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Master thesis

Activity of metabolic key enzymes in early life stages of Cape horse mackerels, *Trachurus trachurus capensis* from the Northern Benguela Current Upwelling System

by

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Abstract

Fish stocks in the Northern Benguela Upwelling System (NBUS) have undergone substantial changes in distribution and abundance over the last decades. Overfishing and the spreading of hypoxic zones resulted in a community shift replacing sardine and anchovy and holding Cape horse mackerel (*Trachurus trachurus capensis*) as the most important pelagic fishery resource off Namibia today. Investigations of metabolic activities and physiological traits of small pelagic fishes from the NBUS are scarce, especially for their early life stages. In this study, activities of metabolic key enzymes, citrate synthase (CS), pyruvate kinase (PK) and lactate dehydrogenase (LDH) were investigated in larvae and juveniles of Cape horse mackerels with respect to adaptive properties to cope with ecosystem changes. The enzyme assay of the electron transport system (ETS) was optimized for fish`s early life stages and ETS activities were estimated giving their potential metabolic rate. Over a broad size range of 4 – 62 mm and 0.002 – 1.056 g gutted wet mass respectively, young Cape horse mackerels showed averaged specific activities of: CS = 6.62 ± 1.34 U g^{-1} WM_m, PK = 128.6 ± 37.1 U g^{-1} WM_m, LDH = 185.7 ± 46.3 U g^{-1} WM_m. Glycolytic enzyme activities, PK and LDH, followed a positive allometric relation with body size. Environmental factors, including temperature and dissolved oxygen concentrations were shown to affect both aerobic and anaerobic enzymatic activities. *T. trachurus capensis* showed different glycolytic activities when reared under normoxic and hypoxic conditions. Comparative analysis with other small pelagic fishes from the NBUS resulted in significantly higher aerobic and anaerobic enzyme activities of Cape horse mackerels which are assumed to be advantageous to tolerate hypoxic conditions and therefore a key property to succeed in the Northern Benguela Upwelling System.

Ac-CoA	acetyl coenzyme A
ADP-K-Salt	adenosine diphosphate potassium salt
ATP	adenosine triphosphate
ANC	anchovy
BCLME	Benguela Current Large Marine Ecosystem
CS	citrate synthase
DO	dissolved oxygen
DTNB	5,5'-dithiobis-2-nitrobenzoic acid (Ellman's reagent)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ETS	electron transport system
GENUS	Geochemistry and Ecology of the Namibian Upwelling System
HMC	Cape horse mackerel
HOM	homogenization buffer
INT	iodonitrotetrazolium chloride
KCl	potassium chloride
LDH	lactate dehydrogenase
$MgSO_4$	magnesium sulfate
NADH	nicotinamide adenine dinucleotide
NBUS	Northern Benguela Upwelling System
OML	oxygen minimum layer
OMZ	oxygen minimum zone
Ox-Ac	oxaloacetate
PEP	phosphoenolpyruvate
pers. comm	personal communication
PK	pyruvate kinase
PVP	polyvinylpyrolidone
Pyr	sodium pyruvate
SAR	sardine
T	temperature
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
WM	wet mass

Content

1 Introduction

The Benguela upwelling system is, besides the Humboldt system, the Canary and the California system, one of the four major eastern boundary current systems of the world. As other upwelling systems it is characterized by high primary production, short trophic cascades and high abundances of small pelagic fishes making it to one of the most productive fishery grounds in the world. Threatened by anthropogenic influences, mainly overfishing but also global climatic change, the ecosystem structure has changed significantly over the past decades. The composition of the predominant pelagic fish species has changed in the Benguela Current Large Marine Ecosystem (BCLME) between 1970 and 1990. This regime shift, by definition a sudden shift in structure and functioning of a marine ecosystem, was initiated and sustained by different processes including bottom-up, top-down and wasp-waist forces. Contrasting ecosystem changes occurred in both sub-systems, the Northern and Southern Benguela, following different progresses in terms of their actual structure and functioning as summarized by Cury and Shannon (2004). The Southern Benguela shifted from a bottom-up controlled ecosystem (control by primary producers) to a wasp-waist ecosystem (control by dominant species) today, where the recruitment of the dominant species is linked to physical processes and intra- as well as inter-specific competition. Overexploitation of dominant pelagic fishes and “fishing down the food web” (depletion of higher trophic levels including predatory fish species) caused a shift in the Northern Benguela between 1970 and 1990. Additional environmental anomalies in the mid-late 1990s lead to an ecosystem shift from top-down control (control by predators) to bottom-up control. In the 1970s the Northern Benguela was dominated by two planktivorous species, anchovy and sardine. The system changed to one holding horse mackerels as dominant pelagic fish in the 1980s and 1990s (Heymans, 2004). Today, dominant species in trawl catches are cape hake (*Merluccius capensis*) and Cape horse mackerels (*Trachurus trachurus capensis*), contributing to the majority of the pelagic fishery in South-West Africa (Bianchi *et al.*, 1999; Cury and Shannon, 2004).

Pelagic fish stocks already suffered a noticeable decline since the 1970s in the northern part of the Benguela Current. The reasons for a failed recovery are not understood yet but the increased occurrence of hypoxic zones within the upwelling system is thought to be a crucial factor. In particular the Northern Benguela Upwelling System (NBUS) is naturally hypoxic to

even anoxic at depth. This oxygen depleted water masses flow southwards at subsurface depths, whereby hypoxia is enhanced by further oxygen depletion simultaneously (Shannon and O`Toole, 2003). During some years, this oxygen depletion of shelf waters in the Benguela is unusually severe resulting in widespread hypoxia and anoxia (Shannon and O`Toole, 2003). This is a consequence of increased primary production and subsequent decay of phytoplankton blooms coinciding with quiescent conditions which follow periods of sustained and enhanced upwelling. Large scale hypoxia and anoxia result in massive mortalities of marine organisms and changes in distribution and abundance of e.g. juvenile cape hakes (Hamukuaya *et al.*, 1998). Numerous studies have demonstrated the ability of adult fishes to avoid or respond to low oxygen concentrations by e.g. changing their metabolic activity, vertical or horizontal migrations and increased use of air breathing or aquatic surface respiration (Chabot and Claireaux, 2008; Cooper *et al.*, 2002; Kramer, 1987). However, the behavioral responses of larvae and juveniles are less understood. In particular, early life stages of fishes are thought to react very sensitive to environmental changes. Ambient oxygen levels are the most important factor governing metabolic activities in marine species. Even at low dissolved oxygen levels fishes rely on oxygen. In order to compensate for adverse environmental conditions they are expected to show physiological and biochemical adaptations (Ekau *et al.*, 2010).

Estimations of metabolic potentials were frequently conducted on various fish species (Sullivan and Somero, 1980; Hickey and Clements, 2003) by measuring the activity of metabolic key enzymes. Activities of these enzymes in different metabolic pathways can be further used as condition indices to describe the physiological status of an organism. To estimate metabolic activity, enzyme capacities of aerobic and anaerobic metabolism can be analyzed. Aerobic metabolic potentials are indicated by activities of oxidative enzymes. Here, measurements of the respiratory electron transport system (ETS) activity, introduced by Packard (1971), serve as a biochemical measure of an organisms potential metabolic rate. Additionally the activity of citrate synthase (CS), one of the marker enzymes for the mitochondrial aerobic metabolism (Clarke *et al.*, 1992), can be analyzed. Glycolytic potential is further indicated by the activities of enzymes such as lactate dehydrogenase (LDH) and pyruvate kinase (PK) (Berges *et al.*, 1990; Berges and Ballantyne, 1991) representing anaerobic potentials. Using spectrometric analysis, key enzyme activities can be determined

to detect species-specific enzymatic responses to a changing environment. Clarke *et al.* (1992) investigated the effect of temperature and nutrition on enzyme activities in larval red drum and lane snapper, showing that LDH activities increased with rearing temperature and that both LDH and CS activities were up to 4-fold higher in well-fed than in starved fish. Furthermore, organisms living in or passing through oxygen minimum layers (OML) are expected to be adapted to some extent in their energy metabolism to the hypoxic environment, in which anaerobic metabolism may also play an important role. Former studies mostly linked specific enzyme activities to the ability of vertical migration of an organism, such as for krill (González and Quiñones, 2002), while others focused on the dependence to parameters like body size and habitat depth (Thuesen and Childress, 1994; Sullivan and Somero, 1980). Especially measurements of the ETS are thought to serve as an index of an organism`s potential metabolic rate, rather than giving actual rates derived from respiration experiments. The respiratory electron transport system (ETS), where oxygen is required to generate ATP from organic compounds, was already used to measure potential metabolic rates in many species, including phytoplankton (Packard, 1971), zooplankton (Gómez *et al.*, 1996; Owens and King, 1975), benthic macrofauna (Cammen *et al.*, 1990; Madon *et al.*, 1998) and fishes (Gopalan *et al.*, 1996; Ikeda, 1989; Lannig *et al.*, 2003; Yamashita and Bailey, 1990).

In order to understand how the Benguela ecosystem and its key species cope with changing environmental conditions, the international GENUS project (Geochemistry and Ecology of the Namibian Upwelling System) aims to clarify overall relationships between a changing climate, biogeochemical cycles and the ecosystem structure. Embedded in the GENUS project, the objective of the present study is to investigate physiological and biochemical characteristics of key species to understand adaptive traits to cope with changing environmental conditions.

The study is focused on metabolic capacities and activities of metabolic key enzymes of young Cape horse mackerels, *Trachurus trachurus capensis*. This species as well as Cape hake, *Merluccius capensis*, can tolerate lower oxygen levels (down to 10 % saturation) than anchovies or sardines (Kunzmann, pers. comm.). Since tolerance to hypoxia seems to be a key property to succeed in the Benguela upwelling system a set of basic aerobic (CS, ETS) and anaerobic (PK, LDH) enzyme activity data will be measured in specimens from various

locations with distinct temperature and oxygen regimes. Additionally, inter-specific comparison with other small pelagic fishes will be made to investigate possible differences in their metabolic properties. By comparing activity levels of these samples with those derived from stress experiments at different hypoxic conditions, the impact of low oxygen levels on the enzyme activities of Cape horse mackerels can be determined. Previously determined respiration rates (Geist *et al.*, 2012; submitted for publication) will be related to activities of the respiratory electron transport system, resulting in a R:ETS ratio that can be used to estimate ambient metabolic rates (Gopalan *et al.*, 1996).

