2007; Yang et al. 2006; Wang et al., 2013), immune and antioxidant function (Wang et al., 2008), physio-ecological parameters (such as rates of ingestion, defecation, growth, respiration and excretion) (Yuan et al., 2007, 2013),

scavenging capacity (Yuan et al., 2013), heat shock protein expression (Wang et al., 2008; Wang et al., 2013; Shao et al., 2015), and also metabolite synthesis (Shao et al., 2015). These studies clearly indicated that the energetic scope for growth in *A. japonicus* increased with temperature, reaching its peak between 14 – 16 °C (Yuan et al., 2007, 2009; Yang et al., 2005). Above this optimal range, the percentage of energy allocated to growth decreased, while the expenditure for excretion and respiration increased, until negative growth started between 20 – 25 °C, depending on body size and maturation status (Yang et al., 2005, 2006). By no later than 30 °C animals discontinued feeding and faeces discharge (Yuan et al., 2007, 2009, 2013). Zamora and Jeffs (2012, 2015) described similar patterns for the species *Australostichopus mollis*. These authors showed that a temperature elevation from 15 – 21 °C increased this sea cucumber species metabolism, but lessened food intake as well as nutrient selection efficiency and growth. For *H. scabra* a clear positive correlation between water temperature and burrowing behaviour was detected (Mercier et al., 1999; Wolkenhauer, 2008). Mercier et al. (1999) reported that a constant water temperature of 29 °C prevented juvenile *H. scabra* from burrowing, which led to extended periods of activity and food intake. Increased growth of juvenile *H. scabra* was observed up to a temperature of 31 °C (Lavitra et al., 2010). At temperatures below 24 °C, however, *H. scabra* showed distinctly lower activity (Purcell et al., 2006; Wolkenhauer, 2008) and extended periods of being buried (Wolkenhauer, 2008). Shao and co-workers (2015) measured a decreased ATP synthesis and increased glucose levels in *A. japonicus* muscle tissue, starting at 4 °C above optimal temperature, and concluded that high temperature stress is the key influencing factor concerning disturbances of the energy metabolism in *A. japonicus*. To date the effects of temperature stress have been extensively studied in *A. japonicus*. So far, however, these studies have only looked at acclimation patterns associated with short-term temperature changes (Wang et al., 2008; Zhang et al., 2013; Shao et al., 2015) and fluctuations (Dong et al., 2008), or on the effects of long-term acclimation on critical thermal maxima values (Meng et al., 2009; Wang et al., 2013). What has not been accounted for to date is information concerning the actual changes in energy expenditure and available energy reserves, that can be associated with long-term acclimation processes to sub-lethal thermal stress conditions. Moreover, comparative studies on thermal stress effects in other sea cucumber species, in particularl from tropical regions, are very limited.

The species *Holothuria scabra* (Jaeger, 1833), commonly known as sandfish, is an Aspidochirotid sea cucumber, which has been well studied (reviewed by Hamel et al. 2001),

mainly due to its importance in artisanal and commercial fisheries in the tropical Indo-Pacific region (Hamel et al. 2001; S. Purcell et al., 2012). Sandfish are deposit-feeders, inhabiting oligotrophic environments such as sandflats linked to seagrass and mangroves, or in the vicinity of fringing reefs (Purcell et al., 2012). Previous studies about the ecological relevance of sea cucumbers such as *H. scabra* have shown that these animals promote the productivity of ecosystems (such as sea grass meadows) significantly through bioturbation, nutrient recycling and dispersion of calcium carbonate (Uthicke and Klumpp, 1998; Uthicke, 1999; Uthicke, 2001; Hamel et al., 2001; Wolkenhauer et al., 2010; Schneider et al., 2011). Moreover, because of its daily burrowing cycle, where sandfish dig through the upper sediment layers, it is assumed that *H. scabra* influences sediment displacement and bioturbation to a much greater extent than other sea cucumber species, which feed on surface sediments exclusively (Wolkenhauer et al., 2010). Sandfish are broadcast spawners with separate sexes, which are indistinguishable until the initiation of spawning (Hamel et al., 2001). The size at sexual maturity differs between regions (Hamel et al., 2001; Purcell et al., 2012) Conand (1990) reported a length of 16 cm and a total weight of 184g for spawning sandfish in New Caledonia, whereas, Indriana and Hilyana (2014) documented a body weight-range of 70 – 100g for first spawning sandfish adults on Lombok, Indonesia. Approximately 14 days after larvae forming, Sandfish larvae reach the doliolaria-stage in which they settle on seagrass leaves (Indriana and Hilyana, 2014), before adopting a benthic mode of life as juveniles. *H. scabra* inhabits preferably shallow muddy-sand milieus (Mercier et al., 2000), where it exhibits a daily burrowing behavior (Wolkenhauer, 2008). During grow out, adult animals often inhabit also deeper zones (<20m) (Mercier et al., 2000; Purcell et al., 2012). *H. scabra* is generally slow moving and habitat bound (Hamel et al., 2001). This lifestyle, together with their high market value (Hamel et al.. 2001; Purcell et al., 2014), make these species prone to overfishing, which has led to severe population declines in the past. In 2010 H. scabra was listed as an endangered species in the IUCN Red List (IUCN, 2015). Consequently, fishing for this species was banned temporarily in the most impacted zones, such as Papua New Guinea (Hair et al., 2016) and Tanzania (Mgaya and Mmbaga, 2007; Eriksson et al., 2010), in order to let wild stocks recover and to implement new management practices (Hair et al., 2016). Sea cucumber aquaculture has recently been implemented in several tropical countries (Eeckhaut et al., 2008; Eriksson et al., 2012; Purcell et al., 2013). Whereby, sandfish is regarded as the most promising candidate for a sustainable aquaculture (Raison, 2008; Robinson et al. 2013), with the potential to provide livelihood options for

remote coastal communities as well as to reduce fishing pressure on wild populations (Rasolofonirina et al., 2004; Eeckhaut et al., 2008; Eriksson et al., 2012; Purcell et al., 2013; Hair et al., 2016). The commercialized farming of *H. scabra* in Madagascar (Eeckhaut et al., 2008) and pilot studies on the co-cultivation of *H. scabra* with the seaweed *Kappaphycus striatum* in Tanzania (Beltran-Gutierrez et al., 2014), are successful examples of such sustainable culture approaches.

The main goal of this study was to evaluate the metabolic balance in juvenile *H. scabra* when brought to sub-optimal temperature conditions for a period of 30 days. Changes in energy expenditure and metabolic shifts were measured to improve the understanding of energetic costs associated with internal defence mechanisms, triggered by temperature.

Materials and Methods

Experimental Design

Juvenile sea cucumber *Holothuria scabra* (Jaeger, 1833) were sourced from the hatchery facilities of the Indonesian Research Centre for Oceanography (LIPI) on Lombok, Indonesia and transported to the Alfred Wegener Institute, Helmholtz-Centre for Polar and Marine Research (AWI) in Bremerhaven, Germany. Upon arrival 63 test animals were sorted by wet weight into three weight classes (<10g, 10–15g and >15g). Subsequently, the animals were randomly assigned, making sure that the weight distribution was uniform, to three separate HDPE tanks (n = 21) filled with 100L aerated seawater (27 °C, 33 ppt). The photoperiod was set to 12h:12h (light:dark) and sea cucumbers were fed *ad libitum*, with a the commercial feed `Algamac´, with the nutrient composition 39% protein, 20,4% lipid and 20,6% carbohydrate (www.aquafauna.com). After an acclimation period of 28 days, the temperature in one tank was increased incrementally (+1 °C/day), using electric aquaria heaters, and another tank was cooled down (−1 °C/day), using a cooler, to the designated temperatures 33 °C (warm treatment) and 21 °C (cold treatment), respectively. The water temperature in the third tank remained at 27 °C to serve as control treatment. According to Lavitra et al. (2010) *H. scabra* increases its growth performance up to a temperature of 31 °C. Whereas, at temperatures below 24 °C *H. scabra* showed a clearly decreased overall activity and metabolism (Purcell et al., 2006; Wolkenhauer, 2008). Hence, for this study we chose the two treatment temperatures 21 °C and 33 °C, in order to create a mild temperature stress, at which we expected no physical dysfunctions in the animals. When the experimental temperatures were reached (t0), the animals were kept at constant conditions for 30 days. At t0, day 15

(t15) and day 30 (t30), six animals from each treatment (n = 6) were removed and frozen immediately at −80 °C for further analyses. Additionally, for each of the sampling days, three animals (n = 3) from each treatment were placed into gas-tight chambers for respirometry measurements.

Tissue Preparation

For the biochemical and metabolic measurements, longitudinal muscle (muscle), and the respiratory tree of sea cucumbers were removed from the organisms, and homogenized in a potassium -phosphate buffer (0.1M, pH = 7.4) in a 1:4 proportion (w/v). The muscle tissue was used for measuring LDH and IDH activities as well as to quantity the energy reserves content and ETS activity, whereas the respiratory tree was used for the measurement of ETS activity (150 µl of homogenized tissue). The homogenate solution of muscle tissue was divided by transferring 150µL for each of the following content determinations: 1) total protein and carbohydrate, 2) total lipid, and 3) ETS activity. The remaining homogenate was further centrifuged for 5 min, at 3000g, at 4 °C and the supernatant was aliquoted for LDH and IDH activity measurements. All different tissue fractions were stored at -80°C for further analysis.

Cellular Energy Allocation Assay

The Cellular Energy Allocation method was applied to detect thermal stress in juvenile *H. scabra*. Cellular energy allocation represents the ratio between the available energy reserves (Ea; total carbohydrate, protein and lipid content) and the energy consumption (Ea: measured through the electron transport system (ETS) activity), within an organism. The relationship between available energy and the rate of energy consumption was then integrated into the cellular energy allocation value and presented as mJ/mg of organism wet weight. The procedures for the measurement were adopted and optimized from De Coen and Janssen (1997, 2003). An evaluation of the sensitivity of cellular energy allocation was achieved in the present study through measurements in muscle and the respiratory tree, to detect metabolic shifts initiated by cold and warm temperature stress in juvenile *H. scabra*.

Energy available (Ea)

Total protein, lipid and carbohydrate content were measured spectrophotometrically in the muscle homogenates, to evaluate the status of available energy reserves in the muscle tissue

at each time point. According to the approaches outlined by De Coen and Janssen (1997, 2003), the total protein content was determined using the Bradford method (Bradford, 1976), with bovine serum albumin as standard, measuring absorbance at 600 nm. The total carbohydrate content was determined with phenol 5% and H2SO4 (95-97%) with glucose as standard, measuring absorbance at 490nm (De Coen et al., 1997). The total lipid content was assessed according to Bligh and Dyer (1959), using tripalmitine (Sigma) as standard and measuring absorbance at 400nm. All samples were measured in triplicate in a Synergy H1 Hybrid Multi-Mode microplate reader (Biotek® Instrument, Vermont, USA). Finally, each energy reserve fraction measured in 150 µl of sample was extrapolated for the weight of homogenized muscle, and then transformed into their energetic equivalents using enthalpy combustion (24 kJ/g proteins, 17.5 kJ/g carbohydrates, and 39.5 kJ/g lipids), following De Coen and Janssens (1997, 2003) procedure.

Energy consumption (Ec)

Energy consumption was measured in form of the maximal oxygen uptake capacity, represented by the ETS activity, at each time point. ETS activity was measured in both muscle and respiratory tree tissues. According to De Coen and Janssen (1997), the ETS activity was measured spectrophotometrically by adding NADPH solution and INT (*p* iodo-nitro-tetrazolium) (Sigma) to the sample. The resulting increase in absorbance was measured at 490nm for 3min. All samples were measured in triplicate. As described by De Coen and Janssen (1997), the formation of 2µmol formazan equals the consumption of 1µmol O_2 in the ETS. The resulting oxygen consumption rate was then converted into caloric values using oxyenthalpic equivalents of 484 kJ/molO_2, which accounts for an average carbohydrate, lipid, and protein mixture (Gnaiger, 1983).

Cellular Energy Allocation (Ea/Ec)

To determine the cellular energy allocation value, the total Ea in the muscle of each organism, at each time point (t0, t15, t30) and in each treatment (21 °C, 27 °C, and 33 °C), is given by the sum of the three energy reserve caloric values. The Ec, for same conditions, was calculated as the ETS activity transformed into caloric values. The cellular energy allocation, representing the total net energy budget of the organism, was then calculated with equation 1, according to Verslycke, et al. (2004a, 2004b).

Cellular energy allocation = Ea/Ec (1)

With Ea = Protein + lipids + carbohydrates (mJ/mg of sample)
Ec = ETS activity (mJ/mg of sample)

Direct Measurements of Energy Metabolism

In addition to cellular energy allocation, thermal acclimation processes were addressed through the measurement of oxygen consumption rate, and the activity of two key metabolic enzymes: iso-citrate dehydrogenase (IDH) and lactate dehydrogenase (LDH). In this way, the temperature effect on cellular energy allocation could be analysed in relation to the immediate energy demand (oxygen consumption rate) and the prevalent type of energy turnover (LDH/IDH).

Oxygen consumption rate

For the respirometry measurements, four gas tight Acrylic Chambers (AC) (V = 1L) were placed into each of the three experimental tanks. This enabled the measurement of organism respiration under exact test conditions. From each experimental tank, three singularly re-identifiable sea cucumbers (the same animals were used throughout the experiment) were placed individually in the AC; one AC per treatment remained empty and served as a control. All ACs were closed for one hour and during this period, a pump (300L/min) circulated the water within the AC to ensure homogeneous water conditions. The oxygen concentration within the closed chambers was measured using a Firesting-system (Pyroscience GmbH, Germany), consisting of optical cables connected to sensor spots. The oxygen concentration was measured in one sec. intervals. The data were directly logged on a computer using the Pyroscience software. The rate of oxygen consumption in the chambers was determined by adding a linear trendline over the measurement period of 1 hour. The oxygen consumption rate was calculated using the resulting linear regression equation in $mgO_2\ g^{-1}\ h^{-1}$.

Lactate and Isocitrate Dehydrogenase (LDH and IDH) activity

LDH activity was measured following the method described by Vassault (1983), with adaptations of Diamantino et al. (2001), using NADH and pyruvate as energy supplier and substrate for the reaction, respectively. IDH was measured following the method described by Ellis et al., (1971) and adaptations of Lima et al., (2007). In this reaction, $NADP^+$ is

oxidized to NADPH when isocitrate is decarboxylated by IDH. For both assays, the samples were transferred to microplates for spectrophotometrical analysis in a Synergy H1 Hybrid Multi-Mode microplate reader (Biotek® Instrument, Vermont, USA). The reactions were measured at 340 nm for 5 min (LDH) and 3 min (IDH). Referring to the molar extinction coefficient (Ɛ) of $6.3x10^3$ $muscle^{-1}$ cm^{-1} for both enzymatic reactions, the results were calculated and expressed in nmol min^{-1} mg^{-1} protein.

Statistical Analysis

Significant differences between temperature treatments (21 °C, 27 °C, and 33 °C) and time points (t0, t15 and t30) were determined with a two-way ANOVA, after testing data for normality and homogeneity of variance using the Kolmogorov–Smirnov and Levene tests, respectively. When differences were found, the Holm-Sidak post-hoc test was used for multiple comparisons using Sigma Plot software for Windows, version 11.0 (SigmaPlot, 1997). Where applicable, results are presented as mean ± SE. For all statistical tests, the significance level was set at $p \leq 0.05$.

Results

Energy available (Ea)

Of the three measured available energy fractions (Fig. 1), carbohydrates represented the reserve with the highest level of fluctuation over the experimental period of 30 days (Fig. 1). At t0 the sea cucumbers in the warm treatment exhibited significantly lower carbohydrate levels compared to the specimens that were in the cold ($p = 0.0024$) and control ($p = 0.0386$) treatments. From t0 to t15 the warm acclimated sea cucumbers increased their carbohydrate reserves significantly ($p = 0.0001$), whereas their levels in the control and cold treatments decreased significantly compared to t0 ($p = 0.0111$; $p = 0.0192$) and exhibited significantly lower levels relative to the warm conditions ($p = 0.0001$; $p = 0.0006$). At t30 no significant differences in carbohydrates, between treatments, were found.

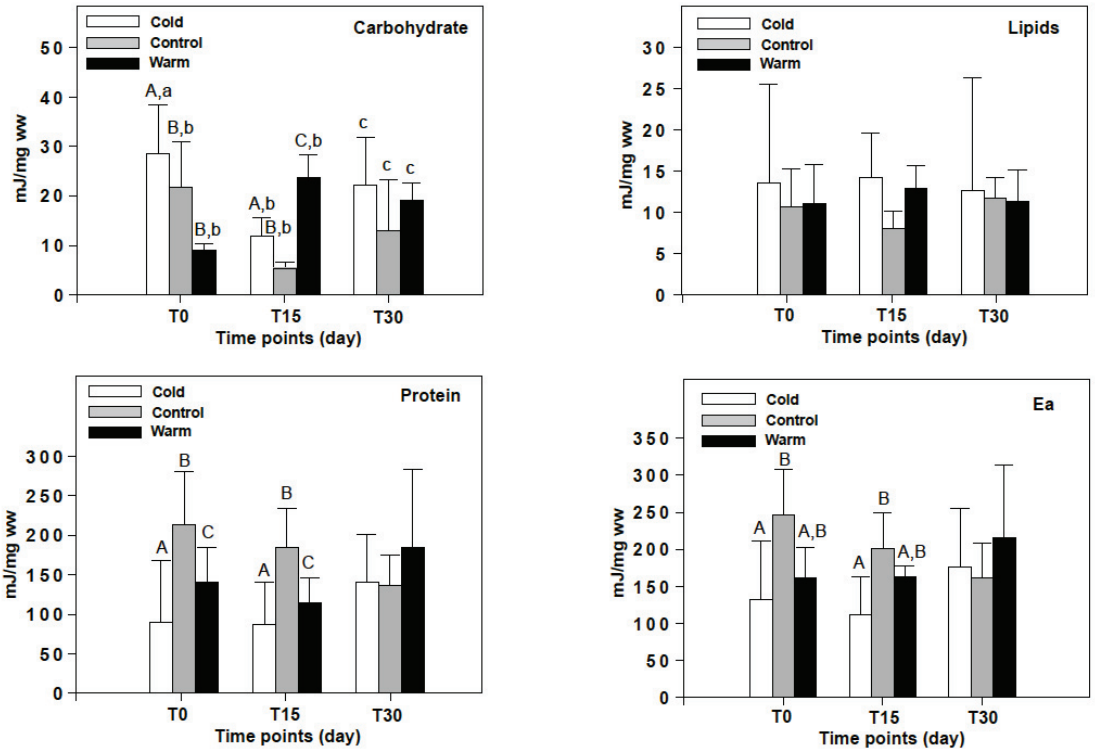

Fig. 1: Effects of thermal stress on the available energy of juvenile *Holothuria scabra*. Carbohydrates, Lipids, Proteins and total available energy (Ea) levels, measured at each time point (t0, t15 and t30) and for each temperature treatment (21 °C, 27 °C and 33 °C). $^{A, B}$ indicates significant differences between temperature treatments within each time point (two-way ANOVA, Holm-Sidak, $p<0.05$). $^{a, b}$ indicates significant differences between time points within temperature treatments (two-way ANOVA, Holm-Sidak, $p<0.05$).

Significant differences in protein levels between treatments occurred only at t0 and t15 (Fig. 1). At the first two time points the control animals had higher protein levels compared to the levels measured in animals in cold (t0: $p = 0.0223$; t15: $p = 0.0087$) and warm (t0: $p>0.05$; t15: $p = 0.0486$) treatments. At t30 the significant differences between the three treatments were no longer observed. The lipidic fraction showed no significant differences between time points or between treatments (Fig. 1). As consequence of the changes in carbohydrate and protein levels at t0 and t15, the total Ea in the sea cucumbers cultured under control conditions were also significantly higher compared to the cold treatment (t0: $p = 0.0281$; t15 = 0.0107) in those two time points. No significant differences were seen between treatments at t30.

Energy Consumption (Ec)

The ETS activity was generally higher in the respiratory tree than in the muscle tissue (Fig. 2). Therefore, it was decided to use the ETS activity measured in respiratory tree as the

indicator for cellular Ec. At t0 Ec was significantly higher in the warm exposed sea cucumbers compared to the animals in control (p = <0.001) and cold (p = <0.001) conditions. Over the experimental time the general alterations in Ec followed a similar trend as the variations in Ea, namely no significant differences between treatments at the latter two measurement times.

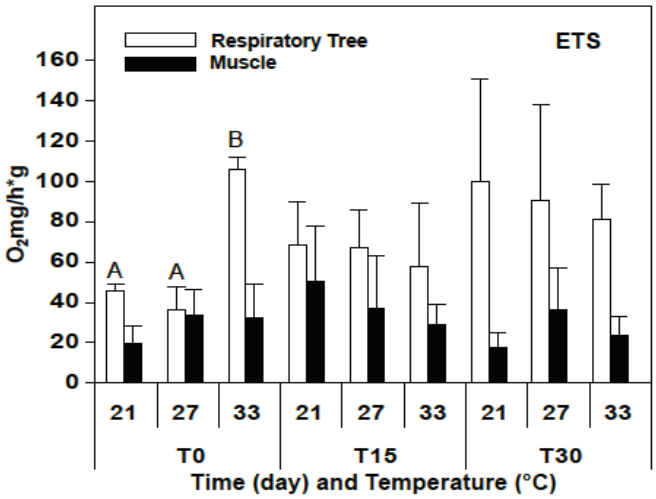

Fig. 2: Cellular energy consumption (Ec) with the electron transport system (ETS) activity in muscle (M) and respiratory tree (RT) of juvenile *Holothuria scabra*, measured at each time point (t0, t15 and t30) and for each temperature treatment (21 °C, 27 °C and 33 °C). Different letters $^{A, B}$ indicates significant differences between temperature treatments within each time point (two-way ANOVA, Holm-Sidak, p<0.05).

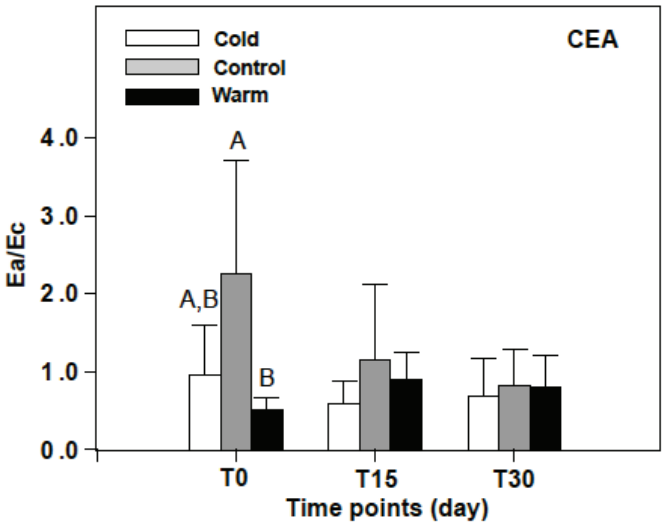

Fig. 3: Results of the cellular energy allocation in juvenile *Holothuria scabra* given by the ratio of total available energy fractions (Ea) in muscle tissue and the ETS activity (Ec) in the respiratory tree calculated for each time point (t0, t15 and t30) and for each temperature treatment (21 °C, 27 °C and 33 °C). $^{A, B}$ indicates significant differences between temperature treatments within each time point (two-way ANOVA, Holm-Sidak, p<0.05).

Cellular Energy Allocation

As for the individual analyses of Ea and Ec, the only significant differences in cellular energy allocation between treatments were measured at t0, followed by diminishing differences over the experimental period of 30 days (Fig. 3). At t0, cellular energy allocation was significantly lower in the warm acclimated sea cucumbers compared to the control animals ($p = 0.045$) (Fig. 3).

Oxygen Consumption Rate

Measurement of direct oxygen uptake ($n = 3$) revealed that the warm exposed specimens had consistently higher oxygen consumption rates and that the cold had usually lower rates (Fig. 4). This trend is clearly visible throughout the experiment, although significant differences between treatments occurred only at t15 (33 °C/21 °C: $p = 0.0002$, 33 °C/27 °C: $p = 0.0065$, 21 °C/27 °C: $p = 0.0111$). When comparing differences between the three measurement times, within the temperature treatments, those sea cucumbers that were exposed to warm and control conditions showed similar oxygen consumption rates. Opposed to that, the cold acclimated specimens reduced their oxygen consumption rate significantly between t0 and t15 ($p = 0.0419$).

Energy Metabolism Related Enzyme Activities (LDH/IDH)

At t0 and t15 no significant differences in LDH levels were observed between treatments (Fig. 5). However, at t30 LDH activity was significantly lower in the sea cucumbers exposed to warm conditions when compared to the control animals ($p = 0.0291$). Over time LDH activity in the warm acclimated animals showed a clear regression over time, with a significant decrease between t0 and t15 ($p = 0.0368$) as well as between t0 and t30 ($p = 0.0029$). IDH activity showed no significant differences, neither between time points nor between treatments (Fig. 5). However, at t30 IDH activity surpassed LDH activity in those sea cucumbers that were cultured for 30 days at 33 °C.

