ALFRED-WEGENER-INSTITUT
HELMHOLTZ-ZENTRUM FÜR POLAR- UND MEERESFORSCHUNG

POLMAR
HELMHOLTZ GRADUATE SCHOOL FOR POLAR AND MARINE RESEARCH

The effect of acute warming on protein turnover and oxygen demand in stenothermal and eurythermal eelpouts

Dissertation

Zur Erlangung des akademischen Grades

Dr. rer. nat.

Vorgelegt Dem Fachbereich 2, der Universität Bremen von

Nina Krebs

Bremen, Mai 2024

Universität Bremen

Gutachter: Prof. Dr. Hans-Otto Pörtner

Alfred-Wegener-Institut Helmholtz-Zentrum für Polar-und Meeresforschung

Head of section Integrative Ecophysiology

Am Handelshafen 12

27570 Bremerhaven, Germany

Gutachter: Dr. Marian Yong-An Hu

Christian-Albrechts-Universität zu Kiel

Research Group Leader, Kiel University

Hermann-Rodewald-Straße 5

24118 Kiel

Prüfer: Prof. Dr. Marko Rohlfs

Universität Bremen

Fachbereich 2 Biologie/ Chemie

Leobener Str. 5 (NW2)

28359 Bremen, Germany

Prüfer: Prof. Dr. Hans-Otto Pörtner

Alfred-Wegener-Institut Helmholtz-Zentrum für Polar-und Meeresforschung

Head of section Integrative Ecophysiology

Am Handelshafen 12

27570 Bremerhaven, Germany

Prüfer: Dr. Marian Yong-An Hu

Christian-Albrechts-Universität zu Kiel

Research Group Leader at Kiel University

Hermann-Rodewald-Straße 5

24118 Kiel, Germany

Prüfer: Dr. Gerlien Verhaegen

Alfred-Wegener-Institut Helmholtz-Zentrum für Polar-und Meeresforschung

Helmholtz Young Investigator Group “ARJEL”

Am Handelshafen 12

27570 Bremerhaven, Germany

Datum des Prüfungskolloquiums: 25.06.2024

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Summary

The increase in CO_2 in the atmosphere due to the burning of fossil fuels is the main cause of climate change and thus global warming. Climate change has fundamental consequences for all organisms, including marine life. Ectothermal organisms, such as fish, are particularly affected, since their body temperature is equal to their environment. Fish have adapted to diverse temperature conditions depending in different climatic regions and thus exhibit a range of temperature adaptations. Fish in polar regions, such as Antarctic fish species, are adapted to constantly cold temperatures, while fish in temperate regions are adapted to seasonal temperature fluctuations and can survive in wide temperature windows.

Thermal adaptation can be investigated by acclimating fish to certain temperatures over a long period (long-term acclimation) or during acute temperature changes (acute warming). This study investigates the differences and similarities in thermal adaptions of stenothermal and eurythermal fish during acute warming events. For this comparison, the closely related eelpout species *Pachycara brachycephalum* from the Southern Ocean and *Zoarces viviparus* from the North Sea were selected as experimental animals. During these experiments, water temperature was increased from 0°C to 10°C at a rate of 2°C day^{-1} for *P. brachycephalum*. For *Z. viviparus*, the temperature was increased from 4°C to 22°C at a rate of 3°C day^{-1}. Isotopically labelled phenylalanine was injected intraperitonially at several temperature levels (*P. brachycephalum*: 0, 2, 4, 6, 8 and 10°C; *Z. viviparus*: 4, 10, 13, 16, 22°C) and after 1.5- and 3-hours gill and white muscle tissues were sampled. Protein synthesis rate was determined in both tissue types and protein degradation analyzed in the muscle by measuring cathepsin D activity and via untargeted metabolic profiling using NMR (nuclear magnetic resonance). Furthermore, oxygen consumption was measured during acute warming to draw conclusions about energy requirements.

In *P. brachycephalum*, the rate of protein synthesis in white muscle did not change with increasing temperature. In contrast, the protein synthesis rate in *Z. viviparus* increased up to 16°C and then decreased slightly at 22°C. Comparing the two species at a common temperature (4 and 10°C), the protein synthesis rate in *P. brachycephalum* was 2-3-times higher, while the activity of cathepsin D was 10 times higher in white muscle. Both the rate of protein synthesis in the gills and whole animal oxygen demand increased exponentially in both species and no differences were found between the species when compared at a common temperature.

In conclusion, these data suggest that protein synthesis in *P. brachycephalum* is cold-compensated and is maintained at a high rate at low temperatures. The protein synthesis rate in the white muscle of *Z. viviparus* only reached that of *P. brachycephalum* near its thermal optimum. This cold adaptation may enable *P. brachycephalum* to survive in the world's coldest ocean. In contrast, *Z. viviparus* responds very quickly to temperature differences, which occur regularly in the North Sea.

Zusammenfassung

Der Anstieg des CO_2 in der Atmosphäre durch die Verbrennung fossiler Brennstoffe ist die Hauptursache für den Klimawandel und damit die globale Erwärmung. Der Klimawandel hat grundlegende Folgen für alle Organismen, einschließlich der Meereslebewesen. Ektotherme Organismen wie Fische sind davon besonders betroffen, da ihre Körpertemperatur der ihrer Umgebung entspricht. Fische haben sich je nach Klimaregion an unterschiedliche Temperaturbedingungen angepasst. Während Fische aus Polarregionen, wie z. B. antarktische Fische, an konstant kalte Temperaturen angepasst sind, sind Fische aus gemäßigten Regionen an saisonale Temperaturschwankungen angepasst und können in einem breiten Temperaturfenster überleben.

Die Temperaturanpassung von Fischen kann einerseits nach langer Akklimierungsphase bei einer bestimmten Temperatur untersucht werden (Langzeitakklimierung), andererseits während schnelleren, akuten Temperaturänderungen (akute Erwärmung). In dieser Studie werden stenotherme und eurytherme Fische während der akuten Erwärmung untersucht, um Unterschiede und Gemeinsamkeiten als Folge von Temperaturanpassung zu ermitteln. Für diesen Vergleich wurden die eng verwandten Aalmutterarten *Pachycara brachycephalum* aus dem antarktischen Ozean und *Zoarces viviparus* aus der Nordsee als Versuchstiere ausgewählt. Während dieser Versuche wurde die Temperatur für *P. brachycephalum* von 0°C bis 10°C (+2°C pro Tag) und für *Z. viviparus* von 4°C auf 22°C (+3°C pro Tag) erhöht. Isotopisch markiertes Phenylalanin wurde den Tieren bei verschiedenen Temperaturen injiziert (*P. brachycephalum*: 0, 2, 4, 6, 8 und 10°C; *Z. viviparus*: 4, 10, 13, 16, 22°C). Nach 1,5 und 3 Stunden wurden Kiemen und weißes Muskelgewebe entnommen, und die Proteinsyntheserate bestimmt. Darüber hinaus wurde der Proteinabbau im weißen Muskel durch Messung der Cathepsin-D-Aktivität und durch ungezieltes metabolisches Profiling mittels NMR (nuclear magnetic resonance) analysiert. Der Sauerstoffverbrauch der Tiere während der akuten Erwärmung wurde ebenfalls gemessen, um Rückschlüsse auf den Energiebedarf zu ziehen.

Bei *P. brachycephalum* änderte sich die Geschwindigkeit der Proteinsynthese im weißen Muskel mit steigender Temperatur nicht. Im Gegensatz dazu stieg aber die Proteinsyntheserate bei *Z. viviparus* bis auf 16°C an und sank danach bei 22°C leicht ab. Vergleicht man die beiden Arten bei einer gemeinsamen Temperatur (4 und 10 °C), so war im weißen Muskel die Proteinsyntheserate in *P. brachycephalum* 2-3-mal höher, und die Aktivität von Cathepsin D sogar 10-mal höher als in *Z. viviparus*. Sowohl die Proteinsyntheserate in den Kiemen als auch der Sauerstoffbedarf stiegen bei beiden Arten exponentiell an und beim Vergleich bei einer gemeinsamen Temperatur wurden keine Unterschiede zwischen den Arten festgestellt.

Diese Daten deuten darauf hin, dass *P. brachycephalum* kältekompensiert ist und auch bei niedrigen Temperaturen eine hohe Proteinsyntheserate aufweist. Die Proteinsyntheserate im weißen Muskel von *Z. viviparus* war nur in der Nähe ihres thermischen Optimums mit der von *P. brachycephalum* vergleichbar. Diese Kälteanpassung ermöglicht es *P. brachycephalum*, im kältesten Ozean der Welt zu überleben. Im Gegensatz dazu reagiert *Z. viviparus* sehr schnell auf Temperaturunterschiede, da diese auch in der Nordsee regelmäßig auftreten

Figures and Tables

Abbreviations

%	Percentage
°C	degrees Celsius
^{1}H-NMR	^{1}H- nuclear magnetic resonance
ACC	Antarctic Circumpolar Current
ATP	Adenosine triphosphate
AWI	Alfred Wegener Institute
CO_2	Carbon dioxide
CSR	Cellular Stress Response
D_2O	Deuterized water
DIA	Data-Independent Acquisition
ETS	Electron Transport Chain
FAD	flavin adenine dinucleotide
FWHM	Full Width at Half Maximum
G	Units of gravity
GHG	Greenhouse Gases
$GtCO_2$/yr,	Carbon dioxide in gigatons per year
HCl	Hydrochloric acid
HSI	Hepatosomatic index
IPCC	Intergovernmental Panel on Climate Change
IQR	Inter-Quartile Range
Ks (% day^{-1})	Protein synthesis rate per day
MeOH	Methanol
MilliQ	Ultrapure water
MS	Mass Spectrometry
MO_2	Oxygen consumption (µmol/h*g wet weight)
NAD	Nicotinamide Adenine Dinucleotide
O:N ratio	Ratio of atomic Oxygen and Nitrogen
OCLTT	Oxygen- and Capacity-Limited Thermal Tolerance
PBS	Phosphate Buffered Saline
Phe	Phenylalanine
PSU	Practical Salinity Units
Q_{10}	measure of the rate of change driven by a temperature change of 10 °C
RV	Research Vessel
SMR	Standard Metabolic Rate
SPE	Solid Phase Extraction
SSP	Shared-Socio-economic Pathway in Wm^{-2}
T_{crit}	Critical Temperature
TMAO	Trimethylamine oxide
T_{opt}	Optimum temperature
T_{pej}	Temperature in pejus range

1 Introduction

1.1 Climate change

The Earth's climate has changed over the last 800,000 years between warm interglacial periods and cold glacial periods (Neugebauer, 1988). In the past, the main reason for warming were changes in solar radiation and volcanic activity (Scotese et al., 2021). Today, the term climate change typically refers to the human-induced climatic changes that have occurred during the past two centuries. Since the 18th century, the Industrial Revolution increased emissions of greenhouse gases (GHGs), mainly CO_2, due to the combustion of fossil fuels such as coal and oil (Cowell, 2000). GHGs retain heat in the atmosphere, leading to higher temperatures and a wide range of climate change impacts.

Compared to the period between 1850 and 1900, global surface temperatures have increased by 1.1°C with larger increase over land (1.59°C) than in the ocean (0.88°C) (IPCC, 2023). The Intergovernmental Panel on Climate Change (IPCC) has published various scenarios predicting future temperature increases as a function of the release of GHGs into the atmosphere ($GtCO_2$/yr, Figure 1) using the model of Shared-Socio-economic Pathways in Wm^{-2} (SSP). The more extreme SSPs project that if the combustion of fossil fuels continues unabated, global temperature will increase by more than 4°C (SSP5-8.5, Figure 1) by the end of this century. A reduction of GHG emissions to 0 $GtCO_2$/yr by the end of this century would still cause a temperature increase of 2.5°C (SSP2-4.5, Figure 1). In the Paris agreement from 2015, the United Nations have agreed to limit the global temperature increase to far less than 2°C above pre-industrial level. In order to reach that goal, CO_2 emission should become negative until the end of the century (SSP1-1.9 or SSP1-2.6, Figure 1).

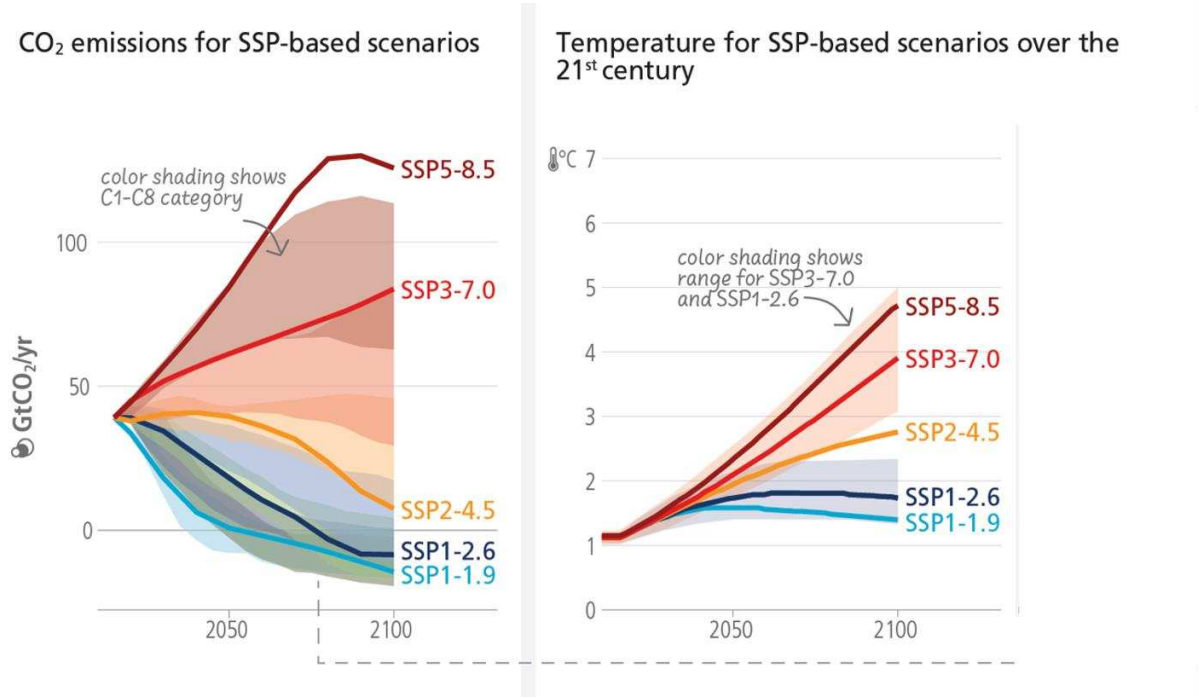


Figure 1. CO_2 emission and related temperature development until 2100 based on the SSP (Shared-Socio-economic Pathway in Wm^{-2}) scenarios. The figure shows 5 scenarios; very low greenhouse gas (GHG) emission (SSP1-1.9, light blue), low GHG (SSP1-2.6, dark blue), intermediate GHG (SSP2-4.5, orange), high GHG (SSP3-7.0, light red) and very high GHG (SSP5-8.5). On the left side, these scenarios are expressed as the emission of CO_2 in Gt per year ($GtCO_2/yr$). The right figure shows the projection of temperature for 2100 in the same 5 categories. The different scenarios clearly show that further increase of CO_2 emission would lead to a temperature increase of 4-5°C (SSP5-8.5 and SSP3-7.0) until the end of this century. A reduction of CO_2 emissions to 0 until the end of the century would still increase the global temperature by about 2.5 °C (SSP 2-4.5). The temperature increase only remains below 2°C when the CO_2 emission is negative until the end of the century (SSP1-2.6 and SSP1-1.9) (adapted from IPCC, 2023).

The scenarios above describe the changes of the global surface temperature. In what follows, I will concentrate on the effects of climate change on marine habitats, since this thesis focuses on the impact of temperature on two related marine fish species. About 71% of the earth surface is covered by water. Oceans are present in all climatic zones with different thermal regimes from tropic to polar. The coldest ocean is the Antarctic Ocean, which is also called Southern Ocean. The Southern Ocean circulates eastwards around Antarctica driven by the Antarctic Circumpolar Current (ACC) with only little exchange with the neighboring oceans. This circulation keeps the Southern Ocean at temperatures as low as 2°C (Kennett, 1977). Furthermore, the Southern Ocean takes up a large proportion of atmospheric heat. It stored 35-43% of the global heat gain between 1970 and 2017 in the upper 2000 m of the water column. In the timespan between 2005 and 2017, this amount increased up to 45-62% (IPCC, 2019). Although the Southern Ocean absorbs a large proportion of heat, models project the lowest degrees of warming compared to the global average. However, models are less predictive for temperature changes of the Southern Ocean since the interactions of ocean, atmosphere, and cryosphere are poorly understood and not included in climate models (IPCC, 2021). Increasing temperature affects ocean circulation and ocean currents (IPCC, 2019). Changes of the ocean circulation increase the stratification of the upper ocean and, together with rising temperature, cause a reduction in oxygen levels (hypoxia) (Keeling et al., 2010). Furthermore, rising atmospheric CO_2 is

entering the oceans, thereby altering its carbonate equilibrium and acidifying the ocean (Doney et al., 2009). Synergistic effects of these abiotic factors make future predictions for marine ecosystems even more challenging.

Despite uncertainties in the projected outcome, many studies have shown negative effects of anthropogenic climate change on biodiversity, species and ecosystems services (Cavanagh et al., 2021; Pörtner et al., 2023; Smale et al., 2019). For example, warming caused a change in migration patterns of ectotherms to higher latitudes, leading to a decline in species richness around the equator and increasing richness in polar areas (Chaudhary et al., 2021). Changes in food web compositions, in particular of economically important species, might affect local fisheries with dramatic consequences for the human population (Pörtner et al., 2023).

1.2 Temperature as dominating factor for organismal performance

Temperature has been identified as one of the major stressors for marine organisms. This is due to the fact that most marine organisms are ectotherms, meaning that they cannot regulate their body temperature and thus, their organismic performance depends on ambient seawater temperatures (Hochachka and Somero, 2002; Kassahn et al., 2009; Pörtner, 2021). Higher temperatures enhance metabolic processes and accordingly the organism's oxygen demand, while colder temperatures reduce metabolic processes. As a rough estimate, a 10°C temperature change alters metabolic processes by a factor of 2 to 3 (Hochachka and Somero, 2002). Thus, warming causes a thermodynamic increase in energy expenditure due to the elevation of resting metabolic rates (Q_{10}).

1.2.1 Oxygen and Capacity Limited Thermal Tolerance (OCLTT)

The relationship between temperature and oxygen in ectotherms defines the organism's performance which can be described in the Oxygen- and Capacity-Limited Thermal Tolerance (OCLTT) (Pörtner, 2021). This concept builds on the assumption that the performance of an organism has to be fully powered by aerobic metabolism for long-term survival (Figure 2).

Within a species-specific thermal optimum (T_{opt}), oxygen demand and supply to tissues remains balanced. Excess aerobic energy provides sufficient energy for growth, storage, or reproduction (Sokolova et al., 2012). Suboptimal temperatures (T_{pejus}, pejus Latin for "worse") leads to a progressive mismatch of oxygen demand and supply, which causes reduced oxygen availability in different tissues and organs (Pörtner et al., 2017). To ensure survival under suboptimal conditions, organisms cope by increasing their cardio-ventilatory performance at the expense of growth and reproduction, which, in the long-term, reduces aerobic performance. When thermal conditions worsen, the discrepancy between oxygen demand and supply becomes so drastic that organisms close this gap through anaerobic energy production. This point defines onset of a critical range where survival becomes strictly time-limited as anaerobiosis provides less energy compared to aerobic energy production (T_{crit}). Additional stressors such as environmental hypoxia and/or hypercapnia reduce the aerobic performance of an organism and narrow the thermal window (Pörtner and Farrell, 2008).

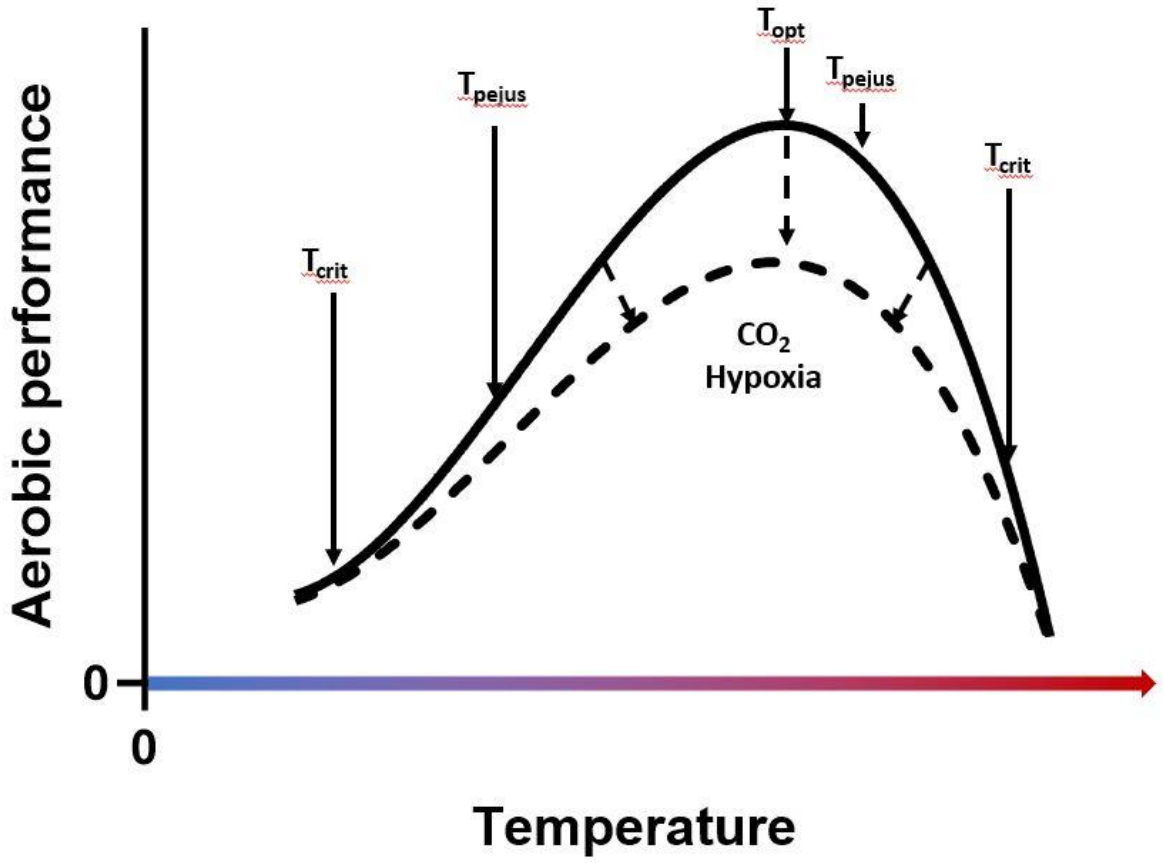


Figure 2. Thermal window of aerobic performance. Ectothermal organisms are affected by temperature and show the highest aerobic performance at their thermal optimum (T_{opt}). Temperatures besides the thermal optimum resulting in loss of aerobic performance are first marked at the T_{pejus}, and then T_{crit}. Additional stressors such as elevated CO_2 levels and/or hypoxia reduce the aerobic performance (dashed line). Schematic after Pörtner and Farrell, 2008.

1.2.2 Thermal adaptation

Ectotherms live in all climatic zones and are either adapted to constantly cold polar regions, to the variable temperate zones, or to the constant warmth of the tropics. This diversity of habitat specific temperatures is reflected in the different thermal tolerance windows. Species adapted to a constantly cold environment are known as stenotherms, meaning that they have a narrow thermal window. In contrast, species adapted to daily and seasonal temperature fluctuations have a wide thermal window and are defined as being eurythermal (Pörtner et al., 2017, Figure 4).

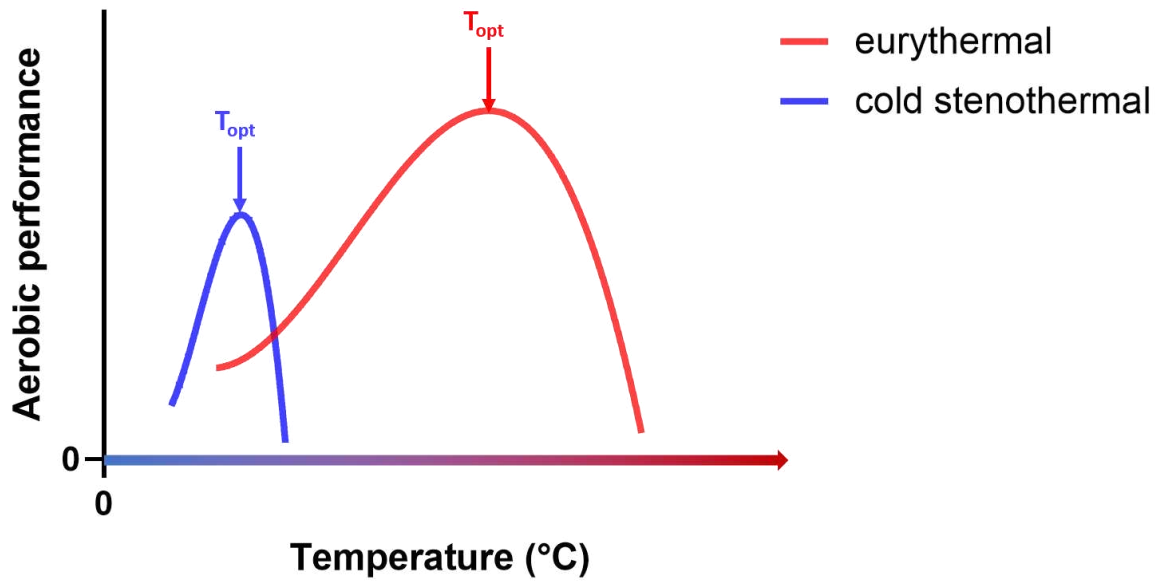


Figure 3. Aerobic performance of cold stenothermal and eurythermal organisms. The cold stenothermal organisms (blue) have a narrow thermal window with high slopes besides its thermal optimum. The eurythermal organisms (red) have a wider thermal window with lower slopes besides its thermal optimum. Schematic after Pörtner and Farrell, 2008.

Eurythermal organisms have a much wider thermal window as a result of high seasonal fluctuations of temperature and food. These seasonal changes are particularly relevant for sessile benthic organisms. Unlike mobile pelagic species, they cannot migrate to areas with favorable conditions (Park et al., 2020). During spring and summer, temperatures are close to the organism's optimum temperature and, at the same time, food is typically abundant. This combination of higher energy availability and efficient metabolic processes provides an energy surplus, which can be spent on growth and reproduction (Lowerre-Barbieri et al., 2011; Stratoudakis et al., 2007). Less food is available in winter, when for example, the plankton community changes (Boyce et al., 2017), temperatures drop and the internal processes of ectothermal organisms are slowed down according to the Q_{10} rule (Wittmann et al., 2008). Various physiological processes occur to compensate for the Q_{10}-dependent slowdown in winter. This is referred to as cold compensation and encompasses all of the physiological aspects of an organism that enable it to survive in the cold (Clarke, 1991). These include adjustments to energy storage (Fernandes and McMeans, 2019), activity of mitochondria enzymes (St-Pierre et al., 1998), ion and acid-base regulation (Pörtner et al., 1998) and translation efficiency (Lucassen et al., 2003). Although cold compensation helps them to survive the winter, the combination of cold temperatures and food shortages can seriously reduce growth rates and increase mortality (Conover, 1992; Martino et al., 2019; Roussel et al., 2011).

An even higher degree of cold compensation is found in stenothermal organisms that live at temperatures constantly below 0°C. The maximal aerobic performance, e.g. growth, is lower in polar stenotherms, even lower than expected on the basis of the Q_{10} relationship (Peck, 2018, 2016). Slow growth rates are likely caused by limited food supply due to seasonal changes and by functioning in a

consistently cold environment around 0°C (Peck, 2018). In addition, thermal trade-offs can be found when comparing components of the energy budget, such as ventilation and circulatory capacity, muscle activity, and cellular and mitochondrial energy costs (Pörtner, 2006).

Differences in the thermal adaptation between stenothermal and eurythermal organisms can be detected by investigating oxygen consumption from the point of view of the OCLTT concept. The most common measurement is the standard metabolic rate (SMR), which reflects the oxygen demand for homeostasis without exercise and digestive activity. When temperature increases over a relatively short period of time, usually within hours to a few days (acute warming), SMR increases exponentially. In both stenothermal and eurythermal organisms, the SMR increase until a breakpoint is reached at which oxygen consumption cannot be further increased. The SMR during acute warming of stenothermal organisms is characterized by a steep slope and high Q_{10}, but a low capacity for oxygen supply. In contrast, Q_{10} value and slope are relatively low in eurythermal organisms, but the capacity of oxygen supply is higher compared to stenothermal organisms during acute warming (Figure 5, after Pörtner et al., 2017).

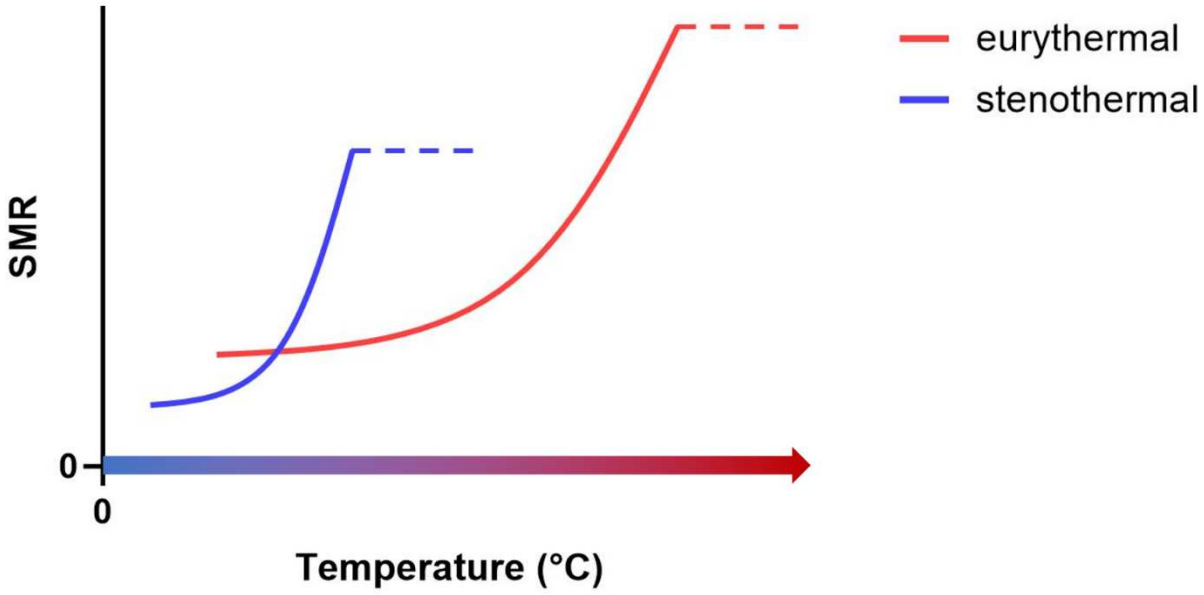


Figure 4. Standard metabolic rates (SMR) during acute warming in stenothermal (blue) and eurythermal (red) organisms. The SMR increases exponentially until a breakpoint is reached at which SMR no longer increases. However, in stenothermal organisms the thermal window is narrow with a steep slope while the thermal window in eurythermal organisms is wide with a shallow slope. This results in a high Q_{10} value for stenothermal organisms and a low Q_{10} value for eurythermal organisms (after Pörtner et al., 2017).

1.3 Aerobic energy production

A functional energy metabolism is essential for the survival of all organisms. However, the speed at which energy can be obtained and converted, as well as the ability to store it, are limited (Sokolova et al., 2012). Energy can be converted into various forms, e.g. ATP (adenosine triphosphate), NADH (nicotinamide adenine dinucleotide) and $FADH_2$ (flavin adenine dinucleotide), all of which are involved in oxidative energy production. However, in cells, energy mostly refers to chemical energy in the form of ATP. In eukaryotic aerobic cells, mitochondria are the main site of ATP synthesis by oxidative

phosphorylation. Mitochondria consist of a matrix space, an inner and outer membrane, and an interstitial space. The matrix space contains the enzymes of the Krebs cycle (Figure 3) which provides redox equivalents (NADH, $FADH_2$). These are necessary to build up the proton motive force by the electron transport chain (ETS) located in the inner membrane (Friedman and Nunnari, 2014). The proton motive force is used for ATP generation called oxidative phosphorylation. In total, one turn of the Krebs cycle gains 10 ATP as 2.5 ATP for each NADH, 1.5 ATP for $FADH_2$ and 1 ATP is produced in the cycle itself. While the Krebs cycle by itself does not need oxygen, the regeneration of NAD^+ and FAD within the ETS depends on oxygen as final electron acceptor.

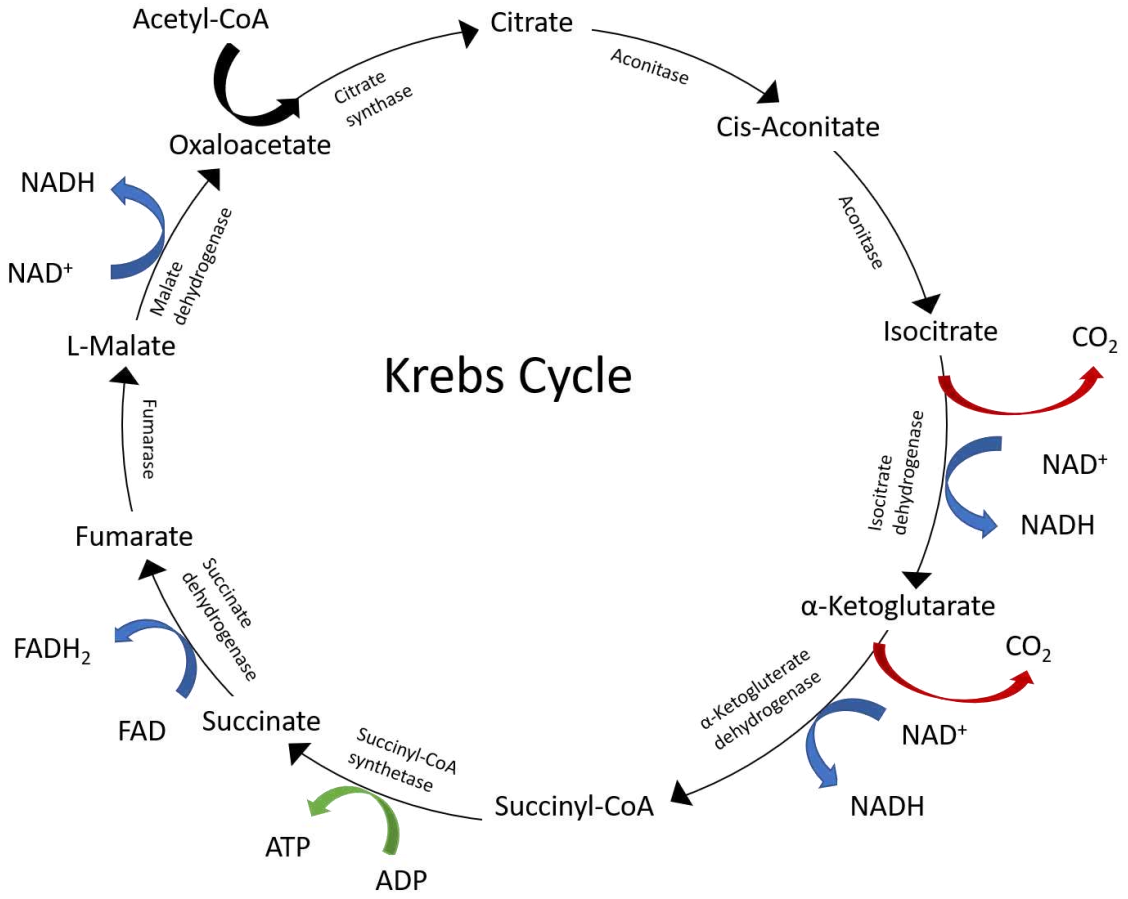


Figure 5. Krebs Cycle and its intermediates. The Krebs cycle usually begins with the entry of Acetyl-CoA into the cycle. Acetyl-CoA is the product of lipid oxidation or glycolysis and citrate synthase catalyzes Acetyl-CoA and Oxaloacetate to Citrate in the first step. After several steps, e.g. oxidation and decarboxylation, the cycle ends with oxaloacetate as the end product and the cycle can start again with the entry of Acetyl-CoA. However, the Krebs cycle is not a closed loop, and intermediates can leave or enter the cycle at any point as required (For a recent review see Arnold and Finley, 2023). For example, adaptations of the Krebs cycle in response to cold adaptation are primarily detected by an increase in the activity of enzymes involved in the Krebs cycle (Pörtner et al., 2005).