The main objectives of this study were:

1) to adjust metabolic enzyme measurements to early life stages of small pelagic fishes
2) to achieve basic data on mitochondrial and glycolytic enzyme activities in young Cape horse mackerels
3) to estimate the relation of metabolic enzymes to physiological traits and
4) their dependence on environmental factors, including temperature and dissolved oxygen concentrations
5) to compare species-specific aerobic and anaerobic activities in small pelagic fishes from the BCLME

Hypotheses:

1. Enzyme activities are correlated to body mass parameters of the fish larvae.

2. Temperature and dissolved oxygen levels influence metabolic enzyme activities.

3. Cape horse mackerel larvae show higher anaerobic capacities than early life stages of anchovies or sardines.

2 Material

2.1 Study Area

The Benguela Current Large Marine Ecosystem (BCLME) is located in the south-east Atlantic off the South African and the Namibian coasts. It extends from the Cape of Good Hope (34 °S) in the south to the Angola-Benguela Front in the North (15 °S) (Bianchi *et al.*, 1999). Intense equator-wind stress pattern, generated by the onshore moving South Atlantic High Pressure System and its northward deflection along the west coast of southern Africa, result in an offshore flow of surface waters. However, as these waters have to be replaced, deep offshore water masses upwell to the surface. This cold upwelled coastal water brings nutrient-rich water to the surface and forms the basis of the high primary production sustaining large zooplankton and pelagic fish stocks in the BCLME. Compared to other upwelling systems, the Benguela system is inimitable in terms of its contact with warm water regimes at both ends (Shannon and O`Toole, 2003): in the north the Benguela mixes with the southward flowing warm tropical Angola Current and in the south with the Indian Ocean`s Agulhas Current and its retroflection area (Fig 1). The major upwelling area is situated near Lüderitz (27 °S, 15 °E) in southern Namibia. The upwelling processes continue throughout the year and separate the Benguela system into two subsystems, the northern and the southern Benguela system. The first extends from 15 °S to 29 °S covering an area of approximately 179.000 km^2 (Cruickshank and Hewitson, 1993). In

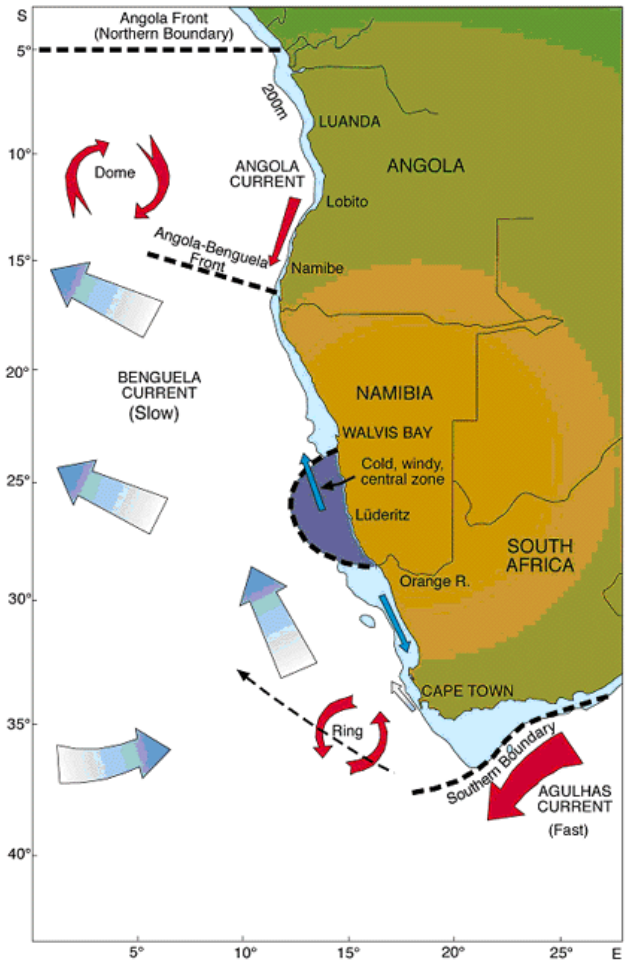

Fig 1: External and internal boundaries of the Benguela Current Large Marine Ecosystem (BCLME), bathymetric features and surface currents (O`Toole *et al.*, 2001)

the central and northern parts of the Benguela system upwelling is most pronounced during winter and spring where winds have a distinct diurnal character (Shannon and O`Toole, 2003), with air flowing from sea towards the land during day (sea breeze) and vice versa during the night (land breeze). In the southern part of the Benguela system upwelling is more seasonal and present mainly in spring and summer.

2.2 Study Organism

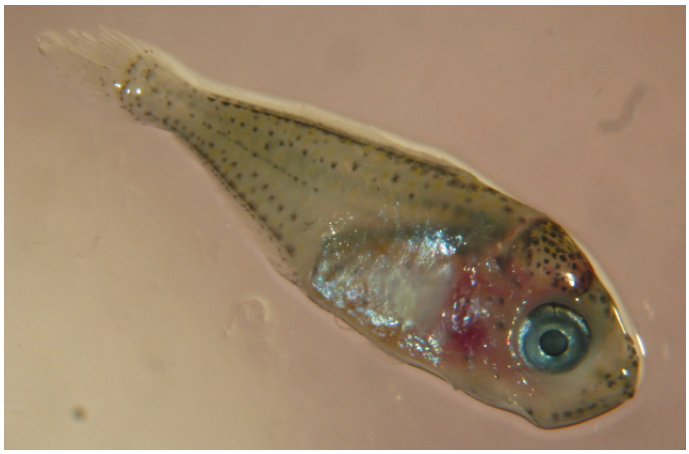

Fig 2: Cape horse mackerel larva. Picture made by Stefanie Bröhl (ZMT).

The Cape horse mackerel (HMC), *Trachurus capensis* (synonym: *Trachurus trachurus capensis*; Castelnau, 1861) belongs to the family of Carangidae (jacks and pompanos) within the order of Perciformes (perch-likes). This mackerel-like pelagic species has a common length between 15 and 30 cm but may reach maximum length of up to 60 cm. It reaches sexual maturity at 3 years at approx. 20 cm length. It is a schooling species often found over the continental shelf or sand bottoms, inhabiting surface waters as well as deeper waters down to 300m. Cape horse mackerels are opportunistic feeders. Their early stages (Fig 2) feed preferentially on copepods while mature specimens take advantage of a wide range of other mesopelagic fishes and invertebrates. Their distribution area extends in the Eastern Atlantic from the Gulf of Guinea to South Africa (Fig 3). In Namibian waters possibly 11 species and 8 genera of the family Carangidae are present (Bianchi *et al.*, 1999) including pelagic and bentho-pelagic life forms. As horse mackerels are very abundant in South African and Namibian waters they often account for high by-catch rates in the offshore demersal trawling, primarily targeting deepwater hake.

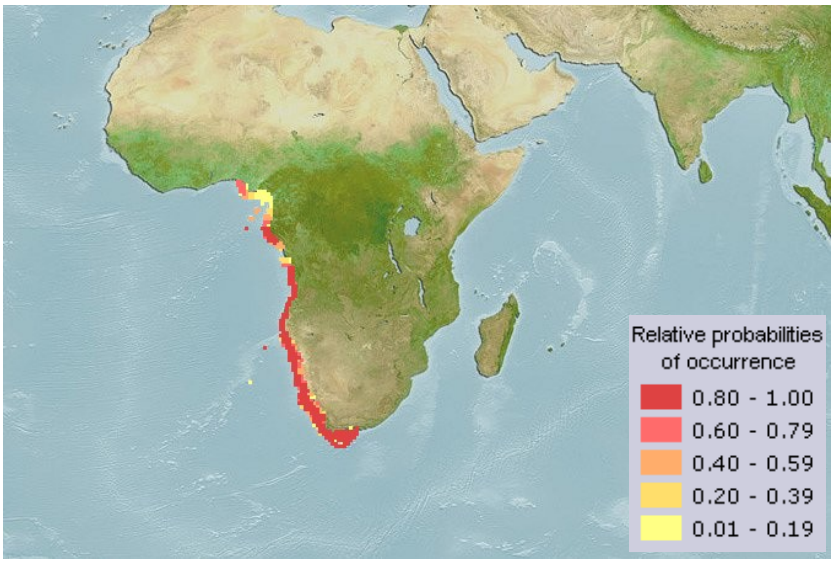

Fig 3: Computer generated distribution map of *Trachurus capensis* (un-reviewed). www.aquamaps.org, version of August 2010.

2.3 Sampling

Larvae and juveniles of Cape horse mackerel were collected between 2008 and 2011 within six GENUS research cruises (Tab 1). Samples of the pelagic communities in the northern Benguela upwelling system were taken on coast normal transects (perpendicular to the coast) at 17.5 °S (Kunene), 19 °S (Rocky Point) and 23 °S (Walvis Bay) next to additional non-permanent transects. The numbers and the positions of sample stations varied between cruises. Hydrographical data were recorded with a CTD-probe (Conductivity, Temperature and Depth) and/or Multinet-Sensor technique, giving station specific environmental depth profiles. Early life stages of pelagic fishes were caught with different plankton nets (Tucker Trawl, MOCNESS/Double MOCNESS, Multinet, Ring Trawl) possessing variable mouth areas (0.25 – 2 m^2) and mesh sizes (500 – 1000 µm). Fish larvae were sorted immediately after catch and kept in aquaria. After sorting the larvae were briefly rinsed with distilled water and frozen and stored at -80 °C for subsequent biochemical analysis. During some cruises, respiration experiments and hypoxic stress experiments were conducted on board with *Trachurus* larvae and juveniles. The samples were also stored at -80 °C and shipped to Bremen for enzymatic analysis.

Tab 1: Overview of the GENUS research cruises from 2008 to 2011.

Year	Month	Cruise	Research vessel
2011	Jul/Aug	MSM 18/4	*RV Maria S. Merian*
	Jan-Mar	MSM 17/3	*RV Maria S. Merian*
2010	Sep/Oct	D-356	*RRS Discovery*
2009	Dec	258	*FRS Africana*
2008	Feb/Mar	MSM 07/2a+b	*RV Maria S. Merian*
	Mar/Apr	MSM 07/3	*RV Maria S. Merian*

2.4 Sample Selection

For this study, samples of Cape horse mackerel larvae were selected according to the following criteria. First, they had to be large enough (≥ 9 mm) to allow for optimal enzyme extraction and analysis. Smaller larvae were pooled with others from the same station. From the large pool of available Cape horse mackerels, 104 samples were used for basic enzymatic analysis from different cruises but similar sampling positions to allow for seasonal and environmental comparisons.

Another 33 samples from hypoxic and normoxic stress experiments on board of the MSM 18/4 cruise were measured for comparative enzymatic capacity. Additional 12 samples of other small pelagic larvae, such as sardines (SAR) or anchovies (ANC) were used for an inter-specific comparison of key enzyme activities. The origins of the selected samples for this study are presented in Tab 2, summarizing the geographical location, depth layer and abiotic parameters.

Tab 2: Overview of the selected HMC samples showing station locations and depth specific environmental parameters. South/East (dez) = station position in decimal degrees; net type = Ring trawl (RT, Ring), Multinet (MN), Tucker trawl (TT, Tuccer), MOCNESS (MOC) and Double MOCNESS (D-MOC); depth interval net = catching depth; T and DO at net = temperature and dissolved oxygen levels of the sampled depth intervals.