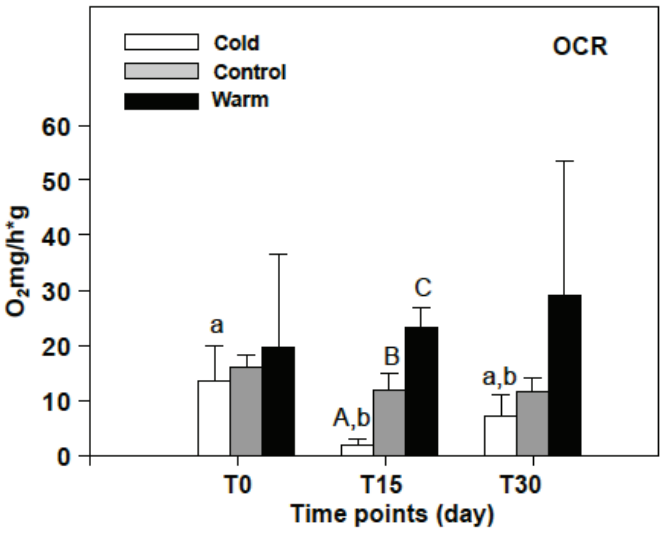

Fig. 4: Oxygen consumption rate of juvenile *Holothuria scabra* (whole organism) measured at each time point (t0, t15 and t30) and for each temperature treatment (21 °C, 27 °C and 33 °C). [A, B, C] indicates significant differences between temperature treatments within each time point (two-way ANOVA, Holm-Sidak, $p<0.05$). [a,b] indicates significant differences between time points within temperature treatments (two-way ANOVA, Holm-Sidak, $p<0.05$).

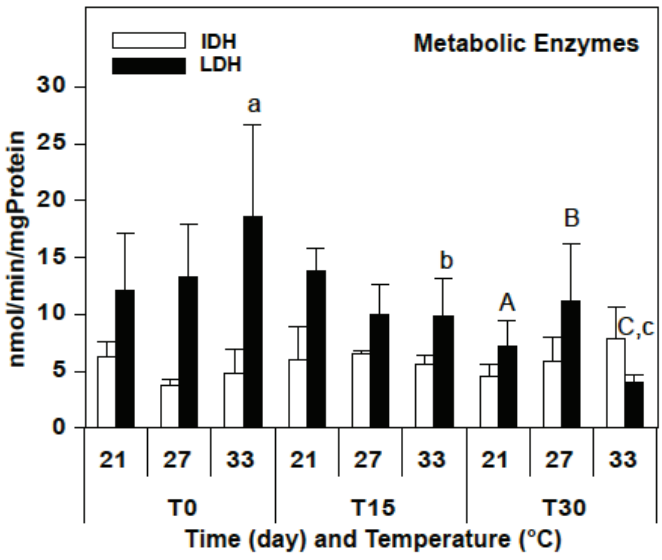

Fig. 5: Activity of the two metabolic enzymes, lactate dehydrogenase (LDH) and iso-citrate dehydrogenase (IDH), measured in muscle tissue of juvenile *Holothuria scabra* at each time point (t0, t15 and t30) and for each temperature treatment (21 °C, 27 °C and 33 °C). [A, B] indicates significant differences between temperature treatments within each time point (two-way ANOVA, Holm-Sidak, $p<0.05$). [a,b] indicates significant differences between time points within temperature treatments (two-way ANOVA, Holm-Sidak, $p<0.05$).

Discussion

Generally, ambient water temperature is considered as one of the most important limiting factors in tropical and temperate sea cucumber aquaculture (Wolkenhauer et al., 2009; Lavitra et al., 2010; Shao et al., 2015). Therefore, knowledge about how candidate species respond to thermal stress in culture is crucial. Mercier et al., (1999) reported that the behaviour of juvenile *H. scabra* did not change at temperatures ranging from 24 °C to 27 °C. Kumara et al., (2013) defined an optimal temperature range of 26 °C – 29 °C for the rearing of *H. scabra* juveniles and larvae. Concerning thermal maxima values it is known that in *H. scabra,* feeding and overall activity decreases distinctly below 24 °C (Purcell et al., 2006; Wolkenhauer, 2008). But, above 27 °C scavenging activity and growth rate of *H. scabra* increases with temperature (Mercier et al., 1999;), up to 31 °C (Lavitra et al., 2010). To our knowledge the upper temperature limit, at which growth and feeding activity collapses, has not been defined yet for *H. scabra*. In this study juvenile *H. scabra* were cultured at 27 °C (control treatment) and exposed to sub-lethal cold (21 °C) and warm (33 °C) temperature stress, over a period of 30 days. To characterize the physiological responses to thermal acclimation, cellular energy allocation in combination with oxygen consumption rate and metabolic enzyme activity was measured. This novel approach provides first insights into the energy budget and biochemical processes underlying the thermal stress physiology of *H. scabra*. In previous studies cellular energy allocation has been successfully used as sensitive marker to assess alterations of the energetic status in animals from diverse environments, from aquatic daphnids (De Coen and Janssen, 1997, 2003), shrimps (Verslycke et al., 2004a, b), mussels (Smolders et al., 2004), fish (Smolders et al., 2003) and snails (Cabecinhas et al., 2015), to terrestrial oligochaets, such as *Enchytraeus albidus* (Novais et al., 2013; Novais and Amorim, 2013). In the latter studies, alterations in cellular energy allocation could also be linked with changes at lower and higher biological levels, such as gene transcription or behaviour, thus conferring this integrative biomarker a high ecological relevance. In the present study, Ea was measured in muscle tissue, and Ec in muscle and respiratory tree tissues. The comparison of the Ec levels in both tissues indicated that ETS activity was generally higher in respiratory tree, showing a clearer response to temperature changes. Hence, respiratory tree was identified to be the most suitable tissue in sea cucumbers to measure Ec for further quantifications of cellular energy allocation. This positive result indicates that the determination of cellular energy allocation in highly conserved tissue is likely to be transferrable to other commercially valuable echinoderms.

The current results show that cellular energy allocation is a sensitive marker to evaluate the metabolic status in juvenile *H. scabra*. Both cellular energy fractions Ea and Ec changed in response to the two temperature treatments. Ea levels in the muscle tissue were negatively affected by cold temperature stress at t0 and t15 whereas differences in Ec and cellular energy allocation were observed after the warm treatment at t0. It is important to note that the lower cellular energy allocation levels in the warm treated animals were a consequence of a significantly higher ETS activity, whereas, the lower cellular energy allocation levels in the cold animals (although not significant) were caused by significantly lower Ea levels (mainly carbohydrates). Hence, it is critical to interpret the Ea and Ec fraction individually first, before drawing conclusions from the cellular energy allocation value alone. The general trend shows that Juvenile *H. scabra* were strongly affected by the initial temperature change of ± 6 °C (1 °C/day) prior to the start of measurements (t0). However, they were quickly recovering their overall energy balance within the experimental period, at both testing temperatures.

Similar to the prevalent energy sources identified for *A. japonicus* (Dong et al. 2006), protein was also the dominant energy source in *H. scabra*, followed by carbohydrates and lipids. Hence, changes in total Ea were greatly influenced by changes in muscle protein. However, it is worth mentioning that the lipid fractions do not seem to be affected by the temperature stress, whereas high fluctuations were measured in the carbohydrate fractions, between treatments and measurement times. This is in accordance with the fact that carbohydrates are the most rapidly available source of energy (Smolders et al., 2003). Our results show that *H. scabra* metabolized carbohydrates as an immediate response to counteract the initial temperature increase (+ 6 °C), which also correlated significantly with higher ETS activity (Pearson´s correlation, $R^2 = -0.571$, $p = 0.03$) measured in respiratory tree. This is opposed to the observation that the initial decrease in temperature (- 6 °C) caused a reduction in protein levels as well as lower ETS activities. This shows distinct differences in metabolic acclimations to cold and warm temperature extremes. Between t_0 and t_{15} the carbohydrate levels increased significantly in the animals exposed to the warm treatment conditions. In previous studies *H. scabra* expressed enhanced growth (Lavitra et al. 2010) and foraging activity (Mercier et al., 1999) with increasing temperatures. Thus, the distinct carbohydrate increase between the first two measurement times at warm conditions, might have been the consequence of increased feeding and/or increased digestion activity. In contrast, the carbohydrate levels in the cold treated animals decreased significantly over the same time period, which might indicate that the feeding rate was inhibited by low temperature.

However, the carbohydrate level in the control animals also decreased between the first two measurement times, indicating that other factors, apart from temperature, can cause small changes in the carbohydrate reserves. Overall, the carbohydrate depletion seemed to occur in cycles of consumption and reallocation, which was also observed in *Enchytraeus albidus* under pesticide exposure (Novais and Amorim, 2013).

In order to compare cellular energy allocation with direct indices of energy metabolism, the oxygen consumption rate of the whole organisms as well as the activities of the two metabolic key enzymes IDH and LDH, were measured. ETS activity represents the theoretical maximum cellular oxygen uptake potential of a particular tissue (King and Packard, 1975). In contrast to that, the oxygen consumption rate represents the true and actual oxygen uptake of the whole organism. The oxygen consumption rate has been determined in several other sea cucumber species to detect metabolic changes. For juvenile *H. scabra* and adult *H. parva*, oxygen consumption ranged from 9 – 12 µg $O_2 gww^{-1} h^{-1}$ (Collard et al., 2014) under optimal conditions, whereas for adult *H. leucospilota* an oxygen consumption rate of 17 µg $O_2 gww^{-1} h^{-1}$ was measured (Yu et al., 2013), under optimal conditions. Wang and co-workers (2013) measured an oxygen consumption rates ranging from 14 – 23 µg $O_2 gww^{-1} h^{-1}$ in juvenile *A. japonicus* at three different temperatures, 15, 20 and 26 °C, and detected a strong positive correlation between oxygen consumption and temperature. The results of this study are in line with the latter findings and confirm a clear pattern of increasing oxygen consumption with elevated temperature, 3-12 µg $O_2 gww^{-1} h^{-1}$ (21 °C), 11–16 µg $O_2 gww^{-1} h^{-1}$ (27 °C) and 20-29 µg $O_2 gww^{-1} h^{-1}$ (33 °C). The oxygen consumption rates measured in this study are well in line with the Q10 law, in which metabolism increases with temperature (Arrhenius, 1889), which indicates that the animals were within their normal range of respiration activity. Interestingly, the significant correlation between oxygen consumption rate and temperature (Spearman correlation t15: $R^2 = 0.95$, $p = <0.0001$; t30: $R^2 = 0.685$, $p = 0.021$) was not in accordance with the ETS activity, neither in respiratory tree nor in muscle tissue. Astall and Jones (1991) measured respiration in eviscerated and non-eviscerated sea cucumbers of the species *H. forskali,* and showed that there were no differences in the oxygen consumption rates between these groups. This provides evidence that oxygen uptake is not directly linked to the presence of the respiratory tree. This could be an explanation for our contradictory findings between immediate oxygen demand (oxygen consumption rate) and theoretical maximal oxygen uptake efficiency (ETS), and implies that sea cucumbers possess multiple pathways to take up oxygen.

LDH plays a key role in the anaerobic pathways of organisms and changes in this enzyme activity can usually be interpreted as a switch in energy metabolism due to a higher energy demand, e.g. due to stressful conditions (Dahlhoff, 2004). For example Guo et al. (2014) measured increased LDH activity in *A. japonicus* in response to hypoxic stress during transportation and Rodrigues et al. (2014) detected increased LDH activities in response to metal exposures in the estuarine crab, *Carcinus maenas*. In this study the analyses of the metabolic enzyme activity revealed that throughout the experimental period, the activity of LDH was higher than IDH activity, in both temperature treatments as well as under control conditions. This indicates that the increased LDH activity was not associated with thermal stress, but rather reveals that juvenile *H. scabra* were favouring an anaerobic metabolism, matching their sluggish motility and preference for burrowing behaviour in sediment. However, under warm conditions the LDH activity decreased significantly over time, reaching the lowest value at t_{30}, where it was even lower than the IDH activity. Mercier et al. (1999) monitored a clear effect of temperature on the burrowing behaviour of *H. scabra*, where warmer temperature conditions prevented animals from burrowing, which eventually led to a prolonged feeding activity and higher growth rates. This is in line with our observations, where those animals exposed to 33 °C spent distinctly more time foraging on top of the sediment than the cold exposed animals. This recognition also strengthens our assumption, that the fast replenishment of carbohydrates in the warm exposed animals was driven by increased food intake. This provides evidence, that under warmer temperature conditions a more aerobic energy metabolism (driven by IDH) is preferred, which enables a more efficient metabolism for *H. scabra* than the usual anaerobic pathways (LDH driven). Overall we see a temperature driven switch in prevalent energy turnover. In a previous study by Lavitra et al. (2010) juvenile *H. scabra* cultured at 31 °C showed a significantly higher growth rate and foraging activity compared to animals reared at 28 °C. This indicates that increased temperature can lead to higher aquaculture production, apparently without stress. However, there are limits, and at a certain temperature level the heat stress will certainly lead to a disturbed homeostasis, which will ultimately cause cellular damage, as well as inhibited growth and reproduction (Sokolova et al., 2012). The current results show that juvenile *H. scabra* were able to cope well up to a water temperature of 33 °C within 30 days, by a continuously increased oxygen consumption rate and a switch in energy metabolism, without showing signs of an imbalanced energy budget. Therefore, it can be assumed that even water temperature as high as 33 °C will be favourable for juvenile *H. scabra* in terms of feeding

activity and growth rate and thus, might lead to increased aquaculture production. Future studies should target the maximum temperature threshold level and exposure time at which energy balance and growth rate is starting to decrease.

Conclusions

In this study we optimized the method cellular energy allocation for the first application on isolated muscle and respiratory tree tissue of a sea cucumber species. The comparison of the two tissues revealed that the respiratory tree could be considered as more suitable tissue to measure ETS activity, for further quantification of cellular energy allocation. In general, cellular energy allocation proved to be a sensitive marker to detect changes in metabolic status in juvenile *H. scabra,* as an early response to temperature acclimation. The analysis of cellular energy allocation revealed that: a) juvenile *H. scabra* were strongly affected by the initial temperature changes of ± 6 °C (1 °C/day) prior to the start of the experiment (t0); b) the animals were quickly recovering their energy balance (day 15 to 30) at both temperature extremes. The analysis of direct oxygen consumption on the other hand, indicated an enhanced metabolism at warmer temperature conditions, throughout the experimental period. This is supported by the measured shift from a more anaerobic to a more aerobic energy metabolism, and the observed increase in foraging activity in the warm acclimatized animals. The combined outcome of cellular energy allocation, oxygen consumption and energy metabolism related enzyme activities indicate that the increased metabolic activity in juvenile *H. scabra*, as response to warmer temperature, was well within the homeostatic range. Thus, a rearing temperature of 33 °C might presumably be favourable for the aquaculture of *H. scabra.*

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Chapter 2:
Thermal acclimation effects
on the antioxidant- and immune- system

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Effects of thermal stress on the immune and oxidative stress responses of juvenile sea cucumber *Holothuria scabra*

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Abstract

Holothuria scabra is the most valued and cultured tropical sea cucumber, given the great demand of this species for human consumption. However, despite its ecological and economic relevance, little is known regarding its immune responses under thermal stress. Here, the main goal was to study the response of sea cucumbers to temperature stress, assessing sub-organismal alterations and acclimation capacities of juveniles to temperature changes. After changing temperature (1 °C/day) for 6 days, organisms were exposed to temperature conditions of 21 °C (cold), 27 °C (control), and 33 °C (warm) over a 30 day period. At each 15-day interval (t0, t15, and t30), six replicates per condition were sampled for biochemical analysis. Immune responses were addressed by studying the activity of phenoloxidase (PO) and prophenoloxidase (ProPO) in the coelomic fluid. Antioxidant defence responses—catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR) enzymatic activities—were measured in the muscle and respiratory tree tissues, whereas oxidative damage was evaluated by measuring levels of superoxide radicals (ROS), DNA-strand breaks and lipid peroxidation (LPO). Juvenile *H. scabra* increased SOD and PO activities when temperature was elevated, and revealed low levels of ROS and damage in both cold and warm treatments throughout the experiment, confirming the organism's moderate thermal stress. After the short acclimation period, the immune and antioxidant responses prevented damage and maintained homeostasis. This multi-biomarker approach highlights its usefulness to monitor the health of *H. scabra* and to gain insight concerning the use of this high-valued species in global-scale aquaculture from different temperature regions.

Keywords: *Biomarkers, Tropical aquaculture, Climate change, Environmental stress, Antioxidant responses, Acclimatio*

Introduction

Water temperature is a crucial factor influencing the physiological status of organisms in terms of growth rates, oxygen consumption and metabolism, or moulting process (e.g. Sierra et al., 1999; Zdanovich, 1999; Dong et al., 2006). While it has been shown that changes in water temperature can evoke acute or chronic stress in a variety of organisms (Cheng and Chen, 2000; Cheng et al., 2004; Coates et al., 2012), there is still some ambiguity concerning the effects of temperature variations. One common strategy to monitor the effects of temperature stress at lower biological organizational levels is the use of biomarkers (Peakall, 1992; Menezes et al., 2006). Some of the most studied and applied biomarkers are parameters related with oxidative stress defense against reactive free radical production, such as superoxide anions ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2) (Dröge, 2003). The ability to regulate the production of these reactive oxygen species (ROS) and maintain "redox homeostasis" determines the health status of an organism (Ames et al., 1993). It is generally known that temperature stress induces the generation of ROS (Valavanidis et al., 2006). Disturbing the balance between endogenous and exogenous ROS can cause a consequent incapacity of the antioxidant defences to respond, which may lead to oxidative damage in different target biomolecules and tissues (Sohal et al., 2002; Valavanidis et al., 2006). Superoxide radicals, for example, are known to have negative impacts on antioxidant vitamins (e.g. tocopherol, ascorbate) and enzyme activities [e.g. catalase (CAT), glutathione reductase (GR) and peroxidases], which can in turn result in DNA damage, enzymatic inactivation, or peroxidation in important cellular biomolecules, especially lipids (Kono and Fridovich, 1982; Blum and Fridovich, 1985; Valavanidis et al., 2006). Thus, antioxidants play a crucial role in the maintenance of cell integrity, homeostasis, and in prevention of oxidative damage (Vigo-Pelfrey, 1990; Dix and Aikens, 1993). Superoxide dismutase (SOD) and CAT provide the first line of defence in responses to oxidative damage. Initially, SOD converts the superoxide radicals to O_2 and H_2O_2 and CAT in the next step transforms the H_2O_2 into O_2 and H_2O (Howcroft et al., 2009). Another important enzyme to protect the cells is GR, which reduces glutathione disulfide (GSSG) into two molecules of glutathione (GSH), which act as a non-enzymatic antioxidant (Saint-Denis et al., 2001).

Aside from enzymes involved in oxidative stress responses, environmental stress in marine invertebrates can also evoke immune responses through for instance the activity of phenoloxidase (PO) enzyme (Gomez-Jimenez et al., 2000). Phenoloxidase is responsible for the process of melanization, which is involved in wound healing and cellular defence

responses (Ratcliffe et al., 1984; Rodriguez and Le Moullac, 2000; Cerenius et al., 2008). Due to the cytotoxic nature of PO, this enzyme is usually stored in its inactive precursor form—pro-phenoloxidase (ProPO)—being activated only after external stimuli (Söderhäll and Cerenius, 1998; Rodriguez et al., 2014). The ProPO activating system is described for many invertebrates and consists of a cascade of interactions between enzymes and their zymogens, inducing the production of PO as final product. Both PO and ProPO are well studied in arthropods such as crustaceans (Söderhäll and Unestam, 1979) and insects (Laughton and Jothy, 2011), but many open questions regarding their function and dynamics remain for non-arthropod invertebrates, including sea cucumbers.

Holothuria scabra is economically the most valuable tropical sea cucumber, given the high interest for the food industry (bêche-de-mer), as well as for pharmaceutical purposes (i.e. bioactive compounds) (Battaglene and Bell, 1999; Hamel et al., 2001; Venugopal, 2009; Bordbar et al., 2011). In addition, concerning their anatomy, sea cucumbers have unique organs/tissues with diverse functions (e.g. cellular aeration, locomotion, metabolism and regenerative processes), suitable for the study of oxidative stress and immune responses (Garcia-Arrarás and Dolmatov, 2010), which make them good target tissues in the study of stress responses and oxidative and immune-related analysis. The respiratory tree, for example, is a well-developed structure responsible for cellular aeration and waste excretion (Spirina and Dolmatov, 2001). Muscular system and body wall of sea cucumbers are also interesting organs to analyse since they are involved in the organisms' locomotion and in the contraction movements in response to environmental stimuli (Motokawa and Tsuchi, 2003). These organisms also play an important ecological role as bioturbators (Uthicke, 2001; Purcell et al., 2012). As shallow, bottom dweller species, they undergo seasonal and daily temperature fluctuations. Some studies demonstrate that *H. scabra* (Wolkenhauer, 2008) and *Apostichopus japonicus* (Dong et al., 2006) seem to be adapted to temperature changes in terms of their burying and feeding habits, but very little is known regarding their mechanisms of adaptation and consequences for fitness in the long term.

Therefore, the main objective of the current study was to determine the effects of temperature stress (i.e. cold and warm) on immune and oxidative stress responses of juvenile *H. scabra*, using biochemical biomarkers involved in such processes, in order to understand the capacity of these organisms to cope with thermal stress and to find suitable markers for effect assessment on those levels.

Materials and Methods

Test Organisms

Holothuria scabra (Jaeger, 1833) originated from the hatchery facilities of the Indonesian Research Centre for Oceanography (LIPI) on Lombok, Indonesia, were transported to the Alfred Wegener Institute, Helmholtz-Centre for Polar and Marine Research (AWI) in Bremerhaven, Germany, where they were maintained in recirculation systems for 14 days at 27 °C with a photoperiod of 12:12 h (light:dark) for acclimation. Sea cucumbers were observed and fed every second day with Algamac (Aquafauna—Bio Marine Inc.). To ensure optimal water quality, the aquaria water was continuously filtered and aerated. The water quality parameters, ammonia, pH and salinity were monitored regularly.

Experimental setup

Experimental design followed previous work from Kühnhold et al. (2017). Briefly, after the acclimation period (14 days), 18 individuals were randomly assigned to each of three water temperature treatments: 21 °C (Cold), 27 °C (Control), and 33 °C (Warm). To achieve such temperatures, seawater temperature was decreased (for cold treatment) or increased (for warm treatment) by one degree per day over 6 days. Once the desired temperatures were reached, six individuals per tank were killed for further analysis, corresponding to day zero of the experiment (t0). Sampling was then performed at 15 days (t15) and 30 days (t30) of exposure to the different temperatures, with six replicates. Before killing the organisms, their coelomic fluid was collected using a 2-ml sterile syringe inserted through the body wall, for the assessment of immune responses. The procedure took no more than 20 s to ensure minimum effects of sampling on the immune responses. Then, muscle, respiratory tree, and body wall tissues were sampled for the oxidative stress-related endpoints (see below for sample processing details). All samples were subsequently stored at − 80 °C until further analysis.

Tissue Preparations

Immune response

Following Jiang et al. (2014), two different fractions of coelomic fluid were prepared: Coelomocyte Lysate Supernatant (CLS) and Cell Free Supernatant (CFS). After centrifugation of extracted coelomic fluid at 500g for 10min (4 °C), the supernatant (CFS)

was stored at − 80 °C, whereas the pellet was suspended with 1X PBS buffer to prepare the CLS fraction. After sonication for 5 min at 30-s intervals (UTR 200, Hielscher, Germany), the re-suspended pellets were centrifuged at 12,000g for 10 min (4 °C) and the obtained supernatant (CLS) was stored at − 80 °C until further analysis.

Oxidative stress

According to different protocols and procedures, the oxidative stress-related parameters (except ROS) were measured in the muscle and respiratory tree tissues of sea cucumbers adapting the protocols more thoroughly described in Alves et al. (2016) and Silva et al. (2016). Both tissues were homogenized in K-phosphate buffer (0.1M, pH 7.4) in a 1:4 proportion (w/v). Part of the homogenized tissue. (150µl) was transferred to a microtube containing 4 % BHT solution (2,6-dieter-butyl-4-methylphenol) to prevent tissue oxidation for further determination of lipid peroxidation (LPO), and another portion (50µl) was separated for quantifying DNA-strand breaks. Samples were then centrifuged at 10,000g, for 20min (4 °C). The resulting post-mitochondrial supernatant (PMS) was stored at −80 °C for further protein quantification and activity measurement of SOD, CAT and GR. For the determination of superoxide free radicals, as a measurement of ROS production, 50mg of sea cucumber body wall was separated and kept at −80 °C until further analysis.

In all assays, K-phosphate buffer (0.1M, pH 7.4) was used as blank. The spectrophotometric measurements were done at 25 °C in a synergy H1 Hybrid Multi-Mode microplate reader (Biotek® Instrument, Vermont, USA) and the enzymatic reactions were all previously optimized to ensure zero-order kinetic reactions (substrate in excess).

Biochemical analysis

Immune responses: phenoloxisase and pro-phenoloxidase

PO (monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1) and ProPO (zymogen form) activities were measured using the method partially described by Söderhäll (1981) with modification made by Laughton and Jothy (2011) and Jiang et al. (2014). The activities of ProPO and PO were measured in both coelomic fluid fractions, i.e. CLS and CFS. PO activities were measured by adding 5mM l -DOPA (l l-3, 4-dihydroxyphenylalanine; Sigma, USA), dissolved in sodium cacodylate buffer (0.01M, pH 7.4), to each fraction of the sample (CLS or CFS). For the blank reactions, seawater was used instead of the sample. The procedure for measuring ProPO was similar with the minor difference that chymotrypsin

(0.25 mg/ml) was added to the sample, with a 10-min incubation prior to the addition of **l** -DOPA, to allow the activation of all phenoloxidase. The conversion of L-DOPA into dopachrome was determined spectrophotometrically at 490nm (25 °C) with readings every 10s for 5min, giving an estimation of the enzyme activity. The final PO and ProPO activities were expressed as U/mg of protein, where 1U is defined as the amount of enzyme in the sample that, by converting the substrate, increases the absorbance by 0.001 per min.