Besides generation of electrons for the ETS, the Krebs cycle provides intermediates for many biochemical processes including protein synthesis. The Krebs cycle is an open cycle. It can only function if there is a balance between the intermediates that leave the cycle (cataplerosis) and the intermediates that refill the cycle (anaplerosis). If energy requirements exceed the energy that can be provided aerobically, organisms may temporarily switch to anaerobic metabolism with the risk of

fatigue or depressing their metabolic requirements e.g. at temperatures beyond T_{pej} (Guderley and Pörtner, 2010; Pörtner et al., 2017).

1.4 Fish as model organisms to investigate thermal adaptation

Fishes (Pisces) are a high diverse class of species that have existed for more than 500 million years. They can be mainly divided into jawless fish (Myxini and Petromyzontida), cartilaginous fish (Chondrichthyes) and bony fish (Osteichthyes) (Facey et al., 2023). The Osteichthyes include the subclasses of Sarcopterygii (lobe-fined fishes and tetrapod descendants) and Actinopterygii (ray-finned fishes, mostly Teleostei). The infraclass Teleostei, better known as teleosts, are numerous and divided into several clades and orders. They are the class most people define as typical fish. Many basic physiological functions of fish are similar to those of other vertebrates, including mammals (Wootton, 2012). Unlike mammals, most fish are ectothermal. Changes of water temperature affects physiological processes at all organizational levels (Alfonso et al., 2021; Kassahn et al., 2009; Little et al., 2020). Furthermore, fish are found in almost all waters of the world, from warm freshwater systems with low oxygen level in the tropics to the highly oxygenated consistently cold saltwater with temperatures below 0°C, making them an ideal organism for studying thermal adaptation (Wootton, 2012).

1.4.1 Comparative fish models for studying thermal adaptation

Various fish models are used for physiological investigations, but the zebrafish (*Danio rerio*) is most frequently studied. It is naturally occurring around the Ganges and Brahmputra river basins. Its preference as a model organism is due to easy maintenance and breeding and its relatively short lifespan. Major research fields that use zebrafish include developmental biology, cancer research, toxicology, drug discovery, and molecular genetics (Silva Brito et al., 2022; Spence et al., 2008). Various studies have also investigated the effects of temperature on *D. rerio* and provided insight into the thermal biology of fish (López-Olmeda and Sánchez-Vázquez, 2011). However, due to decades of selective breeding in laboratories, it is difficult to transfer results from laboratory studies on *D. rerio* to the natural context.

Another approach to studying thermal adaptation is the comparison of species with different natural thermal adaptations. This comparative method is widely used in biology and is based on the idea that the system of an organism can be better understood by comparing and contrasting organisms (Sanford et al., 2002). For this method, is important that the studied species differ only in certain characteristics, e.g. habitat temperature. Thus, closely related eurythermal and stenothermal species are typically selected for comparative studies (Dahlke et al., 2020; Fraser et al., 2022; Gamperl and Syme, 2021; Storch et al., 2005; Van Dijk et al., 1999).

A series of studies on the physiological aspects of thermal adaptation have been carried out on different species of the zoarcid family. The zoarcids, or eelpout family, is a group of Perciformes within the Teleost, consisting of about 220 species from four subfamilies. Zoarcids most certainly developed in the North Pacific and spread out from there, as far as the Southern Ocean (Anderson, 1994). Today,

most eelpout species are found on the continental shelf and on the slopes of boreal seas. They are also highly abundant benthic fishes in the Southern Ocean, along with liparids and notothenoids (Anderson, 1994; Brodte et al., 2006b). The boreal waters and the Southern Ocean differ largely in terms of temperature regime. Adaptation to different temperature windows and the fact that it is quite similar ecotype makes this fish family ideal for comparative studies on their thermal adaptation. Over the last two decades, a basis of comparative studies has been established with the stenothermal Antarctic eelpout *Pachycara brachycephalum*, which lives in the Southern Ocean (Brodte et al., 2006b), and the eurythermal common eelpout *Zoarces viviparus*, which inhabits boreal waters from the southern North Sea along the Norwegian coast up to the White Sea (Lajus et al., 2003; Yershov, 2005; Zakhartsev et al., 2003).

Although they live in different climatic regions, both fish are benthic carnivores and closely related (Brodte et al., 2006a; Windisch et al., 2012). The differential thermal adaptation of these two-eelpout species provides a great opportunity to study the response to temperature at different physiological levels. These include differences between thermally acclimated fish at different temperatures in terms of growth rates, energy distribution (Brodte et al., 2006a), lipid content (Brodte et al., 2008), protein synthesis capacity (Storch et al., 2005) and mitochondrial respiration (Lannig et al., 2005). Other studies focused on the effects of acute warming comparing these two fish species in terms of oxygen consumption (Mark et al., 2002; Van Dijk et al., 1999), changes in intracellular pH (Bock et al., 2001; Sartoris et al., 2003; Van Dijk et al., 1999), mitochondrial proliferation (Lucassen et al., 2003) and uncoupling protein response (Mark et al., 2006).

1.4.2 Antarctic eelpout - *Pachycara brachycephalum*

Pachycara brachycephalum (Pappenheim 1912) is one of 24 species of Antarctic eelpout endemic to the Southern Ocean. It is most frequent at a depth of 400 m around the Antarctic Peninsula, but also occurs at greater depths in high Antarctic waters (Anderson, 1994, Brodte et al., 2006b). *P. brachycephalum* become sexually mature at the age of 5 years and can reach a maximum age of at least 14 years. Their main prey organisms are krill and amphipods (Brodte et al., 2006b). Most commonly, *P. brachycephalum* is found at temperatures between 0 and 0.6°C (Brodte et al., 2006b), -1°C (Windisch et al., 2014) and at even -1.9°C (DeVries and Cheng, 2005) due to the ability to produce antifreeze peptides (Bernardi and DeVries, 1994). However, the thermal optimum of *P. brachycephalum,* according to growth performance studies, is between 3 and 4°C (Brodte et al., 2006a; Windisch et al., 2014). The upper thermal limit for long-term sustainability is around 6°C (Windisch et al., 2014) and 12°C for acute warming (Mark et al., 2002).

1.4.3 Common eelpout - *Zoarces viviparus*

The common eelpout, *Zoarces viviparus* (Linnaeus 1758), is a boreal eelpout species that is distributed between the English Channel in the south and the White Sea in the north (Lajus et al., 2003; Yershov, 2005). *Z. viviparus* is found in shallow waters near the coast, but can also be found at depths of up to 40 m and has a life span of about 5-14 years (Kristoffersson and Oikari, 1975). Similar to its counterpart in the Southern Ocean, *Z. viviparus* is a carnivore that feeds on invertebrates and small fishes (Ojaveer

et al., 2004). It exhibits stationary behavior and does not migrate over long distances (Kinitz et al., 2013). Moreover, *Z. viviparus* is eurythermal with a wide thermal window and can be found at temperatures of -1°C in the White Sea and more than 20°C in the North Sea (Lajus et al., 2003; Pörtner and Knust, 2007; Zakhartsev et al., 2003). The thermal optimum of the North Sea population of *Z. viviparus* lies between 12 and 15°C (Brodte et al., 2006a; Fonds et al., 1989; Pörtner and Knust, 2007). In acute warming experiments they can even survive temperatures of up to 24-26°C (Heise et al., 2006; Pörtner and Knust, 2007; Van Dijk et al., 1999).

1.5 Thermally dependent physiological performance of fish

Changes in ambient temperature can induce stress in organisms. Their ability to cope with it depends largely on their ability to maintain performance and oxidative metabolism (Pörtner, 2021). As complexity in structure and function increases, heat tolerance decreases. E.g., unicellular organisms are more heat tolerant than multicellular eukaryotes (Storch et al., 2014). In more complex animals, temperature induced stress can be detected at all different organizational levels, including the organismal (e.g. growth, oxygen consumption, ventilation, circulatory capacity, and excretion rates), organ and tissue (e.g. liver, gills and muscles), cellular (e.g. proteomic), and molecular level. Responses on the organismal level, e.g. a reduction of growth rates may occur prior to the onset of cellular and/or molecular stress (Kassahn et al., 2009). Therefore, it is important to focus on multiple levels of organization to understand the effects of temperature change in more complex organisms such as fish.

1.5.1 Thermal response of the whole animal

The whole-animal is the highest and most complex organization level of an animal, which includes e.g. growth, oxygen consumption, ventilation and excretion (Kassahn et al., 2009). Depending on the whole-animal parameters studied, different conclusions can be drawn about the effects of temperature on an organism.

Many studies have focused on growth rates to investigate the aerobic performance of an organism because its measurements are comparatively easy over a long period of time without harming the fish. Fish growth can be expressed as absolute growth, relative growth, and specific growth, which can be measured by weight gain or increasing length. These growth parameters vary greatly within and between individuals, depending on age, with higher growth rates in juveniles and slower growth rates in adults (Hopkins, 1992; Lugert et al., 2016). However, fish grow throughout their life span and growth rates decline at both sides of the species-specific temperature optimum (Enzor et al., 2017; Pörtner and Knust, 2007; Windisch et al., 2014).

In contrast, oxygen consumption and ventilation increase with increasing temperature due to higher oxygen demand within the whole organism (Franklin et al., 2013; Mark et al., 2002; Maus et al., 2021). When the temperature increases, all processes occur faster due to the Q_{10} effect and more energy is required as described above. Thus, aerobic energy production increases and more oxygen is consumed. However, the effect of temperature on oxygen consumption depends on the time the fish have spent at that temperature. When the temperature is increased, oxygen consumption initially

increases to cope with the higher energy demand. After a while the fish become accustomed to the new temperature as long as it is within its thermal window. Oxygen consumption decreases again and can even return to control levels (Robinson and Davison, 2008).

The excretion rate of nitrogen provides information about the digestion of an organisms and the respective food conversion. In marine fish, most nitrogenous waste is released as ammonia (Walsh et al., 2001). It has to be directly excreted due to its toxic properties. It substitutes K^+ ions in ion transporters which may disrupt the electro-chemical gradients in the nervous system (Cooper and Plum, 1987). The amount of ammonia excreted depends on the amount of protein digested, as protein-rich food increases proteolysis and thus ammonia excretion (Yang et al., 2002). Temperature can also influence the digestion of proteins and thus ammonia excretion (Jobling, 1981; Sun and Chen, 2009). The ratio of nitrogen excretion (N) and oxygen consumption rate (O), the so-called O:N ratio, can be used to determine whether an organism prefers carbohydrates, lipids or proteins as metabolic fuel. Higher O:N rations indicate more lipid-based metabolism, while lower O:N ratios suggesting a more protein-based metabolism (Mayzaud and Conover, 1988). In the cold, fish prefer a more lipid-based metabolism while in the warmth a carbohydrate-based metabolism prevails (Brodte et al., 2008; Pörtner et al., 2005; Windisch et al., 2014). Less well known, however, is protein metabolism in the cold, which is described separately under 1.5.3.

1.5.2 Thermal response at the organ/tissue level

Higher organisms, including fish, consist of specialized organs and distinct tissues that vary greatly in function and metabolic activity thereby interacting with each other. In order to function properly, all of these organs/tissues require oxygen, which is taken up by the gills. Gills are involved in many processes (Houlihan et al., 1986; Lyndon and Houlihan, 1998) and have evolved to be the first vertebrate gas exchange organ, but gills are also the main organ for nitrogen excretion and osmoregulation. Gills have a highly complex vasculature. They are surrounded by an epithelium with a high surface area, enhancing the gas exchange and ion flux between the extracellular fluids and the aquatic environment (Evans et al., 2005). When environmental conditions change e.g. due to rising temperatures, the gills must react quickly to maintain their function and supply the fish with oxygen. This includes changes within the ion- and acid base regulation (Kreiss et al., 2015; Michael et al., 2016), protein synthesis (Storch et al., 2005) and cell organization (Buckley et al., 2006). This leads to a higher energy demand and thus higher oxygen uptake in the gills (Kreiss et al., 2015).

After the gills have absorbed oxygen, it binds to the hemoglobin of the red blood cells (erythrocytes) and circulates through the body to all organs and tissues (Soldatov, 2005). The number of erythrocytes in the blood can be determined by measuring hematocrit. It varies greatly depending on species, as well as biotic and abiotic factors (Ahmed et al., 2020). Higher temperatures can increase hematocrit in order to facilitate the oxygen transport through the body (Beers and Sidell, 2011; Joyce et al., 2018; Windisch et al., 2014). However, not only the quantity of erythrocytes, but also the lifespan of erythrocytes in fish is influenced by temperature (Hofer et al., 2000). In fish, erythrocytes are produced by the head kidney (pronephros) and stored in the spleen (Fänge and Nilsson, 1985). The storage of

erythrocytes in the spleen has the advantage that they can be released quickly when required e.g. during intensive exercise (Brijs et al., 2020) or when temperature increases (Islam et al., 2020).

The majority of blood-bound oxygen is consumed by the muscles which make up the largest part of the fish's body mass. The musculature is essential for locomotion of the fish and other functions including the peristaltic movements of organs within the body. Fish possess three major muscle types; first the skeletal muscles which are attached to bones allowing for conscious movement, second the smooth muscle located in various internal structures and third the cardiac muscle for the heart. The skeletal muscle is subdivided into the white muscle, the red muscle along the skin and an intermediate pink muscle (Listrat et al., 2016). The white muscle is further divided in myotomes consisting of muscle fibers. These muscle fibers are tightly packed with myofibrils which again are divided into sarcomeres which mostly consists of proteins. These proteins are the contractile proteins myosin (60%) and actin (20%) as well as minor proteins such as troponin and tropomyosin (<20%). (Johnston et al., 2011; Ochiai and Ozawa, 2020). Due to its high protein content, white muscle can act as a protein store and release large amounts when needed, e.g. during starvation (Houlihan et al., 1995; Kiessling et al., 1995). There is also evidence that muscle proteins can function as an energy source during elevated temperature indicated by lower protein synthesis rates and higher protein degradation rates (Fraser et al., 2022; McCarthy et al., 1999). Furthermore, elevated temperature above the thermal optimum can have several other effects on white muscle. It can affect muscle performance in terms of mobility (James and Tallis, 2019), it can alter lipid content and composition (Brodte et al., 2008), protein synthesis capacity (Storch et al., 2005), and intracellular pH (Van Dijk et al., 1999). All of these cellular processes can influence muscle performance and in particular muscle growth. Muscle growth in fish contributes greatly to body growth. Therefore, fish are an important food source for many organisms, including humans.

1.5.3 Thermal response at the cellular and molecular level

Increase in temperature can lead to a cellular stress response (CSR) to protect and restore the macromolecular system, modulate the Krebs Cycle and thus energy metabolism, regulate cell proliferation, or induce programmed cell death (apoptosis) when cells cannot be repaired (Kültz, 2020; Somero, 2020). These CSRs include several processes, such as the restoration of protein homeostasis, including the synthesis and degradation of proteins (Somero, 2020). Protein synthesis is an essential process in all living organisms and accounts for up to 42% of the total available energy in ectotherms, a reason why it is highly regulated (Fraser and Rogers, 2007). The highest protein synthesis rates in fish can be measured in the gills with an overall protein synthesis rate of up to 12% day^{-1} *in vivo* (McMillan and Houlihan, 1989) and 18.7% day^{-1} *in vitro* (Storch et al., 2005). Lyndon and Houlihan (1989) postulate that the high rates of protein synthesis in gills are due to a high turnover rate of collagen, epithelia cells, chloride cells, mucus glycoproteins and musculoskeletal elements (Lyndon and Houlihan, 1998). As an adaptation to changing environmental conditions, the rate of protein synthesis in gills was found to change with temperature (Storch et al., 2005) and oxygen levels (Cassidy et al.,

2018), which seems to be essential for maintaining adequate oxygenation, nitrogen excretion and ion regulation for the entire fish body.

A large proportion of the nitrogen excreted via the fish gills derives from the muscle as a degradation product of protein turnover (Wilkie, 1997). Generally, the protein synthesis rate in white muscle is lower than in the gills but varies greatly depending on species and growth rates. For example, protein synthesis rates in white muscle are relatively high during development when fish are growing rapidly. Values range up to 4.4% day^{-1} at high growth (Foster et al., 1991) and are relatively low in adult Antarctic fish, with values between 0.04 and 0.2% day^{-1} (Smith and Haschemeyer, 1980). Up to 76% of the protein synthesis rate in white muscle contributes to fish growth. Changes in protein synthesis rates in white muscle are therefore indicative of changes in fish growth (Houlihan et al., 1986; McCarthy et al., 1999) and thus animal performance. Temperature appears to have a greater influence on protein synthesis rate in eurythermal fishes (Katersky and Carter, 2007; Lewis and Driedzic, 2007; Watt et al., 1988) than in stenothermal Antarctic fishes (Fraser et al., 2022; Storch et al., 2005).

While the protein synthesis rate only describes the amount of newly synthesized proteins over time (mostly in days), it is equally important to measure the amount of proteins degraded during that time. The ratio of protein synthesis and protein degradation determines the protein turnover. If more proteins are synthesized than degraded in e.g. the muscle, it grows; conversely, if more proteins are degraded than synthesized, the muscle loses mass and shrinks. Moreover, even at balanced protein synthesis and degradation, huge differences may exist for protein turnover, if large amounts of damaged proteins have to be replaced. Protein degradation is more variable than protein synthesis, as there are several different pathways for protein degradation, which may differ depending on species and tissue (Nemova et al., 2021). This dissertation will concentrate only on the degradation pathway in one tissue, the skeletal muscle, since this was the only tissue in which protein degradation was investigated.

First, there are the calcium-dependent proteinases of the calpain family. They mainly degrade short-lived proteins of the cytosolic fraction and while doing so are involved in processes of intracellular signaling, cell differentiation and cell death (Goll et al., 2003; Nemova et al., 2016). Second, there are lysosomal proteinases (cathepsins) that degrade cytoplasmic proteins or organelles of the cell during autophagy at low pH. There are at least 10 different cathepsins, of which cathepsin D is the most important in the skeletal muscle of fish. Cathepsin D is an asparagine proteinase that cleaves native proteins such as myosin heavy chain, actin and tropomyosin, particularly during autophagy (Nemova et al., 2016; Nielsen and Nielsen, 2001). The third degradation pathway, the ubiquitin-proteasome system, is responsible for up to 90% of degradation in vertebrates and is involved in various pathways ranging from cell quality control to apoptosis (Ciechanover, 2013; Nemova et al., 2016). However, the ubiquitin-proteasome degradation plays only a minor role in the overall protein degradation in fish muscle, most likely because the ubiquitin proteasome degradation is more energy demanding than the degradation by calpain or cathepsin (Seiliez et al., 2014). Similar to protein synthesis, temperature affects protein degradation in fish (Lamarre et al., 2009; McCarthy et al., 1999), with different patterns

in eurythermal and stenothermal fish (Fraser et al., 2022). There is evidence that protein degradation occurs at higher rates in stenothermal Antarctic fish than in temperate fish, as their levels of ubiquitinated proteins and proteasome activity are higher in gill, liver, heart and spleen tissues (Todgham et al., 2017, 2007). However, evidence for higher protein degradation rates in white muscle of Antarctic fish has not yet been investigated.

1.6 Rationale and Aims

All fish have a species-specific thermal window with a thermal optimum in which aerobic performance is highest. The thermally-dependent aerobic performance of fish is commonly measured as growth rate after they have been acclimated to a certain temperature for several weeks or months. This is time-consuming as growth can only be measured in fish that are acclimated to a certain temperature and, due to adequate maintenance and diet control of the animals, can be quite challenging. The same applies to another indicator of aerobic performance, reproduction. Moreover, it can only be determined in adult fish and, depending on the species, only at certain times of the year. To determine aerobic performance during acute warming, aerobic exercise is often used as a proxy. During exercise, however, the fish require more oxygen than during the resting phase. Therefore, less oxygen is available to adjust to the thermal changes during acute warming, resulting in lower thermal tolerance (Blasco et al., 2020).

Using an alternative proxy to measure aerobic performance within hours at the resting phase would certainly help to promote a deeper understanding of the effects of temperature on an animal´s physiology. In this way, open research questions in the interplay between aerobic performance and oxygen consumption during acute warming can be investigated to determine the differences and/or similarities between fish with different thermal adaptations.

The experimental approaches carried out for this thesis are embedded in the conceptual framework of the oxygen- and capacity-limited thermal tolerance (OCLTT) of organisms. The OCLTT concept is intended to serve as a bridge between ecology and physiology and states that thermal tolerance is related to the oxygen and capacity limitation of the cardiocirculatory system (reviewed by Pörtner, 2021). In ectothermal organisms, most of the available energy at rest is used for protein synthesis (Fraser and Rogers, 2007), but less is known about the rate of protein synthesis at increasing temperature in terms of thermal adaptation. While fish from temperate zones are adapted to large temperature fluctuations between the seasons, temperatures in the polar regions change very little and remain constantly low. Therefore, various processes of fish that live in the polar region are cold-compensated to ensure that they function at temperatures below 0 °C and grow and reproduce. This specialization has led to a narrow thermal window in comparison to wide thermal windows of temperate fish.

In order to understand evolutionary cold adaptations and related higher sensitivity to warming, I investigated and compared the protein turnover in the cold adapted stenothermal Antarctic eelpout (*Pachycara brachycephalum*) inhabiting the Southern Ocean and its confamilial from the North Sea,

the common eelpout (*Zoarces viviparus*) during acute warming. Both species are closely related, but adapted to different thermal regimes and are therefore perfect candidates for comparative studies and for testing the following hypotheses.

1) **The protein synthesis rate in slow-growing Antarctic eelpout can be measured *in vivo* in white muscle tissue and is comparable with previously published growth data.**

The protein synthesis rate is most commonly measured with radioactively labelled tracers, since the tracer can be detected at relatively low concentrations. Particularly in organisms with a slow protein synthesis rate, such as Antarctic fish, sensitive methods for the precise determination of protein synthesis rates are essential. However, radioactive tracers are potentially harmful to the environment. Therefore, this is the first study in which isotopically labelled tracers are used to determine the protein synthesis rate in the white muscle of Antarctic fish. In addition, the protein synthesis rates measured here were compared with previously published growth data to validate the methodology and determine how much the protein synthesis rate contributes to growth.

2) **During acute warming, the protein synthesis rate changes accordingly to the species specific and temperature-dependent growth rates of the common eelpout (*Zoarces viviparus*) and Antarctic eelpout (*Pachycara brachycephalum*).**

Each ectotherm has its species specific thermal optimum temperature at which aerobic performance is highest. During acute warming, temperature is increased daily and thus changes of growth cannot be measured. Therefore, this thesis aims to use the protein synthesis rate in white muscle as a proxy for aerobic performance during acute warming.

3) **As a result of cold compensation, *P. brachycephalum* is able to maintain a high protein turnover at colder temperatures compared to *Z. viviparus***

In the cold, all processes are slower due to thermodynamic rules. Antarctic fish have to compensate for these cold conditions not only to survive, but also to grow and reproduce in the coldest ocean on earth, the Southern Ocean. This thesis thus targets to identify the combination of protein synthesis and degradation, summarized as protein turnover, at different temperatures during acute warming. The comparison between the stenothermal *P. brachycephalum* and eurythermal *Z. viviparus* will help to identify further indicators for cold compensation as a consequence of thermal adaptation.

4) **The temperature-dependent changes during acute warming caused by a mismatch between temperature dependent increase in energy demand and the limited capacity of oxygen supply as a result of different patterns of thermal adaptation.**

All ectotherms are adapted to a specific thermal window and have the highest aerobic performance at its thermal optimum. At temperatures above the optimum, the aerobic performance decrease. This study demonstrates that limiting oxygen supply leads to a reduction in aerobic performance during acute warming, but different patterns emerge depending on the thermal adaptation of the eelpout.

2 Material and Methods

2.1 Animals

The Antarctic eelpout *Pachycara brachycephalum* (Pappenheim, 1912) were caught in the Southern Ocean at Admiralty Bay, King George Island (62°11' S, 58°20' W), Antarctica. For this purpose, baited fish traps were used at a depth between 430 and 530 m on the RV Polarstern expedition PS112 in March 2018. After 52 hours, the fish traps were recovered from the seafloor and the fish were kept on board at 0°C until they arrived at the Alfred Wegener Institute (AWI) in Bremerhaven, Germany. Before the experiments, the fish were kept at AWI in well-aerated, re-circulating seawater at 0.0° ± 0.5°C and 34 ± 1 practical salinity units (PSU) under a 12:12 light:dark cycle. Once a week, *P. brachycephalum* was fed with frozen blue mussel, *Mytilus edulis* (Erdtmann, Salzwedel, Germany).

The common eelpout, *Zoarces viviparus* (Linnaeus, 1758), were caught in the North Sea near the island of Helgoland and brought to AWI by RV Uthörn in autumn 2020. The fish were kept in well aerated, re-circulating seawater at 12°C for an acclimation period of at least 3 months before cooling the water slowly to a temperature of 4.0 ± 0.5°C, at 34 ± 1 PSU on a 12:12 light/dark cycle. The fish were fed frozen blue mussels, *Mytilus edulis* (Erdmann, Germany), 2-3 times weekly.

2.2 Chemicals

All chemicals are purchased from Sigma-Aldrich, St. Louis, MO, USA if not stated otherwise.

2.3 Experiment 1: Establishing protein synthesis measurements non-radioactively in Antarctic fish

The measurement of the protein synthesis rate by using isotopically labeled tracer (phenylalanine $^{13}C_9H_{11}{}^{15}N_1O_2$) was first established in *P. brachycephalum* at the temperature of 0°C. For this experiment, the weekly feeding rate was stopped 5-7 days prior the experiment started. Afterwards, the fish were first weight and immediately injected intraperitoneal with 0.7 mL/100g body weight of 75 mM labeled phenylalanine (Phe) in PBS buffer (pH 7.4, 4°C) by using a 1 mL Syringe and a 0.4 x 20 mm cannula (Braun Melsungen, AG, Melsungen, Germany). After the injection, the fish were released back into the water. Shortly after the injection, the fish showed no visible stress response as the procedure lasted less than five minutes. At different time points post injection (1.5, 3, 4.5 or 6 hours), six randomly selected fish were sacrificed to measure protein synthesis rates and were compared with a control group. For this, the fish were first stunned with a blow to the head and killed by cutting the spinal cord closely behind their head before collecting white muscle tissue and storing it at -80 °C until further use. Additionally, the hepatosomatic Index (HSI) was calculated for all individuals (HSI= liver weight/body weight * 100). The work was approved by the German authority (Freie Hansestadt Bremen, reference number 160;500-427-103-7/2018-1-5).

2.4 Experiment 2: Protein turnover during acute warming

The acute warming experiments were conducted with both eelpout species, *P. brachycephalum* (acclimated to 0°C) and *Z. viviparus* (acclimated to 4°C). About 4 weeks before the start of the

experiments, the feeding rate of *P. brachycephalum* was increased from once per week to 2-3 times per week, similar to *Z. viviparus*, to allow a better comparison of the two species. For both species, feeding was stopped 5-7 days before the experiment. For *P. brachycephalum*, the experiment started at 0°C and was increased by +2°C day^{-1} until 10°C was reached, while for *Z. viviparus* the experiment started at 4°C and was increased by +3°C day^{-1} until 22°C was reached. At several temperature steps (*P. brachycephalum*: 0, 2, 4, 6, 8, and 10°C; *Z. viviparus*: 4, 10, 13, 16 and 22°C), fish were injected with 0.7 mL/100g body weight of 75 mM labelled Phe in PBS buffer (pH 7.4) and sacrificed at 1.5- and 3-hours post-injection. First, the fish was stunned with a blow on the head before taking blood samples with 1 syringe and a 0.4 x 20 mm cannula (Braun Melsungen, AG, Melsungen, Germany) and sacrificing the fish by cutting the spinal cord closely behind the head. The HSI was calculated and 100 µL of blood was separated and kept on ice for 10 min before centrifugation at 3000G for 10 min (Eppendorf, Hamburg, Germany) to determine hematocrit as the ratio between the light supernatant (blood serum) and the red colored pellet (red blood cells). The rest of the blood, together with various other tissues collected (white muscle, gill, liver, spleen, brain and kidney), was quickly frozen in liquid nitrogen and stored at -80°C until further use.

2.5 Methanol Chloroform extraction

Approximately 50 mg of white muscle tissue and 20 mg of gill tissue were extracted according to Wu et al., (2008) by homogenizing the tissue in 400 µl of methanol (MeOH) and 125 µl of ultrapure water (MilliQ) in 2 circuits of 20 s at 6000 rpm at 4°C using the Precellys tissue homogenizer (Precellys 24 tissue homogenizer, Bertin Instruments, Montigny-le-Bretonneux, France). The homogenized tissue was afterwards transferred into 1.5 mL vials (Eppendorf, Hamburg, Germany) before adding 400 µL Chloroform together with 400 µL ultrapure water and vortexed (Vortex mixer, Scientific Industries, Bohemia, NY, USA) for 20 s. The mixture was then incubated on ice for 10 min before centrifugation for another 15 min at 3000G and 4°C. The three layers, consisting the upper aqueous layer, the lower chloroform layer and the precipitated protein layer in the middle were collected in separate 1.5 mL vials (Eppendorf). The lower chloroform layer containing apolar compounds such as lipids were not used. The upper aqueous layer containing the cytosolic fraction including polar metabolites such as free Phe. The protein fraction containing incorporated Phe were washed two times by adding 1 mL MeOH followed by 20 s vortexing for white muscle tissue and only inverting for gill tissue before centrifugation for 3 min at 13,000G to remove any residual unbound Phe. The supernatants were discarded and the washed protein pellet was dried in a vacuum centrifuge (Speedvac, Thermo Fisher Scientific, Waltham, MA, USA) at 25°C overnight. The next day, the dried protein pellet was hydrolyzed with 100 µL 6M HCL/mg protein pellet for 24 h at 99°C while shaking at 600 rpm (Thermomixer comfort, Eppendorf). The hydrolyzed proteins were then centrifugation at 10,000 rpm for 3 min and the supernatant was collected, frozen and lyophilized for 12 h. The dry hydrolysate was dissolved in 1 mL MeOH and desalted by application to a solid phase extraction (SPE). The aqueous layer including the free Phe was diluted 1:10 with MeOH and also applied to the SPE.

2.6 Phenylalanine quantification

Liquid chromatography high-resolution mass spectrometry (LC-HRMS/MS) analysis was performed with a Vanquish UPLC system coupled to a Q-Exactive Plus mass spectrometer, using a heated electrospray ionization source (all Thermo Fisher Scientific). Separation was performed on a C18 column (C18 BEH, 100 x 2 mm, 1.7 µm particle size, Waters, equipped with guard-column). Positive Ion Calibration Solution (Pierce, Thermo Fisher Scientific) was used for the calibration of the instrument. A blank as well as a quality control standard was injected every five samples to check the instrument's drift and carry-over. A binary solvent gradient was used with a flow rate of 0.35 mL per min on a C18 column at 32 °C, with solvent A = 0.1% formic acid (Roth, VWR, Bruchsal, Germany) in ultrapure water and solvent B = 0.1% formic acid in MeOH. The gradient program was as follows: T0 min: B = 2%, T0.1 min B = 2%, T3.9 min: B = 99%, T4.5 min: B = 99%; T4.7 min: B = 2%. The column was equilibrated for 0.5 min between samples. MS spectra were acquired in full scan mode or data independent (DIA) mode. Full scans were acquired with a resolution of 35,000 (fifty percent of the maximum peak height (FWHM), m/z 200), a scan range of 120 to 250 m/z, automatic gain control (AGC) of 3×10^6 and injection time (IT) of 100 ms. DIA experiments were used to quantify analytes by tandem mass spectrometry utilizing an inclusion list of the accurate masses (m/z 176.11313, m/z 166.08617, m/z 167.07617) at a resolution of 35,000 FWHM (m/z 200), NCE of 30, AGC of 2×10^5 and isolation window of 1.0 m/z. The heated electrospray ionization source was set to 3.5 kV spray voltage, the aux gas to 425 °C (13) and the sheath gas to 50. The capillary temperature was set to 263 °C. The fragment ions m/z 120.0808 ($^{12}C_9H_{11}{}^{15}N_1O_2$—$C_1H_2O_2$) and m/z 129.1047 ($^{13}C_9H_{11}{}^{15}N_1O_2$—$^{13}C_1H_2O_2$) were used for quantification with a mass tolerance of 5 ppm. Quantification was achieved with an external calibration to standard dilution series of standards prepared from $^{12}C^{14}N$ as well as the $_{13}C_{15}N$ phenylalanine ranging from 10 pg/µL to 1000 pg/µL. Calibration curves were measured every 100 samples to evaluate sensitivity changes in the instrument.

Concentrations of protein-bound and free phenylalanine were calculated using an internal standard ($_{15}$N-Phenylalanine: $^{12}C_9H_{11}{}^{15}N_1O_2$) and calibration curve for each analyte (labeled phenylalanine: $^{13}C_9H_{11}{}^{15}N_1O_2$; unlabeled phenylalanine: $^{12}C_9H_{11}{}^{14}N_1O_2$), and outliers (identified by inter-quartile range IQR) were eliminated. Ks was calculated after Garlick et al., 1980 and modified after Krebs et al., 2023a:

$$\text{Ks (\% day}^{-1}) = \left(\frac{\text{Sb labeled } [\frac{\text{pg}}{\mu\text{g}}]}{(\text{Sb labeled}+\text{Sb unlabeled})[\frac{\text{pg}}{\mu\text{g}}]}\right) * \left(\frac{100}{(\text{Sa labeled}[\%]) * \text{t (days)}}\right) * 100$$

with Sb as the protein-bound pool and Sa as the free pool of phenylalanine (pg phenylalanine per µg fresh weight) and t as time in days. Labeled phenylalanine describes the injected $^{13}C_9H_{11}{}^{15}N_1O_2$ phenylalanine and unlabeled the naturally found $^{12}C_9H_{11}{}^{14}N_1O_2$ phenylalanine.