Cruise	South (dez)	East (dez)	Station ID	samples (n)	net type	depth interval net (m)	T at net (°C)	DO at net (ml/l)
D356	-19.07	11.50	28	6	RT2	40-0	15.799	4.894
D356	-19.00	12.18	42	7	RT5	60-0	13.974	4.901
M17-3	-23.00	14.05	222	6	D-MOC R3	50-25	15.185	2.337
M17-3	-17.25	11.72	304	6	TT4	28-0	17.803	2.200
M7-2b	-23.00	13.52	3	9	MNo4	50-25	17.318	4.643
M7-2b	-17.27	11.47	14	11	MOC N4	25-0	18.203	3.357
M7-3	-18.26	11.87	103/229	5	Tuccer	40-0	18.400	2.715
M7-3	-18.00	11.50	104/230	5	MOC N0	220-0	15.516	1.063
M7-3	-19.50	12.52	87/213	10	Ring	60-0	16.636	1.477
M7-3	-19.50	12.43	88/214	5	Ring	60-0	17.572	2.396
M7-3	-19.00	12.35	92/218	10	Ring	30-0	17.992	3.493
M7-3	-19.00	12.25	93/219	5	Ring	60-0	17.372	2.302
M7-3	-19.50	12.43	99/225	18	Ring	80-0	16.888	3.832
MSM18-4			780	7	TT	20-30	19.150	3.208
MSM18-4	-15.16	11.65	799	1	DN	10	18.934	4.731
MSM18-4	-9.43	12.98	818	2	TT	20	16.623	1.276
MSM18-4	-8.80	13.10	824	6	TT	10-20	19.045	4.515
MSM18-4	-8.80	13.17	825	12	TT	10-20	18.153	3.249
MSM18-4	-14.51	9.86	847	2	MN	0-150	17.109	3.383
MSM18-4	-17.25	11.50	850	6	TT	20	15.239	3.849
MSM18-4	-9.43	12.98	818	1	TT	20	16.623	1.276

3 Methods

3.1 Biochemical Analyses

The samples for enzymatic analysis were stored at -80 °C at the Leibniz Center for Tropical Marine Ecology (ZMT, Bremen). The muscle tissue of Cape horse mackerel was dissected following a defined constant procedure and extracted in buffers which were specific for each of the respective enzymes. The activities of pyruvate kinase (PK), lactate dehydrogenase (LDH), citrate synthase (CS) and the electron transport system (ETS) were measured. All chemicals were purchased from Sigma-Aldrich®, except for acetyl coenzyme A (Ac-CoA) and lactate dehydrogenase which were ordered at Roche Ltd. Most substrate solutions were prepared at the beginning of the analysis and stored in aliquots at -20 °C. Solutions of Ac-CoA and NADH (for ETS assay) had to be prepared daily before use. Prepared buffer solutions were stored at 4 °C.

All activity measurements were conducted at 18 °C, considering the *in-situ* temperature at catching depth (~16 °C - 18 °C). All samples were run in triplicate. Blank activities were measured by adding homogenization buffer instead of sample homogenate to the reaction mixture. The increase (CS, ETS) or decrease (PK, LDH) in absorbance was detected in a tempered spectrophotometer (Perkin Elmer, Lambda 35, UV/VIS spectrometer) over a time period of 5 minutes with a time interval of 15 seconds, applying the associated measuring software UV WinLab (Perkin Elmer). Within post-processing steps, the rate was adjusted according to the time range where a linear relationship could be observed, in general between 30 to 270 s or 60 to 240 s. The resulting slope, calculated by subtracting the blank activity from sample activity, was further used to calculate an enzymes activity.

3.2 Sample Preparation

The preparation of muscle tissue followed a defined protocol for all samples. All preparatory steps were conducted on ice in order to maintain enzymatic activity. The frozen samples were placed on an ice-filled petri dish and the standard length (L_s) was measured under the binocular to the nearest 0.5 mm. The gut contents of the larvae were removed and the stomach filling (0 = empty to 5 = full), as well as gutted wet mass (WM_g) of each sample were determined. Muscle tissue samples were dissected and weighed (WM_m) in a pre-cooled 1.5

ml Eppendorf cup on an analytical balance (Sartorius AG, d = 0.001 mg). For larvae of smaller body size, the head was removed and the whole posterior trunk was used for enzymatic analysis. Occasionally, very small larvae (≤ 5 mm) with a comparable environmental background had to be pooled to allow for sufficient biomass in the enzymatic assays. In juvenile HMC, subsamples of white axial muscle were taken for analysis. Tissue samples were kept on ice until subsequent enzymatic extraction.

3.3 Measurement of Metabolic Key Enzymes

3.3.1 Extraction

Extracts for citrate synthase, lactate dehydrogenase and pyruvate kinase were prepared in a 50-fold volume (w:v) of ice-cold homogenization buffer (75 mM Tris-HCl, 1 mM EDTA, pH 7.5). Hence, for 10 mg tissue sample 500 µl buffer were used. The tissues were homogenized manually for approx. 1 min using a plastic pestle and then lysed for another minute via ultrasonication (Bandelin, Sonopuls homogenisator; amplitude: 20%, pulse on: 0.1 s, pulse off: 1.0 s). The homogenates were centrifuged for 10 min in a pre-cooled centrifuge (Eppendorf, 5804R) at 2 °C and 5000 rpm. Supernatants were transferred into new pre-cooled sterile Eppendorf cups and immediately frozen and stored at -80 °C until enzyme analysis. The stability of the enzymatic extracts to freezing was tested in advance (see 4.1).

3.3.2 Citrate Synthase Activity

Citrate Synthase (CS; EC: 4.1.3.7) is an important regulatory enzyme located in the mitochondrion and serves as a quantitative index of citric acid cycle activity (Lannig *et al.,* 2003). It is the initial enzyme of this biochemical cycle and catalyzes the reaction between acetyl coenzyme A (Ac-CoA) and oxaloacetic acid (Ox-Ac) to form citric acid (1).

(1) $$\text{acetyl CoA} + \text{oxaloacetate} + H_2O \rightarrow \text{citrate} + \text{CoA-SH} + H^+$$

The resulting thiol of CoA reacts with the DTNB (5,5`-dithiobis-2-nitrobenzoic acid) in the reaction mixture forming a CoA-S-S-DTNB complex. This yellow product is observed spectrometrically by measuring the increase of absorbance at a wavelength of 412 nm; ε_{DTNB} = 13.61 mM^{-1} cm^{-1} (2), proportional to the enzymatic activity.

(2) $$\text{CoA-SH} + \text{DTNB} \rightarrow \text{CoA-S-S-DTNB}$$

Citrate synthase activity was determined following Sidell *et al.* (1987) with the following modifications: The reaction mixture contained 750 µl Tris/HCl buffer (100 mM, pH 8.2), 50 µl 5,5`-Dithio-bis(2-nitrobenzoic acid) (DTNB, 5 mM), 10 µl acetyl coenzyme A (Ac-CoA, 20 mM), 130 µl distilled water and 50 µl enzyme extract. All substances were well mixed and incubated for 5 min at 18 °C in a cooling-thermomixer (Biotech, HLC-MKR 23). The reaction was started by adding 10 µl oxaloacetate (Ox-Ac, 20 mM) to the reaction mixture. The final concentrations in the assay mixture (1 ml) were 75 mM Tris-HCl buffer (pH 8.2), 0.25 mM DTNB, 0.2 mM Ac-CoA and 0.2 mM Ox-Ac. The increase of absorbance at 412 nm was measured for 5 min.

3.3.3 Pyruvate Kinase Activity

PK (EC: 2.7.1.40) catalyses the final step in glycolysis, the conversion of phosphoenolpyruvate (PEP) to pyruvate with concomitant phosphorylation of ADP to ATP (3). It serves as a principal indicator of glycolytic potential activity.

(3) ADP + phosphoenolpyruvate → ATP + pyruvate

The pyruvate formed from PEP by PK is measured by the formation of NAD^+ in presence of LDH (4). Via spectrophotometric analyses the usage of NADH can be detected at 340nm (ε_{NADH} = 6.31 mM^{-1} cm^{-1}), whereby the decrease in absorbance recorded is proportional to the activity of pyruvate kinase.

(4) Pyruvate + NADH + H^+ → Lactate + NAD^+

The PK activity was determined after Hickey and Clements (2003) with the following modifications for Cape horse mackerels. 828 µl of the assay buffer (containing 60 mM Tris, 60 mM KCl and 6 mM $MgSO_4$ x $7H_2O$, pH 7.6) were mixed with 50 µl ß-Nicotinamide adenine dinucleotide (NADH, 5 mM), 10 µl phosphoenolpyruvate (PEP, 50 mM), 2 µl L-lactate dehydrogenase (LDH from rabbit muscle, 5.5 U/10 µl), 87.5 µl of distilled water and 12.5 µl enzymatic extract. This mixture was incubated for 5 min at 18 °C (see 3.3.2). The reaction was started by the addition of 10 µl adenosine 5`-diphosphate (ADP, 100 mM) to the reaction mixture. The final concentrations in the assay mixture were 50 mM Tris/HCl buffer (pH 7.6) with 50 mM KCl and 5 mM $MgSO_4$ x $7H_2O$, 0.25 mM NADH, 0.5 mM PEP, 1.1 U LDH and 1 mM ADP. The decrease of absorbance at 340 nm was measured for 5 min.

3.3.4 Lactate Dehydrogenase Activity

LDH (EC: 1.1.1.27) is the terminal enzyme of anaerobic glycolysis and serves as an indicator of anaerobic metabolic activity. It converts pyruvate to lactate with concomitant oxidation of NADH (see 3.3.3, (4)). The LDH-activity was determined following Lushchak *et al.* (2001); Lushchak *et al.* (1998) with modifications. Maximum LDH activity was assayed in a medium containing 500 µl Tris/HCl buffer (100 mM, pH 6.8), 15 µl NADH (10 mM), 476 µl distilled water and 5 µl enzyme extract. After 5 min of incubation at 18 °C the reaction was started by adding 4 µl sodium pyruvate (Pyr, 250 mM). The decrease of absorbance at 340 nm was measured for 5 min. The final assay mixture consisted of 50 mM Tris/HCl buffer (pH 6.8) with 0.15 mM NADH and 1 mM Pyr.

3.3.5 Calculation of Enzyme Activity

Mass specific enzyme activities were expressed in units per gram wet mass of the muscle tissue (U g^{-1} WM_m). One unit equals one µmol of substrate converted into product per minute. To calculate enzymatic activity, the slope of each reaction was determined and the following equation applied (6). This calculation was derived on the basis of the Beer-Lambert law (5) describing the relation between the optical density of a solution and its concentration:

(5)
$$A = \varepsilon \times c \times d$$

$$c = \frac{A}{\varepsilon \times d} \quad \leftrightarrow \quad \Delta c/\Delta t = \frac{\Delta A/\Delta t}{\varepsilon \times d}$$

ΔA/Δt = change in absorbance over time
d = path length of the cuvette (1 cm)
ε = molar extinction coefficient [L mol^{-1} cm^{-1}]

Dilution factors (DF) during the enzymatic assays were taken into account to standardize activities to one gram of the muscle tissue.

$$DF_1 = \frac{V_{Assay}}{V_{Aliquot}} \qquad DF_2 = \frac{V_{Extract}}{m_{sample}}$$

V_{Assay} = final volume in the reaction mixture (1000µl)

$V_{Aliquot}$ = volume of the homogenate aliquot used in the reaction mixture [µl]
$V_{Extract}$ = volume of the original homogenate [µl]
m_{sample} = wet mass of the muscle tissue [g]

(6) $$\text{Enzyme Activity } [\text{ U g WM}^{-1}] = \frac{\Delta A\, min^{-1}}{\varepsilon \times \text{d}} \times \text{DF}_1 \times DF_2$$

DF_2 remains constant for CS, PK and LDH, while the DF_1 is specific for each enzymatic assay as well as the molar extinction coefficients (see 3.3.2 – 3.3.4).