Protein quantification

The soluble proteins were quantified according to the Bradford method (Bradford 1976), adapted from BioRad's Bradford microassay set up in a 96-well flat-bottom plate, using bovine γ -globulin as a protein standard. In each well of the microplate, 10µl of each sample was added along with 290µl of Bradford reagent (in quadruplicates). After 15min of agitation at 150 revs/min, absorbance was read at 600nm and results were expressed in mg of protein/mL.

Antioxidant defenses

The activity of SOD (EC 1.15.1.1) was measured performing an adaptation of the method described by McCord and Fridovich (1969), using the xanthine/xanthine oxidase-mediated reduction of cytochrome C. The reduction of cytochrome C was followed at 550nm and SOD activity was expressed in U/mg of protein using an SOD standard of 1.5 U/ml, where 1U represents the amount of enzyme in the sample that causes 50 % inhibition of cytochrome C reduction. CAT (EC 1.11.1.6) activity was estimated following the degradation of H_2O_2 at 240 nm, adapting the method described by Clairborne (1985). CAT activity was expressed in µmol/min/mg of protein, using a molar extinction coefficient of 40 M/cm. The activity of GR (EC 1.8.1.7) was estimated by measuring oxidation of NADPH in the process of reducing GSSG to glutathione (GSH) at 340 nm (Cribb et al. 1989). GR activity was calculated using a molar extinction coefficient of 6.2x103 M/cm and expressed in nmol/min/mg of protein.

Oxidative stress and damage

For the determination of superoxide free radicals production in the body wall of sea cucumber, the method of Drossos et al. (1995) was followed. Briefly, after adding Krebs buffer to the tissue, an incubation with cytochrome C (15µM) was made at 37 °C. The presence of $O2^{-}$ was determined by the capacity of the radicals to reduce cytochrome C, which was measured at 550nm. Using a molar extinction coefficient of 19,000 M/cm (Wu et

al., 2011), the amount of superoxide radicals produced was calculated and expressed in nmol $O2^{-}$ g^{-1} wet weight. Lipid peroxidation levels were assessed by measuring the content of thiobarbituric acid-reactive substances (TBARS), using the method described by Ohkawa et al., (1979) and Bird and Draper, (1984), with modifications made by Wilhelm et al., (2001) and Torres et al., (2002). After the reaction with TBA 0.73 % (2-thiobarbituric acid) reagent, the absorbance of the samples was measured at 535nm. The results were calculated using a molar extinction coefficient of 1.56 × 105 M cm^{-1} and expressed as nmol TBARS mg^{-1} of wet weight. The DNA-strand breaks were measured using the DNA alkaline precipitation assay (Olive, 1988), adapted from De Lafontaine et al., (2000). After the precipitation of SDS-associated nucleoproteins and genomic DNA, the remaining single and double-stranded DNA in the supernatant was mixed with Hoesch dye (1 μg mL^{-1} bisBenzimide, Sigma-Aldrich) and fluorescence was measured using an excitation/emission wavelength of 360/460 nm. Results were expressed as mg of DNA mg^{-1} of wet weight, using calf thymus DNA as standard to extrapolate DNA concentration.

Statistical analysis

Statistics was performed using Sigma Plot software for Windows, version 11.0 (SigmaPlot 1997). Data were first tested for normality and homoscedasticity using Kolmogorov–Smirnov and Levene tests, respectively. To determine statistically significant differences between the treatments and between each time point, a two-way analysis of variance (ANOVA) was applied. When significant differences were found, Holm–Sidak post hoc tests were used for multiple comparisons. Correlations between endpoints in different tissues, at each time point, were performed using Pearson correlations. The results are presented as means + standard error (SE). The significance level for all statistical analysis was set at $p \leq 0.05$.

Results

No mortalities were registered at any treatment at any time point.

Immune responses

PO and ProPO activities in cell-free supernatant (CFS)

Although no significant differences were observed in the activity of PO and ProPO in CFS, the activities in both cases were higher in the warm treatment with a tendency for a decrease

with the experiment duration (Fig. 1). In this CFS fraction, activities of ProPO and PO were found to be similar (within the same order of magnitude) in every treatment.

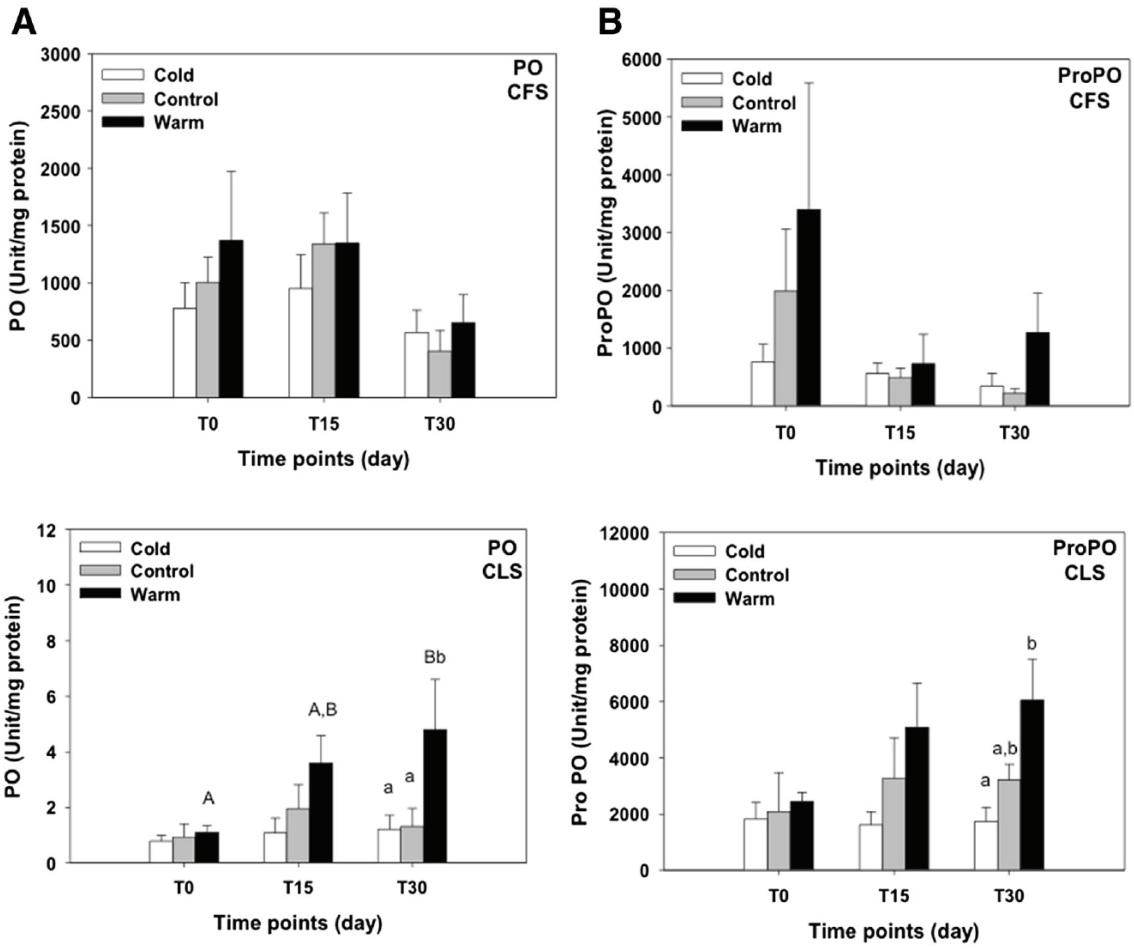

Fig. 1: Immune responses: Phenoloxidase (PO; A) and pro-phenoloxidase (ProPO; B) activities in the cell-free- (CFS; top) and coelomocyte lysate- (CLS; bottom) supernatant fraction of *Holothuria scabra* coelomic fluid exposed to cold (21 °C), control (27 °C) and warm (33 °C) temperatures over different time periods (t0, t15, t30 days). Results express average values + standard error. [a,b]Significant differences between cold, control and warm treatments within each time point (two-way ANOVA, Holm–Sidak, $p<0.05$). [A,B]Significant differences between time points within each temperature treatment (two-way ANOVA, Holm–Sidak, $p<0.05$)

PO and ProPO activities in Coelomocyte lysate supernatant (CLS)

The PO activity in the CLS fraction followed the same pattern as ProPO, with progressively higher activities in the warm treatment over time of exposure (Fig. 1), which in the case of PO was found to be statistically significant at t30 ($p = 0.005$, Fig. 2) Significant differences among different temperature treatments were found at the end of the experiment (t30), both for PO and ProPO ($p = 0.004$, Fig. 2A; and $p = 0.011$, Fig. 2B, respectively). Contrary to the

CFS fraction, in CLS ProPO activities were between 1000 and 2000× higher than PO activities. Despite the increase in the general immune response of the organisms in the warm treatment, the ratio between PO and ProPO remains constant among treatments with no significant differences being observed (Fig. 1).

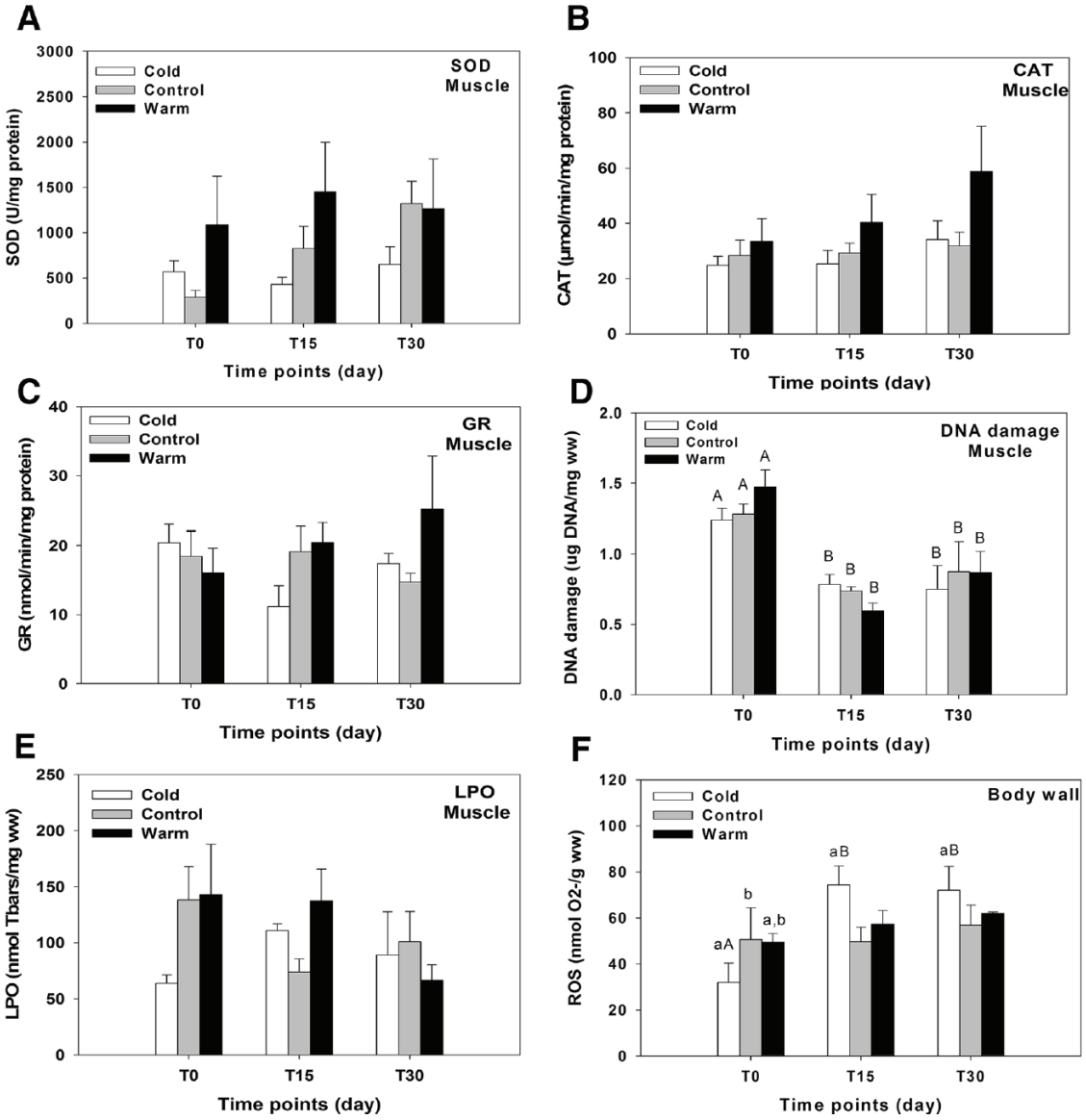

Fig. 2: Oxidative stress-related responses: (A) superoxide dismutase (SOD), (B) catalase (CAT) and (C) glutathione Reductase (GR) enzymatic activities and levels of oxidative damage measured as (D) DNA damage and (E) lipid peroxidation (LPO) in the muscle tissue of *Holothuria scabra* exposed to cold (21 °C), control (27 °C) and warm (33 °C) temperatures over different time periods (t0, t15 and t30 days). (F) Reactive oxygen species (ROS) production in the body wall of *Holothuria scabra* exposed to the same conditions described for muscle. Results express average values + standard error. [a,b]Significant differences between cold, control and warm treatments within each time point (two-way ANOVA, Holm–Sidak, $p < 0.05$). [A,B]Significant differences between time points within each temperature treatment (two-way ANOVA, Holm–Sidak, $p<0.05$)

Correlation analysis between all assessed biomarkers in H. scabra, relative to immune and oxidative stress responses, were performed separately for each time point (Supplementary material Tables S2.1 – S2.3). At all time points, especially in muscle tissue, activities of CAT, SOD and GR correlated with each other positively, indicating that if one of these enzymes is activated or inhibited, the other enzymes follow the same pattern. Similarly, some positive correlations between DNA damage and LPO were observed. Moreover, GR (respiratory tree) and ProPO activating systems (CLS fraction) correlated negatively with ROS, while a positive correlation between DNA damage of the same tissue and ROS is apparent. Furthermore, PO activity of CLS correlated positively with SOD activities from both tissues.

Oxidative stress-related endpoints

No significant changes in the activity of the tested antioxidant enzymes (SOD, CAT and GR) were observed, either in the muscle tissue or in the respiratory tree (Figs. 2, 3). However, although the effects were not statistically significant, in the muscle there was a trend for higher SOD activities in the warm treatment, compared to control and cold treatment (Fig. 2A). Antioxidant enzyme activity levels were usually higher in the respiratory tree (Fig. 3) than in the muscle (Fig. 2), independently of the treatments. In relation to the parameters addressing oxidative damage, no effects of temperature were seen either in peroxidation of lipids or in higher levels of DNA-strand breaks (Figs. 2d, e, 3d, e).

The results of the ROS quantification in the body wall show that in the cold treatment, at t0, the organisms produced significantly less superoxide radicals than in the control treatment ($p = 0.002$—Fig. 2f). However, the levels of ROS in this treatment significantly increased with the duration of the experiment CA ($p = 0.009$), and no further differences were observed with the other temperature treatments.

Discussion

Temperature changes influence the growth rate, susceptibility, and the general health status of invertebrates (Hughes et al., 2003; Cheng et al., 2004; Wang et al., 2008; Purcell and Simutoga, 2008; Hair, 2012). The integrated antioxidant and ProPO activating systems are known as crucial components of invertebrates' self-maintenance (Mathew et al., 2007), but little is known about these responses to different stress levels. The present study is the first to apply combined investigations of immune responses, cellular oxidative damage and

antioxidant enzyme activities to assess stress responses in juvenile *H. scabra* at varying temperatures [i.e. cold (21 °C) and warm (33 °C)], and to assess their potential for easy-to-use, fast, and cost-effective multi-biomarker applications in aquaculture.

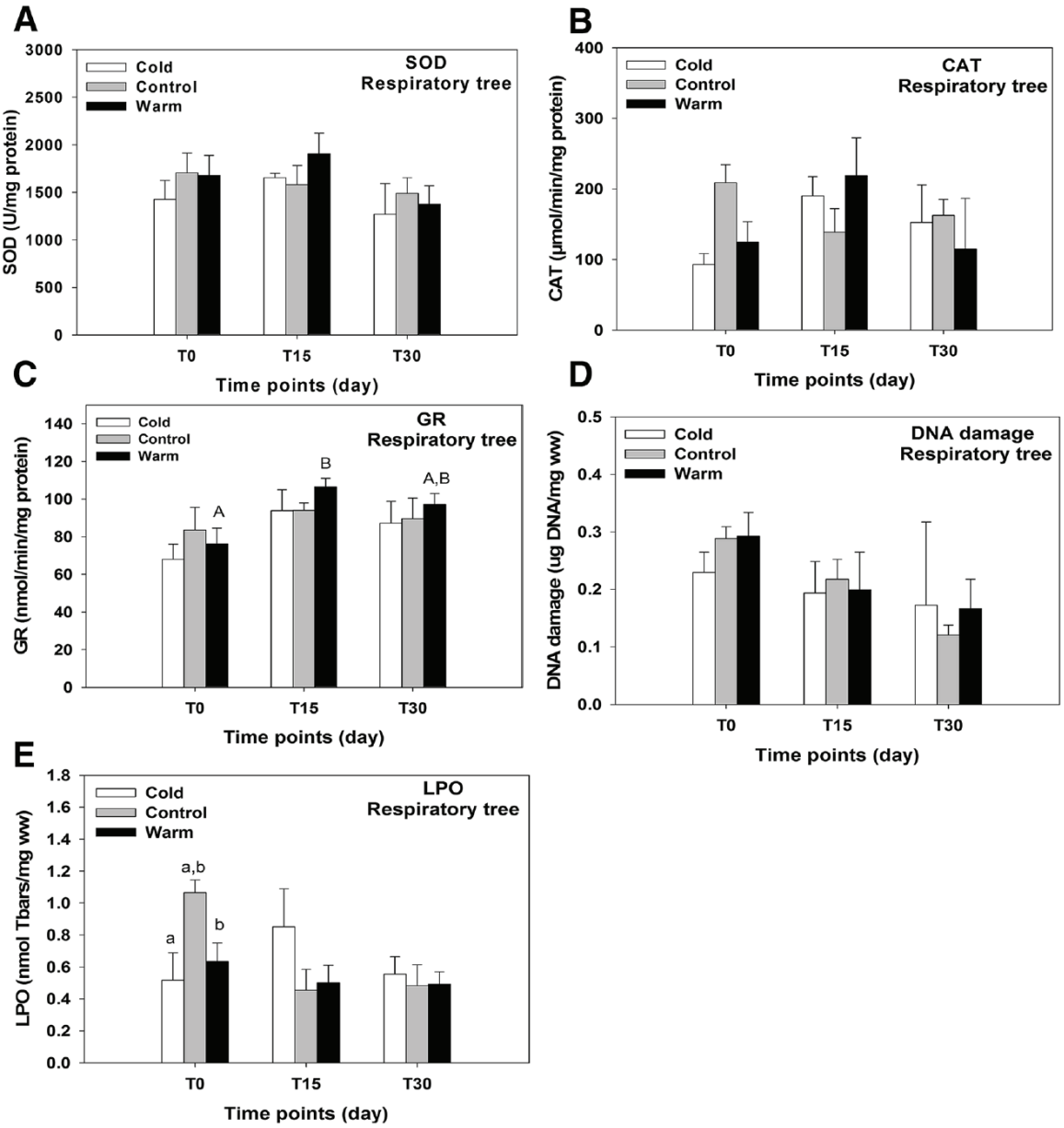

Fig. 3: Oxidative stress-related responses: (A) superoxide dismutase (SOD), (B) catalase (CAT) and (C) glutathione reductase (GR) enzymatic activities and levels of oxidative damage measured as (D) DNA damage and (E) lipid peroxidation (LPO) in the respiratory tree of *Holothuria scabra* exposed to cold (21 °C), control (27 °C) and warm (33 °C) temperatures over different time periods (t0, t15 and t30 days). Results express average values + standard error. [a,b]Significant differences between cold, control and warm treatments within each time point (two-way ANOVA, Holm–Sidak, $p<0.05$). [A,B]Significant differences between time points within each temperature treatment (two-way ANOVA, Holm–Sidak, $p<0.05$)

Immune responses

The nature of sea cucumbers as osmo-conformers (Coteur et al., 2004) indicates that any changes in water temperature may influence their coelomic fluids and particularly affect the activities of coelomocytes (Wang et al., 2008). The ProPO activating system is considered as the first line of defence in the immune response of invertebrates (Sritunyalucksana and Söderhäll, 2000), where any reduction in PO activities affects the cellular defence of organisms (Mathew et al., 2007). This study confirmed that, similar to the Pacific oyster (*Crassostrea gigas*) (Hellio et al., 2007), PO activity is detectable in both fractions of the coelomic fluid in *H. scabra*. Most of PO was activated in the CFS fraction, seen by the similar activity between PO and ProPO, in contrast to the CLS fraction, where most PO remained in the inactive form, as expected at least under control conditions, given the cytotoxic nature of the byproducts of the PO activating cascade (Tujula et al., 2001; Laughton and Jothy, 2011).

In the CFS fraction, which represents the acellular fraction of the coelomic fluid (Gomez-Jimenez et al., 2000), there was a tendency for higher activities of both PO and ProPO activities in the warm treatment, mainly at the beginning of the experiment (Fig. 1). This is in agreement with the findings of a parallel study, where under the same warm temperature condition, the organisms were consuming more energy, possibly indicating the costs of the defence responses by inducing for instance these immune enzymes (Kühnhold et al., 2017). Similarly, Coates et al., (2012) reported that in horseshoe crabs (*Limulus polyphemus*), which have hemocyanin-derived phenoloxidase (Hc-PO), the activity of Hc-PO at the beginning of exposure was initially increased at the warmer treatment, but decreased again over a period of time, suggesting that temperature changes have limited effects on hemocyanin and PO activities. It is important to note that the coelomocytes of sea cucumbers and haemocytes of crustaceans display several common features (Tseng et al., 2009).

In the CLS fraction, this tendency for higher PO activities with increasing temperatures is also seen for both PO and ProPO activities, over the time of exposure. This increase in the total ProPO activity suggests that, along with the PO activation (in a much lower scale), probably more PO is being synthesized (Fig. 1). Although in this study the sea cucumbers responded with an increase in PO in the warmer treatment, the response of this enzyme to temperature changes seems to differ between species. For example, Vargas-Albores et al., (1998) reported that the yellowleg shrimp (*Penaeus californiensis*) had lower PO activity at

higher temperature (32 °C) compared to colder treatment (18 °C). Moreover, Cheng and Chen (2000) and Cheng et al. (2004) reported that the PO and phagocytic activities in the giant freshwater prawn (*Macrobrachium rosenbergii*) and the Taiwan abalone (*Haliotis diversicolor supertexta*) at warmer treatments (34 °C) were lower than the ones reared at colder water (27 – 30 °C).

When comparing the ProPO activities between CFS and CLS factions, it is possible to observe that the activities are higher in the coelomocytes (CLS), indicating a higher potential for PO response in this fraction. These results show the importance of testing both cellular and acellular fractions of the coelomic fluid in order to accurately locate the PO activity. However, the different PO activities found in the two fractions might also indicate different types of PO enzymes (tyrosinase, laccase or catecholase). Although characterization of PO was already done for another holothurian species (Jiang et al., 2014) and this was the base for the methods employed here, it would be important for further studies to understand which specific enzymes are involved in PO activity in each fraction to more precisely target those reactions. Nevertheless, the present study represents already an important indication that PO induction might play an important role in *H. scabra* response to heat stress.

Oxidative stress-related endpoints

Regarding the oxidative stress-related endpoints, results showed that exposure to different temperatures had little impact on *H. scabra*. Various studies demonstrated that free radicals formed by a stressor could enhance the formation of malonaldehyde and therefore increase LPO (Di Pierro et al., 1992). Additionally, the heterogenic DNA molecules are susceptible to breakage and damage inflicted by elevated ROS levels (Cerutti, 1985). In the present study, however, no signs of oxidative damage were observed in any temperature manipulation (Figs. 2, 3). In the beginning of the experiment (t0) specimens from the cold treatment exhibited even lower ROS levels, which resulted in lower levels of LPO in the same condition. These lower ROS levels can also be explained by the lower oxygen consumption rates (OCR) verified in a parallel study with the same exposure conditions (Kühnhold et al., 2017).

Increasing temperatures can stimulate oxidative stress and specific antioxidant responses in different classes of invertebrates. Although the antioxidant activities in *H. scabra* did not show a clear treatment response, induction of SOD was a constant trend observed in the warm treatment. Similar patterns of antioxidant response were observed by Ji et al. (2008) and Wang et al. (2008), in studies with *A. japonicus*, where at the beginning of exposure to

higher temperatures, SOD and CAT activities measured in both body wall and respiratory tree, increased after a short exposure time (12h). The tendency for higher activity of SOD is observed in the warm treatment mainly in the muscle, along with lower activities in the cold treatment (Fig. 2a). A similar pattern was reported for shrimps (Zhou et al. 2010) and the disk abalone *Haliotis discus discus* (Kim et al., 2007), where the activities of manganese superoxide dismutase (MnSOD) and copper superoxide dismutase (CuSOD) increased under heat stress. This SOD induction in the warm treatment in combination with the lack of significant changes in CAT and GR activities over the period of exposure, suggests that this enzyme is the most sensitive antioxidant enzyme among the ones tested, and likely plays an important role in ROS detoxification in juvenile *H. scabra* in response to thermal stress.