2.7 Protein degradation capacity

The protein degradation was determined by measuring the activity of cathepsin D according to Martínez-Alarcón et al., (2018). For this porpoise, 50 mg frozen white muscle tissue from the previous described acute warming experiment was homogenized (1 mg/100 µL) in 50 mM Sodium Acetate (with

5 mM EDTA) buffer (pH 5.0, was adjusted with acetic acid) in 2 circles of 20 s at 6000 rpm at 4°C (Precellys). The homogenate was centrifuged at 3000 rpm and the supernatant was separated into two vials and stored at -80 °C. One vial was used to determine the protein content according to Bradford (Bradford, 1976), the other was used to measure the activity of cathepsin D fluorometrically. Therefore, 70 µL buffer together with 20 µL of 100 mM fluorogenic substrate 7-methoxycoumarin-4-acetyl-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys-(DNP)-DArg-amide (M0938, Sigma-Aldrich) was added onto a black plate. Afterwards, the 10 µL of samples were added (measured in triplicates) starting the reaction and measured fluorometrically at 26°C. All Data are expressed in Units per mg of protein (U mg^{-1}).

2.8 Untargeted metabolic profiling

The metabolic profile was conducted as described in detail in Tripp-Valdez et al., (2017) and Götze et al., (2020). Shortly, we homogenized 50 mg muscle tissue (fresh weight) and extracted the cytosolic fraction by Methanol-Chloroform extraction. Afterwards, the cytosolic fraction was dried overnight in a vacuum centrifuge (Speedvac). The pellet was resolved with D_2O (deuterized water + TSP; 0.075 wt%) in a concentration of 2-fold volume per fresh weight (final concentration: 0.5 g ml^{-1}). For the determination of metabolites using an ultra-shielded vertical 9.4 T NMR spectrometer (Avance III HD 400 WB, Bruker-BioSpin GmbH, Ettlingen, Germany), samples (45µl) were transferred to NMR needle tubes (1.7 mm, FisherScientific, Schwerte, Germany). 1H-NMR spectra were acquired at 400 MHz with a 1.7 mm triple-tuned ^{1}H-^{13}C-^{15}N NMR probe using a Carr-Purcell-Meiboom-Gill (Bruker protocol cpmgpr1d, TopSpin 3.5) sequence including water suppression at room temperature at the following parameters: acquisition time (AQ), 4.01 s; sweep width (SW), 8802 Hz (22 ppm); delay (D1), 4 s; dummy scan (DS), 4; and number of scans (ns), 512.

All spectra were baseline-, shim-, and phase-corrected and calibrated to the TSP signal at 0.0 ppm by using the software Chenomx NMR suite 8.4 (Chenomx Inc.,Edmonton, AB, Canada). Afterwards, the metabolites were assigned and quantified by the chemical shift of their NMR signals based on the TSP signal using Chenomx's internal database and previous NMR studies on polar and marine fish (Rebelein et al., 2018; Schmidt et al., 2017).

2.9 Experiment 3: Oxygen consumption and nitrogen excretion during acute warming

Before the start of the experiment, *P. brachycephalum* was not fed for 10 days and *Z. viviparus* was not fed for 7 days to minimize the effect of the specific dynamic action (SDA). Afterwards, the fish (n=5) were placed in separate respiration chambers, each with a volume of 2 l while one pump circulated the water inside the chamber and the oxygen sensor (Fibox 3 PreSens GmbH, Germany) and another pumped oxygenated water from a 150 L tank into the chambers (Loligo systems, Denmark). Oxygen sensors were calibrated in a separated beaker to 100% in well-aerated water from the water reservoir at 0°C for *Pachycara brachycephalum* and 4 °C for *Zoarces viviparus*. For the calibration of the oxygen content of 0%, nitrogen was bubbled into the water for at least 10 min to drive out all the oxygen. The

calibration was only completed when the oxygen content stopped decreasing and remained stable for several minutes.

For three days, the fish were allowed to acclimatize to their new environment at their species-specific acclimation temperature (0°C for *P. brachycephalum*; 4°C for *Z. viviparus*) in the dark to keep stress to a minimum. On the fourth day, the supply of oxygenated water was interrupted for one hour (*Z. viviparus*) and two hours (*P. brachycephalum*). During this time, oxygen consumption was measured (Software: OxyView, PreSens GmbH, Germany) and water samples were taken at the beginning and end of the measurement. This measurement was repeated 3 (*P. brachycephalum*) to 5 (*Z. viviparus*) for each individual separately. Thereafter, every day at 3 pm the water temperature was increased by +2°C day^{-1} (*P. brachycephalum*) or +3°C day^{-1} (*Z. viviparus*). At each temperature step (*P. brachycephalum*: 0, 2, 4, 6, 8 and 10°C; *Z. viviparus*: 4, 7, 10, 13, 16, 19 and 22°C) we recalibrated the oxygen sensors at 100% saturation. Shortly before reaching the species-specific lethal temperature, the experiment was stopped (10 °C for *P. brachycephalum*; 22 °C; for *Z. viviparus*) and the animals were killed as described for the other experiments.

The water samples (2 mL) taken from each chamber during the oxygen measurement were quickly frozen at -80°C and the ammonia content was then measured photometrically (630 nm; Speedcord S 600, Analytik Jena, Germany) according to the phenol-hypochlorite method of (Solórzano, 1969). In short, 500µl of the water sample was mixed with 750µl H_2O and 100µl phenol solution (the stock solution consists of 0.85 M phenol and 2 mM nitroprusside sodium dihydrate dissolved in 30% ethanol: H_2O solution). After 2 min, 50 µl of citrate buffer (stock solution consisting of 40 M tri-sodium citrate dissolved H_2O) and dichloroisocyanuric acid (DTT) solution (stock solution consisting of 13 mM dichloroisocyanuric acid dissolved in 2M NaOH solution) were added and mixed. The solution was then incubated for 1 hour at 40°C in a thermomixer (400-500 rpm) and measured photometrically at 630 nm. Oxygen consumption (O) (calculated after Boutilier et al., 1984) and nitrogen excretion (N) were then used to calculate the atomic O:N ratio as followed:

$$O : N = MO_2\ NH_4^{+-1}$$

The O:N ratio as an indicator of the composition of lipid and proteins being substrate metabolized e.g. during acute warming (Mayzaud and Conover, 1988).

2.10 Statistical analyses

All data were analyzed using the program GraphPad Prism 9.5.1 and were normally (tested with Shapiro-Wilk test) and homogeneously distributed (chi square test). Statistical differences within one species at the level of 95% were tested by using an ordinary one-way ANOVA (analysis of variance) for non-repeated measurements (protein synthesis rate, protein degradation capacity, hematocrit, spleen weight) and a mixed-effect analysis for repeated measurements (ammonia excretion and O:N ratio) followed by Tukey's multiple comparison test as post hoc. Statistical differences between species at the same temperature at the level of 95% were tested by using an unpaired t-test. Unless otherwise stated, data are visualized as mean ± standard deviation in the figures.

The metabolite data were normalized using the log2 transformation and outliers were identified using unsupervised principal component analyses (PCA) with the online Platform MetaboAnalyst 5.0 (Pang et al., 2022). Significant differences were investigated by using SAM (Significance Analyses of microarray, Tusher et al., 2001) and the distinction between metabolic profiles are presented by using supervised partial least-square discriminant analysis (PLS-DA).

3 Publications

Publication 1: Protein Synthesis Determined from Non-Radioactive Phenylalanine Incorporated by Antarctic Fish

https://doi.org/10.3390/metabo13030338

Publication 2: Evolutionary Adaptation of Protein Turnover in White Muscle of Stenothermal Antarctic Fish: Elevated Cold Compensation at Reduced Thermal Responsiveness

https://doi.org/10.3390/biom13101507

Manuscript 3: Acute warming leads to changes in metabolic processes in the eurythermal common eelpout *Z. viviparus* and stenothermal Antarctic eelpout *P. brachycephalum*.

3.1 Publication 1: Protein Synthesis Determined from Non-Radioactive Phenylalanine Incorporated by Antarctic Fish

Published in Metabolites

DOI: https://doi.org/10.3390/metabo13030338

Contribution of the candidate in % of the total workload

Experimental concept and design	90 %
Experimental work and data acquisition	80 %
Data analysis and interpretation	80 %
Preparation of figures and tables	100 %
Drafting the manuscript	80 %

Article

Protein Synthesis Determined from Non-Radioactive Phenylalanine Incorporated by Antarctic Fish

Nina Krebs [1,*], Jan Tebben [2], Christian Bock [1], Felix C. Mark [1], Magnus Lucassen [1], Gisela Lannig [1] and Hans-Otto Pörtner [1,*]

[1] Department of Integrative Ecophysiology, Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany; christian.bock@awi.de (C.B.); felix.christopher.mark@awi.de (F.C.M.); magnus.lucassen@awi.de (M.L.); gisela.lannig@awi.de (G.L.)
[2] Department of Ecological Chemistry, Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany; jan.tebben@awi.de
* Correspondence: nina.krebs@awi.de (N.K.); hans.poertner@awi.de (H.-O.P.)

Abstract: Direct measurements of temperature-dependent weight gains are experimentally challenging and time-consuming in long-lived/slow-growing organisms such as Antarctic fish. Here, we reassess methodology to quantify the *in vivo* protein synthesis rate from amino acids, as a key component of growth. We tested whether it is possible to avoid hazardous radioactive materials and whether the analytical pathway chosen is robust against analytical errors. In the eelpout, *Pachycara brachycephalum*, $^{13}C_9H_{11}{}^{15}N_1O_2$ phenylalanine was injected intraperitoneally and muscle tissue was sampled before injection and at 1.5 h time intervals up to 6 h thereafter. The incorporation of $^{13}C^{15}N$-labeled-phenylalanine into muscle was monitored by quantification of bound and free phenylalanine through liquid chromatography–mass spectrometry. We found an increase in the pool of labeled, free phenylalanine in the cytosolic fraction that leveled off after 4.5 h. The labeled phenylalanine bound in the proteins increased linearly over time. The resulting protein synthesis rate (Ks) for *P. brachycephalum* was as low as 0.049 ± 0.021% day^{-1}. This value and its variability were in good agreement with literature data obtained from studies using radioactive labels, indicating that this methodology is well suited for characterizing growth in polar fish under in situ conditions in remote areas or on research vessels.

Keywords: polar fish; isotopic labelling; 13C-phenylalanine; slow metabolism; somatic growth

Citation: Krebs, N.; Tebben, J.; Bock, C.; Mark, F.C.; Lucassen, M.; Lannig, G.; Pörtner, H.-O. Protein Synthesis Determined from Non-Radioactive Phenylalanine Incorporated by Antarctic Fish. *Metabolites* **2023**, *13*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s): Mário Araújo, Alexandre M. Campos and Miguel Oliveira

Received: 08 February 2023
Revised: 22 February 2023
Accepted: 23 February 2023
Published: date

1. Introduction

Protein synthesis rates are a key indicator for organismal responses to environmental change [1]. Reliable and accurate methodologies are a prerequisite for analyzing protein synthesis rates in different species and their tissues [2]. Protein synthesis is one of the most energetically costly processes in cell metabolism, amounting to up to 42% of the overall energy expenditure of ectotherms [2]. Protein synthesis in fish white muscle contributes up to 79% to growth performance [3]. Furthermore, the rate of protein synthesis characterizes species and their tissues from various environmental regions as well as their responses to factors such as seasonal changes, temperature and food quantity [4–7]. For example,

protein synthesis quantified as Ks (fraction of total protein synthesized per day) was reported to be higher in temperate zone fish species (4.4% day^{-1} in white muscle tissue of young rainbow trout [8]) than in species found in the tropics (about 0.17 to 0.07% day^{-1} [9]) or in polar regions (0.04 to 0.25% day^{-1} [10,11]). Fractional protein synthesis rates of rainbow trout varied by tissue type, with the highest rates in gills (up to 9% day^{-1}) and the lowest in white muscle (0.5% day^{-1} [3]). The rate of protein synthesis correlates with tissue oxygen demand [12].

Protein synthesis can be measured in vitro or in vivo. In vitro measurements utilize isolated cells or tissues cultured in a "Ringer" medium that contains labeled amino acids. The in vitro incorporation of labeled amino acids reflects the cellular protein synthesis rate and the study of isolated cells excludes possible (and important) influences on the whole animal and its metabolism [12,13]. In vivo measurements mainly derive protein synthesis rates from constant infusion, stochastic endpoint studies or the flooding dose technique [2]. A constant infusion provides a constant supply of labeled tracers via venous cannulation eliminating the risk of fluctuating concentrations [11,14]. This method, however, is less applicable for mobile animals that require anesthesia to stop them from removing the venous cannulation. It needs to be considered that anesthesia by itself may cause artefactual changes in the rate of protein synthesis [15]. For the stochastic endpoint study animals are fed with labeled tracers, such as proteinogenic amino acids, for days or weeks and the difference between intake and excretion reflects the protein synthesis rate. It integrates the protein synthesis rate across all tissues and it is non-invasive [16,17]. A faster and more specific approach is the injection of a flooding dose of labeled tracers as developed by Garlick et al. [18]. Here, organisms are provided with an intravenous or intraperitoneal injection of a large dose of a tracer in combination with a non-labeled tracer which floods all tissues within a short period of time until terminal sampling of the tissues [3,7,9,18–21]. The sampling is generally undertaken over several time steps and is dependent on several assumptions listed in Fraser and Rogers, 2007 [2]:

1. "The intracellular free-pool specific radioactivities are elevated and stable during the protein synthesis measurement"
2. "Incorporation of radiolabeled" (or isotopically labeled) "amino acid into the bound protein pool should be linear and significant over the time course of protein synthesis"
3. "The injected amino acid should flood the plasma, intracellular, extracellular and aminoacyl tRNA pools. Typically, successful flooding doses will elevate intracellular concentrations of the amino acid by about four- to tenfold."
4. "Injecting the amino acid should not result in an elevation of the rate of protein synthesis"

In contrast to amino acids such as leucine [22], the essential amino acid phenylalanine (Phe) is commonly used as a tracer because it does not influence protein synthesis [18] when injected in high amounts, fulfilling the fourth criterion named by Fraser and Rogers (2007) [2]. Phe, however, can have negative effects on the brain when being enriched over a longer period of time. Findings in mammals indicate that, under such conditions, phenylalanine hydroxylase converts phenylalanine into tyrosine [23]. While working with radioactive tracers it is not possible to differentiate between radioactively labeled phenylalanine and tyrosine. Therefore, Garlick et al. (1980) included large amounts of unlabeled phenylalanine

or included other amino acids in the injection. This should minimize any effect of unwanted radioactively labeled tyrosine [18].

Until today, most studies have used radioactively labeled Phe to calculate protein synthesis rates [7,12,13,18,24,25]. However, the background content of Phe varies as every organism has a different composition of amino acids dependent on environmental factors and metabolism [26]. In Antarctic eelpout, the level of protein-bound Phe is 0.74% of white muscle tissue and 0.42% in gills [13]. Antarctic eelpout are benthic Antarctic fish with a low metabolic rate [27]. Tuna, for comparison, live in warmer waters and have higher swimming rates which are reflected in a very high metabolic rate. Their Phe content amounts to 1–2.5% of the whole animal with some variability during development [28].

Radioactive labeling supports very sensitive and accurate measurements especially needed for animals with low protein turnover rates such as Antarctic species [10,11]. However, radioactivity requires significant radiation protection infrastructure, and is mainly used in laboratories, as field studies are logistically challenging (for a recent review see Cresswell et al. 2020 [29]). In recent years, substances labeled with non-radioactive isotopes were used instead of radioisotopes to limit the use of hazardous compounds [19,30–35]. Isotopically labeled tracers bound into proteins as well as unbound in the cytosolic fraction can be quantified with analytical methods such as NMR spectroscopy [30,35] and mass spectrometry [5,9,19,21]. These techniques have the advantage that the labeled isotope as well as the natural (unlabeled) metabolite can be analyzed within the same sample, thereby increasing the accuracy of the labeled and unlabeled fractions against the background of the existing (unknown) pool of Phe.

The aim of the study was to measure the protein synthesis rate of Ks in vivo in an ectothermic polar fish with very low metabolism and growth rates. Using the Antarctic eelpout (*Pachycara brachycephalum*) as a model organism, we applied an intraperitoneal injection of a flooding dose of Phe labeled with a stable isotope in fish. For the detection of labeled and unlabeled Phe, we used liquid chromatography with high-resolution tandem mass spectrometry (LC-HRMS/MS). By using LC-HRMS/MS, we were able to simultaneously quantify labeled and natural, unlabeled phenylalanine in both the cytosol and the protein-bound fraction, which allowed us to calculate net protein synthesis in vivo and to non-radioactively measure very small changes of labeled phenylalanine in the white muscle. We suggest that this method can be easily deployed on ships and/or in remote locations such as stations in Antarctica, providing opportunities to study, for example, the effects of climate change on polar ecosystems and organisms directly on site.

2. Materials and Methods

The Antarctic eelpout, *Pachycara brachycephalum* (Pappenheim, 1912), were caught in Admiralty Bay, King George Island (62°11′ S, 58°20′ W), Antarctica, by baited fish traps between 430 and 530 m depth on RV Polarstern expedition PS112 in March 2018. The fish traps were recovered from the seafloor after 52 h. On board, the fish were kept at 0 °C for the duration of their transport to the Alfred Wegener Institute (AWI), Bremerhaven, Germany. At the end of the cruise, more than 99.5% survived. At AWI, the fish were kept in well-aerated, re-circulating seawater at 0.0 ± 0.5 °C and 34 ± 1 practical salinity units (PSU) under a 12:12 light:dark cycle that remained unchanged during the experiments.

The fish were fed with frozen blue mussel, *Mytilus edulis* (Erdtmann, Salzwedel, Germany) once a week. All selected animals were not fed for 5–7 days before the start of the experiments.

2.1. Chemicals and Instruments

The chemicals used for the preparation of the saline injection buffer were all HPLC grade (Carl Roth, Karlsruhe, Germany; Sigma-Aldrich, St. Louis, MO, USA or VWR, Radnor, PA, USA). Labeled phenylalanine ($^{13}C_9H_{11}{}^{15}N_1O_2$, 98% purity) was purchased from Sigma-Aldrich. Methanol (MeOH) and formic acid (FA) were HPLC grade (Roth, VWR). Hydrochloric acid was purchased from Merck (Merck KGaA, Darmstadt, Germany). Chromabond C18 Hydra 1 mL 100 mg SPE cartridges (Macherey-Nagel, Düren, Germany) were used for the solid phase extraction. Ultrapure water was generated with an Arium Pro system (Sartorius, Göttingen, Germany). A vacuum centrifuge (Speedvac, Thermo Fisher Scientific, Waltham, MA, USA) and lyophilizer (Martin Christ Gefriertrocknung GmbH, Osterode am Harz, Germany) were used to dry the samples.

2.2. Intraperitoneal Injection and Tissue Collection

Antarctic eelpout (total length: 22.6 ± 2.5 cm, total weight: 41.3 ± 12.8 g, n = 30) were first weighed and the conscious fish then immediately injected with 0.7 mL/100 g body weight of 75 mM of $^{13}C_9H_{11}{}^{15}N_1O_2$ phenylalanine in PBS buffer (pH 7.4, 4 °C) into the abdomen by use of a 1 mL syringe and a 0.40 × 20 mm cannula (Braun Melsungen AG, Melsungen, Germany). Shortly after injection, there was no visible stress response by the animal. To measure the protein synthesis response after phenylalanine injection, fish were kept at 0 °C and sampled at four time points after injection: 1.5, 3, 4.5 and 6 h (and compared against a control group). At each time point, six randomly selected fish were sacrificed. Fish were first stunned with a blow to the head and then killed by cutting the spinal cord closely behind the head. White muscle tissue was then collected and quickly frozen in liquid nitrogen and stored at −80 °C until further use. Handling, injection and killing of the fish were conducted in compliance with German legislation and in line with the recommendations of the American Veterinary Medical Association (AVMA). The work was approved by the German authority (Freie Hansestadt Bremen, reference number 160; 500-427-103-7/2018-1-5)

2.3. Methanol Chloroform Extraction

White muscle tissue was extracted according to Wu et al.[36]. Briefly, 50 mg of the frozen muscle tissue was homogenized in 400 µL of methanol (MeOH) and 125µL of ultrapure water in 2 circles of 20 s at 6000 rpm at 4 °C using a Precellys tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France). The homogenized tissue was then transferred into 1.5 mL Eppendorf tubes and 400 µL chloroform with 400 µL ultrapure water was added. This mixture was vortexed for 20 s and incubated on ice for 10 min before centrifugation for 15 min at 3000× *g* and 4 °C. The three layers comprising the upper aqueous layer, the lower chloroform layer and the precipitated protein layer in the middle were collected in separate 1.5 mL vials (Eppendorf, Hamburg, Germany). The upper aqueous layer (total volume of 800 µL) contained the cytosolic fraction with polar metabolites including the free phenylalanine that was not incorporated in proteins. The lower chloroform layer containing apolar compounds such as lipids was discarded and not used for further analyses. The protein

fraction containing the phenylalanine incorporated in proteins was washed twice to remove any residual unbound phenylalanine by adding 1 mL of MeOH followed by 20 s vortexing (Vortex mixer, Scientific Industries, Bohemia, NY, USA) and centrifugation (Eppendorf) for 3 min at 13,000× *g*. Both supernatants were collected separately. The washed protein pellet was dried in a vacuum centrifuge at 25 °C overnight. The protein pellet was then hydrolyzed with 100 µL 6M HCl/mg protein pellet for 24 h at 99 °C while shaking at 600 rpm (Thermomixer comfort, Eppendorf). The supernatant was collected by centrifugation (10,000 rpm for 3 min), frozen and lyophilized for 12 h. The dry hydrolysate was dissolved by vortexing for 20 s in 1 mL MeOH and desalted by application to a solid phase extraction (SPE). The aqueous, cytosolic layer was diluted 1:10 with MeOH and also applied to SPE without further dilution resulting in samples that contained the equivalent of 0.1 mL per 1 mL.

2.4. Phenylalanine Quantification

Liquid chromatography high-resolution mass spectrometry (LC-HRMS/MS) analysis was performed with a Vanquish UPLC system coupled to a Q-Exactive Plus mass spectrometer, using a heated electrospray ionization source (all Thermo Fisher Scientific). Separation was performed on a C18 column (C18 BEH, 100 × 2 mm, 1.7 µm particle size, Waters, equipped with guard-column). Positive Ion Calibration Solution (Pierce, Thermo Fisher Scientific) was used for the calibration of the instrument. A blank as well as a quality control standard was injected every five samples to check the instrument's drift and carry-over. A binary solvent gradient was used with a flow rate of 0.35 mL per min on a C18 column (C18 BEH, 100 × 2 mm, 1.7 µm particle size, Waters, equipped with guard-column) at 32 °C, with solvent A = 0.1% formic acid in ultrapure water and solvent B = 0.1% formic acid in methanol. The gradient program was as follows: T0 min: B = 2%, T0.1 min B = 2%, T3.9 min: B = 99%, T4.5 min: B = 99%; T4.7 min: B = 2%. The column was equilibrated for 0.5 min between samples. MS spectra were acquired in full scan mode or data independent (DIA) mode. Full scans were acquired with a resolution of 35,000 (fifty percent of the maximum peak height (FWHM), m/z 200), a scan range of 120 to 250 m/z, automatic gain control (AGC) of 3 x 10^6 and injection time (IT) of 100 ms. DIA experiments were used to quantify analytes by tandem mass spectrometry utilizing an inclusion list of the accurate masses (m/z 176.11313, m/z 166.08617, m/z 167.07617) at a resolution of 35,000 FWHM (m/z 200), NCE of 30, AGC of 2 x 10^5 and isolation window of 1.0 m/z. The heated electrospray ionization source was set to 3.5 kV spray voltage, the aux gas to 425 °C (13) and the sheath gas to 50. The capillary temperature was set to 263 °C. The fragment ions m/z 120.0808 ($^{12}C_9H_{11}{}^{15}N_1O_2$—$C_1H_2O_2$) and m/z 129.1047 ($^{13}C_9H_{11}{}^{15}N_1O_2$—$^{13}C_1H_2O_2$) were used for quantification with a mass tolerance of 5 ppm. Quantification was achieved with an external calibration to standard dilution series of standards prepared from $^{12}C^{14}N$ as well as the $^{13}C^{15}N$ phenylalanine ranging from 10 pg/µL to 1000 pg/µL. Calibration curves were measured every 100 samples to evaluate sensitivity changes in the instrument.

2.5. Calculation and Statistics

The phenylalanine concentrations in the protein hydrolysate (protein-bound) and the cytosolic fraction (free pool) were calculated using external calibration curves for each analyte. Outliers (as identified by the Inter-Quartile Range IQR) were eliminated. Ks calculation was

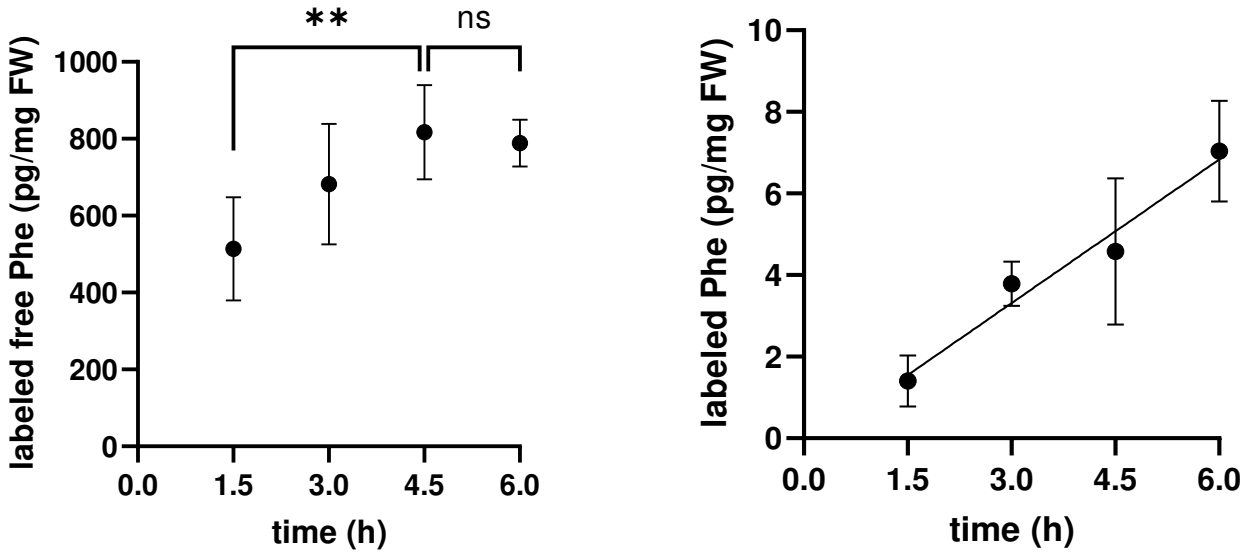
adapted from Garlick et al., 1980 and Ks values are given as means ± standard deviation [18]. Unless otherwise stated, the data were normally (tested with Shapiro–Wilk test) and homogeneously distributed (chi-square test). Statistical differences at the level of 95% were tested by using an ordinary one-way ANOVA (analysis of variance) followed by the Tukey's multiple comparison test as post hoc (GraphPad Prism 9).

$$\text{Ks (\% day}^{-1}) = \left(\frac{\text{Sb labeled } [\frac{\text{pg}}{\mu\text{g}}]}{(\text{Sb labeled}+\text{Sb unlabeled})[\frac{\text{pg}}{\mu\text{g}}]}\right) * \left(\frac{100}{(\text{Sa labeled}[\%])*\text{t (days)}}\right) * 100$$

where Sb is the protein-bound pool and Sa is the free pool of phenylalanine (pg phenylalanine per μg fresh weight) and t is the time in hours. For comparability across measurements, data were calculated per day, from the time between injection and sampling in hours.

3. Results

After the injection of labeled phenylalanine (Phe) into the peritoneum, the fate of the amino acid was followed over time. The concentration of labeled Phe in the free pool of the cytosolic fraction increased over time in white muscle until it reached a plateau approximately 4.5 h after injection. Accordingly, the labeled Phe was linearly incorporated into muscle protein throughout the experimental period of 4.5 h (Figure 1).

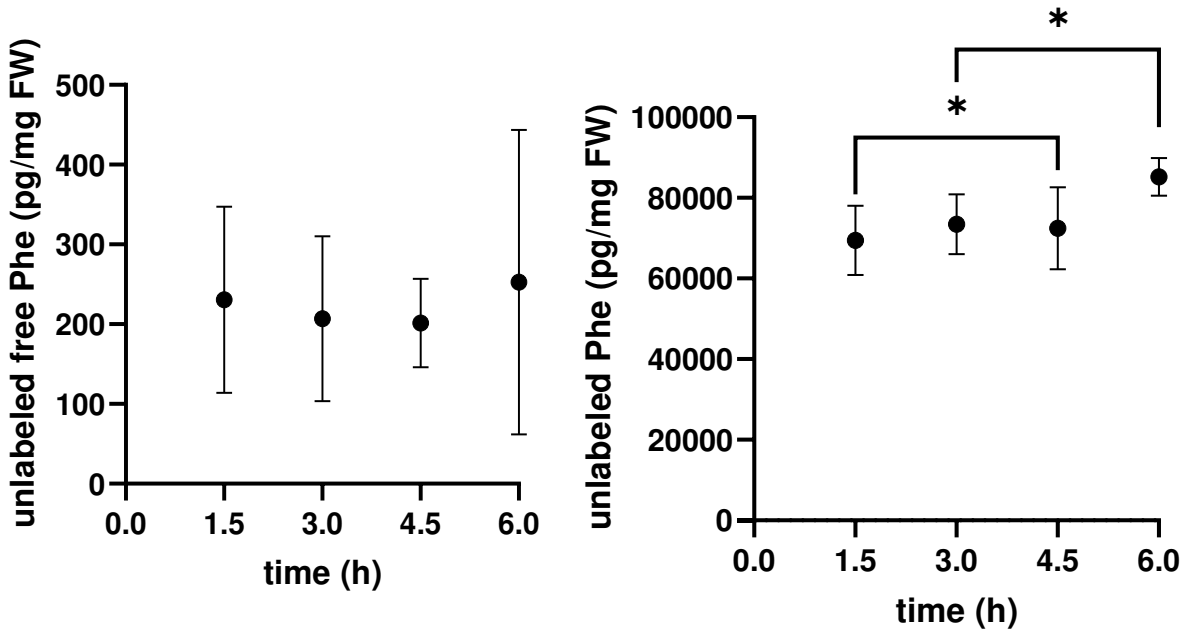


(**a**) (**b**)

Figure 1. Time-dependent concentration of labeled phenylalanine in the free pool (**a**) and protein-bound (**b**) in white muscle tissue extracts of the Antarctic eelpout *Pachycara brachycephalum* kept at 0 °C. The concentration of labeled phenylalanine in the free pool (**a**) increased until reaching a plateau after 4.5 h and at a level of 900 pg/mg FW (Fresh weight) (** $p < 0.005$, *ns* $p > 0.05$). In contrast, the concentration of protein-bound phenylalanine (**b**) increased linearly over time up to 7 pg/mg FW (y = 1.174x − 0.2087; R2: 0.7477).

Additionally, we measured the unlabeled content of free phenylalanine in the cytosol and of Phe bound into proteins (Figure 2). The free pool of unlabeled phenylalanine showed high variability within individuals with no clear time dependence. In contrast, the concentration

of unlabeled protein-bound phenylalanine showed little variability and increased over time.



(**a**) (**b**)

Figure 2. Time-dependent concentration of unlabeled phenylalanine in the free pool (**a**) and protein-bound unlabeled phenylalanine (**b**) in white muscle tissue extracts of the Antarctic eelpout *Pachycara brachycephalum* kept at 0 °C. The unlabeled free pool (**a**) decreased within the first 3 h and reached a plateau thereafter. The unlabeled bound fraction (**b**) increased over time (* $p < 0.05$).

The data between 1.5 and 4.5 h meet all criteria required to calculate Ks after injection of a flooding dose. At the last sampling point (6 h), the free pool of labeled Phe did not continue to increase and the data were no longer normally distributed. Therefore, this sampling point was excluded in the following calculations. Based on these data, the calculation of protein synthesis rate Ks for each specific sampling time point yielded similar Ks values (Table 1), resulting in an average Ks of 0.049 ± 0.021% day^{-1} measured in white muscle of *Pachycara brachycephalum* at 0 °C. Additionally, the percentage of labeled phenylalanine accumulated in the pool of free Phe averaged 75.50 ± 11.07%. Both Ks and the percentage of labeled free Phe (%) remained stable between 1.5 h and 4.5 h.

Table 1. Time-dependent protein synthesis rates Ks and percentage of labeled phenylalanine in the free pool in white muscle tissue of *Pachycara brachycephalum* kept at 0 °C.

Time (h)	Ks (% day^{-1})	Labeled, Free Phe (%)
1.5	0.052 ± 0.029	68.54 ± 12.36
3	0.055 ±0.015	75.58 ± 14.14
4.5	0.041 ± 0.015	80.64 ± 5.57
Total Mean	0.049 ± 0.021	75.50 ± 11.07

4. Discussion

In this study, we determined the protein synthesis rate (Ks) in the white muscle of the Antarctic eelpout *Pachycara brachycephalum* in vivo after an intraperitoneal injection of a flooding dose of labeled non-radioactive phenylalanine (Phe). We analyzed the Phe content using liquid chromatography high-resolution mass spectrometry (LC-HRMS/MS) instead of labeling with radioactive isotopes. As a proof of concept, we were able to determine reliable protein synthesis rates in ectothermal organisms with slow metabolism at freezing temperatures.

To validate the reliability of the method, we quantified labeled unbound and bound Phe and thus tracked its incorporation into proteins over time. The sampling time points 1.5–4.5 h met all criteria for measuring the protein synthesis rate after injection of a flooding dose according to Fraser and Rogers, 2007 [2] (see Introduction) and are therefore considered suitable to calculate Ks. The sampling time point 6 h was excluded from the calculation of Ks because the level of labeled unbound Phe did not increase further after 6 h and the unlabeled free Phe was not normally distributed. After 6 h, no additional injected Phe is thus likely to enter the cytosol and the pre-existing Phe already present has been used to synthesize proteins or converted to tyrosine, by random the use of labeled or unlabeled Phe. Lower intake and random withdrawal of labeled and unlabeled Phe could be the reason for the high variability of the data after 6 h, resulting in a non-normal distribution. The last two criteria were met based on the experimental design and have been successfully tested in previous studies on various fish species.