3.4 Measurement of Electron Transport System (ETS) Activity

3.4.1 Biochemical Background

The electron transport system (ETS) is a multi-enzyme complex in the inner mitochondrial membrane. It acts as a link between the oxidizing organic matter and oxygen, by passing electrons along numerous cytochromes and dehydrogenases to the terminal electron acceptor. The generated energy is used for oxidative phosphorylation and ATP synthesis. The maximum activity of the key enzymes involved in the ETS can be estimated by the use of an artificial electron acceptor under substrate saturated conditions (Fig 4). The rate-limiting step occurs at the cytochrome b UQ complex (complex III) where one electron is transferred to one molecule of cytochrome c. The electron transmission rate can be measured with the artificial electron acceptor INT (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride). The ETS capacity of the mitochondria and the microsomes can be determined individually, as it involves saturating the mitochondrial ETS with NADH and succinate and the microsomal ETS with NADPH (Gómez *et al.*, 1996). The yellow colored tetrazolium salt is reduced to the reddish product formazan (7) which can be detected photometrically at 490 nm (Kenner and Ahmed, 1975). Two moles of INT reduction are equivalent to one mole of oxygen consumed.

(7) NADH-Dehydrogenase: $NADH + H^+ + INT \rightarrow NAD^+ + Formazan + HCl$

UQ-Oxidoreductase: $UQH_2 + INT \rightarrow UQ + Formazan + HCl$

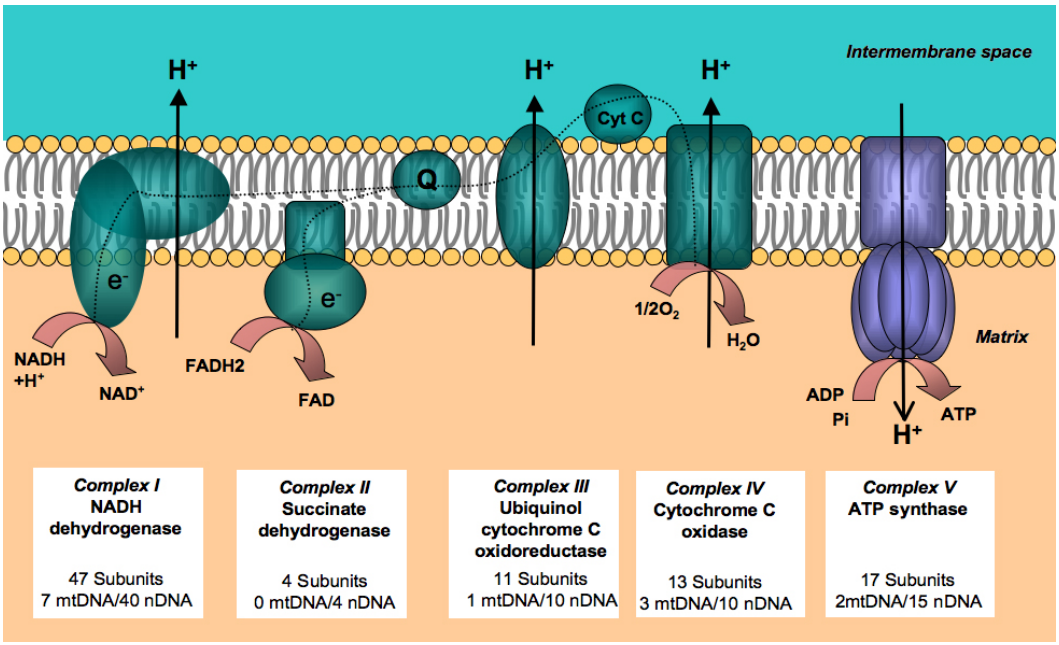

Fig 4: The mitochondrial respiratory chain showing enzyme complexes involved in the electron transport system, ETS (complex I – III).

Only few publications are available which deal with ETS assay in fish larvae (Gopalan *et al.*, 1996; Ikeda, 1989; Yamashita and Bailey, 1990). In this study the method of Packard (1971), modified by Madon *et al.* (1998) and Lannig *et al.*, 2003 was applied and optimized for the investigated fish larvae.

3.4.2 Extraction

The dissected muscle tissue was ground with a plastic mortar for approximately 1 min in homogenization buffer (HOM) containing 1.5 mg/ml polyvinylpyrolidone (PVP), 75 µM $MgSO_4$ x $7H_2O$ and 0.2 % Triton X-100 in 0.1 M phosphate buffer, pH 8.5 (according to Owens & King, 1975). ETS enzyme extracts were prepared in a 200-fold volume (w:v) of homogenization buffer. Hence 5 mg muscle tissue were diluted in 1000 µl HOM obtaining a final concentration of 5mg/ml. After 30 s of tissue lysis by ultrasonication (Bandelin, Sonopuls HD 3100) the homogenates were centrifuged for 10 min at 2°C and 1500g (Eppendorf, 5804 R). The resulting supernatant was transferred into a sterile Eppendorf cup and stored on ice until analyses. Prior to routine analysis, ETS activity was tested to be stable to storage periods on ice (see 4.1).

3.4.3 ETS Assay

ETS activities were determined following Lannig *et al.* (2003) with slight modifications. The final assay volume was adjusted to 1 ml and the reaction mixture was prepared as follows in 1.5 ml single use plastic cuvettes: 500 µl assay buffer (0.1 M phosphate buffer, pH 8.5) were mixed with 250 µl INT-solution (8 mM INT in 0.1 M phosphate buffer, pH 8.5) and 167 µl NADH-solution (7.2 mM NADH with 0.2 % Triton X-100 in 0.1 M phosphate buffer, pH 8.5), stirred with a plastic stirrer and incubated for 5 min at 18 °C in a cooling-thermomixer (HLC, MKR 23). The reaction was started by adding 83 µl of sample homogenate to the assay mixture. The final assay mixture contained 0.09 M phosphate buffer (pH: 8.5), 6.3 µM $MgSO_4$ x $7H_2O$, 0.12 mg/ml PVP, 0.05 % Triton X-100, 2 mM INT and 1.2 mM NADH. The change of absorbance at 490 nm was measured for 5 min at 18 °C in a spectrophotometer (Perkin Elmer, Lambda 35).

3.4.4 Calculation of ETS Activity

As other investigated metabolic enzymes, ETS enzyme activity can also be expressed in units (µmol min^{-1}) per gram wet mass of the muscle tissue (U g^{-1} WM_m), applying the same equation:

$$\text{ETS Activity}\left[\text{U g WM}_\text{m}{}^{-1}\right] = \frac{\Delta A\ min^{-1}}{\varepsilon \times \text{d}} \times \frac{\text{V}_\text{Assay}}{\text{V}_\text{Aliquot}} \times \frac{V_{Extract}}{m_{sample}}$$

$\Delta A\ min^{-1}$	= change in sample absorbance – change in blank absorbance per min
ε	= molar extinction coefficient of INT-Formazan [15.9 mM^{-1} cm^{-1}]
d	= path length of the cuvette [1 cm]
V_{Assay}	= volume of the final assay mixture [1000 µl]
$V_{Aliquot}$	= volume of homogenate used in the reaction mixture [83 µl]
$V_{Extract}$	= volume of the original homogenate [ml]
m_{sample}	= wet mass of the muscle tissue [g]

For a suitable comparison of the calculated muscle specific ETS activity with former data on standard and routine metabolic rates in Cape horse mackerel larvae and juveniles (Geist *et al.*, 2012; submitted manuscript), ETS activity had to be converted into the oxygen consumption rates (8) using the ratio of O_2 to INT of 1 : 2.

(8) $$\text{ETS Activity}\left[\ \mu\text{mol}\ O_2\ \text{min}^{-1}\text{g}\ \text{WM}_\text{m}{}^{-1}\right] = 0.5 \times \text{ETS Activity}\left[\ \text{U g}\ \text{WM}_\text{m}{}^{-1}\right]$$

Beforehand, ETS values were converted to a unit hour by multiplying specific ETS activities with 60.

3.5 Experimental Design

3.5.1 Adjustment of Enzyme Methods

The methods for CS-, PK- and LDH- enzyme activity measurements were already optimized for Cape horse mackerel larvae in a previous student research project (Michalek, 2012). Optimum substrate and cofactor concentrations and pH-values were established for each enzyme. Additional experiments were performed concerning the stability of the sample extracts to freezing, the choice of suitable sample tissues as well as the adjustment of the ETS assay.

Stability of the Crude Extract

To allow for temporal separate preparation and measurement sections, a possible loss in enzyme activities to repeated freezing and thawing had to be examined. For CS, ETS, PK and LDH subsamples of three sample homogenates were directly measured, while remaining extracts were frozen at -80 °C and measured after one and two freezing intervals. Additionally, a time series of 100 min was investigated in three sample extracts which were stored in an ice-water bath to measure the stability of ETS enzyme activities.

Whole Larva vs. Muscle Tissue

Prior to routine analysis, measurements were conducted to select suitable samples (muscle tissue and whole organism) for practicability and best reproducibility of enzyme activity. Enzyme activities of three muscle tissue extracts and three extracts of whole organisms were compared.

Extract Preparation

The common protocols for extract preparation vary considerably in terms of extraction and purification of the ETS. As centrifugation is thought to be a crucial step in purification, three centrifugation protocols (A, B and C) with different centrifugation speeds and temperatures were tested. The centrifugation protocol yielding the highest ETS activity was used for routine analyses.

Substrate Concentration

A saturation curve of the substrate NADH was established for ETS assays, applying NADH concentrations from 0 – 2 mM in the assay. The substrate solution was prepared after Madon *et al.*, 1998 (5.1 mM NADH in 0.1 M phosphate buffer and 0.2 % Triton X-100 (v:v), pH 8.5).

3.5.2 Physiological Effects

Allometric Scaling

Body and muscle tissue mass varied between individual fishes. Accordingly, allometric relationships may influence levels and comparisons of enzyme activities. Enzyme activities were plotted as a function of body mass. Either specific activities (per gram muscle tissue wet mass) or individual activities (per total muscle wet mass) were related to the gutted wet mass of the larvae (WM_g) or the wet mass of the muscle tissue (WM_m) respectively. Simple linear regression analysis was applied and exponential allometric relations were derived (see 3.6)

Respiration : ETS

Routine and standard metabolic rates were previously derived for early life stages of Cape horse mackerels (Geist *et al.*, 2012; submitted manuscript). Comparable to metabolic enzyme activities, allometric equations were used to calculate the range of metabolic rates for five standardized size classes of HMC, ranging from 0.001 g WM in intervals of ten to 10 g WM. Measurements of respiration rates reflect the actual oxygen consumption of an organism, whereas measurements of the electron transport system (ETS) can detect its potential metabolic rate by estimating maximum activities of the key enzymes involved in

the ETS. By correlating ETS activities and *in vivo* respiration of comparable size classes, the proportion of respiratory capacity, HMC larvae and juveniles effectively use, can be estimated.

Interspecific Comparison

As early life stages of Cape horse mackerels are thought to cope better with increasing hypoxic conditions in the northern Benguela Upwelling System, aerobic and anaerobic enzyme capacities were compared with those derived from comparable pelagic fish larvae occurring in this area. Activities of CS and LDH, serving as principal indicators of the respective metabolism, were measured in 12 samples of anchovies (ANC) and sardines (SAR) and compared with those derived for Cape horse mackerels.