However, in the present study, the temperature stress resulted in only marginal increases in the antioxidant activities. This might be explained by the immediate induction of heat shock proteins, which reduce heat stress and oxidative stress in the organism, as reported previously for *Haliotis tuberculata* (Farcy et al., 2007). This cannot, however, be confirmed in the present work, since the expression of heat shock proteins was not studied.

Another observation of the present study was that the respiratory tree in the sea cucumbers had higher antioxidant potential than muscle (higher enzyme activities), similar to the gills in molluscs (Farcy et al., 2007; Box and Sureda, 2009). Considering the functionality of the respiratory tree for oxygen circulation and gas exchange at its surface, and also the close contact with water, similarly to the gills in molluscs, this tissue is ought to be more susceptible to environmental changes. However, a clearer pattern of overall response to the induced thermal stress was observed in the muscle tissue. Further studies featuring more levels and higher intensity of treatment (i.e. lower and higher temperature thresholds) are needed in order to create a better understanding on the physiological thresholds and sensitivity of the antioxidant responses in this species.

In sum, immune and oxidative stress responses indicate that temperature manipulation applied in the present study was not severe enough to cause acute stress in juvenile *H. scabra*. This is in accordance with the general assumption that most of the sea cucumbers, reared in intertidal ponds, can tolerate temperature fluctuations from 20 to 30 °C (Dong et al., 2008). Furthermore, with this study it is possible to infer that immune responses through PO activity, and antioxidant activities (particularly SOD) in *H. scabra* seem to be efficient to reduce ROS production and oxidative damage under thermal variations. This is strengthened by the correlation analysis between biochemical responses throughout the duration of the

experiment (supplementary material Table S2.1 – S2.3), with positive correlations between SOD and PO activities and negative correlations between the activities of GR or PO enzymes and the levels of ROS. Therefore, this study highlights the importance of combining different endpoints into a multi-biomarker approach in order to gain a holistic picture of the processes and mechanisms underlying stress responses.

Conclusions

Juvenile *H. scabra* displayed sensitivity to thermal stress at the beginning of the experiment, especially in the warm treatment, and after a period of time they acclimated to the higher and lower temperatures. From an immune response-related point of view, PO and ProPO activities in the cell-free coelomic fluid were tendentiously increased in the warm treatment at t0, showing an early immune response with the temperature change. Antioxidant and oxidative damage biomarkers indicated that the temperature manipulations applied in the present study were not severe enough to cause significant oxidative damage to *H. scabra*, seen by the low production of ROS and absence of oxidative damage in either lipids or DNA. SOD seems to be the most sensitive enzymatic antioxidant in *H. scabra* in response to thermal stress. The present study highlights the benefits of a multi-biomarker analysis to better understand and interpret biochemical responses to stress, and in particular thermal stress. Assessing changes in the immune and antioxidant biomarker endpoints, particularly in juvenile *H. scabra*, are promising tools for monitoring the health status of the organisms. Also, understanding the impacts of temperature stress on these organisms, provide important insight into the possibility of the use of *H. scabra*, a high-valued species, in global-scale aquaculture from different regions with minimum impact concerning thermal stress.

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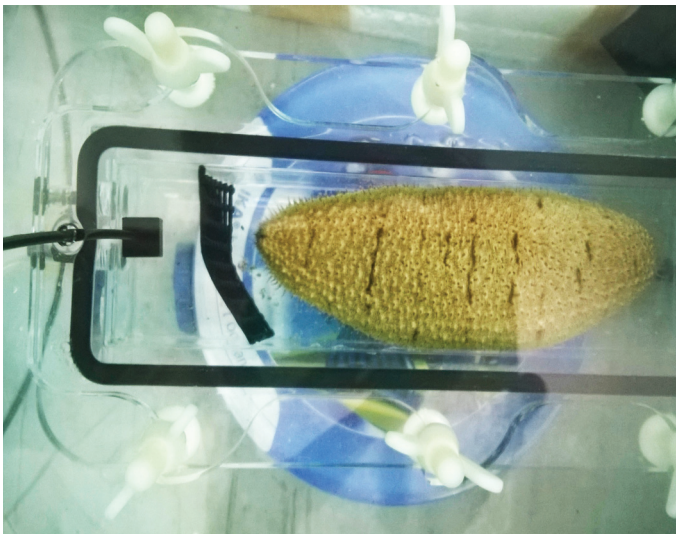
Chapter 3:
Acute thermal limits and aerobic scope



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Temperature-induced aerobic capacity and Hsp70 expression in the sea cucumber *Holothuria scabra*

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Abstract

The aerobic scope, which reflects the functional capacity for biological fitness, is a highly relevant proxy to determine thermal tolerance in various species. Despite the importance of this method, its implementation is often hindered, due to lacking techniques to accurately measure standard- (SMR) and maximal- (MMR) metabolic rates, especially in sluggish marine invertebrates such as sea cucumbers. This study targets temperature-induced maximal- and minimal- O_2 consumption rates in the sea cucumber *Holothuria scabra*, through acute temperature change (2 °C per hour; 17 – 41 °C). In addition to O_2 consumption, mRNA expression of Hsp70 was measured to define critical threshold temperatures on an interlinked basis. O_2 consumption of *H. scabra* peaked distinctly at 38 °C (33.2 ± 4.7 $\mu gO_2\ g^{-1}\ h^{-1}$) and reached a clear bottom line at 22 °C (>3 $\mu gO_2\ g^{-1}\ h^{-1}$). Within the thermal window of 22 – 38 °C *H. scabra* sustained positive aerobic capacity, with theoretic optimal performance in the center of the aerobic window (30.5 °C; 17.5 $\mu gO_2\ g^{-1}\ h^{-1}$). Between 39 – 41 °C *H. scabra* decreased respiration progressively, while Hsp70 levels were significantly expressed, indicating lethal conditions due to functional disruption of homeostasis. At the cold end (17 – 22 °C) *H. scabra* exhibited a narrow minimal respiration range, but no Hsp70 induction. We identified for the first time critical and optimal temperature levels for *H. scabra*, on the basis of aerobic performance. The characterization of temperature-induced maximal- (≈MMR) and minimal- (≈SMR) O_2 consumption rates, may serve as reliable alternative to precisely determine aerobic scope in sea cucumbers and other slow-moving marine species. Moreover, we showcase the important linkage between energy-related physiological markers and molecular defense mechanisms to predict the effects of environmental stress, such as climate change.

Keywords*: energy homeostasis, metabolic rate, aerobic scope, oxygen consumption, heat shock response, temperature threshold, sea cucumber*

Introduction

The determination of species-specific critical temperature limits is highly relevant, to predict stress levels due to global environmental change. Such knowledge is particularly important for tropical marine species, as they are adapted to stable environments, with even narrower temperature ranges than low-latitudinal terrestrial animals (due to smaller temperature changes in water). Hence, the biogeography of marine species conforms closely with optimal temperature windows, especially near equatorial boundaries (Sunday et al., 2011, 2012). Tropical species are, therefore, confined to narrower thermal niches than temperate species, which makes them more susceptible to niche shifts, due to higher metabolic rate changes (Tewksbury et al., 2008; Verberk et al., 2015). A gradual temperature increase of only 2-3 °C caused already significant reductions of thermal tolerance levels in various marine invertebrates (Nguyen et al., 2011), and substantially decreased aerobic performance (Rummer et al., 2014) as well as compromised growth and reproduction for a number of coral reef fish (i.e. Munday et al., 2008; Zarcó-Perello et al., 2012). Consequently, the effects of global climate change – i.e. a temperature increase of 2 – 4 °C for the tropical oceans – projected to occur by the end of this century, might pose a serious metabolic challenge for many equatorial marine species. A comprehensive mechanistic explanation for temperature driven effects on organism performance is provided by the oxygen- and capacity- limited thermal tolerance (OCLTT) (Pörtner, 2001; Pörtner and Knust, 2007; Pörtner and Farell, 2008). This theory is based on the physiological scope for aerobic performance, encompassing the capacity for oxygen uptake, transport and delivery, the so-called aerobic scope. The aerobic scope is defined as difference between maximal performance known as maximal metabolic rate (MMR), and resting metabolism known as standard metabolic rate (SMR) (Fry and Hart, 1948; Priede, 1977). The difference between SMR and MMR represents the accessible respiration energy, which is not required for basal maintenance functions and freely available for functions related to biological fitness (activity, feeding, and reproduction). At a particular temperature (T_{opt}) the aerobic scope peaks, which corresponds to optimal species performance. Above or below T_{opt}, performance declines with a narrowing aerobic scope until the aerobic capacity is delimited by a species-specific lower- (CT_{min}) and upper- (CT_{max}) critical temperature (Pörtner and Farell, 2008). At the cellular level, critical temperature stress can be measured as induction intensity of the universal heat shock response (HSR), which releases heat shock proteins (HSPs) as first line defense mechanism (e.g. Hochachka and Somero, 2002; Dahlhoff, 2004).

Heat shock proteins act as molecular chaperones and regulate the protein folding (Murthy *et al.* 2016). Hsp70 has been detected in complexes with proteins presumably assisting in the recovery from stress by repair of damaged proteins (Jolly *et al.* 2000). With increasing magnitude of temperature-stress, more and more energy resources need to be allocated to fuel cellular- and tissue- protection, such as Hsp70 synthesis, which will ultimately impair the aerobic scope for biological fitness, growth and survival (Sokolova et al. 2012). The aerobic scope has been widely used as model to determine thermal tolerances for various fish species from temperate- (Sylvestre et al., 2007; Farell et al., 2008; Clark et al., 2011) and tropical- (Nilsson et al., 2009; Johansen and Jones, 2011; Rummer et al., 2014) regions. For slow-moving benthic marine species such as sea cucumbers, however, the aerobic scope is difficult to quantify due to lacking procedures to quantify MMR at peak performance (i.e. maximal swimming speed for fish). For benthic invertebrates less specific measures like responsiveness to external stimuli (e.g. Nguyen et al., 2011) or loss of muscle control (Johansson and Laurilla, 2017) are utilized to quantify CT_{max}. The aim of this study was threefold: 1) to determine CT_{max} and CT_{min} for the tropical sea cucumber *Holothuria scabra*, by measuring O_2 consumption along a gradient of acute temperature change encompassing 17 – 41 °C; 2) to measure the mRNA expression level of Hsp70 at the maximum (41 °C) and minimum (17 °C) temperature, in order to link the cellular stress level with the temperature-driven metabolic benchmarks; 3) to discuss the utilization of critical temperatures, on the basis of aerobic capacity, to establish a reliable measure of aerobic scope for a slow-moving marine invertebrate species. *H. scabra* is an ecologically important species (rev. by Purcell et al., 2016), it contributes significantly to the recycling of nutrients and minerals through bioturbation (Wolkenhauer et al., 2009). Moreover, this species serves as prey for many taxa and enhances biodiversity by forming various symbiotic relations. Besides their ecological relevance, *H. scabra* is a high valued aquaculture candidate in the global south (Hamel et al., 2001; Lovatelli et al., 2004; Purcell et al., 2014). Due to *H. scabras* high commercial and ecological relevance, fine-tuned methods to detect thermal stress in this species are crucial to enhance aquaculture production and to assess its susceptibility towards global warming. We predict that: 1) the temperature-induced aerobic peak (CT_{max}; ≈MMR) and bottom line (CT_{min}; ≈SMR) serve as alternative basis to reliably determine aerobic scope in *H. scabra*; 2) the resulting thermal performance window gives important insights into optimal rearing temperatures for *H. scabra*; 3) the combined analysis of O_2 consumption and mRNA expression of Hsp70 enable the detection of functional disruption of homeostasis in *H. scabra*.

Materials and Methods

Study animals and experimental design

This study was conducted at the Indonesian Institute of Science (LIPI), Lombok, Indonesia. In January 2016, a number of 32 *H. scabra* (63 ± 15g) were collected at Pantai Sira di Pagi Hari (8°22´5.42´´S, 116°6´58.37´´E) off the West-Coast of Lombok. After specimen collections, the animals were transported to the LIPI aquaculture facilities and placed in aquaculture tanks (100 l), with flow-through conditions (29 °C; 35 ppt). Natural sediments served as food source. After an acclimation period of 14 days, four animals were placed over a 24h defecation period in a separate tank without sediment (50 l; 29 °C; 35 ppt). Subsequently, the animals were randomly divided into two groups (n= 2) and placed into gas-tight Acrylic Chambers (AC; V = 600 ml), which were submersed in two separate water baths (20 l aerated and filtered seawater; 29 °C; 35 ppt). After one hour acclimation to AC conditions, the temperature in the treatment tank was changed by 2 °C per hour, up to 41 °C and down to 17 °C (two separate experiments for the cold and warm treatment), while the temperature in the second water bath was kept at 29 °C (control). Both experiments were repeated four times (n = 8). For Hsp70 analyses the animals were dissected at the end of the cold and warm temperature treatment to remove respiratory tree (RT) tissue samples, which were immediately snap-frozen and stored in liquid nitrogen.

Temperature induced changes in O_2 consumption

Intermitted-flow respirometry was used to determine resting- and maximum- O_2 consumption for eight *H. scabra* individuals for the respective temperature treatment (cold and warm). Respirometry measurements were conducted always at the same daytime. Submersible pumps (150 l h^{-1}) provided a constant water supply from the water bath through the acrylic chamber (AC), while a peristaltic pump maintained internal water mixing within the AC. The water flow into the AC was stopped for 15 min every 15 min. This interval of continuous flushing cycles and the change rate of temperature (2 °C h^{-1}) enabled one respiration measurement cycle per degree of temperature up- and down- regulation: 1) acute temperature elevation (29°C, 31°C, 33°C, 35°C, 37°C, 39°C, 41°C); and 2) acute temperature decrease (29°C, 27°C, 25°C, 23°C, 21°C, 19°C, 17°C). At each temperature the water flow interruption time was short enough to ensure O_2 concentrations above 80% saturation (see best practices in Clark et al., 2013). The automatically temperature compensated O_2 concentration (mg l^{-1}) over time (s), was continuously recorded (1 s^{-1}) for each AC using oxygen-sensitive

REDFLASH dye on contactless spots (2 mm) glued at the inside of the AC lids and linked to a Firesting Optical Oxygen Meter (Pyro Science e. K., Aachen, Germany) via fibre-optic cables. To warrant consistency, the first and last minute of the resulting respiration slopes were excluded from analyses. The O_2 consumption rate (µg g^{-1} h^{-1}) was then calculated from the average of the eight slopes (n = 8) at each temperature of O_2 consumed per second ($R^2 \geq 0.90$), accounting for background O_2 consumption. The background respiration rate measured through the oxygen depletion in the empty AC´s at the start of each trial (pre-blank) and after removing the animals (post-blank). The initial background respiration rate as well as the additional proportion that accumulated over the experimental time (assumed linear) was subtracted from the respective slope. In this study we define the upper critical temperature limit (CT_{max}) as temperature point where O_2 consumption peaks, and the lower critical level (CT_{min}) as temperature corresponding to the bottom line of O_2 consumption. The exact centre in between these two critical temperature benchmarks, indicate optimal -temperature (T_{opt}) and -performance conditions. To account for temperature effects along the temperature gradients, the temperature quotients (Q_{10}) were calculated using mean O_2 consumption (R_1 and R_2) at control temperature (29 °C; T_1) and at CT_{max} (T_{2max}) and CT_{min} (T_{2min}), using the following equation: $Q_{10} = (R_2/ R_1)^{(10/(T2 - T1))}$

RNA extraction and cDNA synthesis

100mg deep-frozen respiratory tree (RT) samples were immediately homogenized in 1 ml of TRIzol® Reagent (Life Technologies, California, USA). The RNA extraction followed the manufacturers recommendations. The quantity of the extracted RNA was measured in a BioPhotometer (Eppendorf, Hamburg, Germany). Possible contaminations of genomic DNA were removed using DNase (Promega, Madison, WI) digestion according to the manufacturers recommendations. Subsequently, single-stranded cDNA was synthesized at 42°C for 60min, using the GoScript™ Reverse Transcriptase (Promega, Madison, WI) following the manufacurer recommendations. The reaction was based on 4µl RNA template, mixed with 0.5µl oligo$(dT)_{20}$ and 0.5µl of random primers. Revers transcription was conducted.

Primer design for the RT-qPCR assay

Besides Hsp70, two established reference genes 18S rRNA (18S ribosomal RNA) and ACTB (*β*-actin) (e.g. Kozera *et al.* 2013) were targeted in this study. 18S sequences are available for

six sister species of the genus *Holothuria* (AY133470.1 – AY133475.1). These published sequences were used to generate a homologous sequence alignment using BioEdit 7.2.5 (Hall 1999) to identify conserved primer binding sites. Quality control of the primers was conducted using PCR Primer Stats (Stothard 2000). *β*-actin and the target gene Hsp70 were not available for any *Holothuria* species. Therefore this gene was targeted using RACE-PCR (Fig. 1) (Schramm et al., 2000) with adegenerated forward primer in combination with an anchor-primer, introduced at the cDNA synthesis using an anchored OligoT primer (Odt7). The *β*-actin forward primer (ACTB_FW: ACTCTGCTACGTCGCTCTTG) was designed from *Apostichopus japonicas*, whereas, Hsp70 followed the following approach: The known *A. japonicus* sequence (EU930813.1) was utilized to BLAST (Altschul et al., 1997) the Sequence Read Archive (SRA) of *Holothuria glaberrima.* The best 50 hits were imported to the software BioEdit 7.2.5 followed by a contig assembly program (Huang et al, 1999). Three out of seven contigs were selected for further alignments with homologous Hsp70 sequences from animals in various taxa: *Apostichopus japonicas* (EU930813.1), *Parastichopus californicus* (GAVO01016544.1, GAVO01019403.1), *Ciona intestinalis* (AK116745), *Lottia gigantea* hypothetical protein mRNA (XM_009047345), *Psammechinus miliaris* (FN796462) and *Brissopsis lyrifera* (FN667017). The function similarity matrix (for shading) was applied in the alignment window to detect conserved regions, from which the HSP70 primers were designed.

The degenerated forward primer Hsp70_480_fw (CCRGAAGAAATYAGYTCSATGGT) was used in combination with the Odt7 anchor primer at 47°C and 35 cycles. 1µl of the resulting PCR product was used as template in a nested PCR (Fig. 2) using Hsp70_680_rv (TTCTTATCGAGCCCATAGGC) at an annealing temperature of 47°C and 35 cycles. All PCRs used the Opti*Taq* DNA Polymerase kit (Roboklon, Germany) for the amplification. Visible PCR products of the expected size for Hsp70, 18S and β-actin were prepared for sequencing using the ExoSAP-IT™ protocol (USB, Germany), following the manufactures recommendations. The identity of the PCR products was confirmed by Sanger sequencing (StarSEQ GmbH Germany), which was used to pick qPCR primers using Primer-BLAST (Ye et al., 2012). Primer quality was analysed using PCR Primer Stats of the oligo analyser 3.1 software (Owczarzy et al., 2008). For Sanger sequencing the unconsumed primers and nucleotides were eliminated, using ExoSAP-IT® PCR Product Cleanup kit (Affymetrix), according to the manufacturer's instructions. For final qPCR primer design the resulting contig was used as a query sequence in a primer BLAST on NCBI (Ye et al. 2012). The

chosen primer parameters were set to have a PCR product above 70 bp. The melting temperature fell into a range of 57 °C to 6 °C. The Echinodermata Refseq mRNA database was used to exclude amplification of wrong targets. The resulting *H. scabra* primers were tested at three different annealing temperatures (52°C, 54°C, 56°C) and 5% DMSO per PCR reaction was added to reduce primer dimer formation.

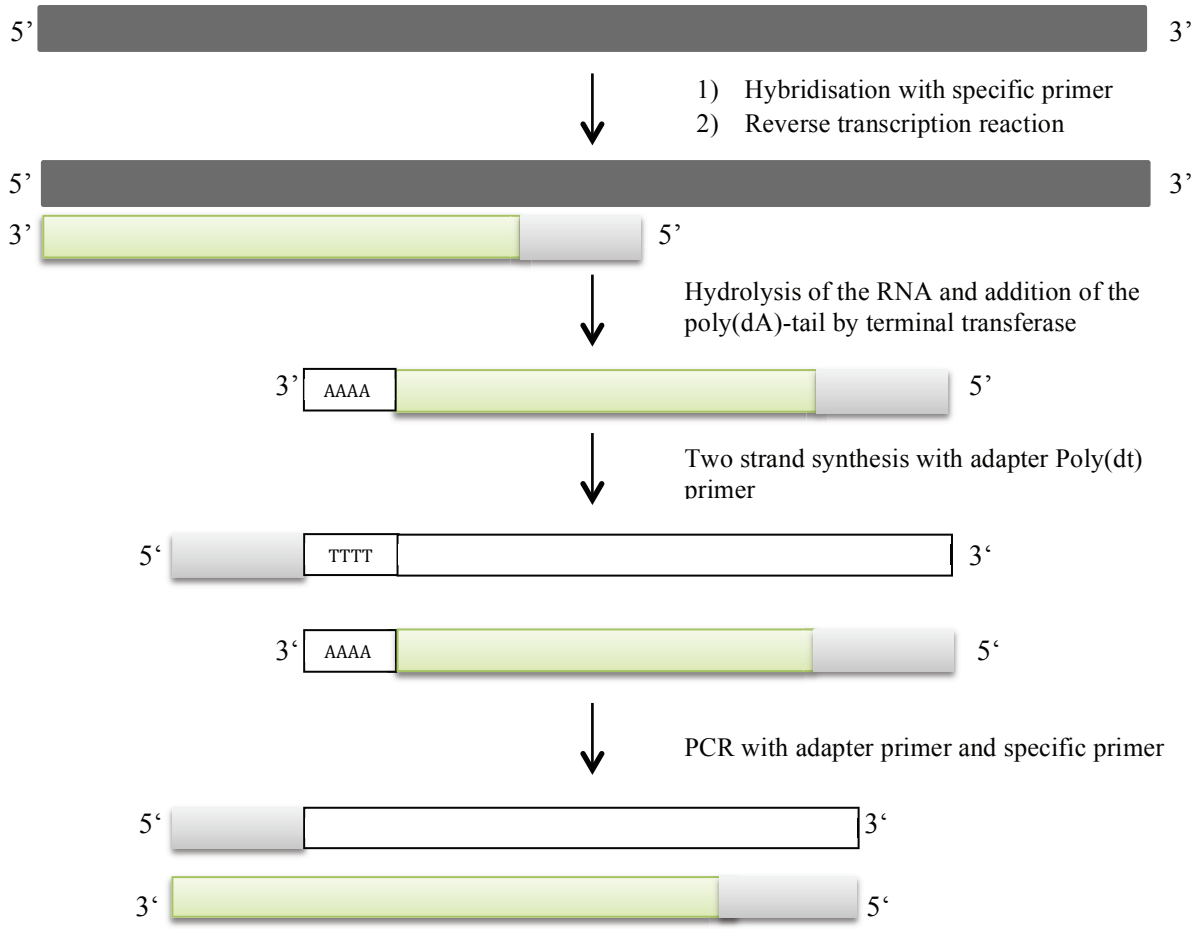

Fig. 1 principle of 5'-RACE-PCR (Jansohn et al. 2007).

RT-qPCR

The expression of Hsp70 was measured relative to the expression level of the two control genes 18S and β-actin in RT tissue. Measurements were conducted in three technical replicates using sterile H_2O as negative control and a confirmed cDNA sample as positive control. Per sample, the final qPCR mixture contained 7.8µl sterile H_2O (sterile DEPC-water, Roth), 10µl iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 0.1µl of the respective forward and reverse primers (HSP70, 18S, β-actin; 500nM in mix) (Biomers GmbH, Ulm, Germany) and 1.5µl cDNA sample. The qPCR reactions were conducted on the CFX

Manager™ Real Time PCR System (Bio-Rad Laboratories Inc., California, USA). The cycling protocol included an initial heating phase (95°C; 3 min), followed by 45 repeats of denaturation (95°C; 10 s) and elongation (60°C; 30 s), and finally the melting curve (95-60°C, 0.2°C s^{-1}) to confirm single amplification products without primer dimers.

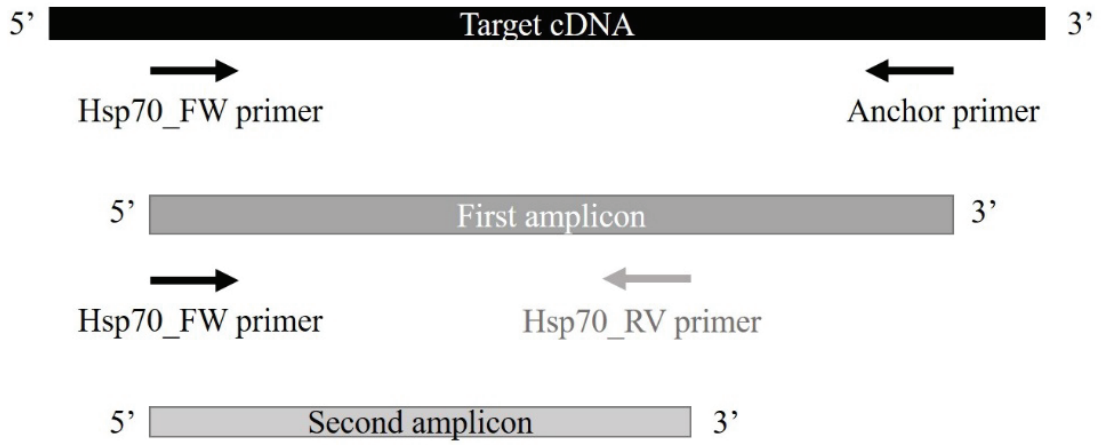

Fig. 2 nested PCR approach.