The amount of labeled unbound phenylalanine varied greatly between individuals and the time of injection. On average, only 75.5 ± 11.1% of the total phenylalanine was labeled, resulting in a mean Ks value of 0.049 ± 0.021% day^{-1}. This rate is about 75-times lower than previously measured in vitro in Antarctic eelpout white muscle at Ks = 3.5–3.6% day^{-1} [13], but similar to in vivo radioactive measurements in other Antarctic fish species (e.g., Smith and Haschemeyer, 1980 Ks = 0.04–0.22% day^{-1} [11]). Indeed, the authors of the in vitro study argued that the higher Ks value in *Pachycara brachycephalum* compared to other Antarctic fish could be due to the inherent differences between in vitro and in vivo measurements (see introduction). The in vitro measurement reflects the maximum capacity of protein synthesis, whereas the in vivo measurement describes a usually lower, more realistic rate. It reflects the response of the entire organism instead of individual tissues or cells [13]. In other fish species, Ks measured in vivo in white muscle varies greatly depending on biotic and abiotic factors such as age, habitat temperature, or food supply, with the highest protein synthesis rates found in well-fed juvenile rainbow trout (Ks = 4.4% day^{-1} [8]) and the lowest rates seen in starving Antarctic fish, *Trematomus hansoni* (Ks = 0.04% day^{-1} [11]). How the mentioned biotic and abiotic factors affect the protein synthesis rate of Antarctic eelpout needs to be investigated in the future.

Up to 79% of the protein synthesis rate (Ks) measured in the white muscle of rainbow trout contributed to growth [3]. On this basis, we estimated the protein growth efficiency of *P. brachycephalum* using literature data on species-specific growth rate measured over several months at 0 °C [37] and compared it with our data for protein synthesis rate $((Ks/\frac{Weight\ gain\ \left(\frac{g}{d}\right)}{weigth\ (g)} * 100) * 100)$. Accordingly, 72–96% of Ks in white muscle contributes to growth in the Antarctic eelpout at 0 °C, an order of magnitude that confirms our approach and data. Measuring growth rates in animals with slow metabolism is time consuming, laborious and can

only be performed in laboratories. However, long-term laboratory experiments of polar organisms are always associated with unpredictable risks, such as water quality degradation, that may affect results or, in the worst case, cooling failure. In addition, it involves costly maintenance of experimental set-ups and of the fish in their maintenance aquaria. The method described here will not only simplify laboratory studies, but also reduce costs and help to draw conclusions on growth rates of delicate organisms in the field, at remote places or on ships within a few hours without using hazardous radioactive material that are difficult to obtain or prohibited to use in some areas. It will also allow the determination of the fractional cost of protein synthesis in the context of the energy budget, e.g., [38]. Energy use plays an important role in setting tolerance to environmental changes, such as those caused by climate change. The proposed technique may help to perform such experiments more easily.

Author Contributions: Conceptualization, N.K., J.T., C.B., F.C.M., M.L., G.L. and H.-O.P.; performing experiments, N.K. and M.L.; methodology, N.K., J.T. and C.B.; data curation, N.K., C.B., G.L. and J.T.; writing—original draft preparation, N.K.; writing—review and editing, N.K., J.T., C.B., F.C.M., M.L., G.L. and H.-O.P.; supervision, N.K., C.B., F.C.M., M.L., G.L. and H.-O.P. All authors have read and agreed to the published version of the manuscript

Funding: This study was supported by the research program "Changing Earth—Sustaining our Future" in the program-oriented funding periods (PoF IV subtopic 6.2 – Adaptation of marine life: from genes to ecosystems) of the Helmholtz Association.

Institutional Review Board Statement: The work was approved by the German authority (Freie Hansestadt Bremen, reference number 160; 500-427-103-7/2018-1-5).

Informed Consent Statement: Not applicable

Data Availability Statement: Data will be uploaded to the public repository PANGEA after acceptance of the manuscript.

Acknowledgments: We would like to acknowledge the technical support of Sandra Götze, Amirhossein Karamyar, Nils Koschnick and Fredy Veliz Moraleda during the experiments. Additionally, we would like to thank Christian Zurhelle for his help during the extraction and measurement of samples.

Conflicts of Interest: The authors declare no conflicts of interest

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3.2 Publication 2: Evolutionary Adaptation of Protein Turnover in White Muscle of Stenothermal Antarctic Fish: Elevated Cold Compensation at Reduced Thermal Responsiveness

Contribution of the candidate in % of the total workload

Experimental concept and design	80 %
Experimental work and data acquisition	90 %
Data analysis and interpretation	90 %
Preparation of figures and tables	100 %
Drafting the manuscript	80 %

Article

Evolutionary Adaptation of Protein Turnover in White Muscle of Stenothermal Antarctic Fish: Elevated Cold Compensation at Reduced Thermal Responsiveness

Nina Krebs [1]*, Christian Bock [1], Jan Tebben [2], Felix C. Mark [1], Magnus Lucassen [1], Gisela Lannig [1] and Hans-Otto Pörtner [1,]*

[1] Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Department of Integrative Ecophysiology, Am Handelshafen 12, 27570 Bremerhaven, Germany; christian.bock@awi.de (C.B.); felix.christopher.mark@awi.de (F.C.M.); magnus.lucassen@awi.de (M.L.); gisela.lannig@awi.de (G.L.)
[2] Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Department of Ecological Chemistry, Am Handelshafen 12, 27570 Bremerhaven, Germany; jan.tebben@awi.de
* Correspondence: nina.krebs@awi.de (N.K.); hans.poertner@awi.de (H.-O.P.)

Abstract: Protein turnover is highly energy consuming and overall relates to an organism's growth performance varying largely between species, e.g., due to pre-adaptation to environmental characteristics such as temperature. Here, we determined protein synthesis rates and capacity of protein degradation in white muscle of the cold stenothermal Antarctic eelpout (*Pachycara brachycephalum*) and its closely related temperate counterpart, the eurythermal common eelpout (*Zoarces viviparus*). Both species were exposed to acute warming (*P. brachycephalum*, 0 °C +2 °C day^{-1}; *Z. viviparus*, 4 °C +3 °C day^{-1}). The *in vivo* protein synthesis rate (Ks) was monitored after injection of ^{13}C-phenylalanine, and protein degradation capacity was quantified by measuring the activity of cathepsin D *in vitro*. Untargeted metabolic profiling by nuclear magnetic resonance (NMR) spectroscopy was used to identify the metabolic processes involved. Independent of temperature, the protein synthesis rate was higher in *P. brachycephalum* (Ks = 0.38–0.614 % day^{-1}) than in *Z. viviparus* (Ks= 0.148–0.379% day^{-1}). Whereas protein synthesis remained unaffected by temperature in the Antarctic species, protein synthesis in *Z. viviparus* increased to near the thermal optimum (16 °C) and tended to fall at higher temperatures. Most strikingly, capacities for protein degradation were about ten times higher in the Antarctic compared to the temperate species. These differences are mirrored in the metabolic profiles, with significantly higher levels of complex and essential amino acids in the free cytosolic pool of the Antarctic congener. Together, the results clearly indicate a highly cold-compensated protein turnover in the Antarctic eelpout compared to its temperate confamilial. Constant versus variable environments are mirrored in rigid versus plastic functional responses of the protein synthesis machinery.

Keywords: protein synthesis rate; protein degradation; polar fish; NMR; metabolic profiling; 13C-phenylalanine; non-radioactive; fish physiology; acute warming

Citation: Krebs, N.; Bock, C.; Tebben, J.; Mark, F.C.; Lucassen, M.; Lannig, G.; Pörtner, H.-O. Evolutionary Adaptation of Protein Turnover in White Muscle of Stenothermal Antarctic Fish: Elevated Cold Compensation at Reduced Thermal Responsiveness. *Biomolecules* **2023**, *13*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Tigran Chalikian

Received: 20 September 2023
Revised: 6 October 2023
Accepted: 9 October 2023
Published: date

1. Introduction

Temperature strongly influences the performance of ectotherms such as in foraging, reproduction and growth. The temperature optimum of fish growth is not only species-specific and dependent on the temperature regime of its environment, it can also vary within species depending on

season, life stage and habitat characteristics [1]. Accordingly, the range of temperatures that fish can thrive in, also known as the width of the thermal window, differs between species, life stages, regions and seasons [1–3]. Stenothermal organisms (e.g., polar and tropical fish) are well adapted to narrow temperature windows, whereas eurythermal organisms (e.g., fish from temperate zones) have a much wider thermal range and are more tolerant to fluctuations in temperature [4]. Polar stenothermal fish are able to maintain all life functions at temperatures constantly below 0 °C. Some functions such as growth are even slower in Antarctic ectotherms than would be expected on the basis of the Q_{10} relationship [5,6]. It is assumed that slow growth is caused by the constantly low temperatures (e.g., seasonal temperature range of Ryders Bay: −1.81–1.7 °C [7]) and a limited food supply due to seasonal changes [5,8,9]. Mechanism-based explanations include thermal tradeoffs between energy budget components such as ventilation and circulatory capacity, muscular activity and cellular and mitochondrial energy costs [10]. Overall, the mechanisms that influence thermal performance and especially the growth of polar ectotherms deserve further study for a deeper understanding of how temperature shapes the functioning of stenothermal fishes as opposed to eurythermal fishes [11].

Growth performance is defined as weight gain per day, which is the result of many different processes, usually measured over a long period of time (weeks to months) particularly in slow-growing Antarctic fish [12–14]. A highly important contributor to growth is protein synthesis. It has been suggested that protein homeostasis is a crucial factor limiting thermal performance in Antarctic organisms [6]. The protein synthesis rate can be measured in the whole organism (e.g., [15,16]) or, especially in fish, it can be approximated by studying protein synthesis in white muscle which contributes up to 79% to fish growth [17] . The protein synthesis rate (Ks) can be determined as the percentage of an incorporated labeled tracer (e.g., phenylalanine) into proteins per day. High Ks values in white muscle have been found in juvenile fish (Ks = 4.4% day^{-1} [18]) and Ks values are low in fish from Antarctica (Ks = 0.04–0.2% day^{-1} [19–21]). Measuring the protein synthesis rate alone may already be indicative of growth performance, but in addition, protein degradation modifies net protein gain. Thus, combined assessment of protein synthesis and protein degradation will help to understand the temperature-dependent protein turnover for more accurate predictions of ectothermal (muscle) growth.

Protein degradation can be calculated as the difference of protein synthesis, food supply and weight gain [19,22]. While the protein degradation rates of fish from temperate waters were found to be higher at the upper end of their thermal window (*Lipophyrs pholis* acclimated for 28 days to minimum 3 °C and maximum 18 °C), fish from polar regions (*Harpagifer antarcticus*, acclimated for 28 days to between −1 °C and 3 °C) did not display any temperature-dependent changes in protein degradation [19]. Compared to protein synthesis, protein degradation is more complex and involves several pathways which may differ between species, life stages and tissue types [23]. Furthermore, the use of pathways can shift, e.g., with temperature as shown in the liver of the Antarctic eelpout, *Pachycara brachycephalum*, where warming induced a shift in protein degradation from the mostly ubiquitin-dependent pathway in the cold towards the lysosomal pathway in the warmth [14]. In contrast to fish liver and mammalian muscle tissue, degradation via the proteasome is less dominant in white muscle of fish, where the main degradation pathway is via the calcium-dependent protease calpain, closely followed

by cathepsin (mainly cathepsin D), which is a lysosomal protease [23]. The maximum protein degradation rate is determined from the capacity of cathepsin D [24,25].

Both the measurements of protein synthesis and degradation quantify the use of a specific pathway. Another approach is non-targeted metabolic profiling which reflects metabolic differences or changes including in the levels of important amino acids, the consequences of shifts in energy budget and the fate of compounds related to protein synthesis and degradation. Metabolic profiling based on NMR (Nuclear magnetic resonance) spectroscopy has been applied to several organisms and results indicate that changes in protein synthesis and/or protein degradation are likely to occur under the influence of various environmental pressures (e.g., [26–28])).

Here we studied all three proxies to assess growth-related metabolic shifts during acute warming in the stenothermal Antarctic eelpout (*Pachycara brachycephalum*) and the eurythermal common eelpout (*Zoarces viviparus*). The two species provide an ideal model system to study evolutionary temperature adaptation due to their high genetic identity [29]. Both species belong to the family of Zoarcidae and most likely evolved in the North Pacific and reached the North Sea and Southern Ocean via the deep sea [30] and have evolved to divergent species-specific thermal windows [12,31,32]. The Antarctic eelpout inhabits the Southern Ocean at temperatures between −1 °C and 1 °C [33,34]. Besides very low temperature variation over the year, high seasonality of primary production is given by the extreme shift between permanent light and the Polar night. As the Antarctic eelpout is an opportunistic feeder of the benthos, high variation in food supply can be anticipated [30,33]. Their optimal temperature of growth was found to be between 3 and 4 °C in whole-animal [12,14] and cellular studies [35]. Antarctic eelpout can survive temperatures up to 12 °C in short-term experiments [36,37], but long-term acclimation indicates a tipping point for successful acclimation at around 6 °C [14]. This narrow thermal window indicates a low ability to adapt rapidly to thermal changes, and thus a low thermal plasticity, which can be defined as rapid adaptation to new thermal conditions.

In contrast, the distribution of the common eelpout ranges from the White Sea in the North, with winter temperatures as low as −1 °C, to the Wadden Sea in the South, at summer temperatures beyond 18 °C [38,39]. In short-term experiments *Z. viviparus* survived temperatures up to 24 °C and their thermal optimum (measured for the Wadden Sea population) ranges between 12 °C and 15 °C indicating a much wider thermal window and thus higher thermal plasticity [12,39,40]. Irrespective of species or habitat, studies of temperature-dependent whole-organism growth performance reveal a kind of bell-shaped curve [12,41–45]. Although growth is generally slow in Antarctic species, the recent literature suggested a lower thermal sensitivity of protein metabolism (synthesis and degradation rates) in Antarctic compared to temperate fish species when exposed to a similar range of warming [19].

In this context, a complete and comparative understanding of the relationship between protein synthesis and degradation rates as well as the associated metabolism in Antarctic and temperate fish is missing. In this study we hypothesize that acute warming affects protein synthesis and degradation rates in both eelpout species according to their thermal window: at the species-specific thermal growth optimum, protein synthesis rates should be high while protein degradation should be lowest.

2. Materials and Methods

2.1. Animals

Antarctic eelpout, *Pachycara brachycephalum* (Pappenheim, 1912), were caught in Admiralty Bay, King George Island (62°11′ S, 58°20′ W), Antarctica, by baited fish traps between 430 and 530 m depth on RV Polarstern expedition PS112 in March 2018. The fish traps were recovered after 52 h from the sea floor. On board, fish were kept at 0 °C for the duration of the transport (2 months) to the Alfred Wegener Institute (AWI), Bremerhaven, Germany. At AWI, fish were kept in well aerated, re-circulating seawater at 0.0 ± 0.5 °C and 34 ± 1 practical salinity units (PSU) under a 12:12 light/dark cycle.

The common eelpout, *Zoarces viviparus* (Linnaeus, 1758), were caught in the North Sea near the island of Helgoland and brought to AWI by RV Uthörn in autumn 2020. The fish were kept in well-aerated, re-circulating seawater at 12 °C for an acclimation period of at least 3 months before cooling the water slowly to a temperature of 4.0 ± 0.5 °C. For an additional three months, fish were maintained at 4 °C, at 34 ± 1 PSU on a 12:12 light/dark cycle.

Both species were fed frozen blue mussels, *Mytilus edulis* (Erdmann, Germany), 2–3 times weekly. Before the start of the experiments, fish were not fed for 5–7 days to minimize/exclude possible side effects like specific dynamic action (SDA). Handling and killing of the fish were conducted in compliance with the German legislation and in line with the recommendations of the American Veterinary Medical Association (AVMA). The work was approved by German authority (Freie Hansestadt Bremen, reference number 160; 500-427-103-7/2018-1-5).

2.2. Acute Warming Experiment

P. brachycephalum was acclimated to 0 °C and exposed to an acute temperature increase of + 2 °C day^{-1} until 10 °C (total experimental duration: 5 days). The temperature of *Z. viviparus*, which was acclimated to 4 °C, was increased by +3 °C day^{-1} until at 22 °C (total experimental duration: 7 days). These acute warming experiments have been performed in other experiments with these fish and were therefore selected (e.g., [36,37]). At several temperature steps (*P. brachycephalum*: 0, 2, 4, 6, 8, and 10 °C; *Z. viviparus*: 4, 10, 13, 16, 22 °C) eelpouts of both species were weighed and injected with 0.7 mL/100 g body weight of 75 mM of $^{13}C_9H_{11}{}^{15}N_1O_2$ phenylalanine in PBS buffer (pH 7.4). Exactly after 1.5 h and 3 h one fish from each species was sacrificed. At first the fish were stunned with a blow to the head and then killed by cutting the spinal cord behind the head before collecting muscle tissue. The collected tissue was flash-frozen in liquid nitrogen and stored at −80 °C until further use. This experiment was repeated four times for *P. brachycephalum* (total n = 47, for each measured temperature step n = 8 except of 10 °C n = 7) and three times for *Z. viviparus* (total n = 30, for each measured temperature step n = 6).

2.3. Measurement of Protein Synthesis Rate

The protein synthesis rate was measured as described in Krebs et al. [20]. Briefly, 50 mg of white muscle tissue was homogenized and extracted with methanol and chloroform to obtain three layers. The upper layer contained the cytosolic fraction in which the free pool of unlabeled and labeled phenylalanine was measured. The middle layer contained the protein fraction, which was subsequently hydrolyzed and used to

measure the protein-bound fraction of labeled and unlabeled phenylalanine, and the bottom layer, containing the lipids, was not used for this analysis. Afterwards, the amount of free labeled and unlabeled phenylalanine as well as the amount of protein-bound labeled and unlabeled phenylalanine were measured with liquid chromatography high resolution mass spectrometry (LC-HRMS/MS).

The protein synthesis rate data were calculated with the phenylalanine concentrations in the protein hydrolysate (bound) and the cytosolic fraction (free pool) using an internal standard and a calibration curve for each analyte. Outliers (as identified by the Inter-Quartile Range IQR) were eliminated.

Ks was calculated after Garlick et al., 1980 [46]

$$\text{Ks (\% day}^{-1}) = \left(\frac{\text{Sb labeled } [\frac{\text{pg}}{\mu\text{g}}]}{(\text{Sb labeled} + \text{Sb unlabeled})[\frac{\text{pg}}{\mu\text{g}}]} \right) \times \left(\frac{100}{(\text{Sa labeled}[\%]) \times \text{t (days)}} \right) \times 100$$

where Sb is the protein-bound pool and Sa the free pool of phenylalanine (pg phenylalanine per µg fresh weight) and t the time in hours. Labeled phenylalanine describes the injected $^{13}C_9$,^{15}N phenylalanine and unlabeled the naturally found ^{12}C, ^{14}N phenylalanine.

2.4. Protein Degradation via Measurements of Cathepsin D Activity

The determination of protein degradation and metabolic profiles was performed only at certain temperature steps, for *P. brachycephalum* at 0, 4 and 10 °C and for *Z. viviparus* at 4, 10, 13, 16 and 22 °C.

First, 50 mg of frozen white muscle tissue was homogenized (1 mg/100 µL) in 50 mM Sodium Acetate buffer (pH 5.0) in 2 circles of 20 s at 6000 rpm at 4 °C (Precellys 24 tissue homogenizer, Bertin Instruments). Afterwards the activity of cathepsin D was measured as described in Martinez-Alarcon et al., 2018. We used the fluorogenic substrate 7-methoxycoumarin-4-acetyl-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys-(DNP)-DArg-amide (M0938, Sigma-Aldrich, St. Louis, MO, USA) and measured the activity of cathepsin D at 26 °C. Data are expressed in units per mg of protein (U mg^{-1}), with protein content determined according to Bradford [47].

2.5. Metabolic Profiling

Metabolic profiling was conducted as described in detail in Tripp-Valdez et al. (2017) and Götze et al. (2020) [27,48]. In brief, we homogenized 50 mg muscle tissue (fresh weight) and extracted the cytosolic fraction by Methanol–Chloroform extraction. Afterwards, the cytosolic fraction was dried overnight in a vacuum centrifuge (Speedvac, Thermo Fisher Scientific, Waltham, MA, USA). The pellet was resolved with D_2O (deuterized water + TSP; 0.075 wt%; Sigma Aldrich, St. Louis, MO, USA) in a 2-fold volume per fresh weight (final concentration: 0.5 g ml^{-1}). For the determination of metabolites an ultra-shielded vertical 9.4 T NMR spectrometer (Avance III HD 400 WB, Bruker-BioSpin GmbH, Ettlingen, Germany) was used. Samples were transferred to NMR needle tubes (1.7 mm, Fisher Scientific, Schwerte, Germany) with a sample volume of 45 µL. ^1H-NMR spectra were acquired at 400 MHz in a 1.7 mm triple-tuned ^1H-^{13}C-^{15}N NMR probe using a Carr–Purcell–Meiboom–Gill (Bruker protocol cpmgpr1d, TopSpin 3.5) sequence including water suppression at room temperature at the following parameters: acquisition time (AQ), 4.01 s; sweep width (SW), 8802 Hz (22 ppm); delay (D1), 4 s; dummy scan (DS), 4; and number of scans (ns), 512

Afterwards the spectra were baseline-, shim- and phase-corrected and calibrated to the TSP signal at 0.0 ppm using the software Chenomx NMR suite 8.4 (Chenomx Inc., Edmonton, AB, Canada). Then, the metabolites were assigned and quantified by the chemical shift of their NMR signals based on the TSP signal using Chenomx's internal database and previous NMR studies on polar and marine fish [28,49].

2.6. Statistical Analyses

The data for protein synthesis and degradation rate were normally (tested with Shapiro–Wilk test) and homogeneously distributed (chi square test). Statistical differences at the level of 95% were tested by using an ordinary one-way ANOVA (analysis of variance) followed by the Tukey's multiple comparison test as post hoc. Interspecific differences were tested with unpaired t-test identifying significant differences at the level of 95% confidence interval.

The metabolomic data were analyzed with the online platform MetaboAnalyst 5.0 [50]. First, the data were normalized using the log2 transformation and outliers were identified using unsupervised principal component analyses (PCA). Significant differences were investigated by using SAM (Significance Analyses of microarray) [51] and the distinction between metabolic profiles are presented by using supervised partial least-square discriminant analysis (PLS-DA)

3. Results

3.1. Protein Synthesis Rate

Overall, the range of protein synthesis rates in white muscle tissue over all temperatures was higher in *P. brachycephalum* (Ks = 0.38–0.614% day^{-1}) than in *Z. viviparus* (Ks = 0.148–0.379% day^{-1}) with significantly higher Ks values in the Antarctic species at the common temperature of 4 °C (Figure 1). While the Ks of *P. brachycephalum* remained unchanged during acute warming despite high individual variation, the Ks of *Z. viviparus* displayed a clear temperature effect with the lowest protein synthesis rate at 4 °C (Ks = 0.148% ± 0.02 day^{-1}) and the highest at 16 °C (Ks = 0.379% ± 0.12 day^{-1}).

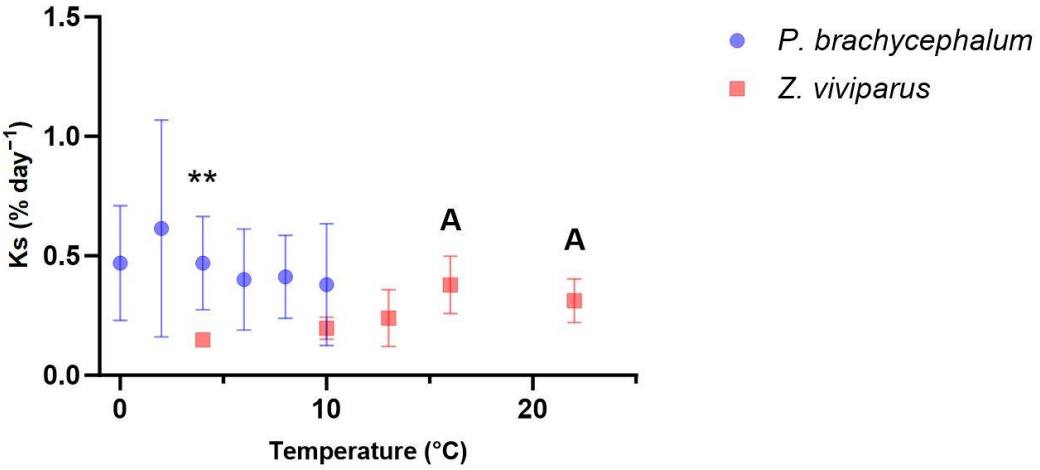


Figure 1. Protein synthesis rate in white muscle during acute warming. The protein synthesis rate in *P. brachycephalum* (blue, data shown as mean ± standard deviation) remained unchanged (One-Way ANOVA, ns = p value > 0.05, n = 8 (2, 6 and 8 °C), n = 7 (0, 4, 10 °C)) during acute warming. In *Z. viviparus* (red, data shown as mean ± standard deviation), protein synthesis differed significantly at

16 and 22 °C from that at acclimation temperature of 4 °C (One-Way ANOVA, A = p value < 0.05, n = 6). The protein synthesis rate was significantly higher in the Antarctic than the North Sea eelpout at 4 °C but not at 10 °C (unpaired t-test ** p < 0.01).

3.2. Protein Degradation

Protein degradation was determined in white muscle tissue by measuring the maximum activity of cathepsin D. The cathepsin D activity was 10 times higher in *P. brachycephalum* compared to *Z. viviparus* when measured at a common temperature (26 °C). When comparing white muscle tissue collected at different temperatures (*P. brachycephalum*: 0, 4 and 10 °C; *Z. viviparus*: 4, 10, 13, 16 and 22 °C), no significant effect on cathepsin D activity (all samples measured at 26 °C) was found in either species (Figure 2), indicating enzyme quantity remaining unchanged during the temperature protocols. Studies of homogenates at various temperatures revealed a Q_{10} for cathepsin activity of 2.3 ± 0.33 (n = 6) for *P. brachycephalum*. No difference was found in Q_{10} values between samples from various temperatures (0, 4 and 10 °C) (see supplementary, Table S1).

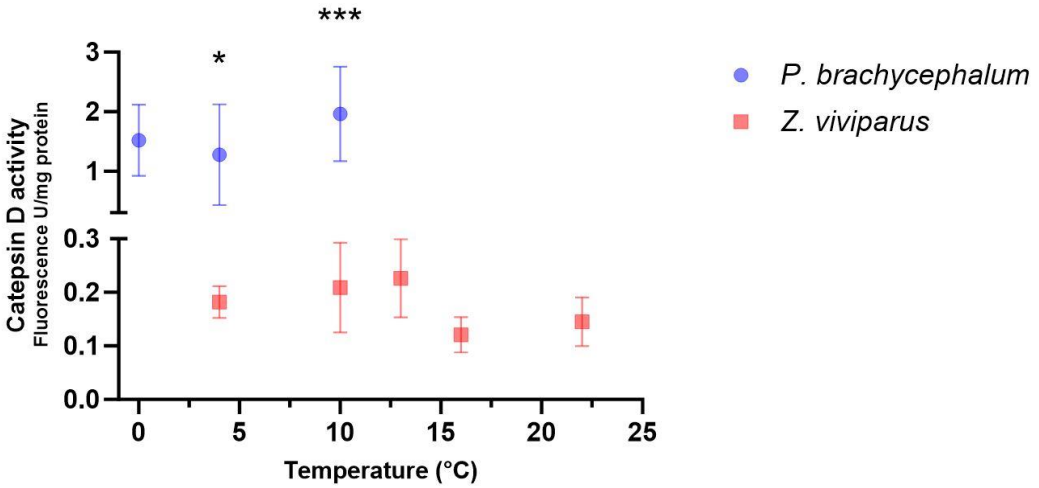


Figure 2. Protein degradation of *Pachycara brachycephalum* and *Zoarces viviparus* measured by the maximum activity of Cathepsin D at 26 °C (data shown as mean ± standard deviation). Acute warming of the animals did not significantly alter the activity of cathepsin D (One-Way ANOVA, ns = p value > 0.05) in either *P. brachycephalum* (blue, n = 8 (4 °C), n = 7 (0 and 10 °C) or *Z. viviparus* (red, n = 6 (16 °C), n = 7 (4, 10, 13 and 22 °C). When measured at a common temperature (26 °C), Cathepsin D activity in white muscle samples collected at 4 and 10 °C was significantly higher in *P. brachycephalum* than in *Z. viviparus* (unpaired T-test; * p < 0.05; *** p < 0.005).

3.3. Metabolites

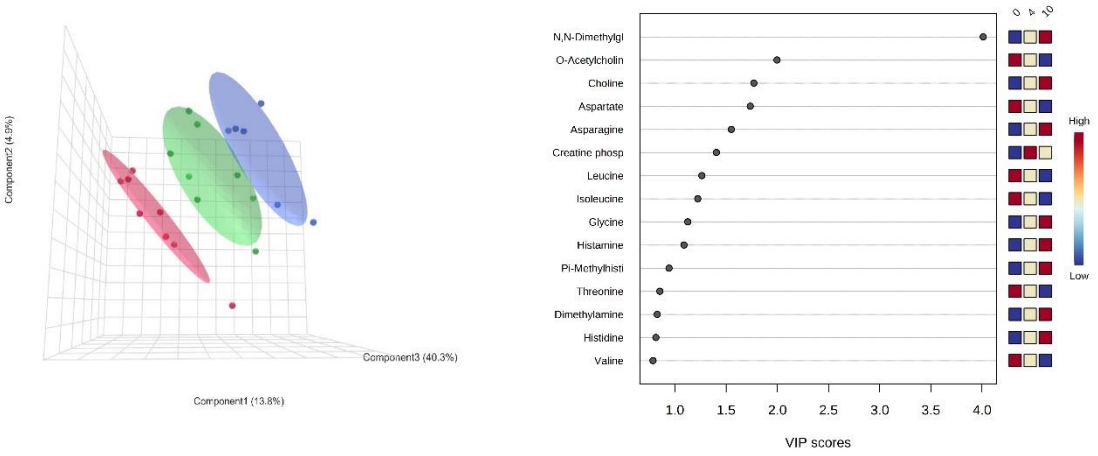
A total of 47 metabolites mainly associated with protein turnover and energy allocation were assigned in white muscle of both species, *P. brachycephalum* and *Z. viviparus*. The metabolic response to acute warming differed between species and was more pronounced in *Z. viviparus* than in *P. brachycephalum*, as reflected in more metabolites changing significantly.

3.3.1. *Pachycara brachycephalum*

The PLS-DA model indicated a clear separation of metabolic profiles in the white muscle of *P. brachycephalum* between the lowest (0 °C, red),

intermediate (4 °C, green) and the highest (10 °C, blue) temperature (Figure 3a). Most importantly, the warming-induced increase in N,N-dimethylglycine levels mainly shaped the PLS-DA, followed by a decrease in acetylcholine levels and the increase in choline levels (Figure 3b) Additionally, mostly amino acid levels changed with warming, with increasing levels in, e.g., asparagine, glycine and histidine, and decreasing levels in, e.g., aspartate, leucine and isoleucine. Therefore, SAM revealed that N,N-dimethylglycine levels increased significantly and linearly with increasing temperature (Figure 4).

(**a**) (**b**)



(**a**) (**b**)

Figure 3. PLS-DA of the metabolic profile of *Pachycara brachycephalum*. The PLS-DA in a 3-component view contained 59% of the data (**a**) and the VIP score described the loadings for the metabolites most important for the description of the model (**b**). A clear separation between the 0 °C (red, n = 8), 4 °C (green, n = 8) and 10 °C (blue, n = 6) groups could be observed.

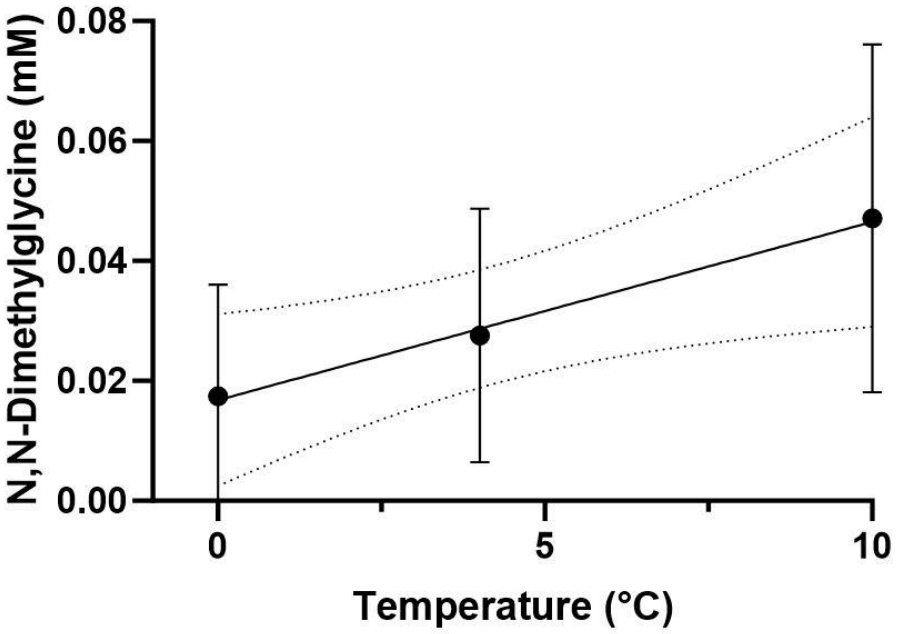
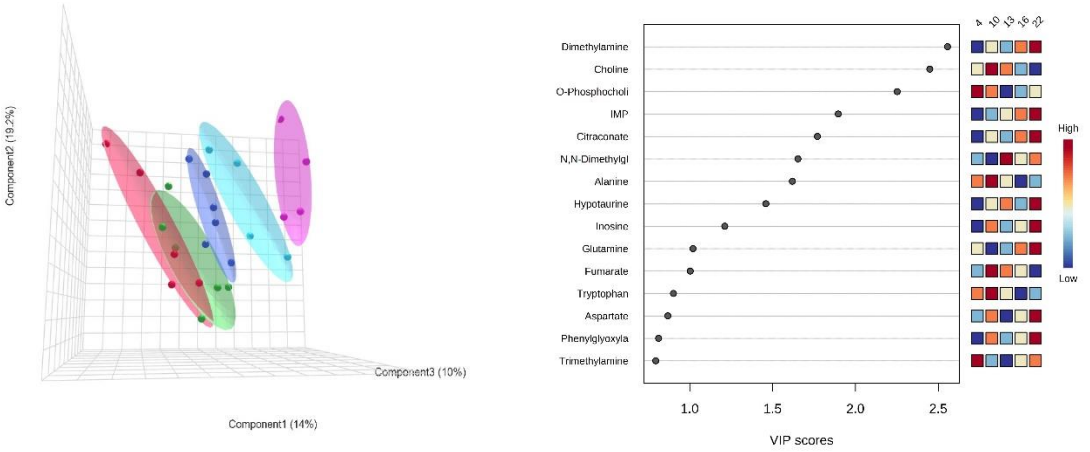


Figure 4. N,N-dimethylglycine level in *P. brachycephalum* during acute warming. The level of N,N-dimethylglycine increased linearly (Y = 0.002969x + 0.01681, R_2 = 0.237, *p*-value < 0.05, data shown as mean ± standard deviation) with temperature (significant difference using Significance Analyses of Microarray SAM (Delta value 0.3, FDR 0.01, False 0.01, n = 8 (0 and 4 °C), n = 6 (10 °C)) with a *p*-value < 0.001.