3.5.3 Physiological and Environmental Effects

Normoxia vs. Hypoxia

Cape horse mackerel samples from the MSM 18-4 cruise, exposed to different hypoxic stress experiments (n = 23) were compared with untreated larvae (n = 9) caught at comparable locations and depth layers. On board various respiration experiments were conducted, in order to determine the effect of hypoxic stress on Cape horse mackerel larvae. Experimental setups varied in terms of oxygen supply and presence or absence of recovery phases for the fish larvae. To determine the effect of lower oxygen concentrations on aerobic and anaerobic enzyme capacities, samples were selected according to different oxygen saturations used, including 30% (Stress II), 50% (Stress I) and 70 to 80 % (Standard; Batch). Additional 6 samples, originally intended to measure a possible accumulation of lactate in the muscle tissue, were used for a comparison of anaerobic enzyme activities with untreated individuals. These larvae, reared without permanent oxygen supply, died due to oxygen depletion and were immediately frozen for enzymatic analysis.

3.6 Data Analysis and Statistics

Unless stated otherwise, data were presented as means ± standard deviation. Statistical analyses were performed with "R statistic package" (R Foundation for Statistical Computing; Vienna, Austria) or SigmaPlot 12 (Systat Software, Inc; San Jose, USA). Statistical comparison of more than two data sets was performed by one-way analysis of variance (ANOVA) when independency of observations, normal distribution of the residuals and equal variances were given. Not-normal distributed data were analyzed with the non-parametric Kruskal-Wallis one-way ANOVA by ranks followed by varying post-hoc analysis. Data sets are considered to be significantly different at a significance level of $p < 0.05$.

To analyze the relationship between enzyme activity and body mass, raw data were *ln*-transformed to achieve a suitable correlation. Simple linear regression analysis was applied and the exponential allometric relation was stated: $Enzyme\ Activity = a \times WM^b$, revealing the normalization constant *a* and the metabolic scaling coefficient *b*. Double logarithmic plots were given for each enzyme including linear regression lines and 95% confidence intervals respectively. Regression coefficients and consequent allometric relations are listed, giving the correlation coefficients (r^2) and significance levels (p - value) of each relationship (Tab 5).

Multiple linear regressions were applied to model the relationship between one dependent variable enzyme activity (Y) and more than one independent variable, such as temperature (X_1) and dissolved oxygen levels (X_2). The parameters to be estimated are represented as coefficients, whereby the intercept ($ß_0$) and the slopes ($ß_1$: temperature; $ß_2$: dissolved oxygen) were named respectively.

$$Y = ß_0 - (ß_1 \times X_1) + (ß_2 \times X_2)$$

$$\text{Enzyme activity} = ß_0 - (ß_1 \times T) + (ß_2 \times DO)$$

4 Results

4.1 Analytical Adjustments

4.1.1 Assays of CS, PK and LDH

Experiments were performed concerning a) the stability of the crude extract to repeated freezing and thawing and b) the differences in activity levels between the whole fish larvae and dissected muscle tissue.

Stability of the Extract to Freezing and Thawing

Frozen extracts did not lose activity compared to fresh extracts (Tab 3). CS and LDH even showed slightly higher activities after freezing (CS: + 2.9 %; LDH: + 8.9 %) while PK-activity slightly dropped (- 4.5 %). However, these differences between the treatments and the immediate measures were not statistically significant for CS ($p = 0.82$, ANOVA), LDH ($p = 0.40$, ANOVA), and PK ($p = 0.48$, ANOVA). As enzyme activities were stable to repeated freezing and thawing, all sample extracts were prepared in advance and stored at -80 °C for batch analysis of enzyme activities.

Tab 3: Comparison of specific enzyme activities of CS, LDH and PK, immediately measured after extraction or after one or two freezing cycles. (Mean ± SD; n = 3).

	CS Activity	**LDH Activity**	**PK Activity**
	mean ± SD	mean ± SD	mean ± SD
direct	5.864 ± 0.548	158.412 ± 25.297	115.109 ± 21.532
1x frozen	6.034 ± 0.615	172.558 ± 25.305	109.908 ± 7.635
2x frozen	5.912 ± 0.651	159.045 ± 22.194	104.486 ± 18.557
p-value	*0.8295*	*0.4016*	*0.4813*
r^2	*0.015*	*0.076*	*0.067*

Whole Larva vs. Muscle Tissue

Both, CS and PK activities were significantly different between extracts of dissected muscle and whole larvae. CS activity was significantly higher in muscle tissue than in the whole organism tissue ($p = 0.007$, ANOVA). Even higher differences were found for PK with nearly

twice as high activities found in muscle tissue extracts compared to extracts of the whole larvae (p = 4.21e-10, ANOVA).

4.1.2 Assay of the Electron Transport System (ETS)

The ETS assay of Lannig *et al.* (2003) was adopted for optimum use with Cape horse mackerel larvae. Additional tests were performed concerning the stability of the extract to freezing and adjustments of substrate concentrations and extract preparations.

Extract Preparation

ETS activities showed highest values at medium centrifugation speed of 1500 x *g* at 2°C (Tab 4). ETS activities were reduced at higher centrifugation speeds (94.78 ± 15.11 %). Centrifugation at 300 x *g* yielded significantly lower ETS-activity (p = 0.006; ANOVA, Holm-Sidak). Therefore, extracts were prepared at 2 °C and 1500 x *g* for routine analysis.

Tab 4: Influence of centrifugation speed and –temperature (T) on ETS Activity (ETS_A), given in % (mean ± SD; n=3). Significant differences were highlighted with asterisks.

Treatment	Speed (x *g*)	T (°C)	Time (min)	ETS_A (%)
A	300	0	10	80.21 ± 7.32 *
B	2500	2	10	94.78 ± 15.11
C	1500	2	10	100.0 ± 14.42

Substrate Concentration

In order to establish suitable NADH concentrations for the ETS, assays were run with 0 to 2 mM NADH (Fig 5). ETS activity showed a plateau of activity at NADH-concentrations between 0.8 and 1.3 mM. Higher NADH concentrations caused slightly elevated ETS activities which, however, were considered as individual scatter. To ensure substrate saturation as well as material-saving work practice, a concentration of 1.2 mM NADH was used throughout all ETS analyses.

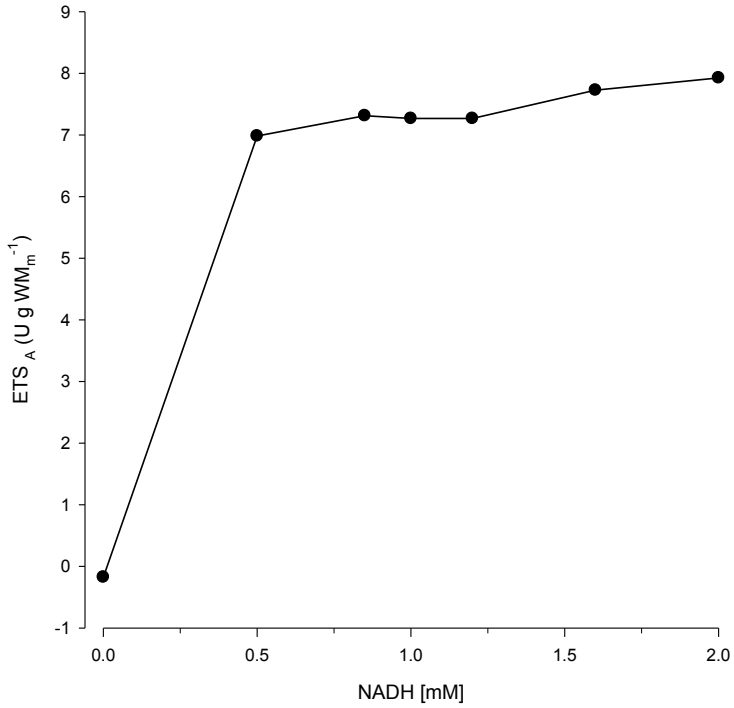

Fig 5: Saturation curve for the NADH concentration [mM] used in the ETS assay (n = 1).

Stability of the Extract

A time series of ETS activity was investigated in extracts which were stored in an ice-water bath (a) and extracts which were measured immediately after preparation or after freezing at -80 °C (b). No loss in ETS activity (ETS_A) was observed when stored in an ice-water bath for up to 100 min. ETS_A changed approximately ± 2.5 % showing no significant decline ($p = 0.40$; ANOVA, Kruskal-Wallis) but a slight increase over time (Fig 6, top).

ETS-activity declined after freezing and thawing. (Fig 6, bottom). The samples lost on average 26% of activity ($p = 0.002$; Mann-Whitney). Accordingly, extracts were measured immediately after preparation to avoid falsification of results.

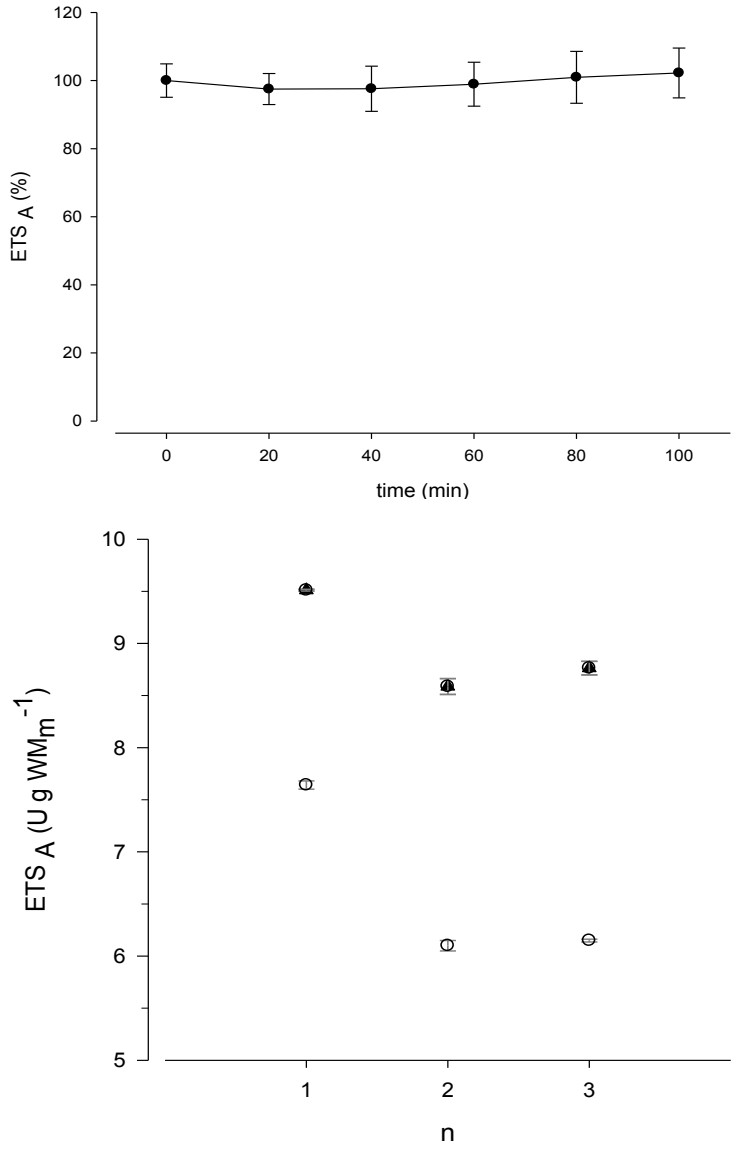

Fig 6: Stability of the ETS Activity (ETS $_A$) at 0 °C (ice-water) for 100 min (**top**). ETS activities of fresh extracts (▲) and after freezing at -80 °C and thawing (○) (**bottom**)**.** ETS $_A$ is either given in percent (**top**) or units per gram wet mass of the muscle tissue (**bottom**). Results are given in mean ± SD, n = 3.