Differential gene expression analysis

The output data (Cq-values) from the RT-qPCR measurements were exported from Bio-Rad CFX Manager™ (Version 3.0) software to qBase+ qPCR analyzing software. The expression levels of the target gene Hsp70 are given in calibrated normalized relative quantities (CNRQ values) ± the 95% confidence interval (CI), which automatically account for replicate variability, amplification efficiency and normalization factors. To test the stability of the two reference genes 18S and β-actin, geNorm (Vandesompele et al., 2002) was utilized. Homogeneity of reference targets was assumed at M values <0.5. Differences between mean (n = 8) relative Hsp70 expression levels and the three temperature treatments: Control (29 °C), cold shock (17 °C) and warm shock (41 °C), was determined by a t-test on the log-transformed data. Significant differences were assumed at $P<0.05$ (*) and highly significant differences at $P<0.01$ (**).

Results

Oxygen consumption

Within the range of 22 – 38 °C, temperature and oxygen consumption rates (OCR) of *H. scabra* correlated significantly positive ($R^2 = 0.99$) (Fig. 2). At 38 °C OCR peaked by 33.2 ±

4.7 $\mu gO_2\ g^{-1}\ h^{-1}$ (n=8), at maximum temperature (41 °C) OCR dropped to 13.3 ± 5.1 $\mu gO_2\ g^{-1}\ h^{-1}$ (n=8). At temperatures ≤ 22 °C *H. scabra* reached minimum metabolic activity with OCR between 1.6 – 3 $\mu gO_2\ g^{-1}\ h^{-1}$ (n=8). The animals cultured at constant ambient (control) conditions (29 °C) exhibited a mean OCR of 13.2 ± 2.7 $\mu gO_2\ g^{-1}\ h^{-1}$ (n=8). The calculated OCR (according to the linear regression between 22 – 28 °C) corresponding to the theoretical optimal temperature (T_{opt}; 30.5 °C) was 17.5 $\mu gO_2\ g^{-1}\ h^{-1}$. The temperature quotient (Q_{10}) reached 11.7 for the OCR change between 22 – 30.5 °C (temperature down-regulation) and 2.3 for the OCR change between 30.5 – 38 °C (temperature up-regulation) (Tab. 1).

Table 1: O_2 consumption rates at the temperature points: Ambient conditions (control), optimal (T_{opt}), critical warm (CT_{max}) and critical cold (CT_{min}). Temperature quotients are given for changes in O_2 consumption rates between T_{opt} - CT_{max} and T_{opt} - CT_{min}.

Temp. point	Temp. (°C)	O_2 consumption ($\mu g\ g^{-1}\ h^{-1}$)	Q_{10}	
			22 – 29 °C	29 – 38 °C
Control	29	13.2 ± 2.7		
T_{opt}	30.5	17.5 (calculated)		
CT_{min}	22	2.2 ± 1.4	13.2	
CT_{max}	38	33.2 ± 4.7		2.8

Table 2: Primer sequences, for the three target genes (Hsp70, β-actin and 18S) in *H. scabra,* and technical details: Number of amplicon base pairs (bp), melting temperature (Tm) and total content of Gs and Cs (GC).

Primer pair	Sequence (5'->3')	Amplicon (bp)	Tm (°C)	GC (%)
Hsp70 (forward)	ATCCCGTTACCCATGCTGTG	145	60.11	55.00
Hsp70 (reverse)	AGCCCATAGGCAATAGCAGC		60.25	55.00
β-actin (forward)	ACTCTGCTACGTCGCTCTTG	143	58.7	55.0
β-actin (reverse)	GGAAGAGTGTCTCTGGGCAA		58.6	55.0
18S (forward)	GCTACTACCGATCGAATGGC	161	57.2	55.0
18S (reverse)	GATCCATCTGCAGGTTCACC		57.5	55.0

Primer design

BLAST results confirmed the identity of control and target genes (Acc. No. XXYYYYYY.1 - XXYYYYYZ.1 of submitted sequences) and Primer BLAST led to the successfully tested primer sequences listed in Table 2. All products were confirmed by sequencing.

Hsp70 gene expression

The animals that were exposed to the acute temperature elevation (+12 °C), up to 41 °C within six hours, showed a highly significant up-regulation of Hsp70 ($p = 0.000028$), in the respiratory tree tissue, compared to the control animals cultured at constant temperature (29 °C), over the same time period. In contrast to that, a temperature decrease of the same magnitude (-12 °C) did not effect Hsp70 expression in *H. scabra* (Fig. 1 and Table 3). During both experiments the two reference genes, β-actin and 18S, exhibited stable homogeneity.

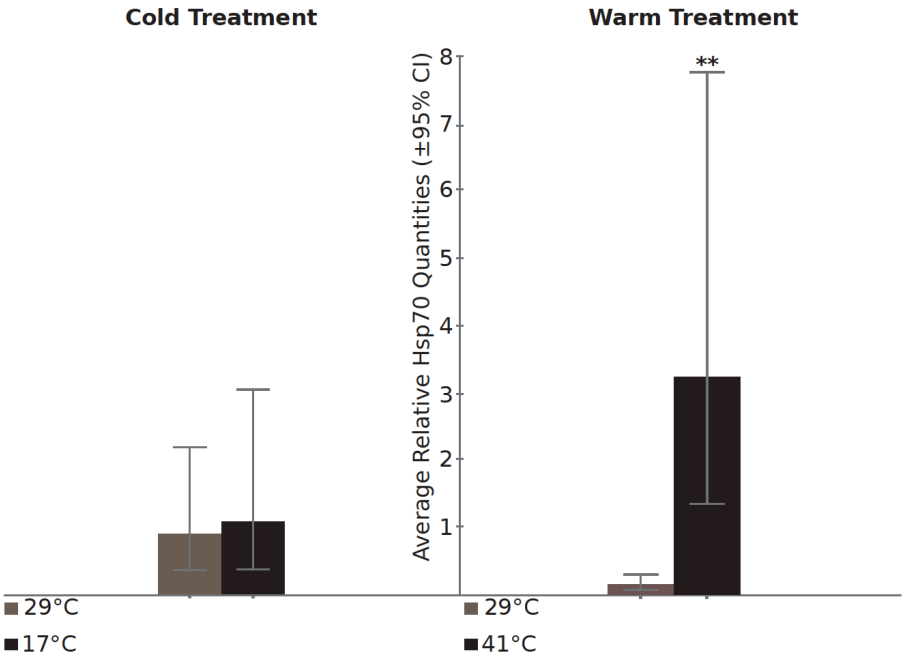

Fig. 3: Average relative quantities of Hsp70 gene expression, for the two temperature manipulation experiments, cold (17°C; n=8) compared to control (29°C; n=8) (left panel) and warm (41°C; n=8) compared to control (29°C; n=8) (right panel). Error bars indicate upper and lower 95% confidence intervals (CI). The statistical analysis was performed on log-transformed data, which led to the non-symmetrical CIs. Differences between treatments were calculated using t-test. Significant differences are indicated for P<0.05 (*) and P<0.01 (**).

Table 3: Hsp70 gene expression levels shown in mean calibrated normalized relative quantities (CNRQ) ± 95% confidence interval (CI), for both temperature manipulation experiments. Significant differences are indicated for P<0.05 (*) and P<0.01 (**).

		Heat shock			
Gene	Temperature (°C)	Mean (CNRQ)	+ 95% CI	- 95% CI	P-value
Hsp70	42 °C	3.24	7.77	1.35	2.81E-5**
	29 °C (control)	0.15	0.3	0.07	
		Cold shock			
Hsp70	17 °C	1.09	3.04	0.39	0.75
	29 °C (control)	0.91	2.19	0.38	

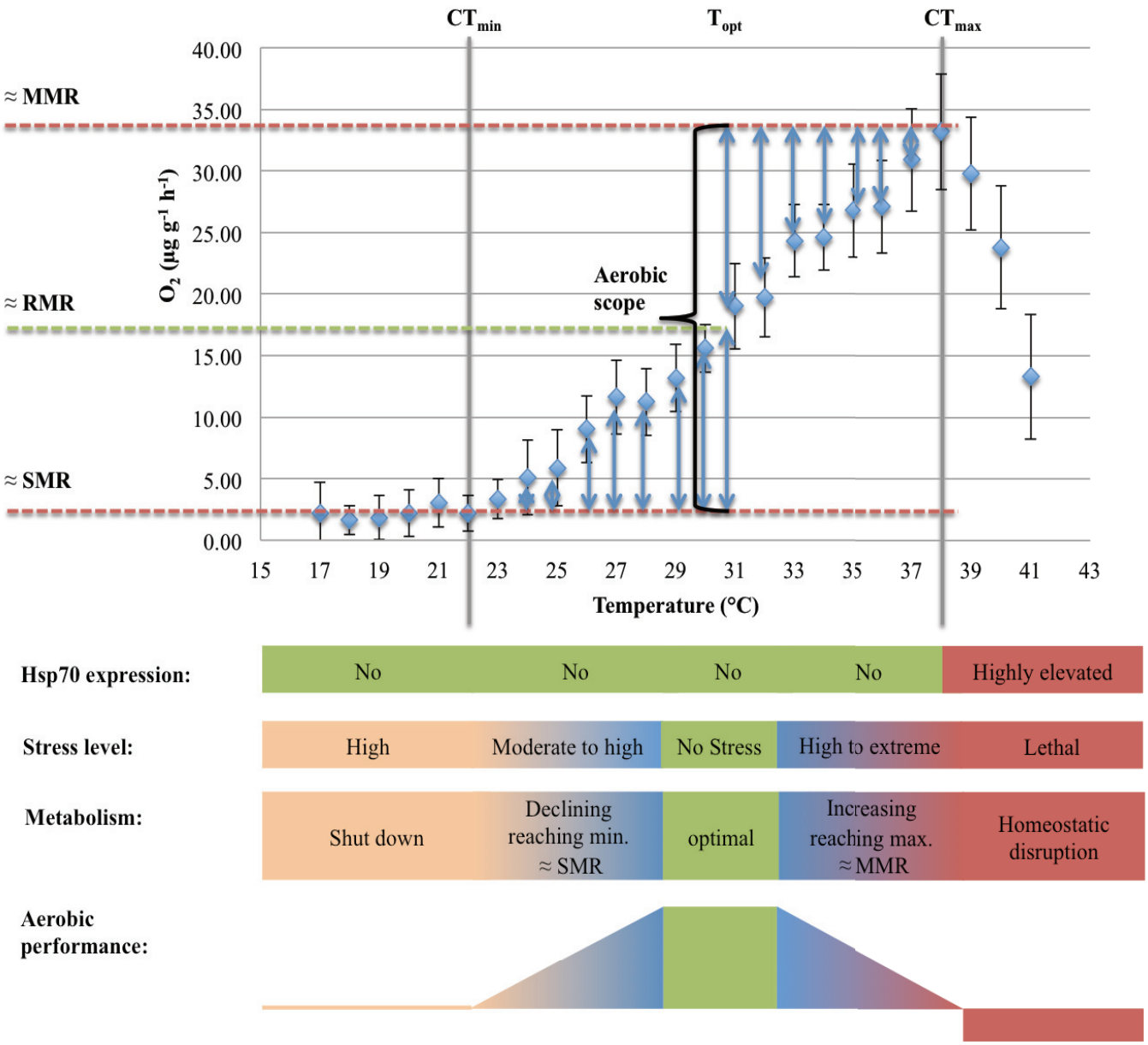

Fig. 4: O_2 consumption rate of *Holothuria scabra* over temperature. Data represent means (n=8) and standard deviation. Upper red dotted line indicate peak O_2 consumption approximately maximal metabolic rate (≈MMR), at the critical warm temperature (CT_{max}; 38 °C), lower red dotted line marks the start of the lowest O_2 consumption range, approximately standard metabolic rate (≈SMR), at critical cold temperature (CT_{min}; 22 °C). The green dotted line marks theoretical optimal performance (17.5 $\mu gO_2\ g^{-1}\ h^{-1}$) at the center of the approximated routine metabolic rate (≈RMR). The optimal temperature (T_{opt}; 30.5 °C) is located in equal distance to CT_{min} (≈SMR) and CT_{max}, (≈MMR) where aerobic scope is assumed (black brace). Above and below T_{opt} the aerobic performance, marked as distance to CT_{max} and CT_{min} (arrows left and right from centre), respectively, decreases. At CT_{max} and CT_{min} the aerobic performance diminishes. Temperatures >38 °C lead to a homeostatic disruption (negative aerobic performance), indicated by reduced O_2 consumption rate, but, elevated heat shock response (Hsp70 expression). Temperatures <23°C *H. scabra* cause stable state conditions, indicating resting metabolism, but, no elevated Hsp70 expression.

Discussion

Definition of SMR, RMR and optimal performance

The capacity to perform aerobically (aerobic scope) is widely considered as key in determining the response of marine species towards future ocean-warming scenarios (Pörtner, 2001; Pörtner and Knust, 2007). The quantification of aerobic scope requires the accurate and species-specific quantification of standard metabolic rate (SMR) and maximal metabolic rate (MMR). In this study we determined SMR and MMR in the sea cucumber *Holothuria scabra* (exemplary for a slow moving bottom-dwelling marine invertebrate species), through acute temperature induced (17 – 41 °C) peak- and base- O_2 consumption. Under acute temperature stress, the energy demand for basal maintenance increases with warming, and decreases with progressively colder temperatures to sustain energy-homeostasis. Basal maintenance is, however, limited by a maximal- and minimal- metabolic capacity (e.g. oxygen uptake- or mitochondrial- efficiency) (Guderly and Pörtner, 2010). *H. scabra* exhibited the distinctly highest oxygen consumption at 38 °C, hence we interpreted this peak performance as close approximation of MMR (≈MMR). Above this temperature, the respiration rate decreased although the intensity of temperature stress increased. At these extreme temperatures (39 – 41°C) we expect survival only over a very short time span, before functional oxygen deficiency (negative aerobic performance) and the high temperature itself will cause fatal damage. Thus, in combination with the significant mRNA expression of Hsp70 we assume the transition from a high stress- to a lethal- level above 38 °C (Fig. 4). At critical cold temperatures metabolic adjustments are limited by minimal basal maintenance costs, which represent minimal energy requirements to sustain life functions. This metabolic bottom-line is equivalent to SMR (Guderley and Pörtner, 2010; Koojimann, 2010). In the present study, *H. scabra* showed metabolic stable-state conditions at temperatures ≤ 22 °C. Hence, we interpret this minimal respiration range as close approximation of SMR (≈SMR) for *H. scabra*. At this resting state (< 3 $\mu gO_2\ g^{-1}\ h^{-1}$), *H. scabra* is presumably suffering from a negative energy balance, where any expenses for somatic growth depend progressively on energy depots. The lack of Hsp70 expression at 17 °C, however, implies no occurrence of acute, damaging temperature effects. Hence, the stress level is interpreted as high, due to seemingly diminished biological fitness, but not yet lethal (Fig. 4).

The temperature induced ≈SMR and ≈MMR forms an alternative basis to analyze aerobic scope in slow-moving species like *H. scabra* to determine energy-homeostasis, and to distinguish between ecologically and physiologically important states (reviewed by Sokolova

et al., 2012). In the current work the two metabolic benchmarks (≈ SMR and ≈MMR) served also to predict optimal temperature (T_{opt}) for *H. scabra*, as temperature that corresponds with the center (30.5 °C) between ≈SMR and ≈MMR (assumed aerobic scope) (Fig. 4). For some reef fish the highest aerobic scope was found at temperatures of 1 – 2 °C above summer maxima values (Rummer et al., 2014). This is in line with our determined optimal (30.5 °C) for *H. scabra* at the assumed aerobic scope. This temperature is slightly above ambient natural conditions at our study side (Lombok, Indonesia), which generally never exceed 30 °C (Firdaus, unpublished; supplementary material S3.5). In previous studies, the oxygen consumption rate of *H. scabra*, at unstressed conditions, ranged from 9 – 12 $\mu gO_2\ g^{-1}\ h^{-1}$ (Collard et al. 2014) and 11 – 16 $\mu gO_2\ g^{-1}\ h^{-1}$ (Kühnhold et al., 2017). In the current study the corresponding O_2 consumption at T_{opt} was calculated and revealed a respiration rate of 17.5 $\mu gO_2\ g^{-1}\ h^{-1}$, as a theoretical value for optimal performance of *H. scabra*. This implies that O_2 consumption increased marginally above normal conditions, at the center between ≈SMR and ≈MMR, which relates to the elevated T_{opt} (30.5 °C), slightly above natural maxima levels.

In the present data, the theoretical O_2 consumption at 30.5 °C (17.5 $\mu gO_2\ g^{-1}\ h^{-1}$) is assumed as center of routine metabolic rate (≈RMR) (Fig. 4), which *H. scabra* was able to adjust as response to acute temperature change within the range of 22 – 38 °C. Similar to many other biological rate functions, the rate of metabolic change, as response to temperature, is predictable through the temperature quotient Q_{10} (approximately a doubling or tripling of a given rate function for every 10 °C of temperature increase, Q_{10} ≈ 2-3) (Schmidt-Nielsen, 1990; Clarke and Johnston, 1999). Within the linear phase of the temperature up-regulation experiment (29 – 38 °C) *H. scabra* exhibited a Q_{10} of 2.8 (Tab. 1), which is within the expected range. Along the linear regression during temperature down-regulation (22 – 29 °C), however, the Q_{10} value was dramatically higher (Q_{10} = 13.2) (Tab. 1). This gives the clear result of a strongly pronounced metabolic rate response due to cold stress. Interestingly, the sharpest decline in O_2 consumption occurs below 24 °C (5.1 to 2.2 $\mu gO_2\ g^{-1}\ h^{-1}$), this point was also defined by Purcell et al. (2006) and Wolkenhauer (2008) as critical temperature, below which growth of *H. scabra* declined. In long-term exposure experiments over 30 days, however, *H. scabra* was able to successfully acclimate to water temperatures of 33 °C and 21 °C, by adjusting respiration rate and metabolic enzyme activity (Kühnhold et al., 2017). These results show that *H. scabra* survived and remained active over a prolonged period at temperatures even below 22 °C. This matches with the present results, which

predict the optimal performance of *H. scabra* to occur at 30.5 °C, and no induction of cellular stress (Hsp70) at temperatures down to 17 °C.

mRNA expression of Hsp70

Hsp70 is a highly conserved cellular chaperon and known as universal stress response in many taxa, ranging from bacteria via plants to mammals (Beere and Green, 2001; Srivastava, 2002; Sørensen et al., 2003). Until now Hsp70 sequences were only available for two *Holothuria* species, *A. japonicus* (Shao et al., 2015) and *H. tubulosa* (Vazzana et al., 2015). For *H. scabra*, however, such sequence data have not been published yet. In this study we successfully targeted, for the first time, the full-length cDNA sequences of Hsp70, 18S and β-actin in respiratory tree tissue of *H. scabra.* Above 38 °C Hsp70 gene expression was highly elevated in *H. scabra*, although basal maintenance, represented by whole-organism respiration, decreased already sharply. This provided evidence that Hsp70 is intensively involved in cellular defence mechanisms, which caused the homeostatic disruption in *H. scabra* at temperatures between 39 – 41 °C. It is known that negative effects such as reduced cell growth rates (Feder et al., 1992) and productivity (Krebs and Loeschcke, 1994) can be associated with enhanced Hsp70 expression. This interaction is in line with our findings, which showed a decline in respiration, while Hsp70 was highly expressed.

The temperate sea cucumber species *Apostichopus japonicus* showed significantly up-regulated Hsp70 levels at an acute temperature increase up to 20 °C and 25 °C, compared to 16 °C (control), were the highest Hsp70 expression corresponded to the highest temperature (Shao et al., 2015). For the tropical gastropod species *Pomacea canaliculata*, the induction temperature for Hsp70 expression was at 36 °C, whereas maximal Hsp70 expression was reached at 42 °C. Cold temperature on the other hand caused only slight reductions in Hsp70 expression at temperatures below 16 °C (Song et al., 2014). This temperature range for *P. canaliculata* is in line with our findings for *H. scabra*, which revealed a highly significant up-regulation of Hsp70 after a fast temperature increase, up to 41 °C, but no significant effect after a fast drop in temperature, down to 17 °C. Although, in this study, we did not define Hsp70 induction temperature for *H. scabra*, the pattern of stable Hsp70 expression at colder temperatures, and the significant Hsp70 up-regulation at warm conditions, show clear similarities between *P. canaliculata* and *H. scabra*. Similar expression pattern were also found for the insect *Drosophila melanogaster* (Sejerkilde et al., 2003), and the two fish species *Danio rerio* (Airaksinen et al., 2003) and *Oncorhynchuss myhiss* (Currie et al., 2000).

For cold resistance, Hsp70 seems to play a minor role (Hoffmann et al., 2003). Acute cold-stress does not affect secondary and tertiary protein structures to the same extend as heat-stress does. Thus, cellular chaperones are not immediately required. Long-term cold acclimation, however, has shown to induce elevated Hsp70 levels, which was associated with the occurrence of chilling injuries (Fujikake et al., 2005). In the present study, those *H. scabra* that were exposed to 17 °C showed no differences in Hsp70 expression levels, compared to control animals (29 °C). This indicates that a water temperature of 17 °C was still above the threshold level at which cellular protection mechanisms were initiated.

Conclusion and perspective

In this study we defined peak- and base- O_2 consumption for the sea cucumber *Holothuria scabra* through acute induction of cold and warm temperature stress. We propose these metabolic benchmarks as accurate and reproducible approximation for the classical standard- (SMR) and maximal- (MMR) metabolic rate, based on physical performance, which enable the quantification of aerobic scope. Moreover, based on the consequential temperature-induced depiction of the aerobic performance window (Fig. 4), we showcase the prediction of optimal temperature- and performance- conditions for *H. scabra*. We suggest our approach, to determine temperature induced metabolic boundaries (≈SMR and ≈MMR), as good proxies for SMR and MMR in various marine benthic species. In classical aerobic scope analyses, however, the aerobic scope is compared between different conditions (e.g. temperature points). This means, to fully explore the potential of our approach, to target aerobic scope trough temperature stimulation, the temperature induced peak- and base-performance need to be quantified at different ambient stress conditions (e.g. different acclimation temperatures). We assume that the total difference between temperature-induced peak- and base- O_2 consumption (≈MMR – ≈SMR) would shrink, under stressful baseline conditions, at similar rates as the classical aerobic scope does (MMR – SMR). This hypothesis, however, remains to be verified in future studies.

The analyses of Hsp70 gene expression and changes in whole-organism respiration rate showed promising synergies for thermal stress detection in *H. scabra*. At the upper temperature end (>38 °C), the transition to the lethal end was clearly visible as defense mechanisms such as the heat shock response (i.e. Hsp70) were fueled for the cost of a progressively disturbed energy balance. At the bottom-most temperatures (17 – 22 °C), *H. scabra* exhibited a narrow, minimal metabolic range, but no signs of homeostatic disruption

neither at the O_2 consumption- nor at the Hsp70 expression- level. These interpretations rely on the assumption that Hsp70 expression was not impaired by cold temperature itself and that the expression level did not peak earlier, during the temperature manipulations, before the lowest and highest temperature endpoints of 17°C and 41°C, respectively, were reached. Future studies should compare Hsp70 expression levels in *H. scabra* at more temperature endpoints and exposure times, to define the exact Hsp70 induction points for this species.

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Chapter 4:
Acclimation performance
in eurytherms and stenotherms

This chapter is in review as:

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Acclimation capability inferred by metabolic performance in two sea cucumber species from different latitudes

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Abstract

The notion that thermal specialists from tropical regions will be more vulnerable to global warming than temperate eurytherms seems to be too generalized. Acclimation potential is primarily a function of metabolic capacity, which can differ distinctly between species. In this study the exposure of a tropical (*Holothuria scabra*) and a temperate (*Holothuria forskali*) sea cucumber species to identical cold- and warm- acclimation stress was compared in terms of key metabolic parameters, encompassing respiration rate, enzyme activity (ETS, LDH, IDH), and energy reserve fractions (lipid, carbohydrate and protein). Present results show much broader respiratory adjustments in *H. scabra* (2 – 30 μgO_2 gww^{-1} h^{-1}) compared to *H. forskali* (1.5 – 6.6 μgO_2 gww^{-1} h^{-1}), accompanied by clear temperature effects on enzyme activity and energy reserves in the tropical species, while energy turnover in the temperate species remains consistent. These findings indicate enhanced metabolic plasticity in *H. scabra*, at the cost of clearly elevated energy expenditures. Beside energetic burden, however, results indicate better acclimation capacity in the tropical stenotherm species. This study reveals the importance to holistically explore metabolic strategies in conspecifics and congeners to predict the heterogeneous effects of climate change across latitudinal gradients.