3.3.2. *Zoarces viviparus*

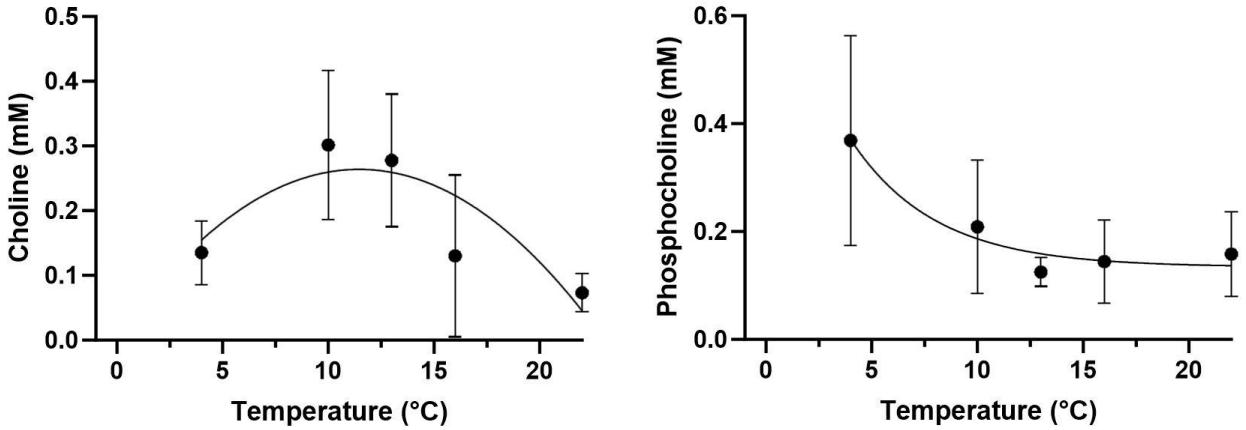
The PLS-DA model values for *Z. viviparus* did not show a clear separation at the lowest temperatures of 4 °C and 10 °C, but they were separated at higher temperatures of 13 °C (dark blue), 16 °C (light blue) and 22 °C (pink) (Figure 5a). Most importantly, warming resulted in an increased dimethylamine content (Figure S1), contributing to the PLS-DA patterns. Choline first increased and then decreased, forming a bell-shaped curve, while the phosphocholine content decreased with increasing temperature (Figure 5b).



(a) (b)

Figure 5. PLS-DA of the metabolite profile of *Zoarces viviparus*. The PLS-DA viewed in a 3D model included 43.2% of the data (**a**) and the VIP score described the loadings of the PLS-DA (**b**). There was no clear separation between values at 4 °C (red, n = 5) and at 10 °C (green, n = 6), but those at 13 °C (dark blue, n = 6), 16 °C (light blue, n = 4) and 22 °C (pink, n = 4) were clearly separated.

The concentration of choline and phosphocholine changed significantly with increasing temperature. Choline doubled its concentration between 4 and 10 °C and remained unchanged up to 13 °C. At higher temperatures the choline concentration decreased and formed a bell-shaped curve (Figure 6a). In contrast, the phosphocholine concentration decreased between 4 and 13 °C and reached a plateau at higher temperatures (Figure 6b).

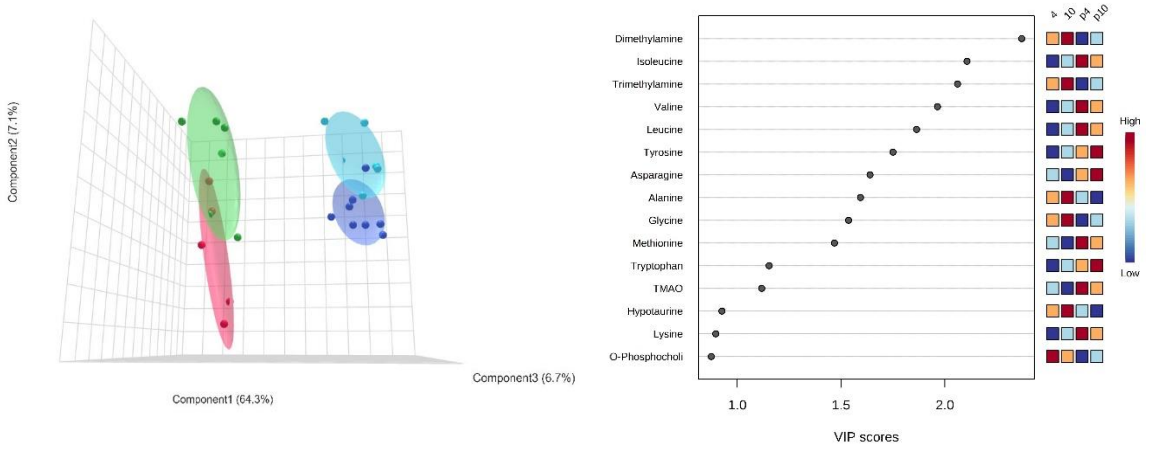


(**a**) (**b**)

Figure 6. Changes in the concentration of choline and phosphocholine during acute warming in *Zoarces viviparus*. Choline (**a**) first increased and then decreased forming a bell-shaped curve ($y = 0.0061x^2 + 0.045x - 0.0020$, $R_2 = 0.39$, data shown as mean ± standard deviation) while phosphocholine (**b**) decreased ($Y = 0.134 - (0.134 - 0.78)^{(-0.25x)}$, $R_2 = 0.39$, data shown as mean ± standard deviation) during acute warming (significant difference using Significance Analyses of Microarray SAM (Delta value 0.5, FDR 0.188, False 0.46, (4 °C (n = 5), 10 °C (n = 6), 13 °C (n = 6), 16 °C (n = 4) and 22 °C (n = 4)) with a p-value < 0.001 (Choline) and $p < 0.05$ (Phosphocholine).

3.3.3. Comparison between the Antarctic and Common Eelpout

The metabolite profiles differed largely between the two species at the same temperatures (4 °C and 10 °C, Figure 7).



(a) (b)

Figure 7. The PLS-DA of the metabolite profile of *P. brachycephalum* in comparison to *Z. viviparus* at 4 and 10 °C. The 3D model of the PLS-DA comprised 78% of the data (**a**) and the VIP score (**b**) describes the metabolites used for the PLS-DA of *P. brachycephalum* (dark blue 4 °C, n = 8; light blue 10 °C, n = 6) compared to *Z. viviparus* (red 4 °C, n = 5; green 10 °C, n = 6).

The PLS-DA indicated a clear separation between *P. brachycephalum* and *Z. viviparus* specimens at 4 and 10 °C (Figure 7a). The VIP score indicated that the main difference was found in the concentration of methylamines (dimethylamine, trimethylamine and TMAO (Trimethylamine-N-Oxide)) and amino acids (e.g., isoleucine, valine, leucine). While di- and trimethylamine were significantly higher in *Z. viviparus*, the concentration of TMAO was higher in *P. brachycephlaum*. Additionally, the concentration of most amino acids was significantly higher in *P. brachycephalum*, but those of alanine, histidine and glycine were significantly higher in *Z. viviparus* (Figure 7b). The SAM (Delta value 5.7, FDR 0.003, False 0.13, $p < 0.005$) identified 23 metabolites that differed significantly between the two eelpout species (Table S1). For better comparison, Table 1 summarizes the significant differences between *P. brachycephalum* and *Z. viviparus* as fold difference between the means at 4 and 10 °C for each species separately, as PLS-DA did not show a clear separation with respect to temperature (Table 1).

Table 1. Overview of the different levels of metabolites in *Zoarces viviparus* and *Pachycara brachycephalum*: Fold differences between *P. brachycephalum* (PB) and *Z. viviparus* (ZV) were calculated using mean values at 4 and 10 °C separately for each species. Metabolites not significantly different according to SAM (see supplementary, Table S2) or with fold differences below 2, and metabolites with values changing within species between 4 and 10 °C, were not considered. Bold numbers indicate a significantly higher concentration of the studied metabolite.

Metabolite (Name)	Concentration in *Z. viviparus* (mM)	Concentration in *P. brachycephalum* (mM)	Fold Difference of Means between PB and ZV
Amino acids			
Isoleucine	0.08 ± 0.04	**0.69 ± 0.34**	9.1
Valine	0.14 ± 0.07	**1.11 ± 0.55**	8
Leucine	0.12 ± 0.05	**0.90 ± 0.43**	7.2
Asparagine	0.11 ± 0.06	**0.56 ± 0.20**	5.6
Methionine	0.07 ± 0.03	**0.31 ± 0.14**	4.6
Tryptophan	0.03 ± 0.01	**0.11 ± 0.04**	3.4
Lysine	0.67 ± 0.26	**1.62 ± 0.59**	2.4
Glycine	**14.59 ± 5.43**	2.43 ± 0.78	0.17
Alanine	**5.96 ± 1.30**	1.11 ± 0.34	0.19
Histidine	**0.86 ± 0.32**	0.38 ± 0.14	0.44
Methylamine			
TMAO	7.54 ± 4.62	**23.15 ± 3.49**	3.1
Dimethylamine	**1.93 ± 1.32**	0.09 ± 0.05	0.05
Trimethylamine	**0.21 ± 0.13**	0.014 ± 0.003	0.07
Others			
Hypotaurine	**0.002 ± 0.001**	0.0007 ± 0.0003	0.35
Taurine	**8.96 ± 2.42**	3.94 ± 0.92	0.44

4. Discussion

In the following, thermal plasticity is discussed separately for both eelpout species, starting with *P. brachycpehalum* and then continuing with *Z. viviparus*. In the last part, the differences between the two species are interpreted to represent consequences of thermal adaptation.

4.1. Thermal plasticity of the cold-stenothermal Pachycara brachycephalum

The protein synthesis rate (Ks) in white muscle of *Pachycara brachycephalum* did not change during acute warming but varied highly between individuals at Ks = 0.5 +/− 0.25% day^{-1}. Most strikingly, it was 10 times higher than in our previous *in vivo* study [20]. Compared to the former study, we increased the food supply from once a week [20] to 2–3 times a week in the present study, 3–6 weeks before the experiment started. In the Southern Ocean, food such as plankton is abundant in summer (December to March), while it decreases dramatically in winter (June to September) [52]. *P. brachycephalum* appears to be adapted to these drastic seasonal changes in food supply by up-regulating protein synthesis in white muscle when food is abundant and drastically reducing protein synthesis in white muscle when less food is available to conserve energy. Smith and Haschemeyer reported a reduction in the Ks by a factor of between 3 (*Trematomus bernacchii*) to 5.5 (*Trematomus hansoni*) in Antarctic fish due to a starvation period of 5 (*Trematomus bernacchii*) to 15 days (*Trematomus hansoni*) at −1.5 °C [21]. Although *P. brachycephalum* was not starved in our previous study, the higher feeding rate used here likely increased the Ks.

Acute warming did not affect the protein synthesis rate in white muscle of *P. brachycephalum*, resulting in a Q_{10} of about 1, far below a Q_{10} value commonly expected at 2–3. This contrasts expectations from long-term studies in thermally acclimated Antarctic organisms where growth below their thermal optimum follows a Q_{10} above 3 [6]. It is important to distinguish results obtained in acute warming experiments from those obtained after acclimation to different temperatures over weeks to months. To our knowledge, this is the first study to investigate protein synthesis rates *in vivo* in white muscle during acute warming in Antarctic fish. Our finding of protein synthesis being non-responsive to temperature is consistent with *in vitro* findings obtained from 3-months-acclimated *P. brachycephalum*, where the capacity for protein synthesis did not respond to temperature changes [32]. In fact, recent *in vivo* measurements on the Antarctic fish *Harpagifer antarcticus*, in which the protein synthesis rate was measured similarly, by use of a flooding dose of phenylalanine, did not reveal any change in the protein synthesis rate as a result of increased temperature, although the fish were acclimatized for 28 days at different temperatures and the protein synthesis rate was measured for the whole fish [19].

Our present results argue that the protein synthesis machinery remains unchanged after acute warming (+2 °C day^{-1}) and operates at low Q_{10}. Further experiments would need to clarify whether protein synthesis capacity is increased by protein expression during long-term cooling or reduced during long-term warming. This leaves the question open whether protein synthesis in white muscle can be thermally compensated in any Antarctic species.

Lysosomal degradation of proteins via cathepsin D did not differ significantly in white muscle samples at 0, 4 and 10 °C when measured at a common temperature (26 °C), indicating unchanged enzyme quantities. When cathepsin D activity was measured at different temperatures (ΔT =

19 °C) the Q_{10} value averaged at 2.3 ± 0.33 (n = 6). Additionally, cathepsin D prefers a pH below 5 as found in the lysosome, even though it is also active at higher pH in extracellular space and cytoplasm [53]. In *P. brachycephalum*, acute warming has been shown to lower intracellular pH [37], which could further increase the cathepsin D activity during acute warming. It is therefore likely that lysosomal degradation *in vivo* was about 2.3 times lower at 0 °C than at 10 °C. This would need to be confirmed by *in vivo* studies, as many regulatory processes of cathepsin D are not yet fully understood, which could further influence cathepsin D activity *in vivo* [54].

To gain a deeper understanding of the metabolic changes, especially those involved in protein degradation, we performed untargeted metabolic profiling of white muscle tissue and identified 47 different metabolites at 0, 4 and 10 °C. A PLS-DA discriminant analysis shows a shift of metabolites towards warmer temperatures, with the metabolite N,N-dimethylglycine responding most strongly to elevated temperatures, as also confirmed by SAM. N,N-dimethylglycine is a derivate of glycine and an intermediate of choline metabolism. It enhances immune responses in salmonid fish [55] and prevents oxidative stress by scavenging free radicals that would otherwise damage cells, proteins and DNA [56]. N,N-dimethylglycine can be formed from choline via betaine to glycine; however, none of these three metabolites changed significantly during acute warming. In addition, PLS-DA revealed the neurotransmitter acetylcholine to decrease, and choline to increase, with acute warming. Acetylcholine is used by the nervous system as a transmitter to activate movement and can rapidly be converted to choline [57]. However, there is no evidence of increased protein degradation as the associated metabolites such as, e.g., 1-methylhistidine and 3-methylhistidine do not change with temperature (see supplementary, Figure S2).

In summary, acute warming has limited effects on *P. brachycephalum* white muscle as neither protein synthesis nor metabolites associated with protein degradation or the maximum protein degradation capacity changed. Similarly, protein degradation, calculated as the difference between protein synthesis, food supply and weight gain, did not change in the thermally acclimated (28 days) Antarctic fish *Harpagifer antarcticus* [19]. Both protein synthesis and protein degradation rates being thermally non-responsive in white muscle, points to other factors that may then lead to reduced growth rates at temperatures above the thermal optimum. For example, in a systemic to molecular hierarchy of thermal tolerance [58], energy-dependent protein synthesis and growth would increasingly be constrained according to OCLTT [1] while degradation might continue unabated. Clearly, further studies are necessary to clarify the limited effect of warming on Antarctic fish within its thermal range. In total, long-term temperature-dependent growths in the Antarctic eelpout [12,14] and other species cannot be explained from the present results of acute thermal changes.

4.2. Thermal Response of the Eurythermal Zoarces Viviparus

In contrast to *P. brachycephalum*, the protein synthesis rate (Ks) in *Z. viviparus* white muscle increased with temperature to a maximum value of 0.38% day^{-1} at 16 °C resulting in a Q_{10} of about 2.2. At higher temperatures (22 °C), protein synthesis did not increase further but decreased slightly (Ks = 0.31% day^{-1}). This suggests that, similar to *P. brachycephalum*, mechanisms such as OCLTT are involved to constrain the

thermally induced increase in processes, to within the thermal window. Overall protein synthesis in white muscle responds to acute warming, supporting previously measured *in vivo* growth maxima between 12 and 15 °C [12,39,40].

As in *P. brachycephalum*, the maximum activity of cathepsin D measured at a common temperature (26 °C) of white muscle exposed to acute warming did not change, indicating that protein expression levels and thus enzyme quantities did not change. In *Z. viviparus* as in *P. brachycephalum,* a simple Q_{10} effect may have increased protein degradation via cathepsin at high temperatures. In fact, a study by Fraser et al. (2022), comparing an Antarctic and a temperate fish, found calculated rates of degradation in the temperate fish to increase towards the upper thermal limit after an acclimation period of 28 days similar to our acute warming approach [19]. Another important protein degradation pathway via calpain was not measured and may have been altered at high temperatures.

Untargeted metabolic profiling revealed several changes in cellular processes during acute warming in *Z. viviparus*. At lower temperatures (4 and 10 °C) the metabolic profile did not change, but at higher temperatures (13, 16 and 22 °C) the metabolic profiles separated the treatment groups from each other (Figure 5). The PLS-DA identified the key metabolites causing this difference to be dimethylamine, choline and phosphocholine as well as metabolites involved in energy production (e.g., IMP, citraconate, inosine and fumarate). The SAM revealed only two metabolites to be changed significantly, namely choline and phosphocholine.

The micronutrient and intermediate choline initially increased between 4 and 10 °C, then stabilized up to 13 °C and decreased thereafter, thereby closely following the long-term temperature-dependent growth curve of *Z. viviparus* [12,39,40]. In skeletal muscle, choline provides its methyl group as a micronutrient for several processes including protein and lipid metabolism as well as autophagy [59]. In contrast to choline, O-phosphocholine, which can be directly synthesized from choline by phosphorylation and is an important precursor of membrane lipids, was found highest in the cold (4 °C) and decreased with increasing temperature. High phosphocholine content was found to improve cold tolerance by increasing the ability to modify phospholipids in membranes to maintain their fluidity in cold conditions [60,61]. In agreement with our results, lipid analyses of acclimatized *Z. viviparus* indicated a high lipid content in the cold, especially for lipids important for membrane fluidity [62].

While all other metabolites did not differ significantly between treatments according to SAM, several metabolites of interest were identified in the PLS-DA indicating metabolic trends relating to energy metabolism. Increasing levels of citraconate could indicate an enhanced Krebs Cycle as it inhibits Cis-Aconitate from leaving the Krebs Cycle [63]. Slightly increasing glutamine and aspartate levels and decreasing alanine levels, as well as an increasing concentration of IMP, a degradation product of ATP, reflect increasing energy deficiency; however, future studies would need to confirm this interpretation. Amino acids of skeletal muscle proteins can be used to fuel other tissues such as the liver as shown for starving mammals [64] as well as migrating and spawning salmon [65,66].

In summary, in the North Sea eelpout, the increased energy demand of white-muscle protein synthesis is within the thermal performance

curve, as indicated by minor changes of metabolites associated with energy production between 4 and 16 °C. The rate of protein synthesis in white muscle of *Z. viviparus* decreasing beyond 22 °C indicates limited energy supply to protein synthesis and growth, in line with the consequences of OCLTT at thermal limits (e.g., [1]). Experiments on gene expression during acute warming (0.08 °C min^{-1}) of the eurythermal fish *Gillichthys mirabilis* support our findings, as genes related to the cell cycle and cell proliferation were reduced in the white muscle, possibly reflecting reduced energy expenditures [67].

4.3. Comparison between the Antarctic and Common Eelpout

When comparing *P. brachycephalum* from the Southern Ocean with closely related *Z. viviparus* from the North Sea at the same temperatures, potentially large differences in protein metabolism became apparent.

The protein synthesis rate was two to three times higher in *P. brachycephalum*, at the same temperatures and feeding rates, indicating cold-compensated *in vivo* activities at their lower optimum temperature. Even more striking, the capacity of the lysosomal protease cathepsin D, an indicator of protein degradation, was found to be more than ten times higher in *P. brachycephalum* compared to *Z. viviparus*, indicating a cold-compensated capacity in the Antarctic species. This also indicates a higher protein turnover in the Antarctic species compared to its temperate confamilial. Higher protein turnover is also indicated from the comparison of metabolic profiles as especially the complex amino acids including branched chain amino acids (leucine, isoleucine and valine) together with asparagine, methionine, tryptophan and lysine are found at significantly higher concentrations in the cytosol of *P. brachycephalum*. These amino acids, which include essential amino acids and are energetically expensive to synthesize, may preferably be recycled and not undergo final oxidation via the Krebs Cycle. Higher cytosolic concentrations as seen in the Antarctic eelpout may indeed enable a higher turnover of proteins. In contrast, glucogenic amino acids like glycine and alanine, which can be synthesized quite simply from the glucogenic pathways, were found at much higher levels in *Z. viviparus*. Storch et al. (2005) found higher concentrations of glycine bound in proteins in *Z. viviparus* compared to *P. brachycephalum* [32], which may be a consequence of its higher abundance in the cytosol. Alanine is an important nitrogen carrier and a major gluconeogenic precursor in fish [68]. Depending on the metabolic state, it is released from skeletal muscle and often a precursor of other non-essential amino acids [69]. Together, these amino acids and their high concentrations may indicate a higher metabolic turnover and a different use of fuels in the two eelpouts, as described earlier [12].

The only essential amino acid found at higher concentration in the temperate eelpout is histidine, but the factorial change is quite low in comparison to the other complex amino acids. The histidine concentration acts as a buffer against changing pH values. For example, it increased in white muscle in salmon before migration to prepare them for the intense exercise [68,70]. Higher histidine concentrations in the temperate eelpout may thus be helpful in the more variable environment of the North Sea including temperature, oxygen and salinity. Overall, the differences in cytosolic amino acids between *Z. viviparus* and *P. brachycephalum* may reflect differences in environmental variability. *Z. viviparus* has a high proportion of simple amino acids that can be metabolized quickly under more variable conditions. In contrast, *P. brachycephalum* experiences stable

temperatures and almost no seasonal variation, but food scarcity may occur. When a high level of food is available, it seems to respond quickly by increasing protein turnover and enhancing growth (as discussed above).

Alternatively, the increased protein turnover rates in Antarctic fish may be due to proteins being less stable in the cold [71], as a consequence of structural flexibility [72]. Several Antarctic fish species possess high levels of ubiquitin-conjugated proteins, suggesting a higher rate of degradation [73]. The hypothesis of higher protein instability would be supported by high level of TMAO in *P. brachycephalum*, while trimethylamine and dimethylamine are higher in *Z. viviparus*. TMAO is an osmolyte important for protein stabilization in marine organisms at depth [74]. *P. brachycephalum* can be found at much greater depth than *Z. viviparus* from the shallow North Sea. Whether TMAO is higher in polar organisms for stabilizing proteins in the cold needs to be investigated in the future.

5. Conclusions

The two eelpout species from the North Sea and the Southern Ocean respond differently to acute warming. The protein synthesis rate in the common eelpout *Zoarces viviparus* was increased between 4 and 16 °C according to the Q_{10} rule, a phenomenon not observed in the Antarctic eelpout. At 22 °C, the protein synthesis rate in white muscle was reduced in the temperate species. As protein synthesis is the largest energy consumer in ectotherms [75] our earlier findings [39] indicate that whole-animal constraints, e.g., through OCLTT, may have set in at upper thermal limits and reduced white-muscle growth. Within the thermal range of the Antarctic eelpout *Pachycara brachycephalum*, the rate of protein synthesis did not respond to acute warming. The increased protein synthesis rate, possibly due to an increased feeding rate (10-fold higher than in our previous study [20], might have masked the thermal response.

Regardless of temperature, protein turnover is significantly higher in *P. brachycephalum* compared to *Z. viviparus* despite the same feeding rate. This indicates cold compensation in *P. brachycephalum*, which can maintain higher metabolic rates than *Z. viviparus* at temperatures around 0 °C. Cold compensation is defined as "the maintenance of an appropriate physiological rate in the face of temperature change" [8]. While protein synthesis and degradation are cold-compensated, other functions such as reproduction and development may appear suppressed in order to enable metabolic down-regulation (cf. [7]). The present study indicates clear cold compensation of both protein synthesis rate and degradation in Antarctic fish. The functional background of this compensation needs further attention.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Q_{10} values of cathepsin D activity in muscle homogenates at various temperatures in *P. brachycephalum*; Table S2: SAM analysis comparison between metabolic profiles of *P. brachycephalum* and *Z. viviparus*; Figure S1: Changes of the concentration of dimethylamine during acute warming in *Zoarces viviparus*; Figure S2: Changes of the concentration of 1-Methylhistidine and 3-Methylhistidine during acute warming in *Pachycara brachycephalum*

Author Contributions: Conceptualization, N.K., C.B., J.T., F.C.M., M.L., G.L. and H.-O.P.; performing experiments, N.K. and M.L.; methodology, N.K., J.T. and C.B.; data curation, N.K., C.B., G.L. and J.T.; writing—original draft preparation, N.K., visualization, N.K.; writing—review and editing, N.K., J.T., C.B., F.C.M.,

M.L., G.L. and H.-O.P.; supervision, N.K., C.B., F.C.M., M.L., G.L. and H.-O.P. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the research program "Changing Earth—Sustaining our Future" in the program-oriented funding periods (PoF IV subtopic 6.2—Adaptation of marine life: from genes to ecosystems) of the Helmholtz Association.

Institutional Review Board Statement: The work was approved by the German authority (Freie Hansestadt Bremen, reference number 160; 500-427-103-7/2018-1-5).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be uploaded to the public repository PANGEA after acceptance of the manuscript.

Acknowledgments: We would like to acknowledge the technical support of Sandra Götze, Amirhossein Karamyar, Isabel Ketelsen, Nils Koschnick and Fredy Veliz Moraleda during the experiments. Additionally, we would like to thank Sandra Götze, Anette Tillmann and Nicole Vogt for their help during the extraction and measurement of the NMR samples. We would also like to thank Christian Zurhelle for his help in measuring protein synthesis rates and Reinhard Saborowski for his assistance and expertise in the analysis of cathepsin D.

Conflicts of Interest: The authors declare no conflicts of interest.

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3.3 Manuscript 3: Acute warming leads to changes in metabolic processes in the eurythermal common eelpout *Z. viviparus* and stenothermal Antarctic eelpout *P. brachycephalum*.

Contribution of the candidate in % of the total workload

Experimental concept and design	70 %
Experimental work and data acquisition	80 %
Data analysis and interpretation	85 %
Preparation of figures and tables	100 %
Drafting the manuscript	80 %

Acute warming leads to changes in metabolic processes in the eurythermal common eelpout *Z. viviparus* and stenothermal Antarctic eelpout *P. brachycephalum*.

Nina Krebs, Felix C. Mark, Jan Tebben, Magnus Lucassen and Hans-Otto Pörtner

Abstract

In the ocean, climate change leads to an increase of water temperatures and thus also the body temperatures of their ectothermal inhabitants, affecting a variety of metabolic processes. The concept of OCLTT postulates that the capacity of oxygen supply mechanisms in relation to demand limits thermal tolerance, with effects depending on the thermal regime and the respective pattern of adaptation. As proxies for potential oxygen limitation we analyzed standard metabolic rate (SMR), gill protein synthesis rate, hematocrit and spleen weight, in the cold adapted stenothermal Antarctic eelpout (*Pachycara brachycephalum*) and the eurythermal common eelpout from the North Sea (*Zoarces viviparus*). Ammonia excretion was determined in *P. brachycephalum* in order to calculate the O:N ratio as an indicator for protein-based metabolism.

Acute SMR increased exponentially with gradual warming in both eelpout species, with a somewhat higher Q_{10} value (Q_{10}=2.46) in *P. brachycephalum* than in in *Z. viviparus* (Q_{10}=1.91). There were no significant differences in SMR between the species at 4 and at 10 °C. Protein synthesis rate (Ks) in the gills also increased exponentially (*P. brachycephalum* Q_{10}=4.2; *Z. viviparus* Q_{10}= 2.8), and was similar in both eelpout species when measured at a common temperature (4 and 10°C). No significant changes in hematocrit or spleen weight were observed in either species during acute warming. However, spleen weight was higher in *P. brachycephalum* (0.219-0.287% of body weight) than in *Z. viviparus* (0.098-0.134% of body weight) regardless of temperature, whereas hematocrit was significantly higher in *Z. viviparus* than in *P. brachycephalum*. The O:N ratio was calculated only for *P. brachycephalum*, and no significant differences were found between temperatures.

In conclusion, both eelpout species increased oxygen uptake and gill protein synthesis rates in similar ways during acute warming, indicating similar levels of energy expenditure in both eelpout species. However, this is in contrast to a previous study which found higher aerobic performance in *P. brachycephalum* in comparison to *Z. viviparus* during acute warming. Therefore, it is likely that *P. brachycephalum* is able to conserve energy for other processes. One of these processes is a reduced number of erythrocytes, which serves to reduce blood viscosity at cold temperatures and thus the energy required for blood circulation.

Introduction

Anthropogenic climate change has already led to a more than 1.1 °C rise in temperature compared to pre-industrial times. It is causing sea level rise, heat waves, ocean acidification, and oxygen depletion in the oceans, with various futures ahead depending on emissions scenarios: under a high emissions scenario global mean temperature will rise by up to 4.3 °C by 2100 (IPCC, 2023). This will particularly affect ectothermal organisms e.g. fish, as their body temperature is the same as that of the surrounding water, they have to acclimatize or adapt to rising temperatures or migrate to higher latitudes. But what about organisms that already live at the poles?

Polar organisms are well adapted and have compensated for the cold in order not only to survive but also to grow at temperatures around and below 0 °C. In general, the term cold compensation encompasses all physiological aspects of an organism that enable it to thrive in polar regions (Clarke, 1991), including various metabolic adaptations e.g. elevated protein synthesis and degradation (Fraser et al., 2022; Krebs et al., 2023a; Storch et al., 2005; Todgham et al., 2017) that enable survival in subzero water (reviewed in e.g. Peck, 2018; Todgham and Mandic, 2020). Otherwise, biochemical processes are slower and less energy is required for homeostasis In the cold, according to the Q_{10} rule (Hochachka and Somero, 2002). However, in polar fish, some functions are even lower than expected from Q_{10} values and the reasons are yet not fully understood (Peck, 2018; Pörtner et al., 2007).

If temperature rises, energy requirement increases according to Q_{10} and with it all metabolic processes providing the energy. When temperature increases over a relatively short period of time, usually within hours to a few days (acute warming), energy demand increases exponentially until a breakpoint is reached. At this breakpoint, energy and thus oxygen demand exceeds the amount of energy that can be produced within the organism. The concept of oxygen and capacity limited thermal tolerance (OCLTT) postulates that this energy discrepancy is caused by a limitation of oxygen supply capacity (e.g. Pörtner et al., 2017). Oxygen is crucial for sustained energy production (Nelson, 2016), thus oxygen consumption (MO_2) and associated metabolic rate mirrors energy demand. Standard metabolic rate (SMR) describes the minimum metabolic rate to sustain life extrapolated to zero motor or digestive activity (Pörtner and Grieshaber, 1993). As it is almost impossible to measure true SMR, it is common to measure routine metabolic rate (RMR), which refers to daily metabolic rate under normal behavior in a fasting animal (Chabot et al., 2016; Metcalfe et al., 2016).

When temperature is increasing, various parameters change to maximize oxygen transport. A larger quantity of erythrocytes can increase the oxygen transport (Soldatov, 2005). Hematocrit varies largely depending on species, biotic, and abiotic factors (Ahmed et al., 2020). Higher hematocrit values were observed in Antarctic fish in response to rising temperatures (Beers and Sidell, 2011; Joyce et al., 2018; Windisch et al., 2014). However, not only the quantity of erythrocytes, but also the lifespan of erythrocytes in fish is influenced by temperature. In summer, heat stress shortens the lifespan and more immature red blood cells are released, while in winter mainly older erythrocytes are present in the blood (Hofer et al., 2000). In fish, erythrocytes are produced by the head kidney (pronephros) and stored in the spleen (Fänge and Nilsson, 1985). The storage of erythrocytes in the spleen has the advantage that they can be released quickly when required e.g. during intensive exercise (Brijs et al., 2020) or temperature changes (Islam et al., 2020). All this indicates an optimization strategy for oxygen transport that could help to overcome thermally induced oxygen deficiency.

Aerobic energy production also uses carbohydrates, lipids or proteins to various degrees (Nelson, 2016). The ratio of oxygen consumption (O) and nitrogen excretion (N) indicates the extent of protein metabolism. In teleosts up to 80 % of the nitrogen is excreted as ammonia, and as urea (Randall and Wright, 1987). Protein catabolism in the liver or muscle produces ammonia, therefore, a low O:N ratio indicates protein metabolism, while a high O:N ratio indicates lipid metabolism (Mayzaud and Conover,

1988). The utilization of metabolic substrates can reflect thermal adaptation, e.g. the metabolism of Antarctic fish in the cold is mainly based on lipids (Brodte et al., 2008; Windisch et al., 2014). Short term temperature changes can also have an impact, e.g. increased proteolysis can be observed in fish at temperatures above the species-specific thermal optimum (Fraser et al., 2022).

Temperature-dependent changes in protein turnover are usually studied in whole fish (Fraser et al., 2022) or in a tissue like white muscle (Krebs et al., 2023a, 2023b). Less is known about protein turnover in the gills. In marine teleosts, gills are one of the most metabolically active organs and play a role in many processes such as oxygen uptake, ammonia excretion, and osmoregulation (Evans et al., 2005; Houlihan et al., 1986; Lyndon and Houlihan, 1998). Rising temperatures cause increased energy demand and thus higher oxygen uptake via the gills (e.g. Mark et al., 2002; Pörtner et al., 2017; Van Dijk et al., 1999a). Genes involved in protein synthesis and repair are upregulated at high temperatures (Akbarzadeh et al., 2018), as are genes associated with growth and cell organization (Buckley et al., 2006). Temperature-dependent changes in protein synthesis capacity differed between eurythermal and stenothermal fish. The capacity for protein synthesis in the stenothermal fish decreased with warming in a low range of temperatures (0°C Ks= 11.8% day^{-1}; 5°C Ks=6.1% day^{-1}). A higher thermal range was visible in the eurythermal fish where protein synthesis increased further and beyond 5°C (5°C Ks= 5.0% day^{-1}; 10°C 18.7% day^{-1}). The authors argued that protein synthesis in the stenothermal fish is cold-compensated to fully function at temperatures constantly at or below 0°C (Storch et al., 2005). Stenothermal fish are characterized by a relatively narrow thermal window and a low capacity for oxygen supply that is highly responsive to temperature, while eurythermal fish have a wider thermal window and a higher, more energetically costly capacity for oxygen supply (Pörtner et al., 2017).

An earlier study found differences in the regulation of energy metabolism and protein turnover in white muscle in *Z. viviparus* during acute warming (Krebs et al., 2023a). White muscle comprises a large part of the fish body and thus has the largest absolute rate of protein synthesis (Houlihan et al., 1986). In ectotherms such as fish, most energy is used for protein synthesis (Fraser and Rogers, 2007), therefore, the protein synthesis rate in white muscle is an important contributor to setting the overall energy demand. In addition to our earlier studies of *in vivo* protein synthesis in white muscle (Krebs et al. 2023a,b) the present study therefore, reinvestigates the oxygen consumption rate in both eelpout species as a proxy for energy demand during acute warming. We included analyses of hematocrit, the rate of protein synthesis in the gills and nitrogen excretion rates for a more comprehensive picture and a better understanding of the mechanisms involved in thermal adaptation, energy and oxygen homeostasis and last not least, the projected consequences of climate change. As thermal breadth plays a key risk in setting extinction risk (Malanoski et al., 2024) we used the thermal response of oxygen consumption and protein synthesis to reconstruct the thermal window of the two species.

Material and Methods

Animals

Antarctic eelpout, *Pachycara brachycephalum* (Pappenheim, 1912), were caught in Admiralty Bay, King George Island (62° 11' S, 58° 20' W), Antarctica, on RV Polarstern expedition PS112 in March 2018 using baited fish traps at depths of 430 to 530m. The fish traps were recovered from the seabed after 52 hours. On board, the fish were kept at 0°C for the duration of the transport (2 months) to the Alfred Wegener Institute (AWI), Bremerhaven, Germany. At the end of the voyage, more than 99.5% of the animals survived. At AWI, the fish were kept in well-ventilated, recirculating seawater at 0.0 ± 0.5°C and 34 ± 1 practical salinity units (PSU) under a 12:12 light: dark cycle.