4.2 Activity of Metabolic Key Enzymes

Activities of CS, PK, LDH and ETS were determined in 104 wild caught Cape horse mackerel larvae and juveniles to achieve baseline data on the enzymatic capacities of this species. The sample size varied for each enzyme as not each larva yielded sufficient material for all assays. The size of the analyzed fish ranged from larval stages of 4.0 mm standard length (L_s) and 0.0018 g wet mass (WM) to juveniles of 62.0 mm L_s and 1.056 g WM.

4.2.1 Effect of Body Mass on Enzyme Activities

CS-, ETS-, PK- and LDH- activities were determined as a function of muscle tissue- or gutted wet mass of Cape horse mackerel larvae and juveniles. Specific citrate synthase activities did not change with increasing muscle tissue or body mass, while specific ETS activities decreased and glycolytic specific enzyme activities, PK and LDH, increased (Fig 7 to 10, a+c). Linear regression analysis and allometric relations (Tab 5) showed no apparent relationships between specific CS activities and body mass (b = 0.004) or muscle tissue mass (b = 0.021) over the studied size range, indicated by metabolic exponents close to zero. In contrast, for CS activity related to individual total muscle weight a high metabolic exponent of b = 1.021 was found, indicating a strong linear relationship (r^2 = 0.890), slightly lower correlations were found for the relationship of individual CS activity and gutted wet mass (b = 0.818; r^2 = 0.795). Specific ETS activities declined with increasing body mass while individual ETS activities increased (Fig 8). This relationship was most pronounced when ETS activity was referred to the muscle tissue wet mass (WM_m) of Cape horse mackerels (b = 0.708, r^2 = 0.300). Contrary to CS and ETS, both glycolytic enzymes, PK and LDH, showed an evident increase in specific activity with increasing muscle tissue and/or body mass (Fig 9 and 10). Highest correlations were observed when the individual activities were related to the wet mass of the muscle tissue in both enzymes. For pyruvate kinase (Fig 9), the best logarithmic fit was achieved for the individual activity as a function of muscle tissue wet mass (b = 1.182, r^2 = 0.838) and was slightly lower when related to the gutted wet mass (b = 1.117, r^2 = 0.811). Specific and individual lactate dehydrogenase activity increased significantly with both wet mass of the muscle tissue and gutted wet mass (Fig 10). Strongest correlations were found for the relation of individual LDH activity to the wet mass of the muscle tissue (b = 1.185, r^2 = 0.875). Linear regression slopes were significantly different from zero ($p <$

0.001; ANOVA), hence significant correlations between glycolytic activities and body mass indices were present in early life stages of Cape horse mackerels.

Specific activities of all enzymes were highly scattered over the studied mass range, indicating potential correlations to additional variables next to mass-specific relationships.

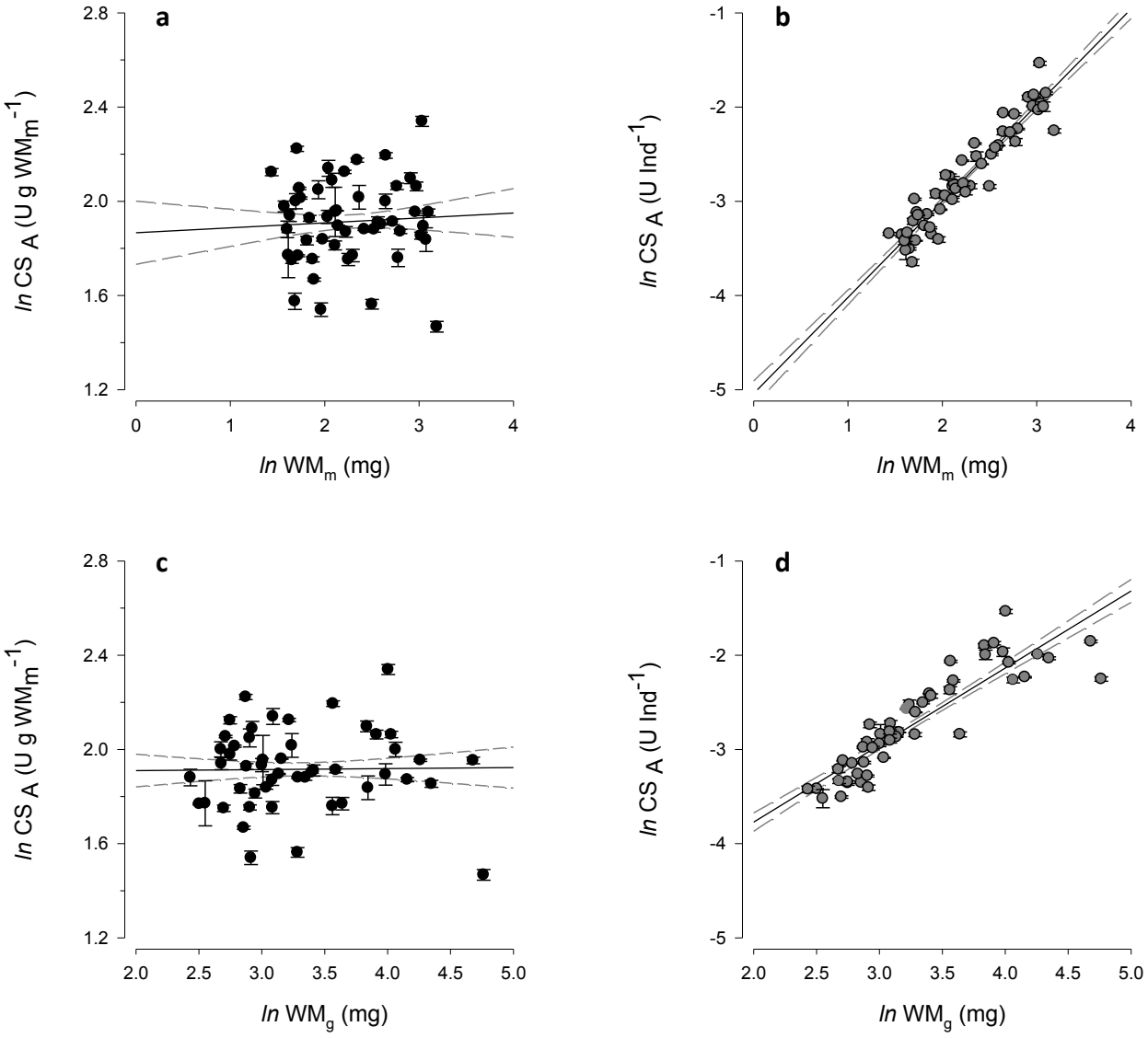

Fig 7 (a-d): Relationships between citrate synthase activity (CS_A) and wet mass of the muscle tissue (WM_m, **a+b**) or gutted wet mass of the larvae (WM_g, **c+d**). Mass specific CS_A is either calculated per gram (black dots, U g WM_m^{-1}, **a+c**) or per individual wet mass of the muscle tissue (dark grey dots, U Ind^{-1}, **b+d**). Confidence intervals (CI = 95%) are shown, linear regressions and allometric relation coefficients are given in Tab 5.

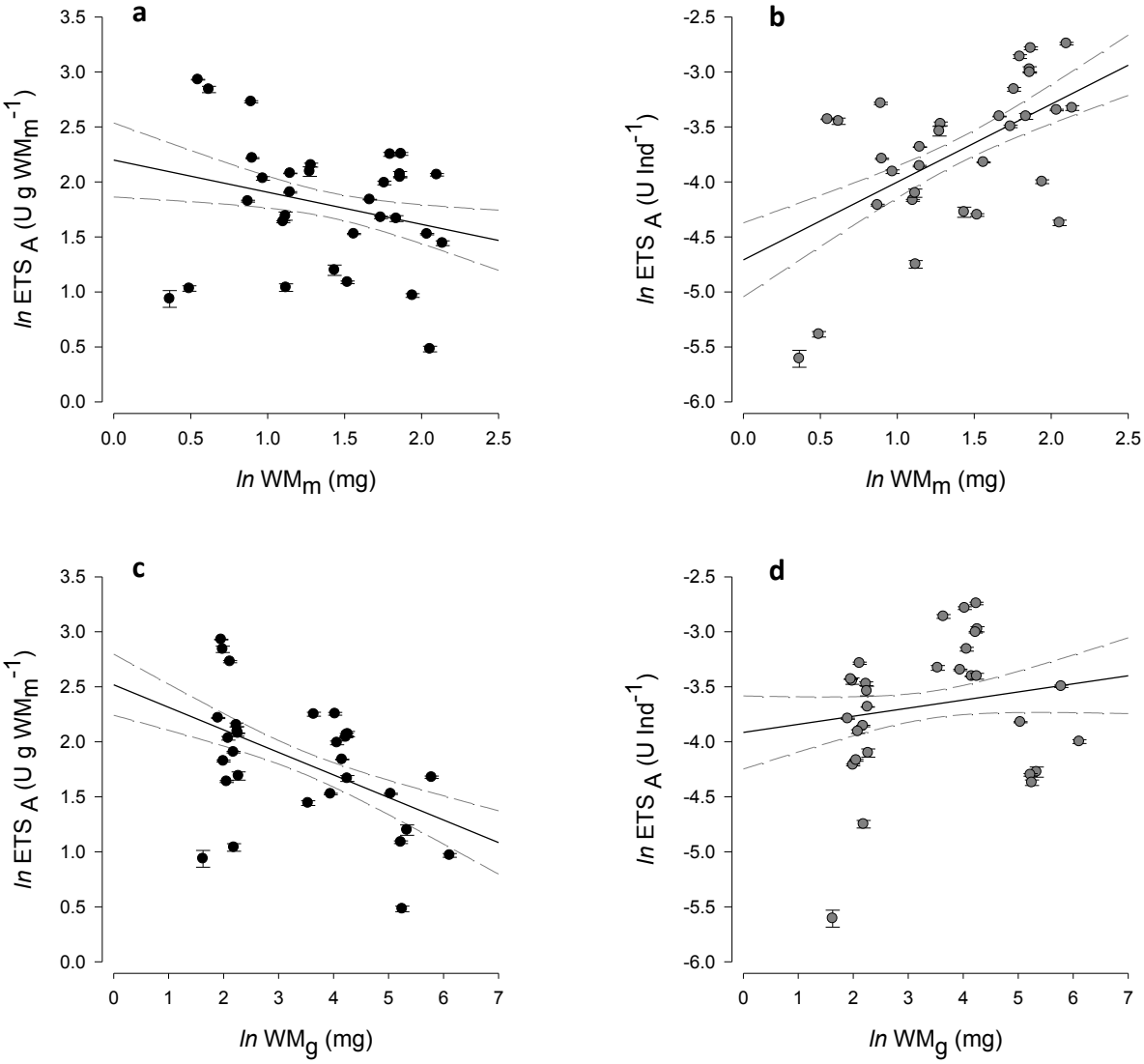

Fig 8 (a-d): Relationships between electron transport system activity (ETS $_A$) and wet mass of the muscle tissue (WM_m, **a+b**) or gutted wet mass of the larvae (WM_g, **c+d**). Mass specific ETS $_A$ is either calculated per gram (black dots, U g WM_m^{-1}, **a+c**) or per individual wet mass of the muscle tissue (dark grey dots, U Ind^{-1}, **b+d**). Confidence intervals (CI = 95%) are shown, linear regressions and allometric relation coefficients are given in Tab 5.