Keywords*: ectotherms, eurytherms, global warming, Holothuria scabra, Holothuria forskali, respiration, stenotherms, temperature stress*

Introduction

The emergence of thermal specialists and generalists implies a fundamental energetic trade-off between peak performance at optimal temperatures and thermal niche breadth (rev. by Verberk et al. 2015). While thermal specialists (stenotherms) have high performance levels

within a very narrow thermal window, thermal generalists (eurytherms) show comparatively lower performance levels across a wide thermal range (Schlichting and Pigliucci, 1998; Hochaschka and Somero, 2002; Somero et al., 2008; Angilletta, 2009). Generally, tropical and polar species are considered stenothermic and temperate species eurythermic. This classification is based on the widely accepted `Temperature Variability Hypothesis´, which assumes that middle-latitude species that are exposed to high seasonal and diurnal temperature variations evolved a stronger thermal tolerance than species from low- and high-latitudes (Janzen, 1967; Addo-Bediako et al., 2000; Sunday et al., 2011). In the context of global climate change this theory predicts that tropical specialists may experience the largest metabolic challenge due to rising temperatures, as they live already close to their upper temperature threshold level (Stillman, 2003; Tewksbury et al., 2008; Dillon et al., 2010; Huey et al., 2012). Consequently, tropical species may be especially vulnerable to oxygen uptake limitations at warmer temperature conditions (Somero, 2010; Rummer et al., 2014). Generalizations are, however, difficult as metabolic plasticity and adaptation capacity to global warming can differ considerably between species.

Findings by Seebacher et al. (2015) showed, though, the potentially adaptive response of thermal specialists to acclimatize to seasonal changes, by optimizing their energy demand through adjustments in respiratory performance. Similar results were found for the acclimatization potential of tropical stenothermic mangrove crabs (Fusi et al., 2014). These studies placed species-specific metabolic plasticity at the center of interest and proposed that actually equatorial stenotherms can possess a higher acclimatization potential than eurytherms. Apparently, the adaptation of thermal generalists to variable environments involves losses in acclimatization capacity (Huey and Berrigan, 1996; Hoffmann, 1999; Van Buskirk and Steiner, 2009; Chown et al., 2010). The debate about `winners and losers´ in future global warming scenarios remains ongoing, and the identification of metabolic plasticity patterns between tropical stenotherms and temperate eurytherms are, therefore, of prime interest to determine acclimation capacities of species from different climatic zones. Future global warming scenarios will intensify acclimatization pressure, especially for sessile and slow-moving benthic organisms like sea cucumbers, as they are unable to exhibit fast distribution shifts. Sea cucumbers can significantly shape benthic communities through their high abundances (Crozier, 1918; Birkeland, 1988; Billet, 1991). Moreover, they provide important ecological functions, such as sediment cleaning, nutrient recycling, enhancing of sea water chemistry, supporting biodiversity as host of many symbiotic associations and

transmission of primary food sources up to higher trophic levels as prey (rev. by Purcell et al., 2016).

In this study, metabolic characteristics of thermal acclimation in two Aspidochirotid sea cucumber species that inhabit similar ecological niches in the tropics (*Holothuria scabra*) and in temperate regions (*Holothuria forskali*) are compared. Previously, Kühnhold et al. (2017) established the combined assessment of key energetic enzymes activity [iso-citrate dehydrogenase (IDH) and lactate dehydrogenase (LDH)], oxygen consumption by direct (changes in whole-organism respiration) and indirect [activity of the electron transport system (ETS)] measurements, and changes in individual energy depots (carbohydrate, protein and lipid), as a comprehensive tool to asses energy-metabolic changes related to thermal acclimation, in the tropical sea cucumber *H. scabra*. In the current study, the same multiple biomarker approach was applied to the sea cucumber *H. forskali*, to assess the thermal acclimation response of a temperate species to a temperature change of identical magnitude. The aim of this study is to compare the new data set of *H. forskali* (eurytherm) with the already published data from *H. scabra* (stenotherm) to investigate whether the thermal acclimation capacity differs consistently in these two congeners from different latitudes. The assessment of thermal acclimation capacities in these two ecologically and commercially relevant sea cucumbers is pivotal to predict the effects of ocean warming, due to global climate change, and to minimize thermal stress in aquaculture scenarios.

Materials and Methods

Model species and sampling area

The sea cucumber *H. scabra* is distributed in tropical to subtropical regions (Hamel et al., 2001) within a preferred temperature window, ranging from 25 to 29 °C (OBIS, 2017). The species *H. forskali* on the other hand, occurs in temperate regions, along the entire Atlantic Coast of Europe and the United Kingdom, and in parts of the Mediterranean Sea (Mercier and Hamel, 2013). Due to factors such as seasonality, *H. forskali* is exposed to a broader thermal variability, hence, its preferred temperature window ranges from 11 to 19 °C (OBIS 2017). In this study, optimal temperature conditions were considered as center of each species-specific thermal window. Therefore, control temperatures of 27 °C and 15 °C where chosen for *H. scabra* and *H. forskali*, respectively. For both species juvenile animals of similar size (10 – 30g), were collected by SCUBA divers close to the shore in water depth between 3 – 10 m. *H. forskali* specimens were collected in February 2015 at Carreiro de

Joannes (39°21´14.3´´N, 9°23´43.6´´W) off the coast of Peniche, Portugal. *H. scabra* were collected in August 2014 at Pantai Sira di Pagi Hari (8°22´5.42´´S, 116°6´58.37´´E) off the coast of Lombok, Indonesia. After specimen collections, the animals were transported to the aquaculture facilities of the Alfred Wegener Institute, Helmholtz-Centre for Polar and Marine Research (AWI) in Bremerhaven, Germany.

Experimental design

The thermal acclimation experiments were conducted sequentially for each species. This allowed the use of the same culture tanks and identical experimental design conditions for both species. Upon arrival the animals were randomly assigned to three separate HDPE tanks (n = 24), filled with 100 L aerated seawater (15 °C, 33 ppt). The photoperiod was set to 12h:12h (light:dark) and sea cucumbers were fed *ad libitum*, with the commercial feed `Algamac´ (39% protein, 20.4% lipid and 20.6% carbohydrate) (www.aquafauna.com). After an acclimation period of 28 days, the cultured animals were exposed to incrementally increasing (+1 °C/day) and decreasing temperatures (-1 °C/day), until the designated treatment temperatures (*H. forskali*: cold: 9 °C, control: 15 °C warm 19 °C; *H. scabra*: cold: 21 °C; control: 27 °C; warm: 33 °C) were reached. Seven animals were sampled after six days, when the desired treatment temperatures were reached (t0), and subsequently after an acclimation time of 15 (t15) and 30 days (t30). At each sample time, longitudinal muscle (for LDH, IDH and energy depots) and respiratory tree (for ETS) tissues were removed (*H. forskali*: n = 7: *H. scabra*: n = 6), immediately shock frozen and stored at -80 °C. In addition, at each sample interval, marked animals (n = 3; photo identified) were placed in gas-tight acrylic chambers, to measure the real-time oxygen consumption of the living animals. The measurements for oxygen consumption as well as the enzyme assays (LDH, IDH, ETS) and analyses of energy depots (protein, carbohydrate and Lipids), were conducted as per Kühnhold et al. (2017).

Statistical analysis

Subsequent to testing the data for normality and homogeneity (Kolmogorov–Smirnov; Levene tests), statistical differences between temperature treatments (cold, control and warm) and measurement times (t0, t15 and t30) were evaluated by two-way ANOVA (Microsoft Excel 2011 with “StatPlus” for Mac). Significant multiple comparisons were determined using the Fisher-LSD post-hoc test. For all statistical tests, the significance level was set at

$p<0.05$. Data are expressed as mean ± minimum and maximum values. For an overall comparison, a `Principal Component Analysis´ (PCA) was also conducted using the statistical program R-Studio (package: "vegan"), where missing replicates were replaced by group means.

Results

Enzymes related to energy metabolism

The specific LDH activity in the temperate species *H. forskali* did not differ significantly between temperature treatments and between time points. In *H. scabra* LDH activity dropped over time with significant differences between t0 and t15 ($p = 0.0111$) and t0 and t30 ($p = 0.0001$), in the warm acclimated animals. The lowest LDH activities were measured under warm conditions after 30 days, with significant differences between warm (33 °C) and control (29 °C) treatment ($p = 0.025$). Specific IDH activities in *H. forskali* exhibited no significant changes over time or between temperature treatments. In *H. scabra*, after 30 days (t30) of acclimation to 33 °C, IDH activities exceeded LDH activities in absolute numbers (IDH = 7.8 ± 2.7 nmol min^{-1} $mgProt.^{-1}$; LDH = 4 ± 0.64 nmol min^{-1} $mgProt.^{-1}$) (Fig. 2). This switch in dominance of energy related key enzymes, was only observed under warm conditions in *H. scabra*.

Direct and indirect oxygen consumption

At the first measurement time (t0) *H. forskali* exhibited significantly lower ETS activities at warmer (21 °C) temperatures, compared to the activities measured at control (15 °C) and cold (9 °C) conditions ($p = 0.0155$; $p = 0.0226$). Thereafter *H. forskali* maintained stable ETS activities at all temperatures and throughout the acclimation time. *Holothuria scabra* initially (t0) showed a significant peak in ETS activity at warm conditions compared to the control ($p = 0.001$) and cold ($p = 0.005$) treatment. Over the remaining experimental time mean ETS activities of *H. scabra* did not vary significantly between treatment conditions. Throughout the experiment, *H. forskali* maintained its OCR within a mean range of 1.5 – 6.6 μgO_2 gww^{-1} h^{-1}, and showed an overall trend of similar oxygen consumption rates for control (15 °C) and warm (21 °C) exposed species, while cold (9 °C) exposed animals showed significantly lower OCR levels compared to control conditions (t0: $p = 0.0376$; t15; $p = 0.0078$; t30; $p = 0.0142$). The experiment with *H. scabra* revealed consistently higher mean OCR levels in warm (33 °C) exposed specimens, and lower consumption rates under cold (21 °C) conditions. This

trend was noticeable throughout the entire experiment, although significant differences were only measured at t15 (33 °C vs. 21 °C; p = 0.019) and t30 (33 °C vs. 21 °C; p = 0.047). For *H. scabra* the overall range of mean OCR levels was between 2 – 30 μgO_2 gww^{-1} h^{-1} (Fig. 2).

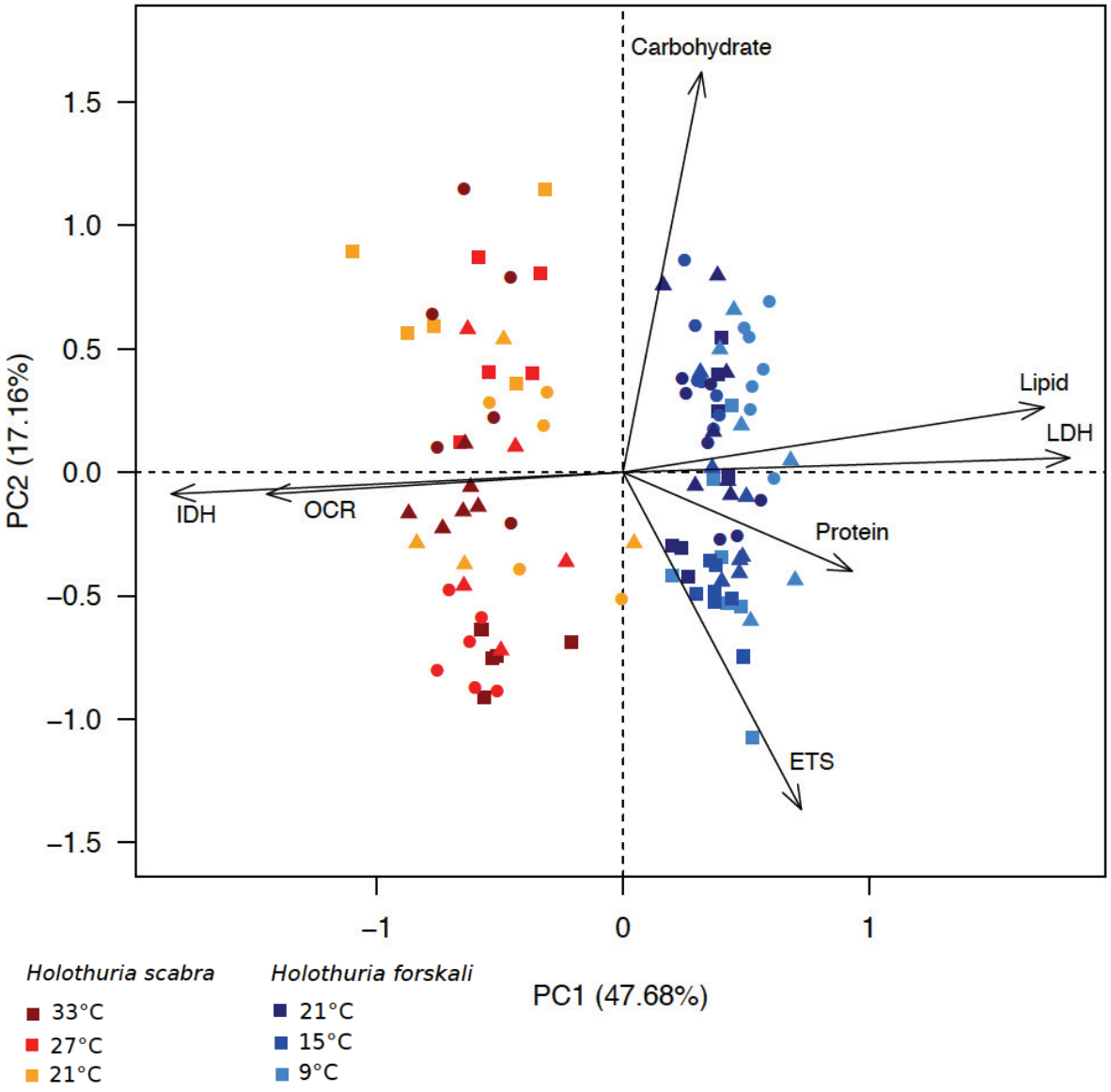

Fig. 1: Principal Component Analysis (PCA). The two species, *Holothuria scabra* and *Holothuria forskali*, and the different temperature treatments are indicated by different colors (see legend). Time points are presented in different shapes: Circle (t0), triangle (t15), and square (t30). Principal component 1 (PC 1) is given in the dimension of the x-axis and explains 47.68% of all differences between the parameters. Principal component 2 (PC2) is represented as y-axis dimension and explains 17.16% of all data variation. This means 64. 84% of all data disparities due to the three variables 1) species, 2) temperature and 3) time can be simplified in a two-dimensional model, visualized through PC1 and PC2.

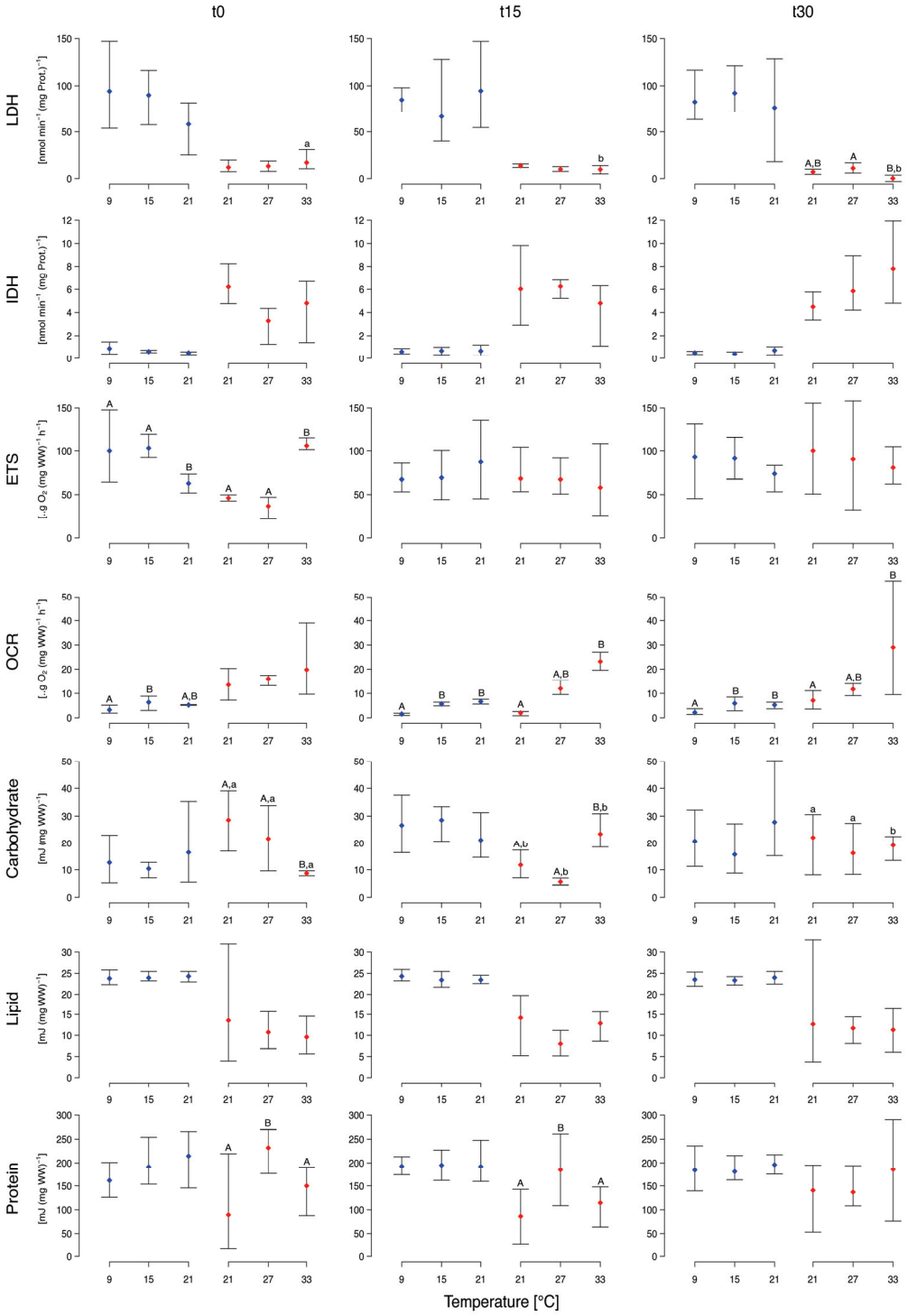

Fig. 2: Data for *Holothuria scabra* (Kühnhold et al., 2017) temperature treatments of 21 °C, 27 °C, and 33 °C (red diamonds).,*H. forskali* temperature treatments of 9 °C, 15 °C, and 21 °C (blue diamonds). Measured parameters, activity of lactate dehydrogenase (LDH), iso-citrate dehydrogenase (IDH), electron transport system (ETS), oxygen consumption rate (OCR), amount of carbohydrate, lipid, and protein. Measurement times, day 0 (t0), day 15 (t15), and day 30 (t30) are shown from left to right. Data points represent mean values with error bars of minimum and maximum values. ^{A,B} indicate significant differences between temperature treatments within one measurement time and ^{a,b} indicate significant differences between measurement times at one temperature (two-way ANOVA, Fisher LSD, p = 0.05)

Energy reserve fractions

Mean carbohydrate levels in the muscle tissue of *H. forskali* did not differ significantly between the three treatment temperatures or between measurement times. *H. scabra* exhibited significant variations in carbohydrate levels over time and between treatment temperatures. At the first sampling (t0), warm (33 °C) treated *H. scabra* exhibited significantly lower carbohydrate levels compared to the specimens cultured under cold (21 °C; $p = 0.00001$) and control (27 °C; $p = 0.0023$) conditions. Over time, *H. scabra* that were acclimated under warm conditions showed a significant increase in carbohydrate level ($p = 0.0004$) between the first (t0) and the second measurement time (t15). Whereas, control and cold animals showed a significant drop in carbohydrate levels between the first two measurement times ($p = 0.0002$; $p = 0.0001$), which led to significantly lower levels relative to the warm conditions ($p = 0.00003$; $p = 0.0043$). After 30 days of acclimation (t30), no significant differences in carbohydrate level in *H. scabra* were observed. Neither *H. forskali* nor *H. scabra* showed significant differences or trends between treatment temperatures and measurement times in the lipid fraction. No significant differences or trends were visible in the protein levels measured in *H. forskali*. Muscle protein levels in *H. scabra* were significantly different at the first two measurement times (t0 and t15), as control animals had higher protein levels compared to the levels measured at cold (t0: $p = 0.0004$; t15: $p = 0.0092$) and warm (t0: $p = 0.0027$; t15: $p = 0.0486$) treatments. At t30, however, the significant differences between the three treatments were no longer observed. In *H. forskali* total mean protein levels ranged from 160 – 220 mJ mg^{-1}, while in *H. scabra* total mean protein varied markedly and ranged from 80 – 240 mJ mg^{-1} (Fig. 2).

Principal component analysis (PCA)

All parameters measured for both species, at each measurement time and temperature treatment, are summarized with a principal component analysis (PCA) (Fig. 1). This data view revealed that PCA 1 (x-axis) is driven primarily by differences between species and explains 47.7 % of all differences between the data. The associated vectors along the x-axis, which tend in opposite directions, indicate that the differences between the species is mainly explained by the parameters IDH, LDH, OCR, and lipids. While IDH and OCR are consistently higher in *H. scabra*, and LDH and the lipid fraction were higher in *H. forskali*. PCA 2 (y-axis) explains merely 17% of the differences between all measured parameters. In this dimension the vectors of ETS and carbohydrate are responsible for most of the variation.

Discussion

Assessing species-specific susceptibility and plasticity towards climate change at different latitudes is key to understanding future biogeographic population shifts and impacts on ecosystem functioning. In the current study we compared two distinctly related congeners from different latitudes to investigate potential connectivity between adaptation to regional thermal variability and metabolic plasticity. It was predicted that the distinct geographical habitats of the two studied sea cucumber species make them suitable model organisms, representing: 1) A tropical stenotherm (*H. scabra*); and 2) a temperate eurytherm (*H. forskali*). Overall, present data confirm this assumption. *Holothuria forskali* exhibited relatively consistent metabolic performance over a broad temperature range (12 °C), which is indicative for a thermal generalist. *Holothuria scabra* on the other hand, showed a pronounced metabolic response over a temperature change of the same magnitude, especially during warm-acclimation, which characterizes a thermal specialist.

The metabolic enzyme LDH is a crucial component in anaerobic energy pathways (Hochachka and Somero, 2002). LDH activity levels have been identified as adaptive trait to distinct thermal variability and geographical latitudes (Fields and Somero, 1998). In sea cucumbers, increased LDH activity can indicate stress response to hypoxia (Guo et al., 2014) and transportation stress (Tonn et al., 2016). The activity of IDH represents the energetic antagonist of LDH, reflecting aerobic energy turnover. *H. forskali* showed no significant trends in metabolic key enzymes activities of LDH and IDH, neither between temperature treatment conditions nor over the acclimation time of 30 days. What is noticeable, albeit not significant, is the elevated IDH level in cold treated (9 °C) *H. forskali* at the first measurement time (t0), as well as at warm conditions (21 °C) after 30 days acclimation (t30). The same pattern was observed in *H. scabra* and may indicate increased aerobic performance after the initial drop in temperature (-6 °C), due to acutely elevated energy requirements. The enhanced IDH activity under warmer conditions at the final measurement time (t30) appears to indicate a growing relevance of aerobic energy turnover over time, due to warm-acclimation in both species. In contrast to *H. forskali*, however, LDH levels in warm treated *H. scabra* followed a reverse pattern relative to IDH over time, with significantly lower LDH expression levels at t15 and t30 compared to t0. Activity changes in LDH represent switches in energy turnover due to challenging conditions such as various stresses (Dahlhoff, 2004). At t30 the LDH activity level of warm acclimated *H. scabra* dropped even below total values of IDH, indicating augmented aerobic metabolism induced by warm-acclimation.

Determining the capability to sustain an appropriate aerobic metabolism under acute thermal variability is a cornerstone principle to understand thermal tolerance in different species (Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Pörtner and Giomi, 2013). The ability of *H. scabra* to switch from an anaerobic (LDH dominated) to an aerobic (IDH dominated) driven metabolism indicates a pronounced acclimation capacity in the form of high metabolic plasticity to respond to anomalous temperature events. Supporting this hypothesis is the difference in total IDH and LDH activity within each species. Throughout the experiment, *H. forskali* exhibited constantly higher mean LDH activity levels (50 – 100 nmol min^{-1} $mgProt.^{-1}$) relative to the activity levels of mean IDH (0.4 – 0.8 nmol min^{-1} $mgProt.^{-1}$). Similar data were obtained by Tonn et al. (2016), IDH 0.4 – 0.9 nmol min^{-1} $mgProt.^{-1}$ and LDH 25 – 100 nmol min^{-1} $mgProt.^{-1}$ for *Holothuria forskali*. *Holothuria scabra* on the other hand showed only slightly lower ranges of mean IDH- (3 – 8 nmol min^{-1} $mgProt.^{-1}$) compared to mean LDH- activities (5 – 18 nmol min^{-1} $mgProt.^{-1}$), with the switch towards an IDH dominated energy turnover under warm acclimation. From this pattern it can be inferred that sea cucumbers are facultative anaerobes, which is in line with their generally slow-moving lifestyle as bottom dwellers. A coherent metabolic strategy across a broad temperature range is typical for a thermal generalist (Fusi et al., 2014). The much more prominent difference between LDH- and IDH activity in *H. forskali* may point towards exclusive LDH dominated energy turnover and, thus, can be interpreted as an energetic attribute of the temperate eurytherm. In contrast, *H. scabra* seems to be able to switch between an anaerobic and an aerobic dominated energy turnover, driven by changes in LDH and IDH activity. This feature would enable *H. scabra* to increase its metabolic efficiency under challenging conditions such as critical temperatures, and more importantly, may reveal pronounced warm-acclimation in the warm tropical stenotherm.