Common eelpout, *Zoarces viviparus* (Linnaeus, 1758), were caught in the North Sea near the island of Helgoland and brought to the AWI in autumn 2020 on RV Uthörn. The fish were kept in well-ventilated, recirculating seawater at 12°C for an acclimation period of at least three months before the water was slowly cooled to a temperature of 4.0 ± 0.5°C. This cooling was adapted to the winter season in order to preserve the seasonality of the fish. Similar to the Antarctic eelpout, *Z. viviparus* were maintained at 34 ± 1 PSU at a 12:12 light-dark cycle.

P. brachycephalum was fed frozen mussel, *Mytilus edulis* (Erdmann, Germany), once a week and *Z. viviparus* twice a week. The different feeding regime has proven to be optimal after 20 years of keeping this fish in our facility, since the metabolism of stenothermal Antarctic fish is much slower compared to eurythermal North Sea fish. Before starting the experiment to measure oxygen consumption and ammonia excretion, *P. brachycephalum* was not fed for 10 days, and *Z. viviparus* was not fed for 7 days to minimize the effect of specific dynamic action (SDA). This is based on a study comparing the SDA of stenothermal fish from the Antarctic (*Notothenia neglecta*) and a fish from the North Sea (*Myoxocephalus scorpius*). The digestion of food took about 250 hours (approx. 10 days) in the fish from the Antarctic Ocean and just over 150 hours (6-7 days) in the cold acclimated fish from the North Sea (Johnston and Battram, 1993). In another experiment (studying protein synthesis, hematocrit, and spleen weight), the food supply was increased to 2-3 times per week for 4-6 weeks for both species. The rate of protein synthesis varies greatly depending on the feeding rate and the time after feeding (reviewed in Fraser and Roger, 2007). Therefore, feeding was stopped 5-7 days before the start of the experiment to minimize the effects of SDA, but not to risk starving the fish and potentially decreasing the protein synthesis rate (see Krebs et al., 2023b). The handling and killing of the fish were carried out following Krebs et al. (2023a) and in accordance with German legislation and the recommendations of the American Veterinary Medical Association (AVMA). The work was approved by the responsible German ethics authority (Freie Hansestadt Bremen, reference number 160; 500-427-103-7/2018-1-5).

Oxygen consumption and ammonia excretion

Five fish were placed in separate respiration chambers, each with a volume of 2 l, while one pump circulated the water inside the chamber and the oxygen sensor (Fibox 3 PreSens GmbH, Germany) and another one pumped oxygenated water from a 150 l tank into the chambers (Loligo systems, Denmark). Oxygen sensors were calibrated in a separate beaker to 100% in well-aerated water from the water reservoir at 0°C for *Pachycara brachycephalum* and 4 °C for *Zoarces viviparus*. For the calibration at 0% oxygen, the beaker was closed to ambient air and nitrogen was bubbled through the water for at least 10 minutes. The calibration was complete when the oxygen content stopped decreasing and remained stable for several minutes.

For three days, the fish were allowed to acclimatize to their new environment at their species-specific acclimatization temperature in the dark in order to keep stress to a minimum. On the fourth day, an

intermittent-flow protocol was started with a measurement period (closed chamber) of one hour (*Z. viviparus*) and two hours (*P. brachycephalum*), respectively. During this time, oxygen consumption was measured (Software: OxyView, PreSens GmbH, Germany) and water samples were taken at the beginning and end of the measurement. This measurement was repeated 3 (*P. brachycephalum*) to 5 (*Z. viviparus*) times for each individual (n=5). Thereafter, the water temperature was increased by +2 °C day^{-1} (*P. brachycephalum*) or +3 °C day^{-1} (*Z. viviparus*) every day at 3 pm. At each temperature step (*P. brachycephalum*: 0, 2, 4, 6, 8 and 10°C; *Z. viviparus*: 4, 7, 10, 13, 16, 19 and 22°C), we recalibrated the oxygen sensors and shortly before reaching the species-specific lethal temperature, the experiment was stopped (10 °C for *P. brachycephalum*; 22 °C; for *Z. viviparus*) and the animals were killed by a blow on the head and subsequent severing of the spinal cord close behind the head.

The water samples (2 ml) taken from each chamber during the oxygen measurement were quickly frozen at -80 °C and the ammonia content was then measured photometrically (630 nm; Speedcord S 600, Analytik Jena, Germany) according to the phenol-hypochlorite method (Solórzano, 1969). In brief, 500µl of the water sample were mixed with 750µl H_2O and 100µl phenol solution (the stock solution consists of 0.85M phenol and 2mM nitroprusside sodium dihydrate dissolved in 30% ethanol: H_2O solution). After 2 minutes, 50µl of citrate buffer (stock solution consisting of 40M tri-sodium citrate dissolved H_2O) and dichloroisocyanuric acid (DTT) solution (stock solution consisting of 13mM dichloroisocyanuric acid dissolved in 2M NaOH solution) were added and mixed. The solution was then incubated for 1 hour at 40°C in a thermomixer (400-500rpm) and measured photometrically at 630nm.

Oxygen consumption (atomic O) (calculated after Boutilier et al., 1984) and nitrogen excretion (atomic N) were then used to calculate the atomic O:N ratio as followed:

$$O : N = MO_2\ NH_4^{+-1}$$

The O:N ratio is an indicator of the composition of lipid and proteins being metabolized e.g. during acute warming (Mayzaud and Conover, 1988).

Measurement of protein synthesis rate in gills, hematocrit and spleen weight

The Antarctic eelpout acclimated to 0 °C were exposed to an acute temperature increase of +2 °C day^{-1} until 10 °C and the common eelpout acclimated to 4 °C were warmed by +3 °C day^{-1} until reaching 22 °C. At each temperature step (*P. brachycephalum*: 0, 2, 4, 6, 8 and 10°C; *Z. viviparus*: 4, 7, 10, 13, 16 and 22°C), individuals of both species (*P. brachycephalum*: n= 8; *Z. viviparus*: n= 6) were weighed and injected with 0.7 mL/100 g body weight of 75 mM of $^{13}C_9H_{11}{}^{15}N_1O_2$ phenylalanine (Sigma Aldrich, USA) in PBS buffer (pH 7.4). Exactly after 1.5 h and 3 h one fish of each species was sacrificed. First, the fish were anesthetized with a blow on the head and blood samples were taken, from which 100 µl of blood was separated and placed on ice for 10 minutes before centrifugation at 3000G for 10 minutes. Subsequently, the ratio of the light supernatant (blood serum) to the pellet was determined as hematocrit. The fish were then killed by severing the spinal cord just behind the head before gill and spleen tissues were removed and quickly frozen in liquid nitrogen and stored at -80 °C until further use. The frozen spleen was weighed and then the ratio of body weight to spleen weight was calculated.

Protein synthesis rate was measured as described in Krebs et al., 2023 a. In brief, 20 mg of gill tissue were homogenized and extracted by methanol chloroform extraction. With this method, the homogenate was separated into the aqueous cytosolic fraction containing the free labeled and unlabeled phenylalanine and the protein fraction containing the labeled and unlabeled phenylalanine bound in proteins. Subsequently, the content of labeled and unlabeled phenylalanine in the protein fraction (after hydrolysis) and in the cytosolic fraction was measured separately by liquid chromatography and high-resolution mass spectrometry (LC-HRMS/MS).

Concentrations of protein-bound and free phenylalanine were calculated using an internal standard (^{15}N-Phenylalanine: $^{12}C_9H_{11}{}^{15}N_1O_2$) and a calibration curve for each analyte (labeled phenylalanine: $^{13}C_9H_{11}{}^{15}N_1O_2$; unlabeled phenylalanine: $^{12}C_9H_{11}{}^{14}N_1O_2$). Outliers (identified by inter-quartile range IQR with the software: Microsoft Excel) were eliminated. Ks was calculated as before (Garlick et al., 1980; Krebs et al., 2023b):

$$\text{Ks (\% day}^{-1}\text{)} = \left(\frac{\text{Sb labeled } [\frac{\text{pg}}{\mu\text{g}}]}{(\text{Sb labeled+Sb unlabeled})[\frac{\text{pg}}{\mu\text{g}}]}\right) * \left(\frac{100}{(\text{Sa labeled}[\%]) * \text{t (days)}}\right) * 100$$

with Sb as the protein-bound pool and Sa as the free pool of phenylalanine (pg phenylalanine per µg fresh weight) and t as time in days. Labeled phenylalanine describes the injected $^{13}C_9H_{11}{}^{15}N_1O_2$ phenylalanine and unlabeled the naturally found $^{12}C_9H_{11}{}^{14}N_1O_2$ phenylalanine.

Statistical analyses

All data were analyzed using the program GraphPad Prism 9.5.1 and were normally (tested with Shapiro-Wilk test) and homogeneously distributed (chi square test). Statistical differences within one species at the level of 95% were tested by using an ordinary one-way ANOVA (analysis of variance) for non-repeated measurements (protein synthesis rate, hematocrit, spleen weight) and a mixed-effect analysis for repeated measurements (ammonia excretion and O:N ratio) followed by Tukey's multiple post hoc comparison test. Statistical differences between species at the same temperature were tested at the level of 95% by using an unpaired t-test. Unless otherwise stated, data are visualized as mean ± standard deviation in the figures.

Results

Oxygen consumption increased exponentially with temperature in both species at Q_{10} values of 2.46 in *P. brachycephalum* and 1.91 in *Z. viviparus* (Figure 1). Differences between the two species were not significant when oxygen consumption was measured at the same temperatures (4 and 10 °C).

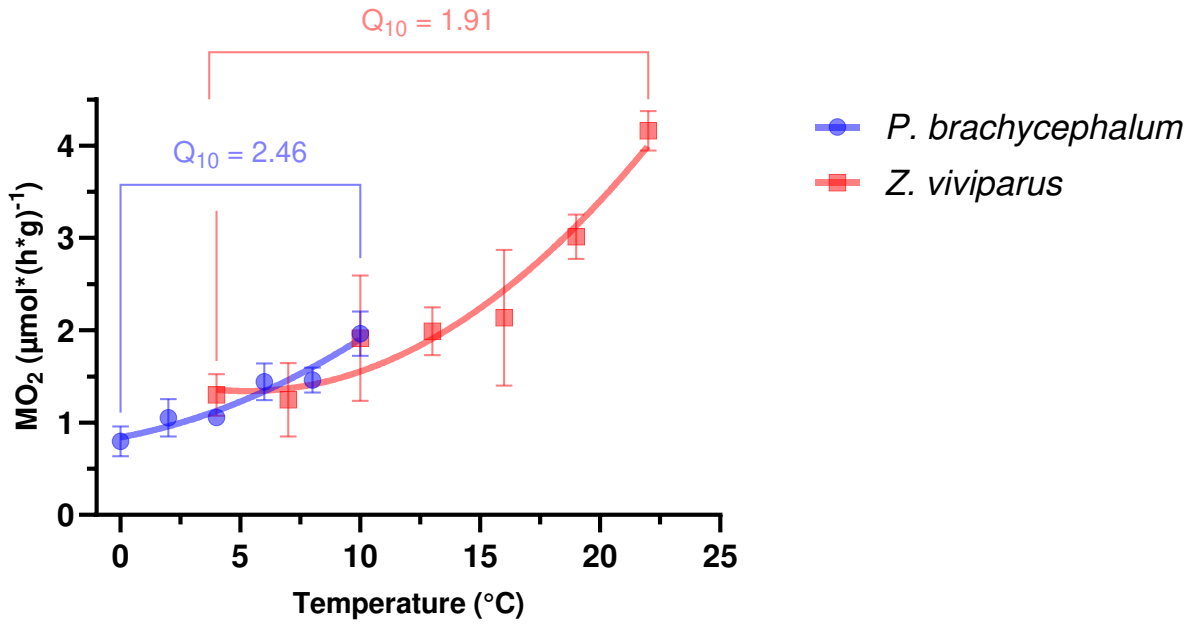

Figure 1: Oxygen consumption during acute warming in *Pachycara brachycephalum* and *Zoarces viviparus*. Oxygen consumption (MO_2) increased exponentially ($Y= 0.005824x^2 + 0.04821x + 0.8402$; $R_2=0.8096$) in *P. brachycephalum* (blue, n = 4) within a temperature range of 0 to 10°C with a Q_{10} of 2.46. In *Z. viviparus* (red, n = 4), MO_2 also increased exponentially ($Y= 0.009503x^2 - 0.1002x + 1.605$; $R_2=0.8208$), measured between 4 and 22 °C with a Q_{10} of 1.91.

Ammonia excretion did not change significantly in *P. brachycephalum* between 0 and 8 °C, but it increased significantly at 10 °C (p-value < 0.05), while the O:N ratio did not change significantly with increasing temperature (Figure 2).

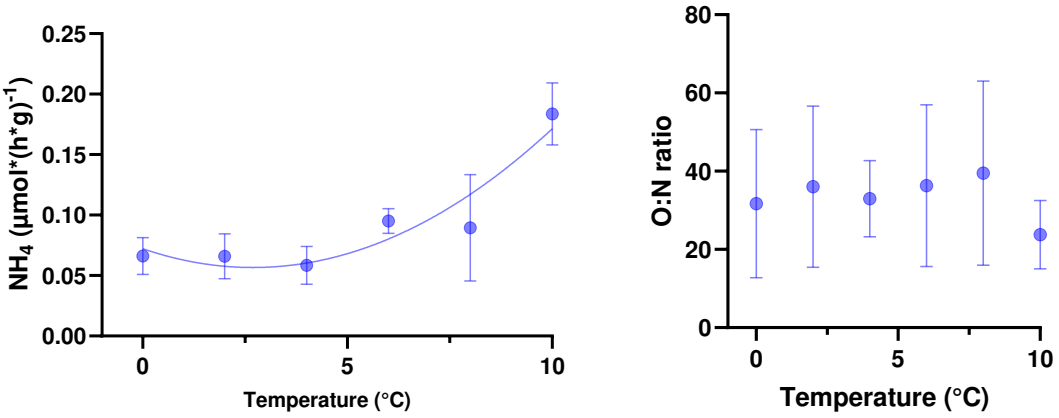

Figure 2: Ammonia excretion (NH_4) and O:N ratio of *P. brachycephalum* during acute warming. The ammonia excretion increased exponentially between 0 and 10°C ($y = 0.072x^2 – 0.012x + 0.002$, $R_2 = 0.7116$) (left). The O:N ratio (right) did not change significantly during acute warming.

During acute warming, hematocrit in *P. brachycephalum* (0 to 10 °C) and in *Z. viviparus* (4 to 22 °C) did not change significantly in both species (Figure 3). However, at 4 °C, the hematocrit of *Z. viviparus* was significantly higher than in *P. brachycephalum*, while at 10 °C no significant difference was detectable between species.

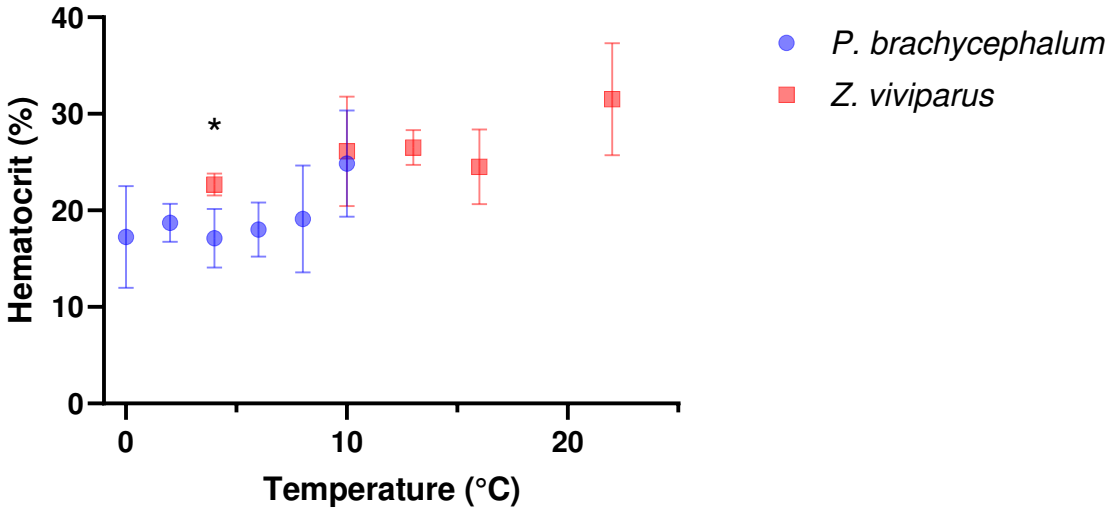

Figure 3. Changes of hematocrit in *P. brachycephalum* and *Z. viviparus* during acute warming. The hematocrit was determined in *P. brachycephalum* (blue, n = 4 (0, 8 °C), n = 5 (2, 4 °C), n = 6 (6, 10 °C)) between 0 and 10 °C without significant differences, albeit with a trend to differ between 0 and 10 °C (p-value < 0.1). For *Z. viviparus* (red, n = 3 (4, 13 °C), n = 4 (16 °C), n = 5 (10, 22 °C)) the hematocrit did not change significantly between 4 and 22 °C. Comparing both species at the same temperature, *Z. viviparus* had a higher hematocrit (* p-value < 0.05) than *P. brachycephalum* at 4 °C, but at 10 °C there were no significant differences between the species. Both species showed a trend towards increased hematocrit at upper thermal limits.

In addition, the percentage of the spleen to body weight was determined to identify possible acute releases of erythrocytes from the main storage tissue. Acute warming did not affect spleen weight in *P. brachycephalum* between 0 and 10 °C or in *Z. viviparus* between 4 and 22 °C. However, the percentage of spleen weight to body weight was significantly higher in *P. brachycephalum* than in *Z. viviparus*.

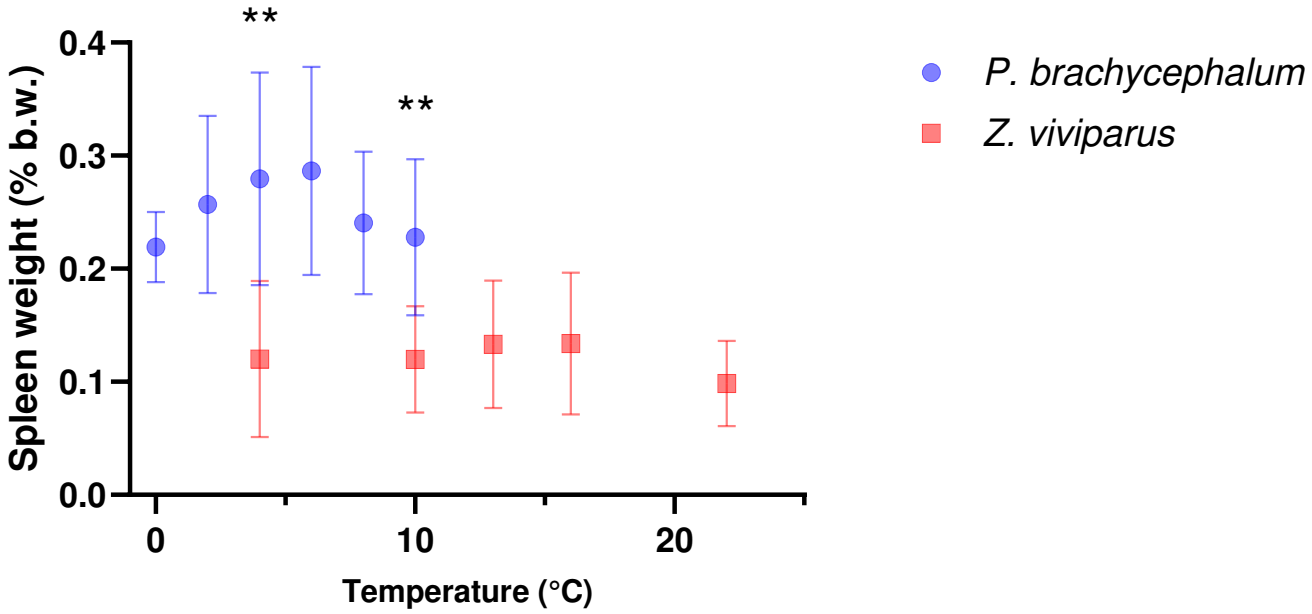

Figure 4. Percentage of spleen weight to body weight (% b.w.) during acute warming in *P. brachycephalum* and *Z. viviparus*. In *P. brachycephalum* (blue, n = 7 (0, 8, 10 °C), n = 8 (2, 4, 6 °C)), the percentage of spleen to body weight did not change significantly between 0 and 10 °C, and in *Z. viviparus* (red, n = 6) the percentage of spleen to body weight did not change between 4 and 22 °C either. However, the percentage of spleen to body weight was significantly higher in *P. brachycephalum* than in *Z. viviparus* (** p- value < 0.01).

The protein synthesis (Ks) in the gills increased significantly in both species during acute warming. In both fish, Ks increased exponentially in gills (*P. brachycephalum* measured between 0 and 10°C: $Y= 0.003762x^2 + 0.2859x + 1.114$; *Z. viviparus* measured between 4 and 22°C: $Y= 0.04971x^2 - 0.5923x + 4.224$). The measured protein synthesis rate at the same temperatures (4 and 10 °C) did not differ significantly between the two species.

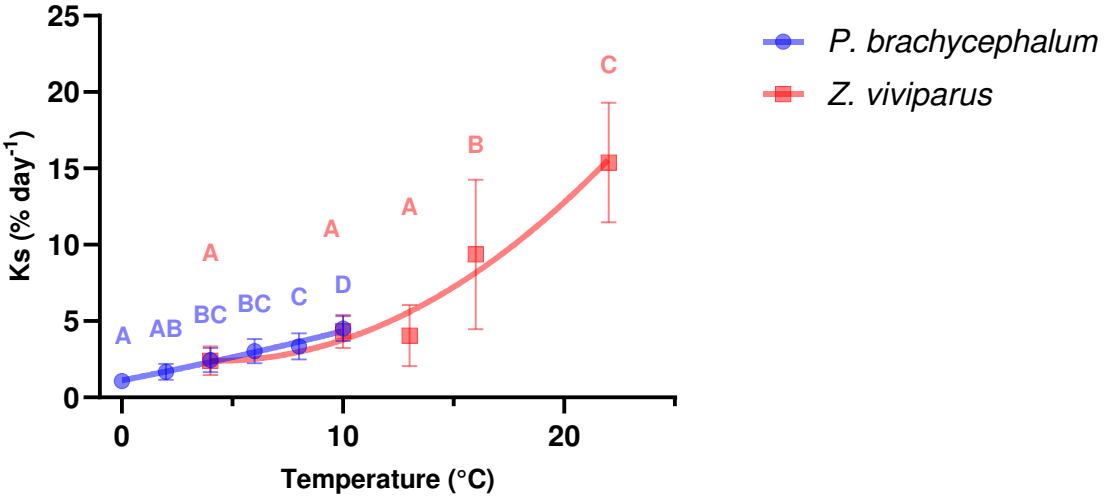

Figure 5. Protein synthesis (Ks) in gills of *P. brachycephalum* and *Z. viviparus* during acute warming. The protein synthesis rate in gills increased in *P. brachycephalum* (blue, n = 7 (0, 10 °C), n = 8 (2, 4, 6, 8 °C)) between 0 and 10 °C ($Y= 0.003762x^2 + 0.2859x + 1.114$, R_2=0.7126) with a Q_{10} of 4.2. In *Z. viviparus* (red, n = 6), the protein synthesis rate in gills increased exponentially ($Y= 0.04971x^2 - 0.5923x + 4.224$, R_2=0.8399) between 4 and 22 °C with a Q_{10} value of 2.8. Different letters indicate significant differences with a p-value < 0.05.

Discussion

Metabolic rate

Upon warming, oxygen consumption (MO_2) increased exponentially in both species with a Q_{10} value of 2.4 between 0 and 10 °C for *P. brachycephalum* and 1.9 between 4 and 22 °C for *Z. viviparus*. This is in line with previous studies that examined Q_{10} values for MO_2 in *P. brachycephalum*. Depending on the warming protocol used, Q_{10} ranged from 2.63 (Mark et al., 2002, 1°C every 12h between 0 and 12°C) to 5.2 (van Dijk et al., 1999, 1°C day^{-1} between 0 and 9°C) as a result of acute warming. After an acclimation period of 3 months to 6°C, MO_2 was back to control level (0°C) and thus fully compensated (Brodte et al., 2006). *P. brachycephalum* can metabolically compensate for slightly elevated temperatures, but the energy turnover may change and be channeled towards maintenance rather than to growth (Brodte et al., 2006; Windisch et al., 2014). In *Z. viviparus*, the Q_{10} value of oxygen consumption in another study varied between 2.4 and 3 during acute warming at a rate of +3 °C per day, depending on previous acclimation temperatures (Van Dijk et al., 1999a; Q_{10} of 2.4 after long term cold acclimation between 0 and 24 °C; Q_{10} of 3 between 3 and 24 °C). Both Q_{10} values of oxygen consumption are in the range of 2 and 3, which is a normal response to increasing temperatures according to thermodynamics. However, the calculated Q_{10} values in the present study were slightly lower than in previous studies, but this may be explained by a smaller temperature range in which MO_2 was measured, as MO_2 increased exponentially. Despite generally lower Q_{10} values in both species compared to previous studies, it is higher in *P. brachycephalum* compared to *Z. viviparus*. In addition, the thermal window is narrower in *P. brachycephalum*, which is consistent with the concept of oxygen and capacity-limited thermal tolerance (OCLTT), since *P. brachycephalum*, being a stenothermal fish, has a lower capacity for oxygen.

Hematocrit

The hematocrit measured during acute warming in both species showed no significant temperature-dependent differences. A previous study carried out by Windisch et al., found increasing hematocrit values above 6°C after 3 months of acclimation (Windisch et al., 2014). In other Antarctic fish, hematocrit varied between 20 and 40 % depending on the fish species and increased during acute warming experiments (Beers and Sidell, 2011; Joyce et al., 2018), whereas the hematocrit remained unchanged in Antarctic fish acclimatized to elevated temperature (Strobel et al., 2012).

Although we did not find a significant temperature dependence of hematocrit in both species, the hematocrit value of *Z. viviparus* was significantly higher at 4°C compared to *P. brachycephalum*. A higher hematocrit supports higher oxygen transport capacities and may indicate a higher need for oxygen. However, the routine oxygen consumption rate did not differ between both species at a common temperature, thus other reasons might cause the difference in hematocrit. Larger amounts of erythrocytes increase blood viscosity and may thereby limit cold tolerance. A temperature of 0°C increased blood viscosity by 40 % over that at 10 °C in *Trematomus bernacchii* (Axelsson, 2005). The lower hematocrit in *P. brachycephalum* may be part of an energy saving strategy in the cold, at the expense of a stronger exponential increase in oxygen demand at warmer temperatures when increased oxygen demand depends on higher circulatory activity than with a higher hematocrit. However, the heart of Antarctic fish as a low pressure but high-volume pump (Tota et al., 1997), may contribute to setting limiting temperatures to lower values, being pre-adapted to a lower energy turnover mode of lifestyle. Various mechanisms thereby contribute to the narrower thermal range of the cold-adapted Antarctic fish.

We also examined the weight of the spleen, since there might be a correlation between spleen weight and hematocrit, as the spleen stores erythrocytes. There were no temperature-dependent changes in

spleen weight, but *P. brachycephalum* had a generally higher spleen weight compared to its size. In other Antarctic fish (*Pagothenia borchgrevinki*), it has been shown that a larger spleen (varying between 0.2 and 0.8% b.w.) is beneficial and can quickly supply the fish with erythrocytes, supporting intensive exercise by increasing the oxygen carrying capacity (Brijs et al., 2020). In temperate fish, spleen weights of about 0.1% b.w seem to be more common as they maintain a higher hematocrit to begin with (e.g. *Salmo salar* (Gamperl et al., 2020) or *Dicentrarchus labrax* (Islam et al., 2020)).

Ammonia excretion

In this study, we additionally investigated the effects of temperature on ammonia excretion. The amount of excreted ammonia was lowest at the thermal optimum of *P. brachycephalum* and highest near its upper thermal limit. Together with the oxygen consumption rate, the O:N ratio can be calculated. The O:N ratio, an indicator of the contribution of protein metabolism, did not change significantly. On average, O:N ratios between 0 and 8 °C varied between 30 and 45, and at 10 °C it was 24. Values between 3 and 16 indicate the use of proteins as the main substrate for metabolism, while values between 50 and 60 indicate a mixture of proteins and lipids (Mayzaud and Conover, 1988). These results suggest a more protein-based metabolism in *P. brachycephalum*, especially at 10 °C with the lowest O:N ratio. In other Antarctic ectotherms, the O:N ratio also indicates a predominantly protein-based metabolism (e.g. Clarke and Prothero-Thomas, 1997; Fraser et al., 2002; Obermüller et al., 2010). Within the same experiment, we found high protein turnover in white muscle, which would explain a more protein-based metabolism in white muscle (Krebs et al., 2023a).

Protein synthesis

Since both oxygen consumption and ammonia excretion occur via the gills, we studied the protein synthesis rate to gain more insight into the involvement of the gills in thermal responsiveness. The rate of protein synthesis in gills increased in both species during acute warming, with Q_{10} being higher in *P. brachycephalum* (4.2) than in *Z. viviparus* (2.8). There were no differences between species in their gill protein synthesis rates at the same temperature. In contrast, a recent study revealed that the protein synthesis rate in white muscle changed during acute warming only in *Z. viviparus*, but not in *P. brachycephalum*. However, white muscle protein synthesis was highly cold-compensated in *P. brachycephalum* and up to three times higher than in the temperate eelpout (Krebs et al. 2023a). The difference in the reaction to acute warming might be related to the different functions of the tissues. White muscle is mainly involved in movement and most of its protein synthesis rate results in growth, while the gills are involved in many processes and most proteins synthesized concern collagen, mucus glycoproteins and musculoskeletal structure and their functions, or are involved in epithelial cell replacement, chloride cell turnover, etc. (Houlihan et al., 1986, Lyndon and Houlihan, 1998). The rate of protein synthesis in muscle can be downregulated at high temperatures (*Z. viviparus*) or remain unchanged during acute warming (*P. brachycephalum*). The latter may balance growth in order save energy (Krebs et al., 2023a), but this is not the case in the gills. Adjustments of its homeostatic function to temperature dependent demand may require increased protein expression activity (this study). This picture is supported by another study which found a decrease of gene transcripts associated with the cell cycle and cell proliferation to conserve energy in white muscle after acute warming, while in gills gene expression important for the cytoskeleton and heat shock proteins are upregulated (Buckley et al., 2006; Logan and Buckley, 2015).

Conclusion

Overall, this study complements the evolving picture of the differences in thermal adaptation between stenothermal and eurythermal fish below their upper critical temperatures. The OCLTT concept postulates that oxygen supply capacity in relation to demand limits thermal tolerance. Increased oxygen consumption during acute warming mirrors energy demand which was similar in both fish species at 4 and 10°C. Accordingly, in both fish the rate of protein synthesis in the gills also increased exponentially. Therefore, the energy cost of protein synthesis in the gills was presumably similar in both fish species when measured at the same temperature.

This is striking considering that aerobic performance, measured as protein synthesis rate during acute warming (Krebs et al., 2023b), and growth performance after long-term acclimation (Brodte et al., 2006b) were very different between the two-eelpout species. At 4°C, the protein synthesis rate in white muscle was 3 times higher in *P. brachycephalum* compared to *Z. viviparus* (Krebs et al., 2023b). Since white muscle makes up a large part of the fish body, changes in the rate of protein synthesis are likely to have an effect on the overall energy budget and thus oxygen consumption. However, as this is not the case, it appears that *P. brachycephalum* is more energetically efficient in protein synthesis at 4 °C than *Z. viviparus*. In line with this, growth rates after long-term acclimation at 4 °C were significantly higher in *P. brachycephalum* than in *Z. viviparus*, but there was also no significant difference in oxygen consumption (Brodte et al., 2006b). At 10 °C, the protein synthesis rate in the white muscle of *P. brachycephalum* was still higher than that of *Z. viviparus*, but only by a factor of 2, while oxygen consumption did not differ between the two-eelpout species. Long-term acclimation to temperatures above 7 °C leads to negative growth rates in *P. brachycephalum* (Windisch et al., 2014), while a temperature between 12 and 15 °C represents the thermal optimum of *Z. viviparus* (Brodte et al., 2006b; Fonds et al., 1989; Pörtner and Knust, 2007).

The high protein synthesis of the white muscle and the high growth at low temperatures in *P. brachycephalum* are the result of an evolutionary adaptation to the constantly cold Southern Ocean. Another adaptation to cold is a higher spleen weight and lower hematocrit in *P. brachycephalum* compared to its counterpart *Z. viviparus*, indicating cold compensation for the lower blood viscosity in cold conditions and a larger reserve of erythrocytes to be released when needed. Although this adaptation clearly saves energy, it cannot fully explain the higher protein synthesis and growth at low temperatures found in *P. brachycephalum* with the same energy expenditure compared to *Z. viviparus*. Future studies would need to investigate further mechanism which might help *P. brachycephalum* to compensate for life in the constantly cold Southern Ocean.

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4 Discussion

4.1 The protein synthesis rate measured in white muscle tissue of slow-growing Antarctic eelpout in vivo is comparable with previously published growth data.

This is the first study in which protein synthesis in white muscle tissue of stenothermal Antarctic fish was measured *in vivo* using isotopically labelled tracers instead of the commonly used radioactive tracers. Radioactive tracers provide a sensitive and accurate measure, which is particularly necessary for organisms with low protein synthesis rates (Smith and Haschemeyer, 1980; Fraser et al., 2022). However, the sensitive high-resolution tandem mass spectrometry (LC-HRMS/MS) provides a precise detection of the isotopically labeled tracer, which in turn, allows determining a protein synthesis rate as low as 0.05% day^{-1} in the white muscle of *P. brachycephalum*. This method has the advantage to avoid the use of hazardous radioactively-labelled tracers and can thus be easily applied in the field and on research vessels in remote areas such as the Southern Ocean.

The protein synthesis rate in white muscle is of particular interest because it provides a measure of the *in vivo* growth rates of fish. Houlihan et al., 1986 showed that up to 79% of the protein synthesis in white muscle contributes to growth in fish (*Oncorhynchus mykiss*). In order to validate whether the protein synthesis measured here reflects growth rates, previously published growth data by Brodte et al., 2006a were compared with the protein synthesis rate measured in white muscle at 0°C. Growth rates vary with age and are lower in older fish (Lugert et al., 2016). However, the study compared used similar size fish (Brodte et al., 2006a: 20.1 cm ± 2.6; my study: 22.6 ± 2.5 cm) which allowed me to estimate that 72-96% of the protein synthesis rate in white muscle contributes into growth.