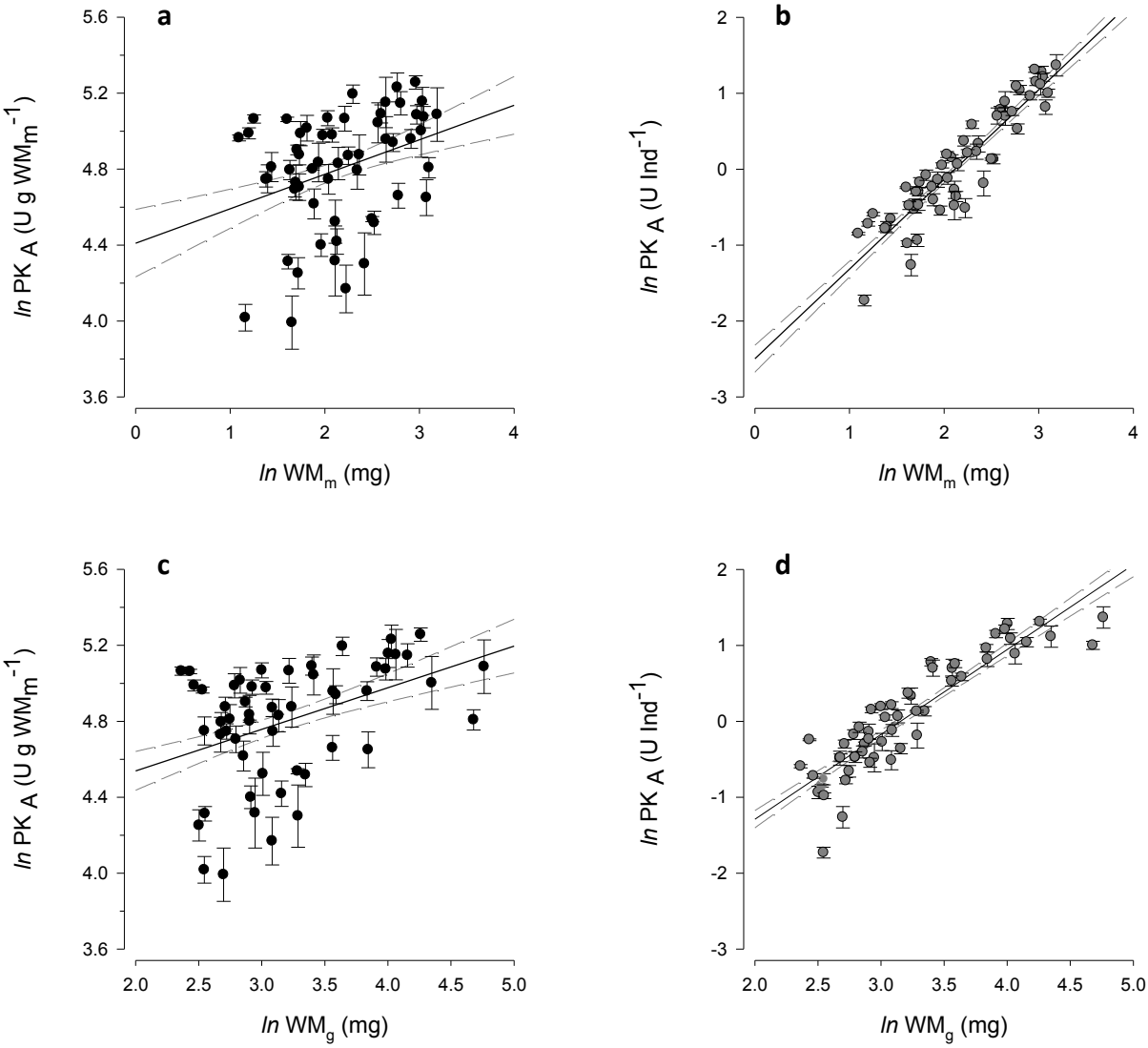

Fig 9 (a-d): Relationships between pyruvate kinase activity (PK_A) and wet mass of the muscle tissue (WM_m, **a+b**) or gutted wet mass of the larvae (WM_g, **c+d**). Mass specific PK_A is either calculated per gram (black dots, U g WM_m^{-1}, **a+c**) or per individual wet mass of the muscle tissue (dark grey dots, U Ind^{-1}, **b+d**). Confidence intervals (CI = 95%) are shown, linear regressions and allometric relation coefficients are given in Tab 5.

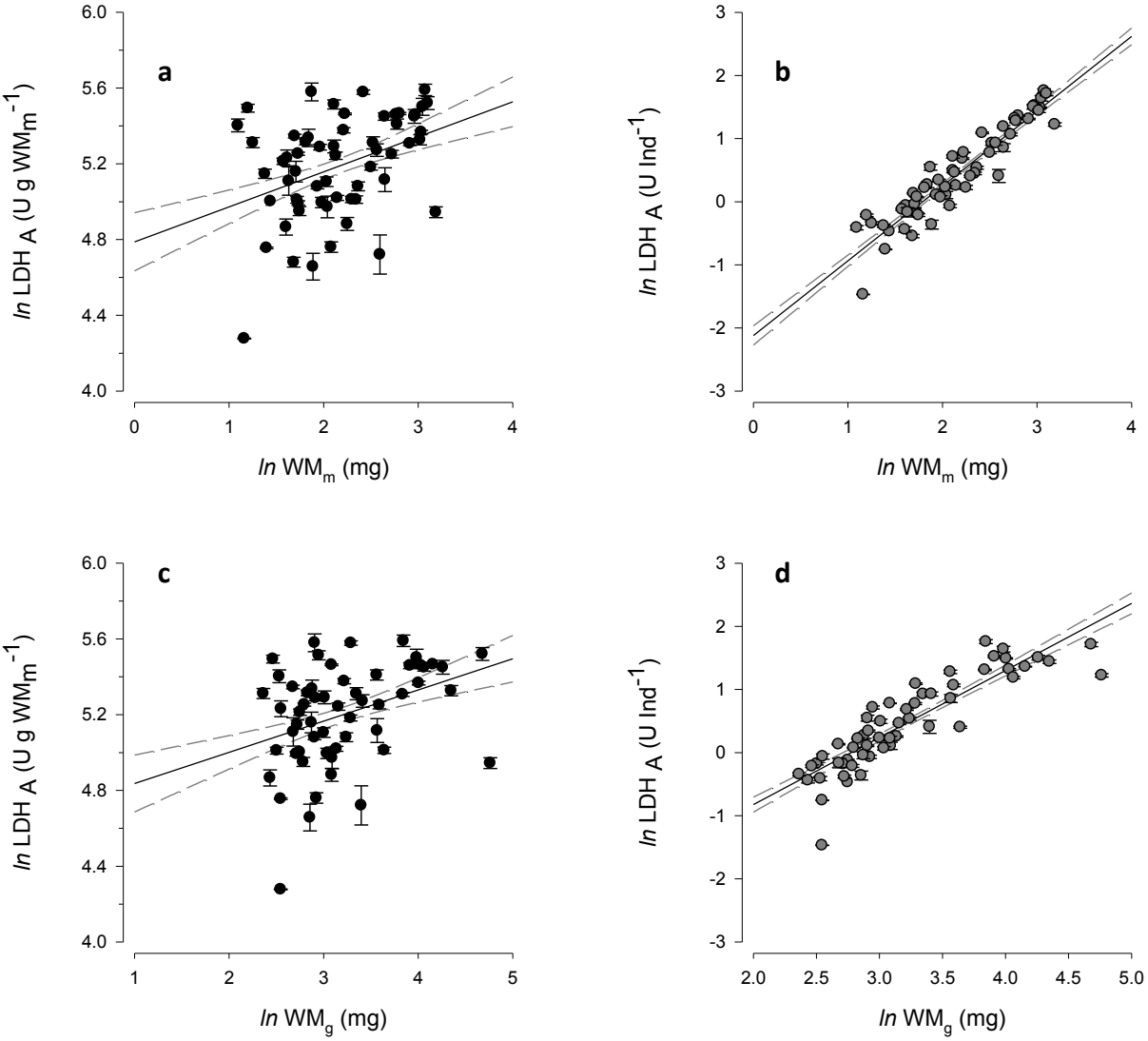

Fig 10 (a-d): Relationships between lactate dehydrogenase activity (LDH $_A$) and wet mass of the muscle tissue (WM_m, **a+b**) or gutted wet mass of the larvae (WM_g, **c+d**). Mass specific LDH $_A$ is either calculated per gram (black dots, U g WM_m^{-1}, **a+c**) or per individual wet mass of the muscle tissue (dark grey dots, U Ind^{-1}, **b+d**). Confidence intervals (CI = 95%) are shown, linear regressions and allometric relation coefficients are given in Tab 5.

Tab 5: Relationship between body mass indices (WM_m: muscle tissue, or WM_g: gutted wet mass) and enzymatic activities (CS, ETS, PK and LDH), expressed as allometric relation. Coefficients are given below, next to sample size (n) and regression statistics (r^2, p - value). Significant regressions are indicated with asterisks. Letters a-d in the first column relate respective equations to regressions shown in plot a, b, c, d for each enzyme.

		allometric relation $Y = a \times WM^b$		regression statistics	
CS *(n=65)*		exp (a)	b	r^2	*p-value*
	a	6.462	0.021	0.003	0.472
	b	0.006	1.021	0.890	< 0.001 *
	c	6.693	0.004	0.000	0.860
	d	0.004	0.818	0.795	< 0.001 *
ETS *(n=31)*					
	a	9.016	-0.292	0.068	0.012 *
	b	0.009	0.708	0.300	< 0.001 *
	c	12.410	-0.205	0.251	< 0.001 *
	d	0.020	0.074	0.030	0.108
PK *(n=69)*					
	a	82.269	0.182	0.110	< 0.001 *
	b	0.082	1.182	0.838	< 0.001 *
	c	60.401	0.219	0.170	< 0.001 *
	d	0.029	1.117	0.811	<0.001 *
LDH *(n=58)*					
	a	120.061	0.185	0.145	<0.001 *
	b	0.120	1.185	0.875	<0.001 *
	c	106.911	0.165	0.133	<0.001 *
	d	0.052	1.063	0.781	<0.001 *

4.2.2 Effect of Temperature and Dissolved Oxygen on Enzyme Activities

Metabolic enzyme activities do not seem to be exclusively related to the size or developmental stage of an organism, but also to their habitat specific environmental regimes. In this study, activities of oxidative (CS) and glycolytic (PK and LDH) enzymes were related to the environmental background of each sample, in this case temperature and dissolved oxygen levels, to detect possible correlations.

Unifactorial analysis was applied to determine the individual effect of either temperature (T) or dissolved oxygen (DO) concentrations on enzymatic activity. Specific and individual activities were grouped in three T-categories (14 – 16°C, 16 – 18 °C and 18 – 20 °C) and DO-categories (1 – 2 ml l^{-1}, 2 – 4 ml l^{-1}, 4 – 5 ml l^{-1}).