The rate of oxygen consumption and adjustments in respiratory physiology are also crucial in buffering environmental stress (Pörtner, 2010; Verberk and Bilton, 2013, Giomi et al., 2014; Verberk et al., 2015). It is predicted that tropical stenotherms face higher energy expenditures than eurytherms especially when approaching their upper temperature limit. At t0 warm treated *H. scabra* showed increased respiration, ETS activity and depletion in carbohydrates and protein reserves showing the initial increase in temperature clearly promoted oxygen demand, which led to higher energy turnover and depletion of energy reserves. The respiration rate of *H. scabra* acclimated to warm conditions remained clearly higher throughout the experiment, while ETS levels and energy reserves stabilized at levels equal to

control and cold conditions. This shows successful warm-acclimation of the thermal specialist through elevated respiration in combination with adjustments in metabolic enzyme activity. Enhanced foraging activity was observed in the warm exposed *H. scabra*, implying increased food intake as another important warm-acclimation factor, not quantified in this study. The eurytherm species *H. forskali* did not show an increased oxygen demand due to increased temperature. At t0 *H. forskali* exhibited the lowest ETS activity under warm conditions. Throughout the experiment *H. forskali* acclimated to control and warm conditions showed very similar respiration and ETS activities. Clear signs of respiratory depression due to cold-acclimation were, however, visible in *H. forskali*. According to Bao et al. (2010), cold induced metabolic loss (hibernation) was much more pronounced in the temperate sea cucumber *Apostichopus japonicus* than reduced energy demand driven by warm temperature (estivation). Hibernation and estivation are well described for sea cucumbers inhabiting the same latitude as *H. forskali*, such as *Holothuria tubulosa* (Coulon and Jangoux, 1993). Hence, our findings of enhanced down-regulation of respiration in cold-acclimated *H. forskali* could be associated to a hibernation behavior, which has not been studied yet in this species. In *H. scabra*, both up- and down –regulation of 6 °C caused clear deflections in respiration, while in *H. forskali* only the 6 °C temperature drop led to a clear adjustment of the respiratory system. Summarized, this might direct towards a broader aerobic scope in *H. scabra* (2 – 30 $\mu gO_2\ gww^{-1}\ h^{-1}$) than in *H. forskali* (1.5 – 6.6 $\mu gO_2\ gww^{-1}\ h^{-1}$), indicative of more advanced metabolic plasticity and, thus, higher acclimation capacity of the thermal specialist *H. scabra.* To confirm this hypothesis, further studies on the thermal tolerance levels such as aerobic scope and associated species-specific critical temperature levels need to be implemented.

The nomination of polar- and tropical- stenotherms as undisputed losers in terms of global warming, due to adaptation to more stable thermal environments appears over-simplified (Giomi et al., 2014; Verberk et al., 2015). As described by Williams et al. (2008), a multilevel integrated approach is essential when assessing species-specific susceptibility and plasticity towards climate change at different latitudes. This requires also the detection of thermal acclimation stress at different organization levels (Kamyab et al., 2017). In the current study different levels of the metabolic system were compared between two sea cucumbers from contrasting thermal environments, to determine distinct patterns in thermal-acclimation capacity. The results of this study, which reveal a prominent metabolic response due to warm acclimation in the tropical species, confirm the prediction that metabolic adjustments come with the burden of increased energetic costs, especially in tropical species

(Stillman, 2003; Tewksbury et al., 2008; Dillon et al., 2010; Huey et al., 2012). However, costly adjustments of the aerobic system have also been associated with enhanced acclimation capacity to cope with extreme temperature events (Fusi et al., 2014; Seebacher et al., 2015). Seen from this perspective, the enhanced metabolic plasticity in *H. scabra* may point towards higher thermal tolerance and, thus, may make the tropical stenotherm more resilient to future global warming scenarios.

We identified whole organism respiration in combination with the key metabolic enzymes activities of IDH and LDH as main drivers of thermal acclimation and detected clear higher warm-responsiveness in the tropical stenotherm *H. scabra*. The total values of oxygen consumption (OCR) and metabolic enzyme activities (LDH, IDH) were also the most prominent factors that determined differences between the two species. The PCA analyses (Fig. 1) revealed a clear dominance of aerobic performance parameters (OCR and IDH) in the tropical species while indicators for an anaerobic metabolism (LDH) were prevalent in the temperate species. The latter analysis was, however, based on the comparison between two separate experiments. We wish therefore to reiterate that potential deviations in the level of technical, chemical and human factors limit the comparison of absolute values between these two experiments. Another critical point is that present findings, which revealed differences in energetic strategies between the two species, may not be a representation of thermal adaptation, but, instead a depiction of other adjustment- or behavioral- patterns matching local environmental requirements. The most obvious example is the daily burrowing behavior that is only exhibited by *H. scabra*. While being buried, *H. scabra* relies entirely on ventilation through its posterior opening, which the animals leave unburied to take up oxygen. In contrast *H. forskali* is always surrounded by water and is known to take up oxygen through ventilation as well as by cutaneous respiration (Newell and Courtney, 1965; Astall and Jones, 1991). In *H. forskali* oxygen uptake via ventilation through its posterior opening accounts only for roughly 60% (Newell and Courtney, 1965), supposedly much less than in *H. scabra*. Consequently, an alternative explanation for *H. scabra's* capability to adjust its aerobic metabolism in such a flexible way might direct towards its burrowing behavior.

In conclusion, the detailed analysis of different metabolic system levels in two sea cucumber species enhances our knowledge on thermally driven metabolic changes. Assumptions on latitudinal effects, however, need to be validated in further studies. We propose population level studies, as suggested by Fusi et al. (2014), with these two sea cucumber congeners. Both species have a wide distribution breadth across different latitudes. Therefore, comparing

metabolic plasticity within one species, between distribution limits, will help to assess thermal tolerance levels along latitudinal gradients and to predict the heterogeneous effects of global warming.

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General Discussion

Key findings and relevance

The accurate prediction of future global warming effects, on ecological key species such as sea cucumbers, is considered an incredibly important cornerstone in environmental science. Moreover, water temperature is the main abiotic driver that determines sea cucumber growth and, hence, aquaculture production efficiency. In this study, we established a selection of synergistic biomarkers to generate a holistic understanding of cold- and warm- temperature effects on juvenile sea cucumbers of the species *Holothuria scabra*. Our analyses encompassed acclimation capacity, critical temperature limits and susceptibility towards global warming.

In the following chapter, key results and their significance are summarized and discussed, in terms of the initial research questions stated in the general introduction.

Thermal acclimation capacity

Acclimation can be described as shift of the thermally driven, optimal performance window, through internal adjustments on different biological organization levels (Schulte, 2014; 2015). In this context, thermal acclimation would mean a shift of the optimal temperature range and/or changes in thermal window widths (Kassahn et al., 2009). This phenotypic plasticity can only occur within certain limits, regarding the rate and magnitude of temperature change. Hence, the measurement of processes that underlie thermal acclimation requires stress detection at sub-lethal temperature levels over time. Moreover, a comprehensive mechanistic understanding is necessary to interpret whether the measured changes are negative, damaging effects or positive adjustments to sustain or regain homeostasis (Sokolova et al., 2012). We, therefore, targeted response parameters at the metabolic-, antioxidant- and immune- level. First, we tested the suitability of two sea cucumber tissues, longitudinal muscle (M) and respiratory tree (RT), for enzyme-specific sensitivity, to establish our planned assays. Overall enzyme activities were higher in RT tissue. This enhanced RT sensitivity reflects the important functional role of this tissue, however, with regards to responsiveness to temperature change only ETS activity showed higher consistency relative to M tissue (**Chapter 1**). All enzymes related to the antioxidant system revealed more reliable activity patterns, due to temperature change, in M tissue (**Chapter 2**). Moreover, we tested immune parameters in the cell free- (CFS) and the

coelomocyte lysate- (CLS) supernatant of the coelomic fluid. The comparison of these two fractions revealed higher ProPO activity in the coelomocytes (CLS) (**Chapter 2**). Similar to previous enzyme analyses conducted on sea cucumbers (Tonn et al., 2016) and other marine invertebrates (i.e. Kim et al., 2007), the present findings indicate the importance of tissue- and stress- specific calibration, to establish reliable biomarker assays.

Animal respiration, measured as whole organism oxygen consumption rate (OCR), in combination with the selected biochemical markers was successfully applied to measure physiological processes, underlying thermal acclimation in juvenile *Holothuria scabra*. Overall the thermal acclimation results showed that the minimum (21 °C) and maximum (33 °C) temperatures did not evoke temperature stress in juvenile *H. scabra*. Initially (t0), the animals exhibited temperature sensitivity, mainly at warm conditions, through enhanced depletion of energy reserves (mainly carbohydrate and protein), and increased ETS, LDH and PO/ProPO (CFS) activity. Throughout the acclimation period, however, the initial differences between temperature treatments disappeared, which indicates successful acclimation of juvenile *H. scabra* to cold and warm conditions. The only continuous trend was the positive correlation between OCR and temperature, accompanied by a growing relevance of an aerobic energy turnover (increased IDH activity) under warm conditions. This implies a temperature driven increase in overall activity and oxygen demand, which correlates positively with our observations of increased foraging activity and shortened burrowing cycles of the warm acclimated *H. scabra* (**Chapter 1**), which was also documented by Mercier et al. (1999). Albeit the clearly elevated OCR levels, warm exposed juvenile *H. scabra* did not exhibit significant up-regulations of the antioxidant system (**Chapter 2**). Thus, we can conclude that the chosen temperatures never reached severe conditions for the animals. On the contrary, we suggest that juvenile *H. scabra* were even profiting from the elevated temperature of 33 °C, at least over the experimental time of 30 days, through a more efficient energy turnover and presumably longer feeding periods and enhanced growth.

Definition of critical temperature limits

Besides knowledge on acclimation potential, the definition of species-specific upper- (CT_{max}) and lower- (CT_{min}) critical temperature limits is crucial to optimize

aquaculture production and to predict future global warming effects. Critical temperatures are commonly defined as point at which the energy requirements for basal maintenance functioning take up the entire aerobic scope of an organism (Sokolova and Pörtner, 2003; Sokolova et al., 2012; Schulte, 2015). The detection of aerobic scope requires exact knowledge on the difference between standard metabolic rate (SMR) and maximal metabolic rate (MMR) (Guderly and Pörtner, 2010; Pörtner et al., 2007). In slow moving benthic invertebrates such as sea cucumbers, however, the classical respiratory endpoints SMR and MMR cannot be defined through induction of physical challenges (i.e. maximal swimming speed in fish). We, therefore, used a novel approach by characterizing temperature driven bottom and peak metabolic performance as reliable approximation of SMR and MMR, respectively, for *Holothuria scabra*. Following this approach, the species-specific aerobic scope was defined as difference between the metabolic rate at a given temperature and the metabolic performance at CT_{max} and CT_{min}, respectively. These two benchmarks determine optimal temperature (T_{opt}) conditions in the exact center between CT_{max} and CT_{min}, where the aerobic scope peaks, with equal distance to the upper and lower performance limits (**Chapter 3**, Fig. 2). Employing this approach we measured animal respiration rates during acute temperature variation, over a temperature spectrum ranging from 17 °C to 41 °C. This revealed a T_{opt} of approximately 30.5 °C, in between a CT_{max} of 38 °C and a CT_{min} of 22 °C reflecting metabolic threshold levels at which juvenile *H. scabra* exhibited maximal- and minimal- respiration rates, respectively (**Chapter 3**). Compared to the upper limit of normally occurring natural habitat temperatures, e.g. 31 °C in Fiji (Lee et. al., 2017) and 30 °C in Lombok, Indonesia (Firdaus, unpublished), our prediction of T_{opt} = 30.5 °C is at the upper end of the normal range. Information on the optimal temperature range for *H. scabra* varies from 26-29 °C (Kumara et al., 2013) and 27-30 °C (Agudo, 2006), with documented growth decline below 24 °C (Purcell et al., 2006; Wolkenhauer, 2008). The latter temperature coincides nicely with the cold induced resting metabolism of *H. scabra*, which occurred at temperatures <23 °C (**Chapter 3**). Opposed to that juvenile *H. scabra* showed positive signs of acclimation and no elevated stress levels during long term exposure to 21 °C (**Chapter 1 and 2**). This indicates that our predicted CT_{min} represents solely the boundary of the aerobic performance window, which does not necessarily coincide with acute stress or the

limit of acclimation capacity. Regarding upper temperatures, recent studies show increased growth and activity of *H. scabra* at 31 °C, during long-term culture experiments (Lavitra et al., 2010), and sustained growth up to 35 °C, whereas, mass mortalities occurred at temperatures above 38 °C (Gamboa unpublished; supplementary material Fig. S3.4 and Table S3.1). These latter data are nicely in line with the results of our study on the acclimation capacity of *H. scabra*, in which juveniles were able to enhance their aerobic metabolism up to 33 °C without showing signs of stress (**Chapter 1 and 2**). Moreover, our measured data of the CT_{max} (38 °C) for *H. scabra*, predicted through acute changes in aerobic performance, are very close to observational data and literature findings.

To detect homeostatic disruption at critical temperature limits, we targeted the gene expression of Hsp70, which is well known as key cellular chaperon under acute temperature stress (e.g. Hochachka and Somero, 2002; Dahlhoff, 2004; Tomanek 2010). For this purpose we established the **first** gene expression assay (RT-qPCR) for *H. scabra*. Again we worked with tissue from longitudinal muscle (M) and respiratory tree (RT), to optimize the protocol. The comparison revealed a reliable detection of expression levels of target and reference genes in RT, whereas, many M tissue samples did not show any expression at all. We observed discolorations of many M tissue samples after the RNA extraction. We suppose that this might have led to unsuccessful first strand cDNA syntheses in many M samples. Besides the discolorations, the RNA quality and quantity was generally lower in samples extracted from M, compared to RT. Consequently, we propose RT as better tissue for gene expression analyses of Hsp70 in *H. scabra*. The combination of respiration and Hsp70 proved to be a well functioning and synergistic approach to determine energy homeostasis. At the upper end of the temperature spectrum above 38 °C, homeostatic disruption was marked by a decreasing basal maintenance function, accompanied by a highly elevated cellular protection machinery (Hsp70 expression). Contrary, at the cold end, the basal maintenance function reached stable state conditions below 23 °C, with no signs of relative Hsp70 mRNA expression at minimum temperature (17°C), compared to control genes (**Chapter 3**).

Global warming susceptibility inferred by metabolic capacity

The energetic level, ranging from key metabolic enzymes activities to whole organism energy- consumption and reserves status is of prime importance, as system components are directly linked with whole organism fitness parameters (rev. by Sokolova et al., 2012). Thus, energetic condition indicators such as oxygen-uptake, energy metabolism and depletion of energy reserves, have the potential to provide highly relevant insights into distinct acclimation patterns of different thermally adapted species (Sokolova et al., 2003). To test this hypothesis, we repeated the experiment from chapter one with the temperate sea cucumber species *Holothuria forskali*. The temperature manipulation for *H. forskali* was an exact replication of the conditions applied to *H. scabra*, in terms of magnitude (6 °C above and below optimal temperature conditions). The literature shows that metabolic adjustments come with the burden of increased energetic costs, especially in tropical species (Stillman, 2003; Tewksbury et al., 2008; Dillon et al., 2010; Huey et al., 2012). This is confirmed by the present study results, which reveal a prominent metabolic response due to warm acclimation in the tropical stenotherm *H. scabra*, but no noticeable reaction in the temperate eurytherm *H. forskali*. On the other hand the adaptation of eurytherms to variable environments involve losses in acclimatization capacity (Hoffmann, 1990; Huey and Berrigan, 1996; Van Buskirk and Steiner, 2009; Chown et al., 2010), which is in line with our findings of distinctly weaker acclimation responsiveness of temperate *H. forskali* compared to tropical *H. scabra* **(Chapter 4)**. This means, the weakness of tropical species due to higher energetic burden offers on the other hand metabolic plasticity, which may lead to enhanced acclimation capacity (Fig. 1). This paradigm shift (also addressed by Fusi et al., 2014 and Veberk et al., 2016) challenges the widely accepted hypothesis of increased vulnerability of thermal specialists (from tropical and polar regions), compared to thermal generalists (temperate regions), towards future global warming. Direct predictions of future global warming effects on the basis of our experiments are difficult. We exposed the animals to a fast temperature change (up- and down-regulation of 1°C/day) and measured their recovery over time (30 days). This does not reflect the predicted scenarios for global warming, which will occur at slow rates over long periods (2-4°C until the end of the century; (IPCC, 2015). From the viewpoint of metabolic plasticity, however, the distinct metabolic adjustments towards aerobic

prevalence, in warm acclimated tropical *H. scabra*, might indicate a higher acclimation potential relative to temperate *H. forskali* (**Chapter 1 and 4**).

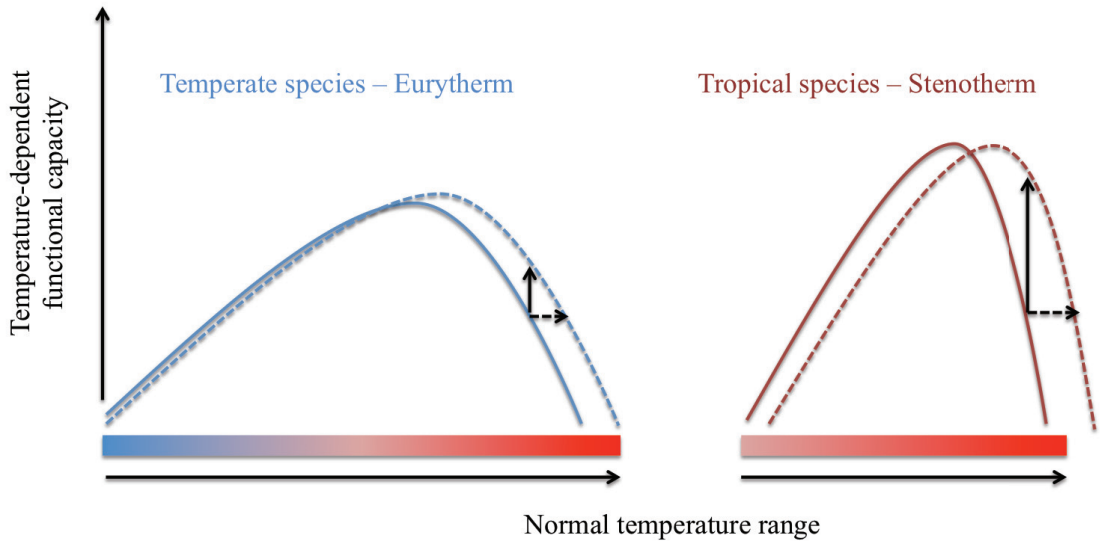

Fig. 1: Conceptual depiction of differences in warming susceptibility between temperate (eurythermal) and tropical (stenothermal) species. Eurytherms (blue line) can sustain temperature-dependent performances across a wide thermal range, but functional capacity is confined by the normally occurring temperature window, which may cause increased vulnerability of temperate species to stochastic anomalous warming. Comparatively, tropical stenotherms (red line), adapted to a more stable climate, may have more energetic reserves to efficiently sustain functional capacity beyond the normal ambient temperature range. Note the distinctly higher increase in functional capacity related energy demand (solid arrows) in the stenotherm compared to the eurytherm, at similar temperature variations (dotted arrows). Modified from Fusi et al. (2014).

Conclusion and future perspective

In this study we holistically assessed the thermal acclimation capability of *Holothuria scabra* through analyses of the metabolic system and quantification of antioxidant- and immunological stress. Moreover, we established a novel approach to determine the aerobic scope for *H. scabra*, which also led to the definition of acute cold- and warm temperature limits. The integration of our data from the short- and long- term temperature manipulation experiments enabled us to define detailed temperature windows for *H. scabra* (Table 1). For the first time we provide a characterization of thermal ranges for *H. scabra* based on stress levels at different biological organization levels, encompassing molecular- biochemical- and physiological- stress markers. With this approach we established the first mechanistic understanding of thermal stress in *H. scabra*, which is of great relevance for the optimization of aquaculture practices. Our data show a high metabolic flexibility of *H. scabra*, which enables this

species to sustain activity across a wide temperature range over prolonged periods, without showing signs of severe oxidative stress or impairments at the immune level. This means temperature could be used for artificial metabolic manipulations without causing lethal stress.

Table 1: Temperature windows for juvenile *Holothuria scabra*: (Center) optimal temperature (T_{opt}), (left) lower-acclimation range (21-29°C) and elevated cold stress range (17-20°C) with temperature of minimal respiration (CT_{min}). (Right) upper-acclimation range (31-33°C), elevated warm stress range (34-38°C), with temperature of maximal respiration (CT_{max}), and lethal range (39-41°C). Key response parameters: Oxygen consumption rate (OCR), iso-citrate dehydrogenase (IDH), lactate dehydrogenase (LDH), heat shock protein 70 (Hsp70) and respiration benchmarks: Standard metabolic rate (SMR) and maximal metabolic rate (MMR) are listed for each temperature window.

	Acute cold	**Acclimation capacity**			**Acute warm**	
Temp. (°C)	17 - 22 (CT_{min}= <23)	23 - 29	30-31 (T_{opt})	32 - 33	34 - 38 (CT_{max}= 38)	39 - 41
Metabolic strategy	Conservation	Compensation	Optimal	Compensation	Conservation	Survival
Aerobic scope	Minimum range	Decreasing	Maximum	Decreasing	Minimum range	Disruption
Stress level	Elevated	Low	No Stress	Low	Elevated	Lethal
Response parameter	Approaching minimal respiration ≈ SMR	Lower OCR and foraging activity, enhanced LDH-activity		Higher OCR and foraging activity, enhanced IDH-activity	Approaching maximal respiration ≈ MMR	Homeostatic-disruption, cellular protection (Hsp70)

A possible application could be a cold induced metabolic reduction, to reduce oxygen consumption and ammonia release during transport of *H. scabra*. On the other hand, directed sub-lethal temperature increases could be used to promote growth. Especially the latter application, however, need to be cautiously assessed over time limited periods, where the exclusion of other environmental stress factors (e.g. elevated pathogen concentrations and food availability) is of prime importance. Concerning the established critical temperatures limits, especially the warm boundary can be of use for farmers. Because it is expected that global climate change will cause more frequently occurring extreme weather events, *H. scabra* farmers need to be aware of

critical warm temperatures, when additional water cooling and/or shading is required. In addition, foremost the uncovered metabolic plasticity of *H. scabra* provides crucial knowledge to predict the vulnerability of this species towards global warming. The comparison of the metabolic system between tropical *H. scabra* and temperate *H. forskali* revealed the unexpected finding of higher acclimation capacity of the tropical species and point towards high thermal tolerance of *H. scabra*. Finally, this work provides a comprehensive `toolbox´, encompassing methods at different biological organization levels, which pave the way for various hypotheses testing in any field of stress detection in *H. scabra*, and also other sea cucumber species, in the future.

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Appendices

Supplementary material Chapter 1

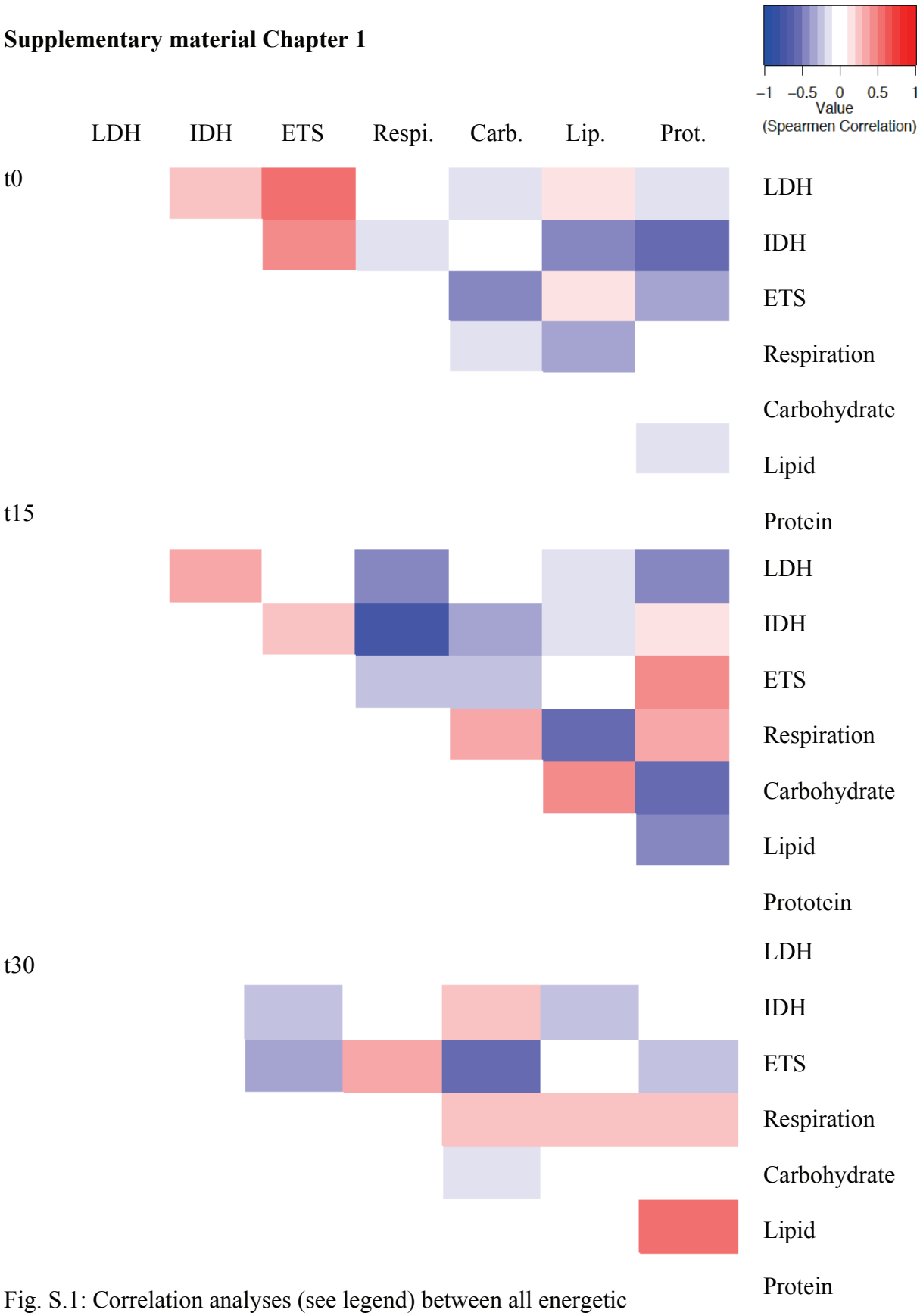


Fig. S.1: Correlation analyses (see legend) between all energetic biomarkers, represented in heat maps for each measurement time (t0, t15, t30).