The protein synthesis rate in the white muscle of *P. brachycephalum* was lower than found in most other studies e.g. in adult temperate fish (*O. mykiss* Ks= 0.5% day^{-1}). Protein synthesis rates as low as in the first experiment of my study (publication 1) have previously been published by Smith and Haschemeyer (1980). The authors measured protein synthesis in white muscle of different Antarctic fish species to investigate the influence of different starvation periods. After 5 days of starvation, protein synthesis was reduced to 1/3 in *Trematomus bernacchii* (from 0.23% day^{-1} to 0.07% day^{-1}) and after 15 days of starvation to about 1/5 in *Trematomus hansoni* (from 0.22% day^{-1} to 0.04% day^{-1}) (Smith and Haschemeyer, 1980). This example clearly demonstrates the relationship between food supply and protein synthesis. Protein synthesis requires a lot of energy and high rates only if sufficient energy is available. The conversion of food into protein increases the fish's energy turnover, measured as the specific dynamic action (SDA). In crustaceans, the total protein synthesis rate is highest at the peak of SDA (Whiteley et al.,2000). There is no data available for the SDA of *P. brachycephalum*, but in other Antarctic fishes the SDA peak was reached after about 2 days and the duration of SDA varied between 8 and 16 days (Boyce and Clarke, 1997, Johnston and Battram, 1992). In experiment 1, the fish were not fed for 5-7 days before the start of the experiment and were likely past the SDA peak with less nutrient supply to protein synthesis.

In the second experiment, protein synthesis in white muscle was also measured after the food had been withheld for 5-7 days. However, the white muscle protein synthesis rate measured at a common temperature of 0°C was 10 times higher (0.47 ± 0.24 % day^{-1}) than in the first experiment. The aim of the first experiment was to establish the method of isotopically labeling the Antarctic fish *P. brachycephalum*. The aim of the second experiment was to investigate the protein synthesis rate during acute warming. Since nutrient availability is crucial for protein synthesis, the food supply was increased from once a week (1st experiment) to 2-3 times a week for a duration of 3-6 weeks (2nd experiment). These fish may thus be better fed than in the first experiment although, prior to injection of the isotope-labelled tracer, all fish in the second experiment were also deprived of food for 5-7 days.

This variability of protein synthesis rate in white muscle might be explained by the fish's ecology. In the wild, food availability changes around Antarctica with the seasons. While a large variety of food is available in summer, its abundance decreases by 68 % in winter (Atkinson and Peck, 1988). This low food supply during winter resulted in negative growth (up to -0.05% weight gain day^{-1}) in juvenile *Notothenia coriiceps*. In summer, growth rates are 5 times higher (up to 0.25% weight gain day^{-1}) (Coggan, 1997). The authors discussed various reason that might lead to increasing growth rates during summer including higher prey abundance. Further reasons include higher temperatures (+2°C), that increase the appetite of fish and a longer photoperiod, which makes hunting easier for the predator *N. coriiceps*, as it uses the optical sense (Coggan, 1997).

P. brachycephalum is most common in the Southern Ocean at depths of about 400 m. Neither temperature nor light conditions change much at that depth. Furthermore, all experiments described in this thesis were carried out under controlled laboratory conditions and all parameters remained unchanged except for the different feeding rates. Therefore, this study postulates, that the feeding impulse before the starvation period alone increased the protein synthesis rate in white muscle of *P. brachycephalum*.

In summary, the comparison of previous growth data (Brodte et al., 2006a) and protein synthesis data revealed that protein synthesis in white muscle can be used as proxy to estimate growth in Antarctic fish. This study further demonstrates that high feeding rates increase the protein synthesis rate in white muscle tenfold whereas in other studies, growth rates in Antarctic fish only increase fivefold (Coggan, 1997). Only part of the protein synthesis rates contributes to growth and another part to general cell maintenance. The muscle can also act as a protein store, especially during periods of starvation or reproduction (Nemova et al., 2016). During starvation periods, the protein synthesis is reduced (Haschemeyer and Smith, 1980). Therefore, the acute protein synthesis rate in white muscle reveals information about the feeding state in the previous past, emphasizing the suitability of this proxy as an ecologically important performance parameter. The protein synthesis rate can therefore serve as an additional parameter to the parameters usually determined for an assessment of fitness such as the hepatosomatic index (HSI). The 3-6 weeks feeding pulse given prior to the second experiment doubled the HSI in *P. brachycephalum* (Supplementary, Figure S1). Liver is used to store lipids in many fish species including *P. brachycephalum* (Windisch et al., 2014). HSI analyses are likely

to be less sensitive compared to protein synthesis measurements since it would take longer to measure changes in liver weight. The rate of protein synthesis increases within hours or days of feeding, depending on the species (Whiteley et al.,2000, Haschemeyer and Smith, 1980). In the future, it could be promising to use the protein synthesis rate together with other proxies such as the HSI in the field to analyze the nutritional status of an organism.

4.2 During acute warming, protein synthesis mirrors species specific and temperature-dependent growth rates

Temperature-dependent growth analyses of both eelpout species were published by Brodte et al. (2006a) (Figure 6), who found a growth maximum for *P. brachycephalum* at 4°C and for *Z. viviparus* at 12°C. Similarly, Windisch et al. (2014) found a temperature-dependent growth maximum for *P. brachycephalum* at 3°C and both Fonds et al. (1989) and Pörtner and Knust (2007) found the growth maximum for *Z. viviparus* at 15°C.

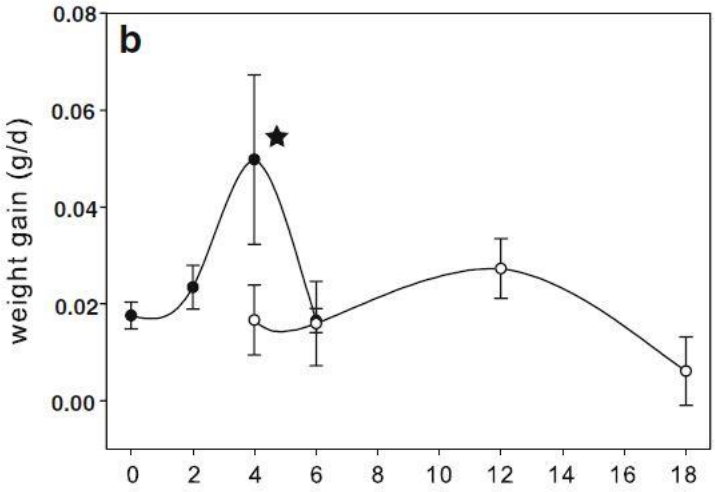

Figure 6. Temperature-dependent growth data published by Brodte et al. (2006). The growth rate of *P. brachycephalum* (filled circles) was at 0, 2, and 6 °C about 0.02 g/d, but highest at 4°C with a weight gain of ~0.05 g/d. In *Z. viviparus* (open circles), the weight gain per day was generally lower with a maximum at 12°C (Brodte et al., 2006a).

The growth data published by Brodte et al., 2006 (figure 6) were used as a basis for quantifying temperature-dependent growth rates and compared to the protein synthesis rate found in white muscle (Publication 2). In this study, similar temperature steps were used for both species to allow for a better comparison. In *P. brachycephalum*, the protein synthesis rate in white muscle did not change during acute warming but averaged at 0.5 ± 0.25% day^{-1} between 0 and 10°C. This is different to previously measured growth rates, which were strongly influenced by temperature (Figure 6, Brodte et al., 2006a). Growth rates are investigated in fish acclimated to a certain temperature for several weeks to months. During this acclimation period, several processes within the fish change in order to cope with the new temperature. At the end of thermal acclimation, the fish is in a steady state and all changes to the processes have been completed. These changes are not hereditary and are reversible in the course of an organism's life (reviewed in Lagerspetz, 2006). However, while thermal adaptation is crucial for an organism's survival, it can result in several tradeoffs such as reduced growth (Pörtner

et al., 2006). An explanation for reduced growth rate at temperatures outside the species specific thermal optimum can be found in the concept of oxygen and capacity limited thermal tolerance (OCLTT) (Pörtner, 2021). The concept of OCLTT focuses on mechanisms that define thermal limits of organism's on the capacity for oxygen supply in relation to its oxygen demand (Pörtner et al., 2017). Oxygen is significantly involved in energy production, thus an imbalance between oxygen supply and demand at temperatures outside the thermal optimum leads to insufficient energy production and cause a reduction in the growth of the fish (Pörtner and Knust, 2007). Another important factor for growth is an adequate supply of nutrients, which are usually ingested through food. In the study by Brodte et al., (2006a), the amount of food consumed did not change within the analyzed thermal window, but the allocation of the energy supplied with the food did. At the thermal optimum of *P. brachycephalum* (4°C), the percentage of energy invested in growth is highest and less energy is required for metabolic processes. At a higher temperature (6°C), the energy expenditure for growth is lower and more energy is required for the metabolism of the fish. This change in energy distribution over several weeks can then be measured in different growth rates.

In contrast, the protein synthesis rate in white muscle described the increase of proteins between the injection of the labelled tracer and the tissue sampling (in this study 1.5 and 3 h). This is completely different from growth measurements, since it is not the summary of several processes that lead to growth changes, but acute changes in protein synthesis, some of which can result in growth. Therefore, several reasons can cause differences between protein synthesis rate and growth at different temperatures. On the one hand, protein synthesis in white muscle varied greatly between individuals, which could mask possible differences between temperature steps. All the fish were kept and fed together, it is likely that the stronger fish received more food than the weaker fish, causing the difference in protein synthesis rate. On the other hand, the rate of protein synthesis in white muscle during acute warming is not limited by oxygen and therefore can produce enough energy to continue protein synthesis regardless of temperature.

It is important to distinguish between the long-term effects of warming, as observed in the growth experiment by Brodte et al. (2006a), and acute warming, as described in this study. As an Antarctic fish, *P. brachycephalum* is adapted to very cold temperatures with only slight temperature fluctuations all year round (Figure 7). Usually, *P. brachycephalum* lives at depths of around 400 m, where the temperature hardly changes over the course of the year, even less than at the sea surface, as shown in Figure 7. In order to survive and grow in these cold waters, a high degree of adaptation and compensation is required. In addition, in the wild, *P. brachycephalum* are no active hunters but scavengers that feed on occasion. This means that there are only limited periods when food is highly abundant. Although life in the Southern Ocean clearly has an effect on the long-term growth rate in *P. brachycephalum*, it could also explain the results of the protein synthesis rates measured here. While *P. brachycephalum* is not confronted with large temperature fluctuations in the wild, it often has to contend with a shortage of nutrients since, for example, there is not much food available in winter. *P. brachycephalum* can only increase protein synthesis in the white muscle and thus grow in

times of high food supply. Hence, this study suggests that protein synthesis in the white muscle of Antarctic eelpout, and finally growth rates, depends on food availability rather than temperature during acute warming. Whether or not the protein synthesis rate of white muscle changes as a function of temperature in a similar way to growth measurements would have to be investigated in future studies.

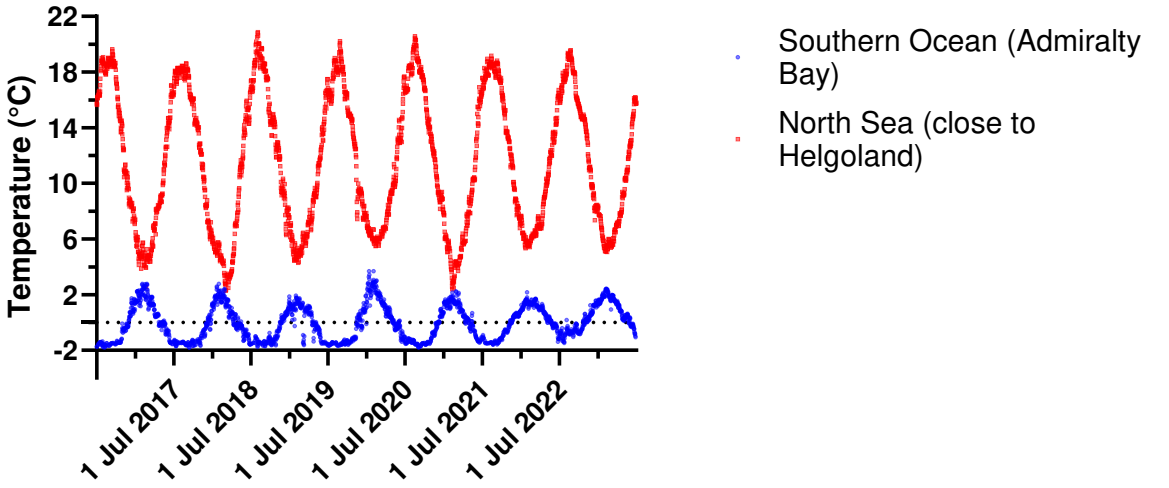

Figure 7. Sea surface temperature at Admiralty Bay in the Southern Ocean (-62.125; -58.375, blue) and in the North Sea off Helgoland (54.125; 7.875, red). The data are taken from the Server of NOAA using the data set: ncdcOisst21Agg_LonPM180. The temperature was measured daily at 12 pm and provides an accuracy of 0.25° (Huang et al., 2020).

In contrast to *P. brachycephalum*, the protein synthesis rate in the white muscle of *Z. viviparus* increased between 4°C and 16°C (4°C Ks= 0.15% day^{-1}; 16°C Ks= 0.38% day^{-1}) with a Q_{10} value of 2.2. This process follows the thermodynamic rule of faster reactions in the warmth. At 22°C the protein synthesis rate in white muscle decreased slightly (Ks= 0.31% day^{-1}) forming a bell-shaped curve. With the maximum protein synthesis rate found close to 16°C, the protein synthesis rate in white muscle responded to acute warming in similar ways as the *in vivo* growth rate of acclimated *Z. viviparus* which displayed a maximum between 12 and 15°C (Brodte et al., 2006a; Fonds et al., 1989; Pörtner and Knust, 2007). The protein synthesis rate does not increase further between 16 and 22 °C, which is likely due to the limitation of oxygen (Publication 2).

Protein synthesis consumes large amounts of energy, and thus oxygen. Therefore, a limitation of oxygen due to increasing thermal stress is likely causing a suppressing of protein synthesis in the white muscle to reduce the muscle's energy requirements. Restriction of oxygen due to thermal stress can lead to an insufficient energy supply, and only the most important processes are maintained in order to survive. In another study conducted on eurythermal fish, acute warming decreased the expression of genes related to cell proliferation in white muscle. At the same time, the expression of genes involved in thermal stress and cytoskeletal components increased (Buckley et al., 2006).

In the North Sea, the water temperature fluctuates between seasons (Figure 7) and *Z. viviparus* is adapted to these changes. The common eelpouts used in this experiment were caught in a period from

summer to autumn and in the incubations, the temperature was slowly lowered to 4°C to simulate winter conditions. After more than three months at 4°C, the acute warming experiment started in late spring (April-May) to maintain the natural seasonality of the fish. Although the temperature was increased faster than in the field, the temperatures were within the temperature fluctuations found in the natural environment (figure 7). Thus, *Z. viviparus* is adapted to seasonal temperature change and increases protein synthesis with temperature.

The response of protein synthesis to temperature in eurythermal and stenothermal fish has been investigated in earlier studies. In line with present findings, the protein synthesis capacity measured *in vitro* did not change with temperature in the muscle of *P. brachycephalum* (0°C Ks= 3.5% day^{-1}; 5°C Ks=3.6% day^{-1}), however, it varied in *Z. viviparus* (5°C Ks= 3.1% day^{-1}; 10°C Ks= 5.3% day^{-1}) (Storch et al., 2005). *In vitro* measurements quantify the maximum capacity of the protein synthesis pathway at saturated substrate concentrations, thus explaining the much higher Ks values found in both eelpouts by Storch et al. (2005) compared to the *in vivo* protein synthesis rates seen in the present study. Fraser et al. (2022) also investigated the protein synthesis rate *in vivo* in the stenothermal Antarctic fish *Harpagifer antarcticus.* They found no change in whole body protein synthesis rate after 28 days of acclimation to different temperatures, but a linear increase with temperature in the eurythermal fish *Lipophrys pholis*. Despite the differences between species and experimental design, the patterns seen by Fraser et al. (2022) in eurythermal and stenothermal fish are similar to those found in the present study.

While the protein synthesis rate describes the amount of newly synthesized proteins, it is also important to measure the degradation of proteins in order to identify the protein turnover and thus net protein gain (Publication 2). Here, the capacity of protein degradation was derived from the activity of cathepsin D. In both eelpout species, protein degradation capacity did not change during acute warming. However, the here described capacity of protein degradation only reflects the maximum activity of the enzyme Cathepsin D at a certain temperature with large amounts of substrates. The activity of enzymes depends on both, the available substrate and temperature. Since the activity of cathepsin D was measured at a common temperature (26°C) it does not reflect the activity at the temperature the samples were taken.

The activity of cathepsin D was thus measured at different temperatures for the white muscle tissue of *P. brachycephalum* (ΔT = 19°C). Cathepsin D activity increased linearly at all measured temperatures, with an average Q_{10} value of 2.3 ± 0.33 (n=6) (Supplementary, publication 2). Therefore, it is likely that *in vivo* activity of cathepsin D was 2.3 times lower at 0°C in comparison to 10°C. However, this would need to be confirmed by *in vivo* studies as many regulatory processes are not yet fully understood that could further influence the activity of cathepsin D *in vivo*.

In addition, 1-methylhistidine and 3-methyhistidine were determined by untargeted metabolic profiling (Supplementary, Figure 2) as indicators of potentially increased protein degradation (Nagasawa et al., 1996). Similar to cathepsin D activity, levels remained unchanged in both eelpout

species. Another indicator of protein degradation is a shift of the O:N ratio. A lower ratio indicates a higher protein-based metabolism, while a higher O:N ratio indicates a more lipid-based metabolism (Mayzaud and Conover, 1988). O:N ratios could only be measured in *P. brachycephalum* and again there were no significant changes with temperature. Similar to measurements of protein synthesis rate, variability between individuals was high. The average O:N ratio varied from 30 to 45 between 0 and 8°C while the lowest value of 24 was recorded at 10°C, which might indicate a higher protein-based metabolism in the warmth. Since fasting was induced for 10 days prior the experiment, a higher protein-based metabolism would indicate an increased protein degradation towards 10°C. However, the O:N ratio is an indicator of protein degradation in the entire animal and says nothing about where exactly more proteins were degraded. Unfortunately, the O:N ratios for *Z. viviparus* could not be determined due to technical errors and a lack of samples. Overall, the protein degradation rate might be increased during acute warming, but this study does not provide enough evidence to draw this conclusion. Future analyses, including *in vivo* measurements of protein degradation, would be necessary to clarify this result.

Fraser et al. (2022) found elevated protein degradation rates in the eurythermal fish *Lipophrys pholis* at the highest measured temperature. In this study, protein degradation of the whole fish was calculated as the difference between growth, protein synthesis, and food intake over a period of 28 days (Fraser et al., 2022). Another study by Lamarre et al. (2009) found higher 20S proteasome activities in white muscle of Atlantic wolfish acclimated at lower temperatures (4°C) than at higher temperatures (12°C). In general, it is difficult to compare protein degradation rates from the available studies, since several pathways may be involved and can differ depending on the species and tissue (Nemova et al., 2021). Such difficulties are also apparent in a previous study where degradation rates are negative at high temperature, which must be considered an experimental artefact (Katersky and Carter, 2007).

In summary, white muscle protein synthesis rate can generally be used as an indicator of growth, but the protein synthesis rates in stenothermal and eurythermal fish respond differently to acute warming. The stenothermal *P. brachycephalum* increases protein synthesis after a feeding pulse but the protein synthesis rate did not change during acute warming. This can be explained by its adaptation to the environmental conditions in the Southern Ocean, where temperature does not change significantly during the year, but food supply does. In contrast, the eurythermal *Z. viviparus* increases protein synthesis rates in response to rising temperature until reaching a maximum beyond which it declines. This is in line with published growth data.

4.3 As a result of cold compensation, *P. brachycephalum* is able to maintain a high protein turnover at colder temperatures compared to *Z. viviparus*

At common temperatures (4 and 10°C), significant differences in protein turnover were found in the white muscle of the two confamilial eelpout species. To our knowledge, this is the first study reporting such high differences of protein turnover (2- to 3-fold higher protein synthesis rates and 10-fold higher

protein degradation capacity) between an Antarctic fish species and a boreal congener. Both eelpout species were fed with the same food and in the same way. At 4°C the protein synthesis rate was 3 times higher in *P. brachycephalum* (0.47 ± 0.195% day^{-1}) than in *Z. viviparus* (0.15 ± 0.02% day^{-1}). At 10°C, the protein synthesis rate was still 2 times higher in *P. brachycephalum* (0.38 ± 0.26% day^{-1}) compared to *Z. viviparus* (0.20 ± 0.05% day^{-1}). However, with increasing temperature, the protein synthesis rate in *Z. viviparus* increased continuously until it reached a maximum at 16°C (0.38 ± 0.12), which corresponds to the same level as in *P. brachycephalum* between 0-10°C. However, *P. brachycephalum* was able to achieve higher protein synthesis rates at very low temperatures, most likely in order to sustain growth in the cold, while *Z. viviparus* was only able to increase its protein synthesis rate to the same rates as *P. brachycephalum* when temperature was close to its thermal optimum. Therefore, white muscle protein synthesis rate is fully cold compensated in *P. brachycephalum* to facilitate survival and growth at low temperatures (Publication 2).

In contrast to the present results, Fraser et al. (2022) found lower protein synthesis rates in an Antarctic fish (*Harpagifer antarcticus*) compared to a eurythermal species (*Lipophyrs pholis*) at a common temperature. The reasons for this deviating result can be manifold. Most importantly, it has to be pointed out that these two fish species are not closely related genetically, but only occupy similar ecological niches in their habitat. In the present study, the close relationship of both eelpout species has been demonstrated at the level of the genome, which is 96% identical. Most of the differences between eelpout species are mainly due to thermal adaptations, which are reflected in the composition of biological macromolecules (RNA, proteins) (Windisch, 2012). Thus, the closely related model species seem to be more suitable for studying evolutionary thermal adaptation. Furthermore, the feeding regime was different in the study by Fraser et al. (2022). Both species were fed with different feed and feeding was stopped only one day before injecting the flooding dose of radioactively labelled tracer (Fraser et al., 2022). However, digestion is temperature dependent and takes more time in Antarctic fish than in temperate fish (Johnston and Battram, 1993). In *Harpagifer antarcticus*, digestion can take up to 16 days and peaks between 2.7 and 55 hours after injection as measured by the rate of oxygen consumption (Boyce and Clarke, 1997). It is therefore possible that the physiological scope for processing food and absorbing nutrients was limited in *H. antarcticus* and hence the rate of protein synthesis was lower compared to *L. pholis*. In addition, the protein synthesis rate was measured in the entire fish and not specifically in the white muscle (Fraser et al., 2022). Meaningful comparative studies require a suitable set of eurythermal and cold-stenothermal Antarctic species.

Apart from the protein synthesis rate, the protein degradation capacity measured as cathepsin D activity in white muscle was 10 times higher in *P. brachycephalum* than in *Z. viviparus* at 4 and 10°C, indicating an exceptional over-compensation. Usually, polar fish compensate for cold temperatures by increased enzyme capacity (Hochachka and Somero, 2002). Therefore, the extremely high enzyme capacity of the lysosomal protease cathepsin D ensures sufficient degradation of the heavy chain in the white muscle of *P. brachycephalum*. Higher protein degradation has also been found in other Antarctic fish (Todgham et al., 2007, 2017), and it has been hypothesized that this is the result of the

reduced stability of proteins (Fields et al., 2015) due to compensation for enhanced structural flexibility in the cold (Feller et al., 2010). The assumption of higher protein instability in the cold is also supported by higher TMAO (Trimethylamine N-oxide) content in *P. brachycephalum* compared to *Z. viviparus*. TMAO is an important metabolite for protein stabilization, a role particularly discussed for deep-sea fish (Treberg and Driedzic, 2002). As the *P. brachycephalum* species originates from the deep sea (Anderson, 1994) and is still found in deeper waters than *Z. viviparus*, the higher TMAO content might simply be related to water depth, but in any case, it is likely, that protein instability is involved in the extraordinarily high degradation capacity.

High proteolysis levels in fish muscle are most commonly caused by starvation, gonadal maturation, and high physical activity (Nemova, et al., 2016). Therefore, the high capacity of protein degradation in the white muscle of *P. brachycephalum* may also be an adaptation to the conditions in the Southern Ocean, including long winters with very limited food availability and declining growth rates (Atkinson and Peck, 1988, Coggan, 1988). In times when food is highly abundant, proteins can be synthesized quickly, as described above. When conditions change and *P. brachycephalum* suffers from starvation, the high activity of the enzyme cathepsin D is able to degrade proteins, which serve as fuel for the organism. Enzyme activity is temperature-dependent, with higher temperatures increasing the activity of cathepsin D and lower temperatures decreasing it. *P. brachycephalum* is adapted to survive at constantly cold temperatures and must nevertheless be able to degrade proteins. It is thus important to have a higher capacity for protein degradation.

A generally higher protein turnover in *P. brachycephalum* is further supported by significantly higher levels of several amino acids including branched chain amino acids (leucine, isoleucine, and valine), asparagine, methionine, tryptophan, and lysine. All of this amino acids are essentially important for growth, are either energetically costly to synthesize or they are essential and thus cannot be synthesized by the fish itself (Wilson and Halver, 1986). It is therefore likely that these amino acids are recycled in the fish and not metabolized to produce ATP. In contrast, the concentrations of the energetically less expensive, non-essential glucogenic amino acids such as glycine and alanine were higher in the cytosol of *Z. viviparus* than in *P. brachycephalum*. Compared to *P. brachycephalum* (Storch et al., 2005), a higher content of glycine bound in proteins was also found in *Z. viviparus*, which is likely due to the larger amount of free glycine in the cytosol. Alanine is an important nitrogen carrier and can be synthesized from almost all other amino acids (Bucking, 2017). It can be released from white muscle and transported to the liver in which it can serve as a precursor for other non-essential amino acids or as substrate for the gluconeogenic pathway (Li et al., 2009, Owen et al., 2002). The transamination of alanine to pyruvate can account for up to 50 % of total ammonia excretion in fish (Bucking, 2017). A high alanine content in the muscle can therefore serve as a source of energy when required, e.g. during temperature changes. Since *Z. viviparus* is probably confronted with environmental changes more often than *P. brachycephalum*, it might be better prepared for this. However, further studies would be necessary to eliminate the uncertainties.

In summary, protein turnover was higher in *P. brachycephalum* than in *Z. viviparus*, which can be attributed to cold compensation. Despite the low temperatures, *P. brachycephalum* was able to increase protein synthesis after a high feeding pulse to reconstitute proteins and to grow. In contrast, *Z. viviparus* was only able to synthesize a similar amount of proteins as *P. brachycephalum* close to its thermal optimum.

4.4 The temperature-dependent changes during acute warming caused by a mismatch between temperature dependent increase in energy demand and the limited capacity of oxygen supply

Oxygen consumption was measured in both fish species during acute warming in order to decipher the energy turnover of the whole fish. Oxygen consumption during acute warming increased exponentially in both species. In *P. brachycephalum*, the temperature coefficient Q_{10} between 0 and 10°C was 2.46 and in *Z. viviparus* 1.91 between 4 and 22°C (Manuscript 3). Previous studies reported Q_{10} values for oxygen consumption rates in *P. brachycephalum* as a result of acute warming from 2.63 (Mark et al., 2002, 1°C every 12h between 0 and 12°C) to 5.2 (van Dijk et al., 1999, 1°C day^{-1} between 0 and 9°C). The difference between the lower Q_{10} of the present study and the somewhat higher one reported by Mark et al. (2002) compared to the higher value reported by van Dijk et al. (1999) may be explained by the fact that the latter experiments were performed on board of a research vessel, while the former were conducted in the laboratory. On board of a ship, there are several additional disturbances and potential stressors such the movement of the ship and a short recovery time of the fish after capture. This likely caused stress to the fish and therefore increased respiration as suggested by Mark et al., (2002). In *Z. viviparus*, the Q_{10} value of oxygen consumption varied between 2.4 and 3 during acute warming at a rate of +3°C per day, depending on the previous acclimation temperature (van Dijk et al., 1999; a Q_{10} of 2.4 in cold acclimated fish a Q_{10} of 3 in warm acclimated fish). Both Q_{10} values are in the normal range according to thermodynamic laws.

Oxygen is transported in the blood via the hemoglobin of the erythrocytes. The proportion of erythrocytes is expressed as hematocrit. In the present study, there were no significant changes in the hematocrit of either eelpout species during acute warming although there was a tendency towards higher hematocrit values at higher temperatures. In *P. brachycephalum*, the hematocrit varied between 17 and 18% between 0 and 8°C but increased to 25 ± 5% at 10°C. In *Z. viviparus*, the hematocrit was lowest at 4°C (23 ± 1%) and varied between 25 and 27% at 10 to 16°C. It was even higher at 22°C, reaching 32 ± 6%. The low number of replicates compared to other parameters is due to the difficulties to obtain enough blood for hematocrit measurements due to low blood volume of the fish and the small size of the blood vessels. But at similar temperatures, the hematocrit was significantly higher in *Z. viviparus* (23 ± 1% at 4°C) than in *P. brachycephalum* (17 ± 3% at 4°C). An increase in hematocrit raises the oxygen carrying capacity, but also blood viscosity. Low temperature also increases blood viscosity. For example, the blood viscosity in *Trematomus bernacchii* is 40 % higher at 0°C than at 10°C (Axelsson, 2005). In addition, the presence of antifreeze proteins in Antarctic fish

further increases viscosity (Axelsson, 2005; Eastmann, 1993; Bernadi and Devries, 1994). The lower hematocrit of *P. brachycephalum* compared to its temperate congener seems to be an adaptation to the cold environment by keeping blood viscosity as low as possible. In this respect, the hematocrit of *P. brachycephalum* increased significantly after long term acclimation above 6°C (Windisch et al., 2014) possibly in response to the onset of oxygen and capacity limitation at elevated temperatures (Pörtner, 2022). In the present short-term exposure, the observed trend of an increased hematocrit at 10°C may thus be the beginning of this acclimation process, which might have become more obvious and probably significant over longer time. Erythrocytes can also serve as a buffer for changing pH in the blood (Woods et al., 1982). Since there is no significant difference in oxygen consumption between the investigated eelpout species, it is possible that higher numbers of erythrocytes in *Z. viviparus* act as a buffer against pH changes. The buffer function is provided by the histidine in the hemoglobin of the erythrocytes, since histidine can accept or donate H^+ ions (Milligan and Wood, 1982, Nikinmaa et al., 2019). However, this conclusion is speculative due to the fact that the buffering function of the blood was not measured in any of the eelpout species.

In contrast, a similar trend of a potentially higher buffering capacity of *Z. viviparus* was observed in white muscle tissue. The histidine content in the white muscle tissue was higher in *Z. viviparus* than in the white muscle of *P. brachycephalum* at 4 and 10°C (Publication 2). In muscle, histidine is the main non-bicarbonate buffer against acidosis caused by e.g. anaerobic metabolism and can also act as an antioxidant (Khan, 2018). An earlier study by van Dijk et al. (1999) demonstrated that acute warming increased anaerobic metabolism and thus decreased intracellular pH (pHi) in both eelpout species towards the upper thermal limit. In *P. brachycephalum*, anaerobic end products such as succinate and lactate only increased at 9 °C, a temperature that was never measured in the area where *P. brachycephalum* was captured (Figure 7). In contrast, the anaerobic end products in *Z. viviparus* increased between 21 and 24°C, a temperature it can experience in summer (Figure 7, Pörtner and Knust, 2007). It could therefore be that the higher histidine concentration in the white muscle acts as a buffer at high summer temperatures. In addition, higher swimming activity increases anaerobic metabolism, and since *Z. viviparus* is more active than *P. brachycephalum* (personal observation), this could additionally lead to increased anaerobic metabolism in *Z. viviparus*. This argumentation would correspond to another study which found 6 times higher histidine concentration in marlin than in trout. The authors also argue that the large differences between these two fish species are due to the difference in thermal adaptation (marlin 25°C, trout 4-15°C) and to the intensive swimming activity of marlin compared to trout (Abe et al., 1985).

The erythrocytes are mostly stored in the spleen and can be released quickly when needed e.g. due to increasing oxygen demand. The relative spleen weight (% per body weight (% b.w.)) is higher in *P. brachycephalum* (4°C = 0.28 ± 0.1%; 10°C = 0.23 ± 0.07%) than in *Z. viviparus* (4°C = 0.12 ± 0.07%; 10°C = 0.12 ± 0.05%) (Manuscript 3). Larger spleens are beneficial during intense exercise and thus higher oxygen uptake. Generally, it seems that the spleen of Antarctic fishes is larger (varying between 0.2 and 0.8% b.w, Brijs et al., 2020) than those of temperate fish (0.1% b.w in *Salmo salar*, Gamperl et

al., 2020, or 0.1% *Dicentrarchus labrax*, Islam et al., 2020). This would lead to the assumption that Antarctic fish generally store more erythrocytes than temperate fish. However, in order to decrease blood viscosity, the number of erythrocytes is reduced in the blood when they are not absolutely necessary. Large quantities of erythrocytes are stored in the spleen and can be released as soon as they are needed (Brijs et al., 2020). But, spleen size did not shrink significantly during the present experiment (even at 10°C), indicating that during the present short-term exposure oxygen limitation was not severe enough to cause a significant release of stored erythrocytes.

To shed light on another important part of the oxygen transport system, the protein synthesis rate of gills was investigated. While gills are only a small part of the body they are involved in many processes including oxygen uptake. Upon acute warming, gill protein synthesis rate increased significantly in *P. brachycephalum* (0°C Ks= 1.07% day^{-1}; 10°C Ks= 4.51% day^{-1}), but also in *Z. viviparus* (4°C Ks= 2.41% day^{-1}; 22°C Ks= 15.4% day^{-1}) (Manuscript 3). Similar to oxygen consumption, gill protein synthesis increased at the same rate in both species (Figure 8).

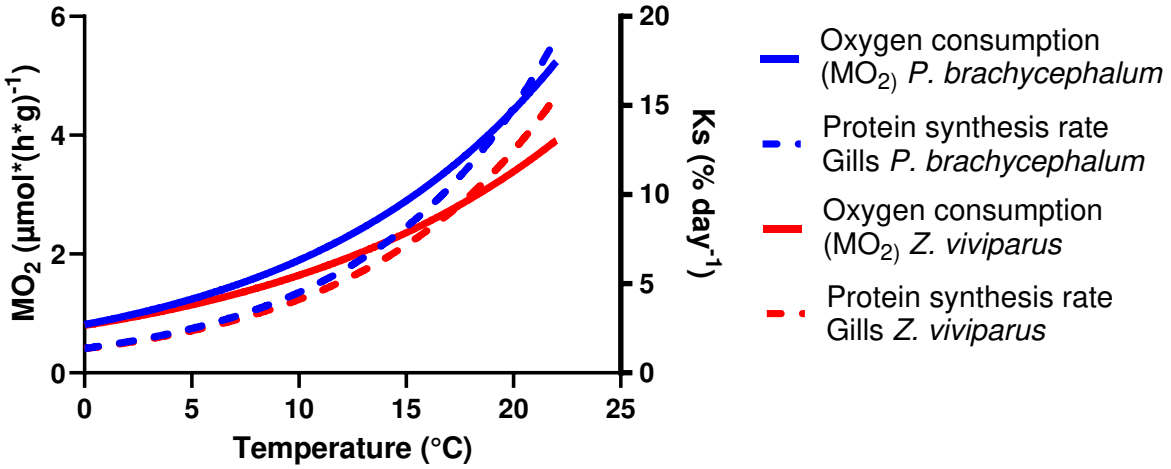

Figure 8. The whole-animal oxygen consumption and protein synthesis rate in gills of *P. brachycephalum* and *Z. viviparus* during acute warming. The oxygen consumption in both, *P. brachycephalum* (blue line, Model: exponential growth equation Y= 0.8129 * $e^{(0.0847X)}$, R_2= 0.8102) and *Z. viviparus* (red line, Model: exponential growth equation Y= 0.7966 * $e^{(0.07236X)}$, R_2= 0.8083) increase exponentially. Also, the protein synthesis rate in gills increase exponentially in *P. brachycephalum* (blue, dashed line, Model: exponential growth equation Y= 1.372 * $e^{(0.1190X)}$, R_2= 0.7013) and *Z. viviparus* (red, dashed line, Model: exponential growth equation Y= 1.359* $e^{(0.1108X)}$, R_2= 0.7163).

Thus, there is no cold compensation in gill protein synthesis of the Antarctic eelpout, resulting in a lower capacity in the gills of the cold-adapted species at its respective optimum habitat temperature. *In vitro* protein synthesis capacities of gills were also similar at 5°C in *P. brachycephalum* (Ks= 6.1% day^{-1}) and *Z. viviparus* (Ks= 5.0% day^{-1}) (Storch et al., 2005).

Gills are essential for survival as they are involved in both oxygen consumption and osmoregulation and nitrogen excretion. It has been shown that temperature affects the cellular structure of gills as cell abnormalities occur more often at elevated temperatures (Amir et al., 2022). At higher temperatures,

the formation of reactive oxygen species (ROS) increases and damages the gill cells. Necrosis can occur, which impairs the gill function (Yang et al., 2023). To minimize the damage of gill cells, heat stress induces gene expression associated with an unfolded protein response to reduce protein damage and thus protein degradation as well as osmoregulation in pacific salmon (Jeffries et al., 2014, 2012). Furthermore, heat stress increases the expression of genes involved in major structural components of the cytoskeleton (Buckley et al., 2006) and ion regulation (Yang et al., 2023). All of this may be necessary to ensure the function of gills because otherwise oxygen transport, osmoregulation, and nitrogen excretion cease and the fish will die. Accordingly, gill function seems to be more vulnerable to warming stress and thus increases the protein synthesis rate. Since the gills are not the primary site of growth and protein storage as discussed earlier for white muscle, the given protein synthesis rate is sufficient to support a balanced protein turnover at cold temperatures. No cold compensation is visible when comparing the cold-adapted and temperate eelpouts.

Generally, the proteins which are mainly synthesized in gills are involved in cartilaginous and bony tissues, epithelia cell replacement, intracellular protein turnover, chloride cell turnover including Na^+/K^+-ATPase and mitochondrial structure, mucus glycoproteins, and musculoskeletal proteins (Lyndon and Houlihan, 1998). Increased protein degradation in gills of Antarctic fish compared to temperate fish indicates lower stability of proteins in the cold (Todgham et al., 2007, 2017). This conclusion was drawn by Todgham et al. on the basis of the higher 20S proteasome activity and the higher number of ubiquitin-conjugated proteins found in Antarctic fish. However, this is only one protein degradation pathway and was measured shortly after capture (48h) at the natural habitat temperature (-1.5°C for Antarctic fish; 12°C for temperate fish; Todgham et al.2007, 2017). Various factors can influence protein degradation. If the fish were caught in the wild, little is known about their recent history and the impact of catching stress. In the present study, fish were kept for several months (*Z. viviparus*) or years (*P. brachycephalum*) under controlled conditions. Since Todgham et al. (2007, 2017) did not measure protein synthesis rates and the present study did not determine protein degradation rates, it remains to be shown, whether cold compensated protein turnover in gills is a common adaptive trait in cold-adapted Antarctic fish.

To gain a deeper understanding of metabolic changes, untargeted metabolic profiling was performed. The PLS-DA model revealed different patterns at the investigated temperature of 0, 4 and 10°C. Most important for the model was the metabolite N,N-dimethylglycine which increased significantly during acute warming. N,N-dimethylglycine is a derivate of glycine and an intermediate of choline metabolism. It enhances immune responses in salmonid fish (Muona and Virtanen, 1993) and prevents oxidative stress by scavenging free radicals (Bai et al., 2016). N,N-Dimethylglycine can be formed from choline via betaine to glycine. However, none of these three metabolites changed significantly during acute warming. Moreover, there was no significant change in energy metabolism with acute warming, neither within the Krebs cycle (Figure 8) nor in pyrimidine or purine metabolism (data not shown). This low response of metabolites to acute warming matches the low response of protein synthesis rate in the muscle during acute warming.

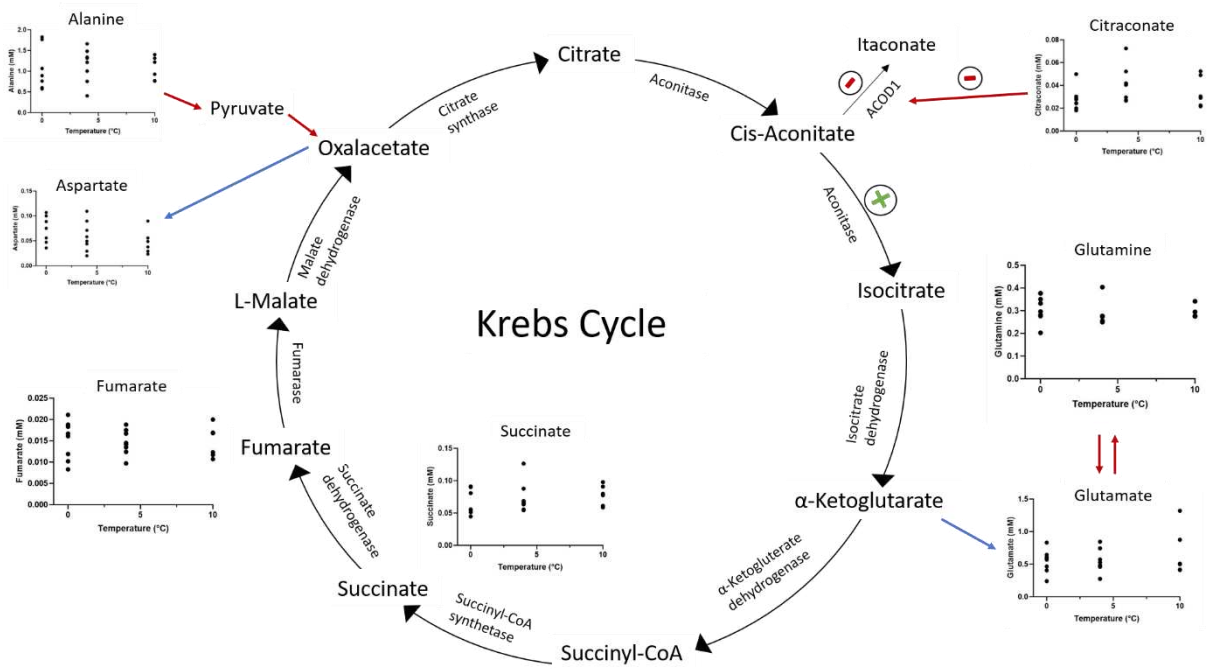

Figure 9. Concentrations of metabolites related to the Krebs cycle in *P. brachycephalum* during acute warming. The minus (red -) indicates the inhibitory effect of citraconate on ACOD1 (aconitate decarboxylase). This inhibition leads to a reduced formation of itaconate, which then promotes the formation of cis-aconitate to isocitrate (green +). The arrows indicate potentially anaplerotic (red arrows) or cataplerotic (blue arrows) function of the different amino acids. In *P. brachycephalum*, concentrations of metabolites associated with the Krebs cycle are not changing significantly during acute warming. Neither do amino acids which can have a cataplerotic or anaplerotic function, nor succinate or fumarate.

In *Z. viviparus*, untargeted metabolite profiling revealed a different picture. The metabolic profiles of *Z. viviparus* at 4 and 10°C were not identified to be separate from each other by PLS-DA, but at higher temperatures, the profiles were separate from each other (13, 16 and 22°C). In more detail, the SAM identified two metabolites (choline and phosphocholine) that responded significantly during the acute warming trial. The micronutrient choline initially increased between 4 and 10°C, then stabilized up to 13°C and decreased thereafter, thereby closely following the long-term temperature-dependent growth curve of *Z. viviparus* (Brodte et al., 2006a; Fonds et al., 1989; Pörtner and Knust, 2007). In skeletal muscle, choline provides its methyl-group for several processes including protein and lipid metabolism as well as autophagy and promotes or inhibits processes of neurotransmitters (Moretti et al., 2020). In fish muscle in particular, there is evidence that choline can stimulate the protein synthesis by activating the eIF4E-binding protein 2 gene (Wu et al., 2011). Similar to the protein synthesis rate measured here in the white muscle of *Z. viviparus*, the choline concentration increased with temperature (publication 2). However, it reached its highest concentration at 13 °C and decreased at higher temperatures, while the protein synthesis rate increased up to 16 °C and did not change further at higher temperatures. Therefore, this study can neither prove nor disprove the relationship between protein synthesis rate and choline.

In contrast to choline, O-phosphocholine, which can be directly synthesized from choline by phosphorylation, is an important precursor of membrane lipids. It was highest in the cold (4°C) and decreased with increasing temperature. A high phosphocholine content was hypothesized to improve cold tolerance by increasing the ability to maintain membrane fluidity in the cold (Jiang et al., 2020; Williams et al., 2014). Similarly, the findings in *Z. viviparus* indicate shifts in membrane lipid composition with temperature. While at 4°C a higher O-phosphocholine content seems advantageous, its concentration and thus importance decreases at temperatures above 13°C. In line with our results, lipid analyses of acclimatized *Z. viviparus* indicated a high lipid content in the cold, especially for lipids relevant for membrane fluidity (Brodte et al., 2008).

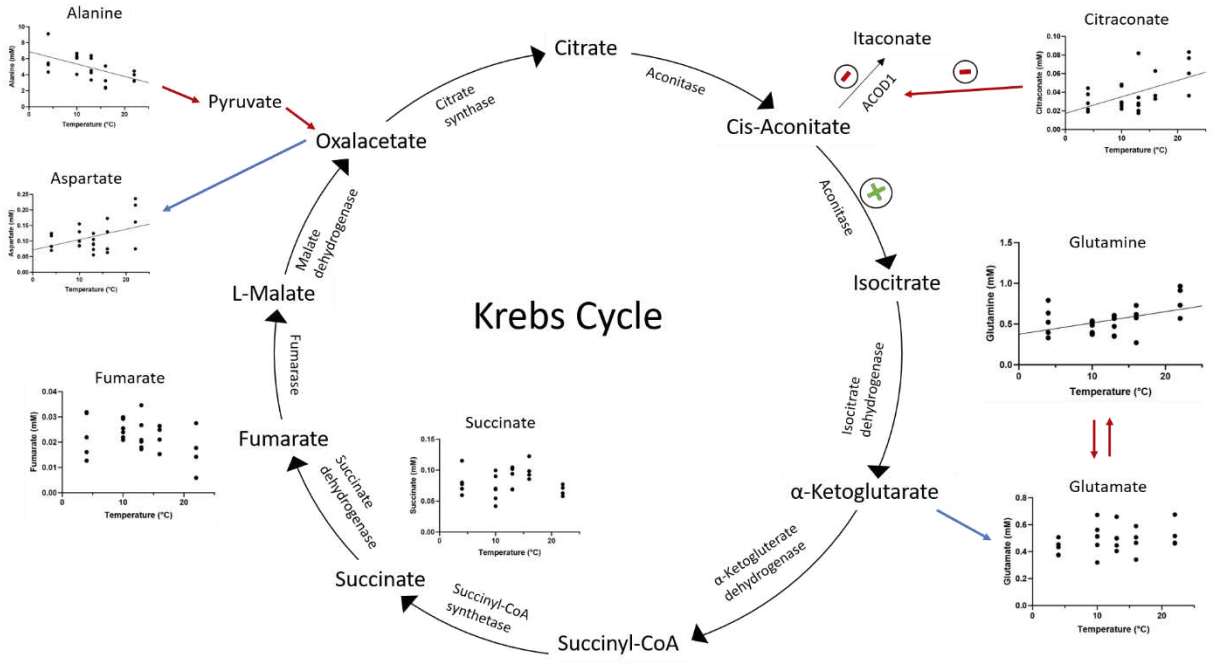

Figure 10. Temperature-dependent changes in the concentrations of metabolites involved in the Krebs cycle in *Zoarces viviparus*. Red arrows are indicating metabolites promoting the Krebs cycle (e.g. due to anaplerosis) while blue arrows are indicating metabolites leaving the Krebs Cycle (cataplerosis). The minus (red -) is indicating an inhibition and plus (green +) a promotion of a pathway. Citraconate (upper right corner) increases with temperature. This might be an indicator for an inhibition of ACOD1 (aconitate decarboxylase). Following, less cis-aconitate is converted to itaconate and thus more cis-aconitate remains in the Krebs cycle. Glutamine (right, middle) is increasing and Glutamate (lower right corner) is not changing. Succinate (in the middle) and Fumarate (left, lower corner) are also not changing with elevated temperature. Aspartate is increasing (middle, left) and alanine is decreasing during acute warming (upper left corner). This would provide additional amino acids that can be distributed and utilized by the muscle or other tissues.

Linear regression analysis revealed changes within the Krebs cycle (figure 9). Citraconate increased between 4 and 22°C. Citraconate is the only known naturally occurring inhibitor of cis-aconite decarboxylase (ACOD1), which converts cis-aconite into itaconate. (Chen et al., 2022). In the Krebs-cycle, cis-aconitate is an unstable intermediate upon formation of isocitrate from citrate by the enzyme aconitase. At this point, ACOD1 can interfere and convert cis-aconitate to itaconate, which is an important metabolite for the immune response (Li et al., 2020). The price of inhibiting itaconate formation could be an impaired immune response. At temperatures above the optimum, there is thus a higher risk of bacterial infections (Albert and Ransangan, 2013). Whether this is partly due to the

reduction in itaconate, however, is speculative. In addition, it was shown that ACOD1 knockout mice increased glucose oxidation and reduced fatty acid oxidation (Frieler et al., 2022). Similarly, ACOD1 inhibited by citraconate could increase the glucose oxidation at high temperatures. For several fish species, including both eelpout species, a shift in fuels upon warming have been demonstrated (Brodte et al., 2008; Windisch et al., 2014). In the cold, fish accumulate lipids and favor a lipid-based metabolism, but oxygen is crucial for the oxidation of lipids. At higher temperatures, the metabolism shifts to the formation of glycogen stores and the carbohydrate-based metabolism. Carbohydrates can also be metabolized at oxygen deficiency as induced by high temperatures (Pörtner et al., 2005). Therefore, changes in citroconate concentration here could indicate the shift from a lipid-based metabolism in the cold to a carbohydrate-based metabolism due to oxygen deprivation as a result of acute warming. Clearly, the presence and function of this regulatory pathway in fish needs to be demonstrated in future.

Furthermore, the amino acid aspartate, which is known for its function as a substrate in the Krebs cycle for several processes e.g. redox shuttles and nitrogen trafficking, increased in the cytosol with warming (Inigo et al., 2021; Owen et al., 2002). Alanine can be utilized from almost all amino acids and converted into pyruvate, which is then supplied to the Krebs cycle for energy production (Owen et al., 2002). Glutamine is also involved in the refilling of the Krebs cycle and thus has an anaplerotic function (Owen et al., 2002), but in skeletal muscle it can also be used for cataplerosis during intense exercise (Cruzat et al., 2018).

The amino acids leaving the Krebs Cycle could then be used for protein synthesis. However, the protein synthesis rate of white muscle of *Z. viviparus* starts to decrease at 22°C which is beyond the optimum temperature (between 12 and 15°C). Thus, the surplus of amino acids may be transferred to other tissues including gills. In gills, the protein synthesis rate increased exponentially and it is possible, that the amino acids are provided by the white muscle. This is speculative and should be investigated in the future. Therefore, it would first be necessary to follow the amino acids through the Krebs cycle. This would be possible by the usage of labeled tracer which can then be followed.

Table 1. Linear regression of Krebs Cycle metabolites of *Z. viviparus*

Metabolite	Equation	R_2	p-value
Alanine	Y=-0.155x + 6.889	0.319	0.033
Aspartate	Y= 0.0033x + 0.0716	0.175	0.0376
Citraconate	Y= 0.00177x + 0.0174	0.278	0.0067
Glutamine	Y= 0.0139x + 0.375	0.202	0.024

5 Conclusions

The two-eelpout species from the North Sea and the Southern Ocean respond differently to acute warming as a consequence of their thermal adaptation to different temperature profiles of their habitats. In the common eelpout *Z. viviparus* the rate of protein synthesis increases during acute warming until it reaches its maximum at T_{opt} and then decreases it thereafter. At the same time, the oxygen consumption increased exponentially as an indicator for high energy turnover within the fish (Figure 10). Although oxygen consumption increases continuously, protein synthesis in the white muscle decreases above the thermal optimum. In ectotherms, protein synthesis consumes most of the energy (Fraser and Rogers, 2007) and the muscle is the largest part of the fish and therefore consumes a large proportion of energy available. A reduction in protein synthesis in the white muscle should lead to a lower energy requirement and thus to lower aerobic energy production. In this study, however, the respiration rate increases despite lower protein synthesis rates in the white muscle. Other unknown processes appear to require large amounts of energy to survive temperatures above the thermal optimum. In order to obtain sufficient energy for these processes, protein synthesis in the white muscle and thus growth is reduced at high temperatures. One of these energy-consuming processes is the rate of protein synthesis in the gills, which increases exponentially during acute warming (Figure 8). This result is consistent with the concept of OCLTT, which postulates that oxygen limits thermal tolerance and aerobic performance (here protein synthesis) is lower due to oxygen and thus energy limitation beyond the thermal optimum.

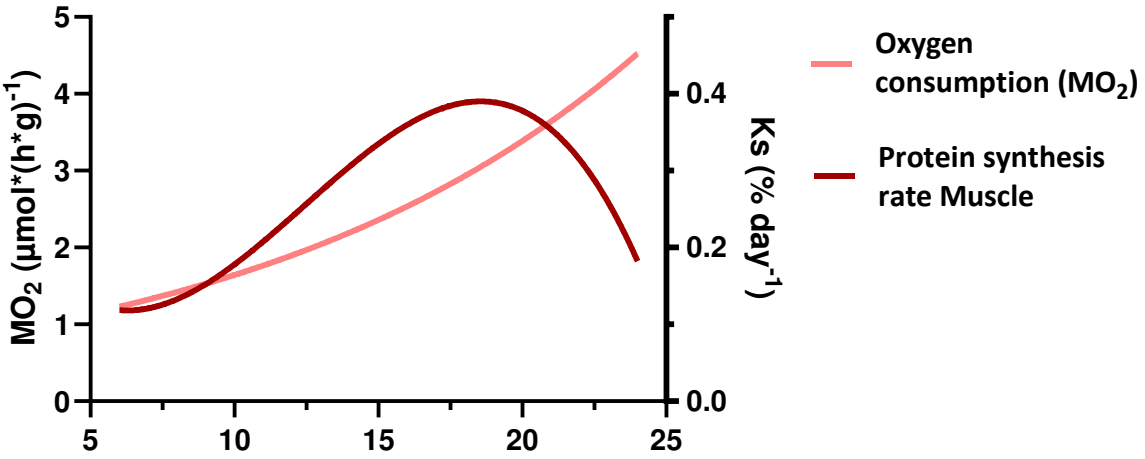

Figure 11. Protein synthesis rate in white muscle and whole-animal oxygen consumption of *Z. viviparus* during acute warming. The protein synthesis rate in white muscle (dark red) is first increasing until 16°C and then decreasing forming a bell-shaped curve (Model: Third order polynomial with the formula Y= 0.4048x3 - 0.1029x^2 - 0.1029x -0.0002941, R_2= 0.4767) In contrast, the oxygen consumption is increasing exponentially (Model: exponential growth equation Y= 0.7966 * $e^{(0,07236X)}$, R_2= 0.8083).

In contrast, protein synthesis in the white muscle of the stenothermal Antarctic eelpout *P. brachycephalum* remained unchanged during acute warming, although oxygen consumption increased similarly to *Z. viviparus* (Figure 11).

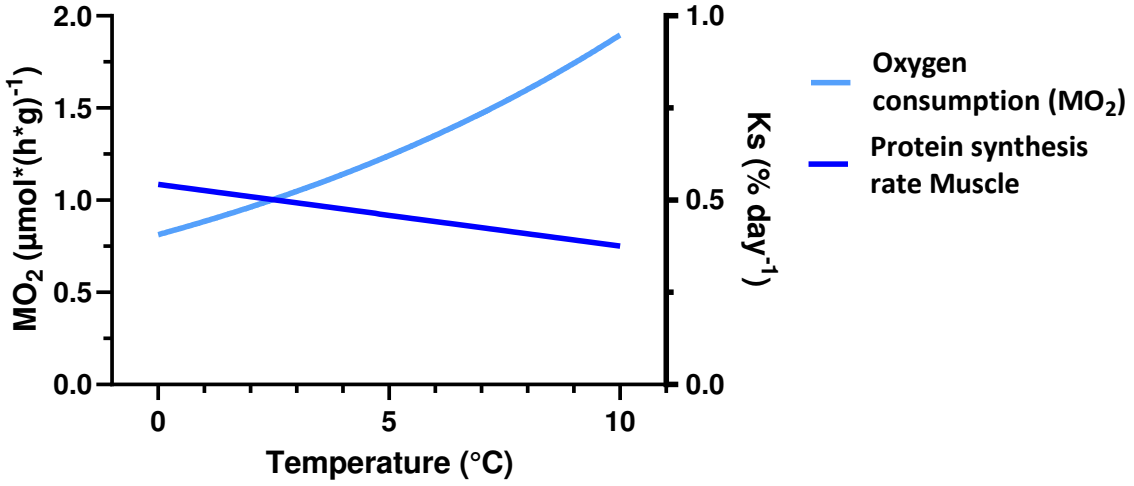

Figure 12. Protein synthesis rate in white muscle and whole-body oxygen consumption in *P. brachycephalum*. The protein synthesis rate in white muscle is slightly, but not significantly decreasing with temperature (Model: linear regression with the equation: Y = -0.01682*X + 0.5432, R_2 = 0.04482, p-value = 0.1627). In contrast, the oxygen consumption increased exponentially with temperature (Model: exponential growth equation: Y= 0.8129 * $e^{(0.0847X)}$, R_2= 0.8102).

The main reason for this difference in the response to acute warming lies in the different adaptation to their natural environment, as *Z. viviparus* is adapted to seasonal temperature fluctuations, while *P. brachycephalum* is adapted to constantly cold temperatures. However, while acute warming had only a limited effect on *P. brachycephalum*, the feeding pulse shortly before the start of the experiment increased the rate of protein synthesis tenfold. This indicates a high degree of cold compensation to achieve such high protein synthesis and despite very low temperatures. *Z. viviparus* was only able to synthesize the same amount of proteins when close to its thermal optimum (here 16°C).

This specialization of *P. brachycephalum* in consistently cold temperatures allows it to survive in the world's coldest ocean. However, if climate change continues and temperatures continue to rise, other species could migrate and survive in the Southern Ocean and compete with *P. brachycephalum*. Models are already predicting the migration of species to the polar regions as a result of climate change, which will intensify competition (Chaudhary et al., 2021; IPCC, 2022), whereby it is uncertain which species will survive. While *P. brachycephalum* cannot migrate to colder areas, *Z. viviparus* could migrate further north and become extinct in the North Sea, especially as the temperatures in the North Sea are already close to the upper temperature limit in summer. Not only is aerobic performance reduced at temperatures above 16°C, but warm summers already cause higher mortality of *Z. viviparus* (Pörtner and Knust, 2007).

Overall, this thesis provides evidence that protein synthesis rate in white muscle can be used as an indicator for growth and thus aerobic performance during acute warming. However, a reduction of the protein synthesis rate towards the upper thermal limit, in parallel to the change in growth rate after long-term acclimation, was only found in *Z. viviparus*. Whether the feeding pulse masks the response to acute warming in *P. brachycephalum* or whether the temperature rise was too rapid for the stenothermal eelpout to track the growth curve would need to be investigated in the future.

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7 Acknowledgement

First, I would like to thank my "Doktorvater" **Prof. Dr. Hans-Otto Pörtner** not only for employing me, but also to believe in me. This PhD was a lifetime experience I will never forget and therefore I am very thankful. Also, thanks to the other committee members for taking the time to read and evaluate this dissertation: **Dr. Marian Hu**, **Prof. Dr. Marko Rohlfs** and **Dr. Gerlien Verhaegen**.

Additionally, I would like to thank my TAC (Theory Advisory Committee) who stood by my side throughout the entire time (in alphabetical order):

Dr. Christian Bock for his help throughout my time at AWI, during lunch breaks or in the NMR lab even though it was forbidden.

Dr. Gisela Lannig for having an open ear whenever I needed it and her moral support during hard times

Dr. Magnus Lucassen for not only helping me with my experiments but for proofreading the dissertation

Dr. Felix Mark for his help with the oxygen consumption measurements, his high expertise of fish physiology and trying to help me to catch eelpouts in the arctic ocean even though it was almost impossible.

With this I would also like to thank the whole team from the covid expedition to Svalbard in 2020 **HE560**! It was 6 weeks of normality in a time of social distancing and limited social contacts. Especially **Ayla Murray**, the Kiwi who not helped to get rid of all the jellyfish, but for proofreading this dissertation. I am looking forward to see you at the next "Die Ärzte"-Concert ;).

I would also like to thank **Dr. Jan Tebben** and **Dr. Christian Zurhelle** for their support with the protein synthesis measurements. It was not always easy to explain chemistry to a biologist, but thank you for never stop trying. I will definitely never eat oysters with you Christian, it could just be too dangerous ;).

Special thanks to all the technicians who are doing an amazing job! I could have never done it without you! Thank you:

Amir Karamyar and **Freddy Moraleda** for taking such good care of all the animals and being there whenever I needed you! Thank you for setting up all the systems and keep them running even at air temperatures of -5°C and water temperatures around 0°C and come for my help when the electricity fell out on weekends!

Isabel Ketelsen not only for her help in the labs and with the fish but for being a great person I always enjoyed talking to and spending my lunch breaks with.

Nils Koschnick for his help with my "Schatzis" and his high expertise on the biology of fish. Thank you for the expedition on the North Sea and to Svalbard to catch eelpouts, even though both were not successful as planned. And thanks to all the other work you perform in the background to keep everything running!

Anette Tillmann for her help with the NMR and in the labs. Without you the labs would look like a big mess where no one would like to work in.

Rolf Wittig for all our lunch breaks in the cafeteria and agreeing with me that fish should not be eaten and for helping and explaining me the NMR. I just hope you found your happy place.

Thank you to all the apprentices who were involved in this dissertation: **Lorenz Eckardt**, **Ruben Lietzau**, **Kira Szidat** but especially **Rebecca Gorniak**. Rebecca, you helped me to get comfortable in Bremerhaven and while I was sea sick. Over the years you have become a good friend and I look forward to our next game night.

Thank you to my fellow PhD students! Some of you have already made it, the others will follow:

Nina Paul and **Dr. Bastian Maus** for all the pre-covid lunch breaks, hat building sessions and sharing their expertise in how to survive a PhD.

Nicole Vogt for sharing the office with me! It was not always easy but we had a great deal of fun, I will miss it! However, I will definitely not miss the long hours in the noisy NMR lab or checking your flat for water damage while you are away or being stuck for a week in a tiny apartment instead of visiting the conference due to covid.

Many thanks to Dr. (or not) **Sandra Götze**! Seriously, I do not know how I would have managed without you! During covid we were the only people NOT working from home and coming to AWI every day. With you working on weekends were actually fun. “What can I say except your welcome!” Over time you have become not only a colleague but a dear friend! THANKS!

I do not want to forget to thank the entire **crab lab** team! Especially, **Dr. Reinhard Saborowski** for helping me with the Cathepsin D measurements, taking me in for the last months of my PhD and proofreading my dissertation. I always enjoyed our discussions even when we disagreed on things. Daphnia are definitely crustaceans!

Thanks to my “**Schatzis**” for participating in my experiments, even though it was not voluntarily. Especially, I need to thank **Herbert** who proofed that eelpouts do exist around Svalbard but I am just not capable of finding them.

Also, thanks to my former colleagues from the **Ruhr-University Bochum**! I have been gone for 5 years but I still think of the amazing fun we had! I remember the first day entering the office when I had to close the door quickly trying not to knock over the ladder as someone was catching a huge butterfly. Without you I would not have gotten the PhD at AWI in the first place! Thank you!

At last but definitely not least, I want to thank my family and friends outside of work. You stood by my side through all this time. You cheered me up and made me the person I am today! Although you would be all worth being mentioned here separately, I will point out two persons:

First, **Jesse** for sharing his life with me for almost 4 years now. I never thought I would find someone I want to spend every day with, especially not during the covid pandemic shortly before taking off to an expedition to the arctic ocean without phone reception or internet connection. You have always supported me and helped me through this difficult time with your love and humor.

And thanks to my **dad**. My dad had a main influence in my life. Together we watched endless hours of documentaries and walked through zoos. You were the first person who I came to when I got a new interesting fact about animals and vice versa. You taught me to be passionate about animals and made me choose to study biology in the first place. I am sorry you cannot be here today, but I miss you every day and hope you would be proud, dad, I did it! I finished this damn PhD!

8 Supplementary

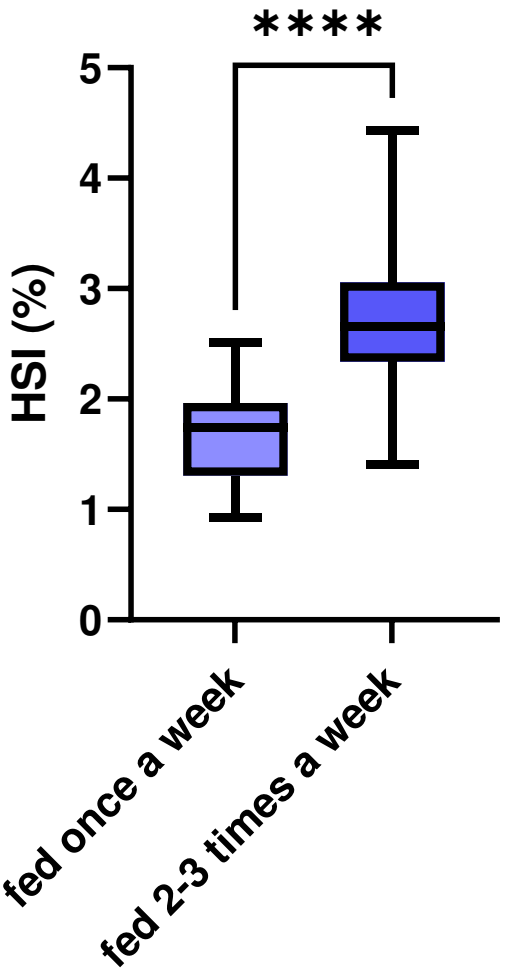

Figure S1: HSI depending on feeding rate in *P. brachycephalum*. The HSI was higher in *P. brachycephalum* which were fed 2-3 time a week for a duration of 3-6 weeks. Significant differences were tested with unpaired T-test (p-value < 0.001 (****))

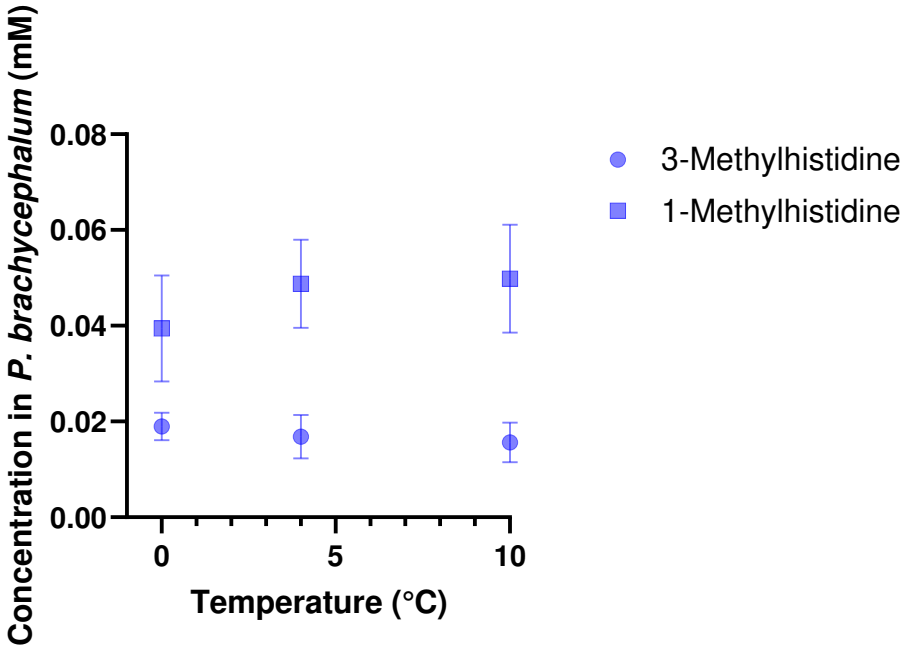

Figure S2: Concentration of 3- and 1- Methylhistidine in *P. brachycephalum*. There is no significant change of 3- or 1- Methylhistidine in white muscle during acute warming (0-10°C) in *P. brachycephalum*.

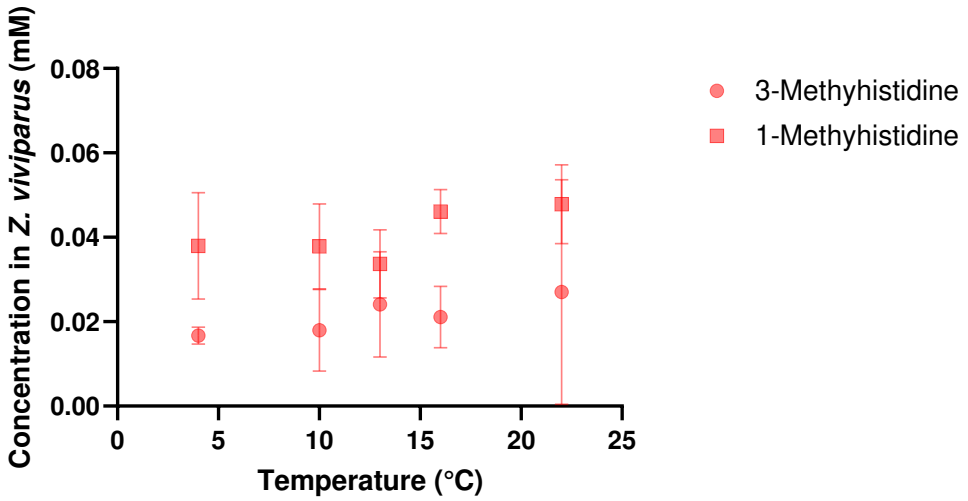

Figure S3: Concentration of 3- and 1- Methylhistidine in *Z. viviparus*. There is no significant change of 3- or 1- Methylhistidine in white muscle during acute warming (4-22°C) in *Z. viviparus*.