Supplementary material Chapter 2

Table S2.1: Correlation analysis (Pearson Correlation) between all oxidative stress related endpoints and immune response enzymes assessed at the beginning of the experiment (t0). The pairs of variables with significant correlations ($p < 0.05$) are highlighted in bold and with asterisks (*).

t0	SOD M	CAT M	GR M	LPO M	DNA M	SOD RT	CAT RT	GR RT	LPO RT	DNA RT	ROS	ProPO CFS	PO CFS	ProPO CLS	PO CLS
SOD-M		**0.823***	**0.560***	0.066	0.443	-0.001	-0.01	-0.264	0.080	-0.254	-0.085	0.01	0.090	0.025	-0.079
CAT-M			**0.745***	0.0827	0.067	0.286	0.093	-0.175	0.438	-0.408	-0.054	-0.007	0.062	0.016	-0.290
GR-M				-0.286	-0.478	0.248	0.074	-0.187	0.395	-0.410	-0.266	-0.209	-0.151	-0.122	-0.317
LPO-M					**0.746***	-0.117	0.037	-0.175	0.262	0.192	0.388	-0.185	-0.173	-0.321	-0.287
DNA-M						-0.468	-0.17	-0.016	-0.146	-0.151	0.094	0.049	0.082	-0.207	-0.253
SOD-RT							**0.53***	0.451	0.356	0.435	0.141	-0.103	-0.101	-0.091	0.002
CAT-RT								**0.644***	0.214	**-0.530***	0.119	0.023	0.03	-0.282	0.006
GR-RT									-0.156	0.433	-0.251	0.014	-0.009	-0.119	0.131
LPO-RT										-0.025	0.370	-0.277	-0.301	0.081	-0.100
DNA-RT											0.373	-0.225	-0.250	-0.321	0.492
ROS												-0.020	-0.0187	-0.037	-0.105
ProPO, CFS													**0.997***	0.371	0.055
PO, CFS														0.0872	-0.067
ProPO, CLS															**0.690***
PO, CLS															

SOD = Superoxide dismutase; CAT = Catalase; GR = Glutathione reductase; LPO = Lipid peroxidation; DNA = DNA damage; M = measured in the muscle tissue; RT = measured in the respiratory tree; ROS = Reactive oxygen species (superoxide radicals) measured in body wall; PO = Phenoloxidase and ProPO = ProPhenoloxidase measured in two fractions of the coelomic fluid: cell free supernatant (CFS) and coelomocyte lysate supernant (CLS).

Table S2.2: Correlation analysis (Pearson Correlation) between all oxidative stress related endpoints and immune response enzymes assessed at day 15 of the experiment (t15). The pairs of variables with significant correlation ($p < 0.05$) are highlighted in bold and with asterisks (*).

t15	SOD M	CAT M	GR M	LPO M	DNA M	SOD RT	CAT RT	GR RT	LPO RT	DNA RT	ROS	ProPO CFS	PO CFS	ProPO CLS	PO CLS
SOD-M		**0.64***	**0.65***	-0.167	-0.291	0.356	-0.009	0.240	-0.121	-0.048	-0.465	0.137	0.158	**0.663***	0.464
CAT-M			0.464	0.316	**-0.57***	0.081	0.219	0.073	-0.199	-0.427	-0.188	0.014	-0.011	0.442	0.345
GR-M				-0.005	-0.202	-0.146	-0.039	0.379	-0.063	-0.060	**-0.53***	0.052	0.394	0.380	0.231
LPO-M					-0.158	-0.075	0.336	0.037	0.301	0.004	0.094	-0.275	-0.061	0.005	0.051
DNA-M						-0.194	-0.206	0.102	**0.578***	0.496	-0.065	-0.206	-0.11	-0.216	-0.27
SOD-RT							**0.518***	0.088	-0.263	0.217	-0.142	-0.133	-0.11	**0.566***	0.475
CAT-RT								0.335	-0.106	0.031	-0.106	-0.285	-0.099	0.259	0.438
GR-RT									-0.007	0.402	-0.382	-0.36	0.251	0.138	0.349
LPO-RT										0.042	0.054	0.119	-0.188	-0.147	-0.231
DNA-RT											-0.343	-0.456	-0.179	00	0.053
ROS												-0.014	-0.157	**-0.575***	**-0.575***
ProPO, CFS													0.234	0.225	0.107
PO, CFS														0.218	0.219
ProPO, CLS															**0.878***
PO, CLS															

SOD = Superoxide dismutase; CAT = Catalase; GR = Glutathione reductase; LPO = Lipid peroxidation; DNA = DNA damage; M = measured in the muscle tissue; RT = measured in the respiratory tree; ROS = Reactive oxygen species (superoxide radicals) measured in body wall; PO = Phenoloxidase and ProPO = ProPhenoloxidase measured in two fractions of the coelomic fluid: cell free supernatant (CFS) and coelomocyte lysate supernant (CLS).

Table S2.3: Correlation analysis (Pearson Correlation) between all oxidative stress related enzymes, end points, and immune enzymes assessed at the end of the experiment (t30). The pairs of variables with significant positive or negative correlation coefficients ($p < 0.05$) are with asterisks (*)

t30	SOD M	CAT M	GR M	LPO M	DNA M	SOD RT	CAT RT	GR RT	LPO RT	DNA RT	ROS	ProPO CFS	PO CFS	ProPO CLS	PO CLS
SOD-M		**0.707***	**0.54***	-0.256	0.066	0.293	-0.374	0.355	0.408	-0.203	-0.066	-0.333	-0.038	0.364	0.114
CAT-M			**0.86***	-0.132	0.115	0.24	-0.369	0.446	0.417	-0.059	-0.059	**0.537***	**0.521***	0.279	0.0933
GR-M				-0.383	-0.431	0.292	-0.376	0.309	0.34	-0.306	0.138	**0.714***	**0.669***	-0.007	-0.050
LPO-M					0.524	-0.473	0.366	-0.079	-0.015	**0.598***	-0.065	-0.217	-0.167	-0.046	0.042
DNA-M						0.116	0.361	0.414	0.548	0.051	-0.61	0.183	0.171	0.021	0.166
SOD-RT							0.203	0.49	0.345	**-0.506***	-0.423	0.285	0.234	-0.226	-0.474
CAT-RT								0.174	-0.142	-0.107	-0.381	-0.147	-0.087	-0.253	-0.354
GR-RT									0.165	-0.201	**-0.69***	0.275	0.213	0.380	0.204
LPO-RT										-0.150	-0.058	0.051	0.0502	-0.062	-0.224
DNA-RT											0.119	-0.1	-0.032	0.043	0.047
ROS												-0.086	-0.109	-0.207	-0.083
ProPO, CFS													**0.976***	-0.177	-0.071
PO, CFS														-0.163	-0.094
ProPO, CLS															**0.801***
PO, CLS															

SOD = Superoxide dismutase; CAT = Catalase; GR = Glutathione reductase; LPO = Lipid peroxidation; DNA = DNA damage; M = measured in the muscle tissue; RT = measured in the respiratory tree; ROS = Reactive oxygen species (superoxide radicals) measured in body wall; PO = Phenoloxidase and ProPO = ProPhenoloxidase measured in two fractions of the coelomic fluid: cell free supernatant (CFS) and coelomocyte lysate supernant (CLS).

Supplementary material Chapter 3

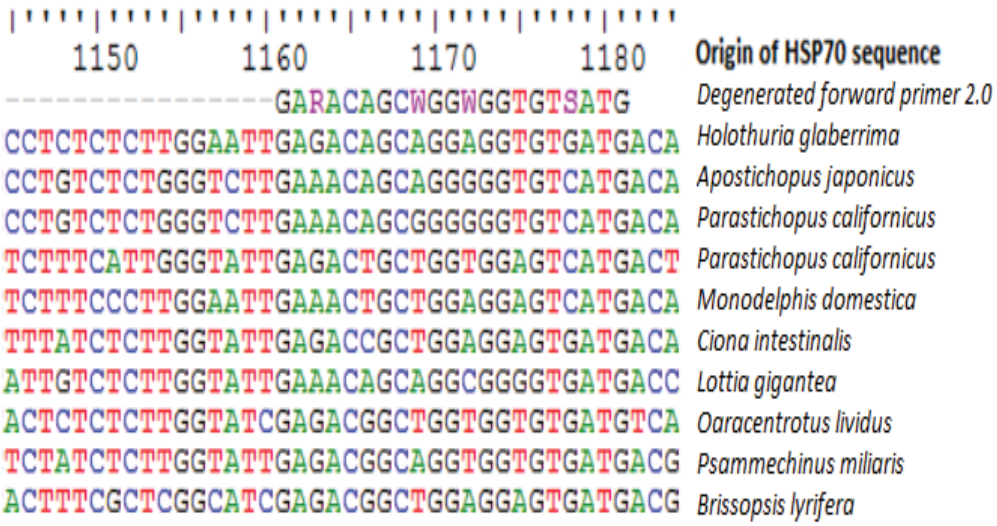


Fig S3.1: Homologues alignment of various species for degenerated primer design to target the Hsp70 sequence in *Holothuria scabra*.

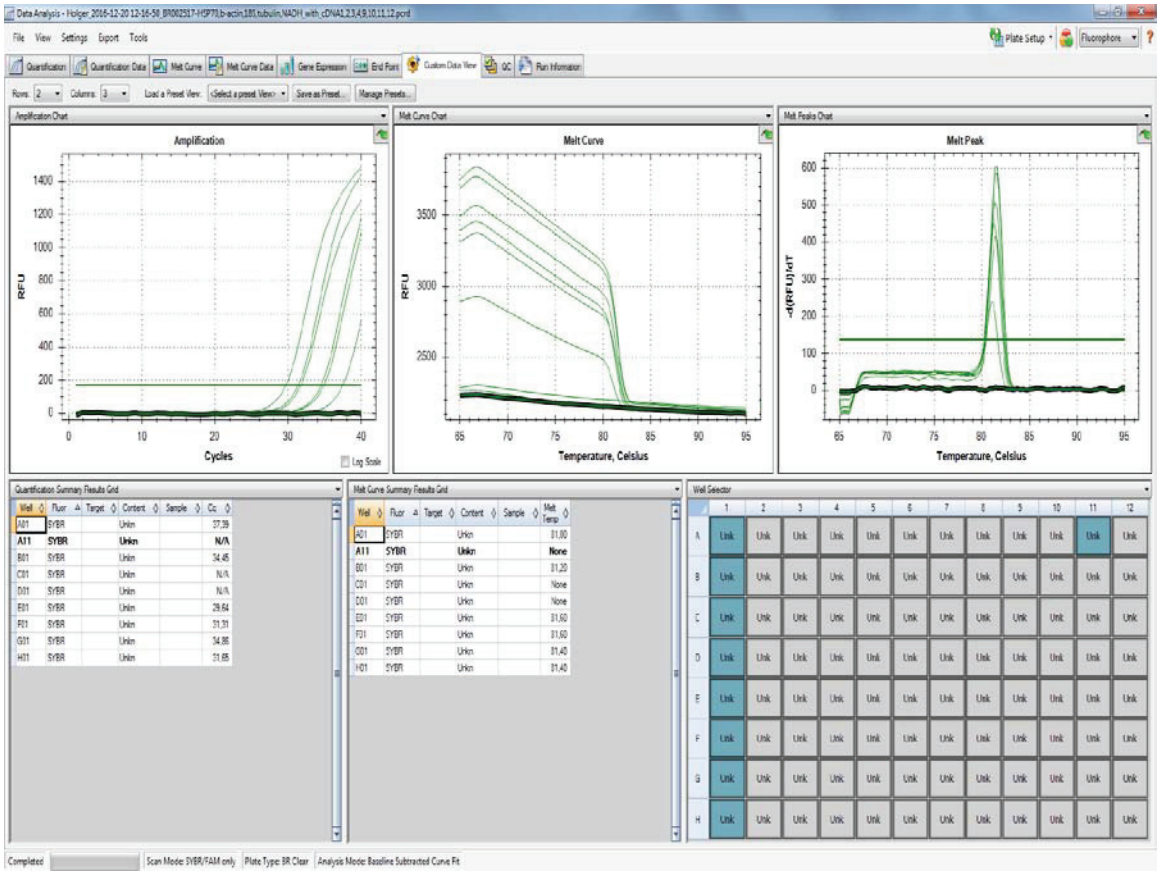
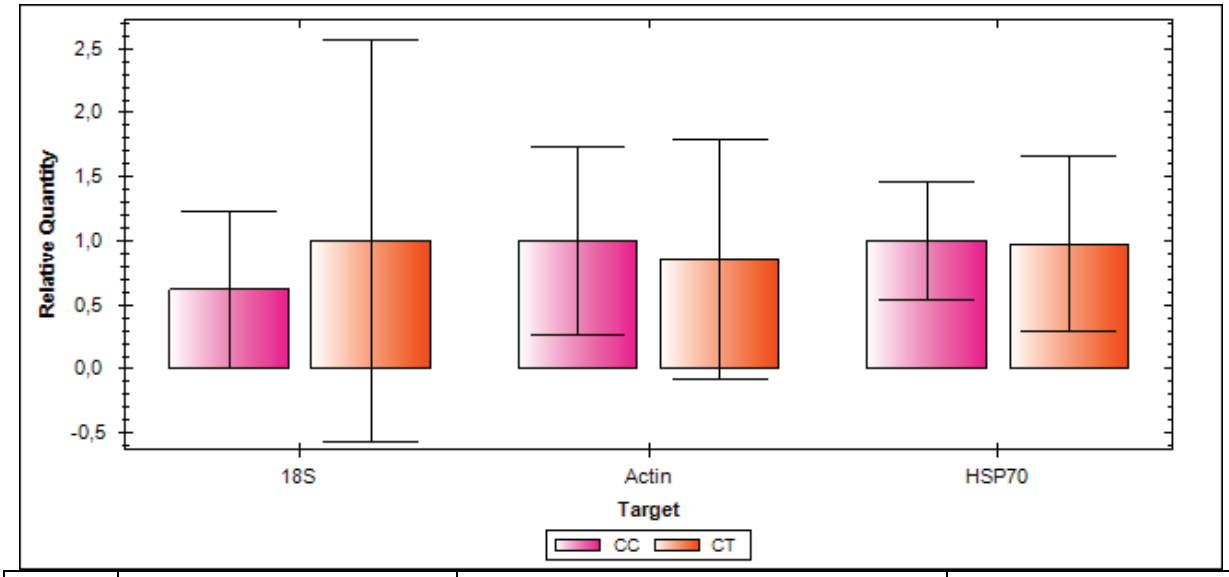
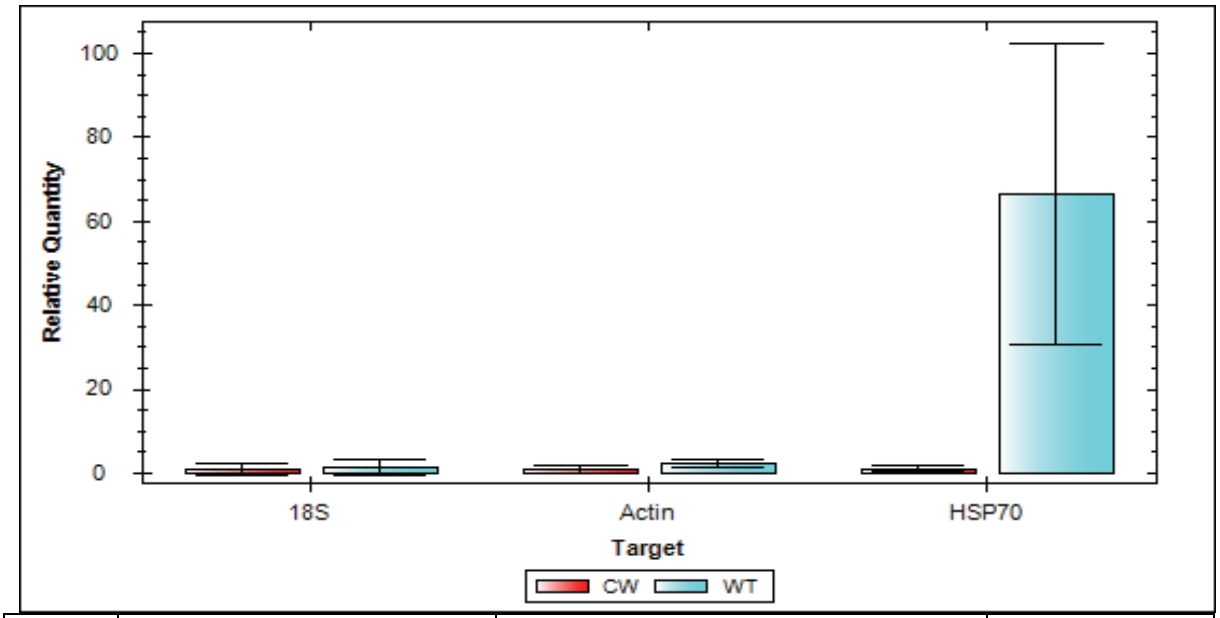


Fig. S3.2: Amplification, melt curve and melt peak of Hsp70 targeted in respiratory tree tissue of *Holothuria scabra* through qPCR.



Target	Sample	Compared to Regulation Threshold	P-Value
18S	Control CC	No change	N/A
18S	Cold Temperature (CT)	No change	0,228389
Actin	Control CC	No change	N/A
Actin	Cold Temperature (CT)	No change	0,462132
HSP70	Control CC	No change	N/A
HSP70	Cold Temperature (CT)	No change	0,424833



Target	Sample	Compared to Regulation Threshold	P-Value
18S	Control (CW)	No change	N/A
18S	Warm Temperature (WT)	No change	0,403676
Actin	Control (CW)	No change	N/A
Actin	Warm Temperature (WT)	No change	0,722409
HSP70	Control (CW)	No change	N/A
HSP70	Warm Temperature (WT)	**Up regulated**	**0,042696**

Fig. S3.3: Differential mRNA expression of Hsp70 relative to the two control genes 18S and actin as response to cold- (upper graph; 17 °C) and warm- (lower graph; 41 °C) shock.

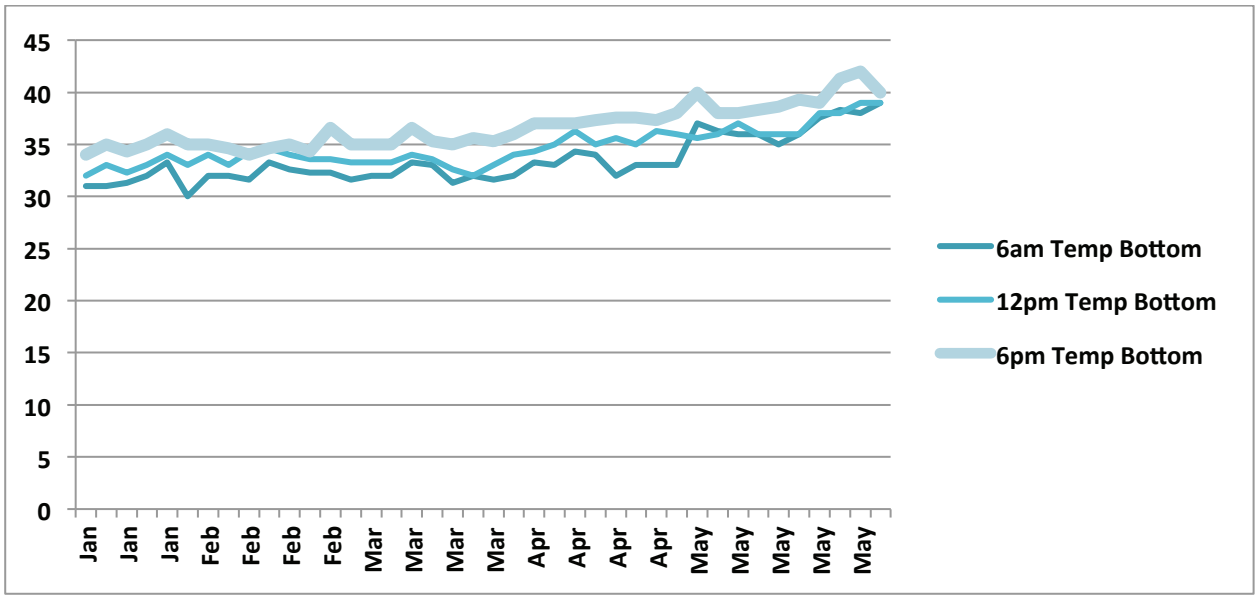


Fig. S3.4: Temperature documentation of an extreme temperature event that occurred in a *Holothuria scabra* pond culture in 2010. Water temperature recorded through a Sea Star® water logger installed 24/7 in at an average depth of 1.0 meter (Gamboa, unpublished).

Table S3.1: Summary of the conditions of the pond and *Holothuria scabra* juveniles throughout the extreme temperature conditions shown in Fig. 1 (Gamboa, unpublished).

Sampling Dates	No. of Juveniles[A]	Temp.[B] (°C)	Growth Rate (g d^{-1})	Body size (mm) Length	Width
Jan 22, 2010	100	34.50	n/a	56.33	17.75
Nov 4, 2010	101	34.0	n/a	41.96	11.51
Feb 18, 2010	73	34.81	0.42	59.79	19.28
Dec 6, 2010	69	35.12	0.42	52.45	15.97
March 22, 2010[C]	76	35.34	0.15	71.13	23.09
Jan 8. 2011	no juveniles recovered; data logger discovered stolen; last retrieved on 26 Dec 2010				
April 25, 2011	Ave temp was 37.01°C. Hard to measure the disfigured and shrinking animals which displayed mucus-secretion in their body wall				
May 3, 2010[D]	14	39.0	'melting' individuals averaging 9.55g (ranging from 2.3-59.8g)		

[A]Total number of juveniles recovered in all five pens.
[B]Average bottom temperature at 6PM
[C]Evisceration of one juvenile was noted.
[D]Juveniles recovered have mucus secretions in their body wall.
pink cells are for the second trial.

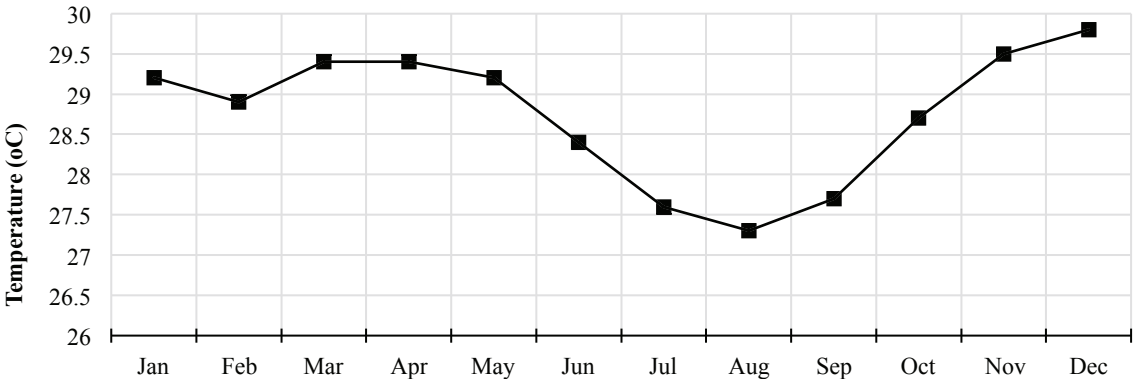


Fig. S3.5: Monthly recorded ocean temperature. Measurement location: Marine science station of the Indonesian Institute if Science (LIPI), located on Lombok, Indonesia (Firdaus, unpublished).

Supplementary material Chapter 4

All data are made public under the following link:

https://doi.pangaea.de/10.1594/PANGAEA.880879

Declaration on the contribution of the candidate to a multi-author article/manuscript which is included as a chapter in the submitted doctoral thesis

Chapter:

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

Experimental concept and design:	ca. _100_%
Experimental work and/or acquisition of (experimental) data:	ca. _80_%
Data analysis and interpretation:	ca. _100_%
Preparation of Figures and Tables:	ca. _100_%
Drafting of the manuscript:	ca. _100_%

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Data analysis and interpretation:	ca. _100_%
Preparation of Figures and Tables:	ca. _100_%
Drafting of the manuscript:	ca. _100_%

Chapter:

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Experimental concept and design:	ca. _100_%
Experimental work and/or acquisition of (experimental) data:	ca. _80_%
Data analysis and interpretation:	ca. _100_%
Preparation of Figures and Tables:	ca. _80_%
Drafting of the manuscript:	ca. _100_%

Date: 06.10.2017

Signatures:

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel:

Thermotolerance of the sea cucumber *Holothuria scabra;*

Towards a systematic understanding of multi-level temperature effects

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit, dass es sich bei den von mir abgegebenen Arbeiten um drei identische Exemplare handelt.

(Unterschrift)