The combined effect of T and DO was analyzed by multiple linear regression models for multivariate analysis and plotted three dimensionally. Activities from samples that provided comparable environmental background information (sampling station and catching depth) were averaged forming 16 groups with different temperature and dissolved oxygen regimes. Relationships between enzyme activity and the combined effect of temperature and oxygen were not statistically significant for all enzymes. However, three dimensional plots are shown as correlations between temperature and DO may not necessarily have to apply for upwelling regions.

Citrate Synthase

CS activity related to the wet mass of the muscle tissue did not differ significantly with temperature ($p = 0.212$, ANOVA). Activity values scattered considerably over the studied size range, as illustrated in Fig 11 (left) and noted in Tab 6 with the respective correlation coefficients (r^2). Standardizing CS activities to either 10 mg or 20 mg wet mass of the muscle tissue, highest activities were found at medium temperatures of (16 – 18 °C) and lowest at higher temperatures (18 – 20 °C). Contrarily, activities increased with decreasing dissolved oxygen levels when related to a standard muscle weight (Tab 7).

From multiple regression analyses ($Y = ß_0 – ß_1 \times X_1 + ß_2 \times X_2$), the following formulas were derived:

$$\text{specific CS}_A = 5.030 – 0.002 \times T + 0.435 \times DO$$

$$\text{individual CS}_A = 0.150 – 0.004 \times T – 0.002 \times DO$$

The model described the correlation between CS activity (Y), T and DO (X_1, X_2), giving the coefficient of the Y-intercept ($ß_0$) and the slopes of T ($ß_1$) and DO ($ß_2$) respectively. The overall goodness of both fits was comparatively low ($r^2 = 0.152$, $r^2 = 0.041$). 15.2 % and 4.1 % of the variance of the specific and individual CS activity could be explained by temperature ($p = 0.404$) and dissolved oxygen ($p = 0.796$). However, at low temperatures, citrate synthase activities moderately declined with decreasing oxygen levels (Fig 12). At very low oxygen levels, activities increased with temperature.

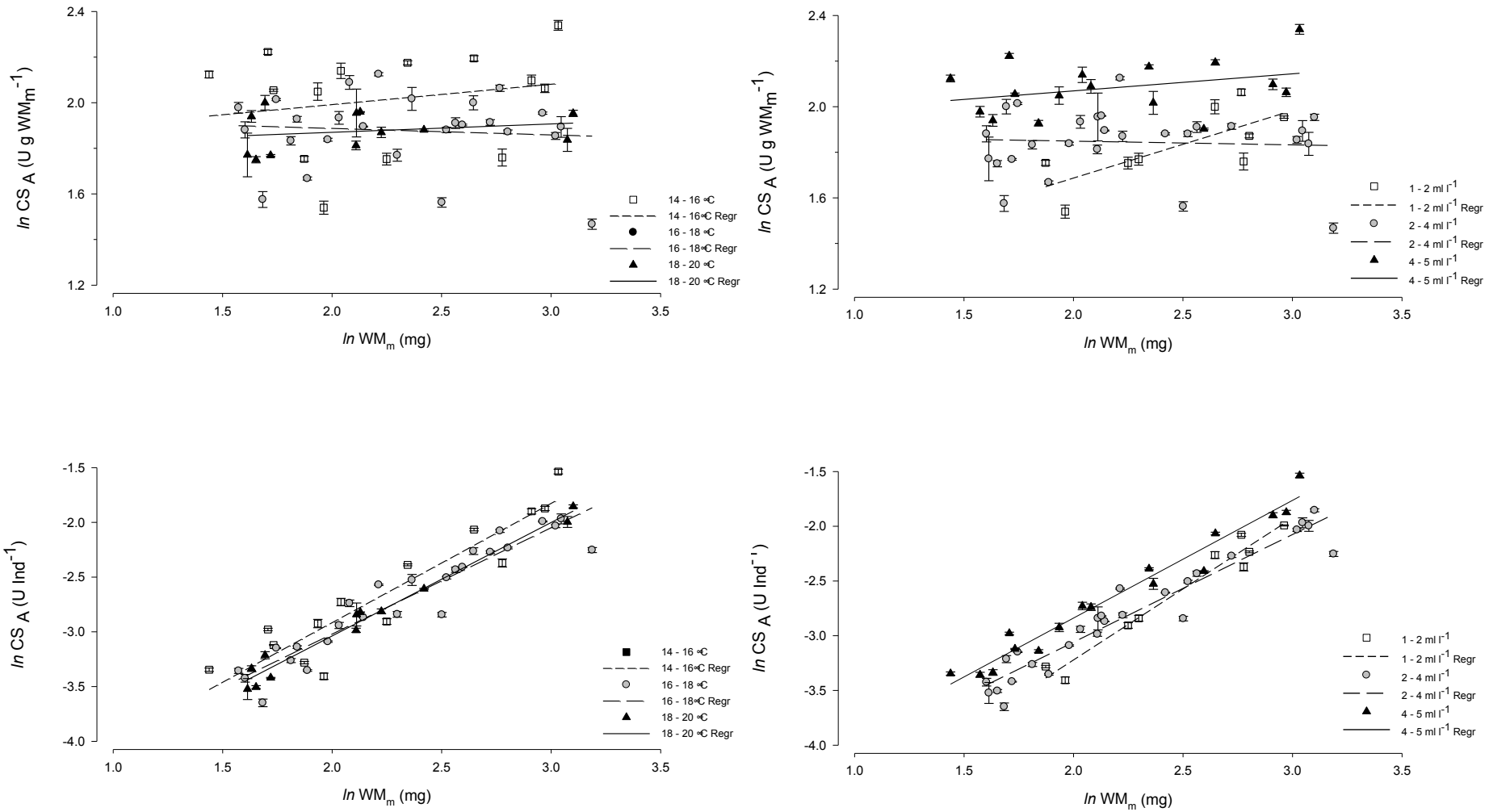

Fig 11: Relationship between citrate synthase activity (CS_A) and wet mass of the muscle tissue (WM_m) at different temperatures (**left**) and dissolved oxygen concentrations (**right**). Sample specific T and DO groups are explained by symbols beneath the graphs. Allometric relations and regression statistics are shown in Tab 6 and 7.

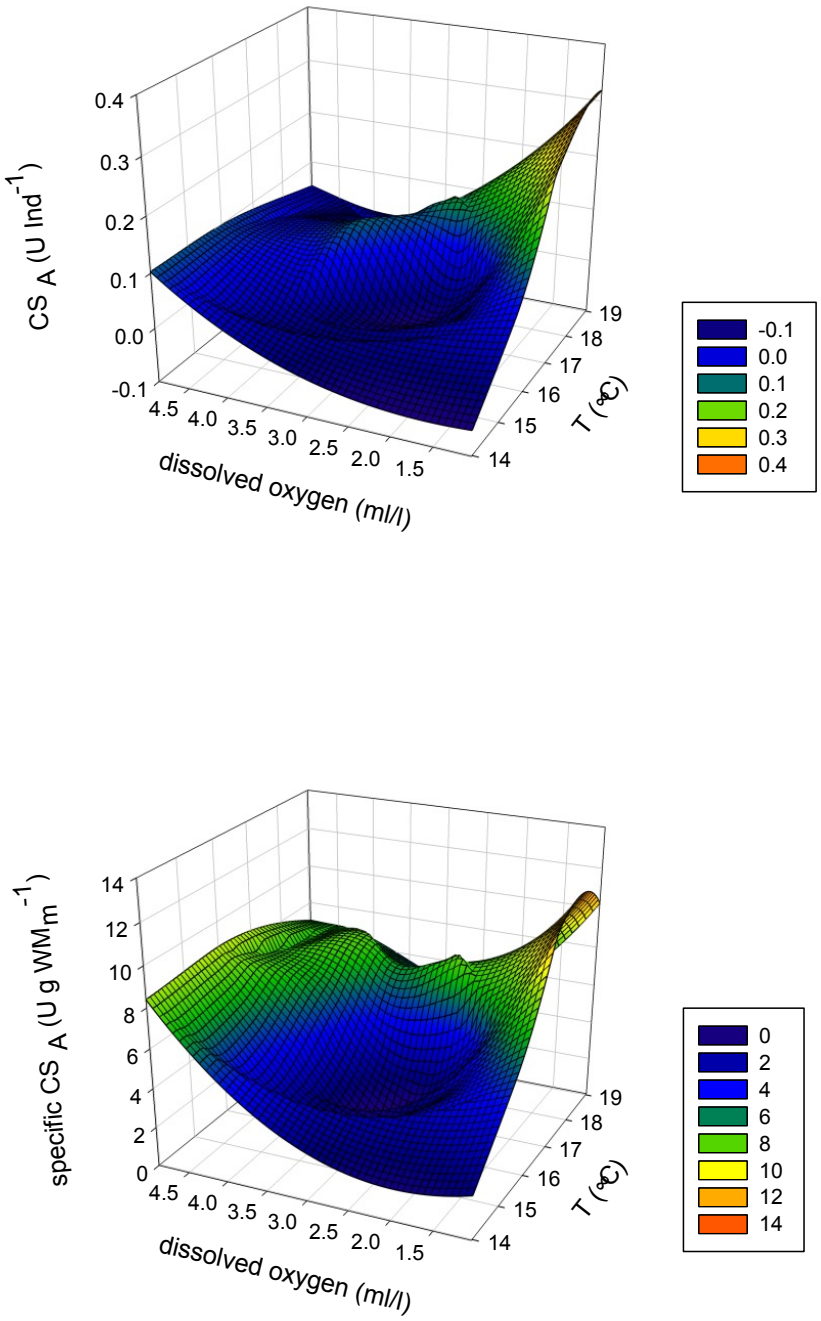

Fig 12: 3D mesh plots showing correlations between citrate synthase activity (**top**: individual; **bottom**: specific activity), temperature (°C) and dissolved oxygen (ml l^{-1}) levels. Different activity values are indicated by coloration as explained in the box beneath the graph.

Pyruvate Kinase

PK activities related to muscle tissue wet mass did not differ significantly with changing temperature (p = 0.769) (Fig 13, left). As for citrate synthase, the correlation coefficients were low for each regression explained by the spread of the data (Tab 6). PK activity related to a standardized muscle wet mass of either 10 or 20 mg was lowest at highest temperatures. Contrarily to CS, PK activities increased with decreasing dissolved oxygen concentrations when related to a standard muscle mass (Tab 7). When comparing the median values among the temperature groups, even a significant increase in PK activity can be observed with decreasing DO concentrations (p = 0.047, Kruskal-Wallis ANOVA), but allometric relationships were neglected.

Investigations of a combined effect of temperature and dissolved oxygen concentrations on PK activities resulted in the following multiple regression models:

$$\text{specific PK}_A = 208.970 - 4.848 \times T + 1.089 \times DO$$

$$\text{individual PK}_A = 4.107 - 0.132 \times T + 0.118 \times DO$$

Compared to CS, the overall goodness of both applied fits was even lower ($r^2 = 0.065$; $r^2 = 0.051$). These models describe that either 6.5 % or 5.1 % of the activity were described by abiotic factors such as temperature and dissolved oxygen. According to this the activity of pyruvate kinase could not be predicted statistically significant from a linear combination of temperature (p = 0.40) and dissolved oxygen concentrations (p = 0.87) (Fig 14).

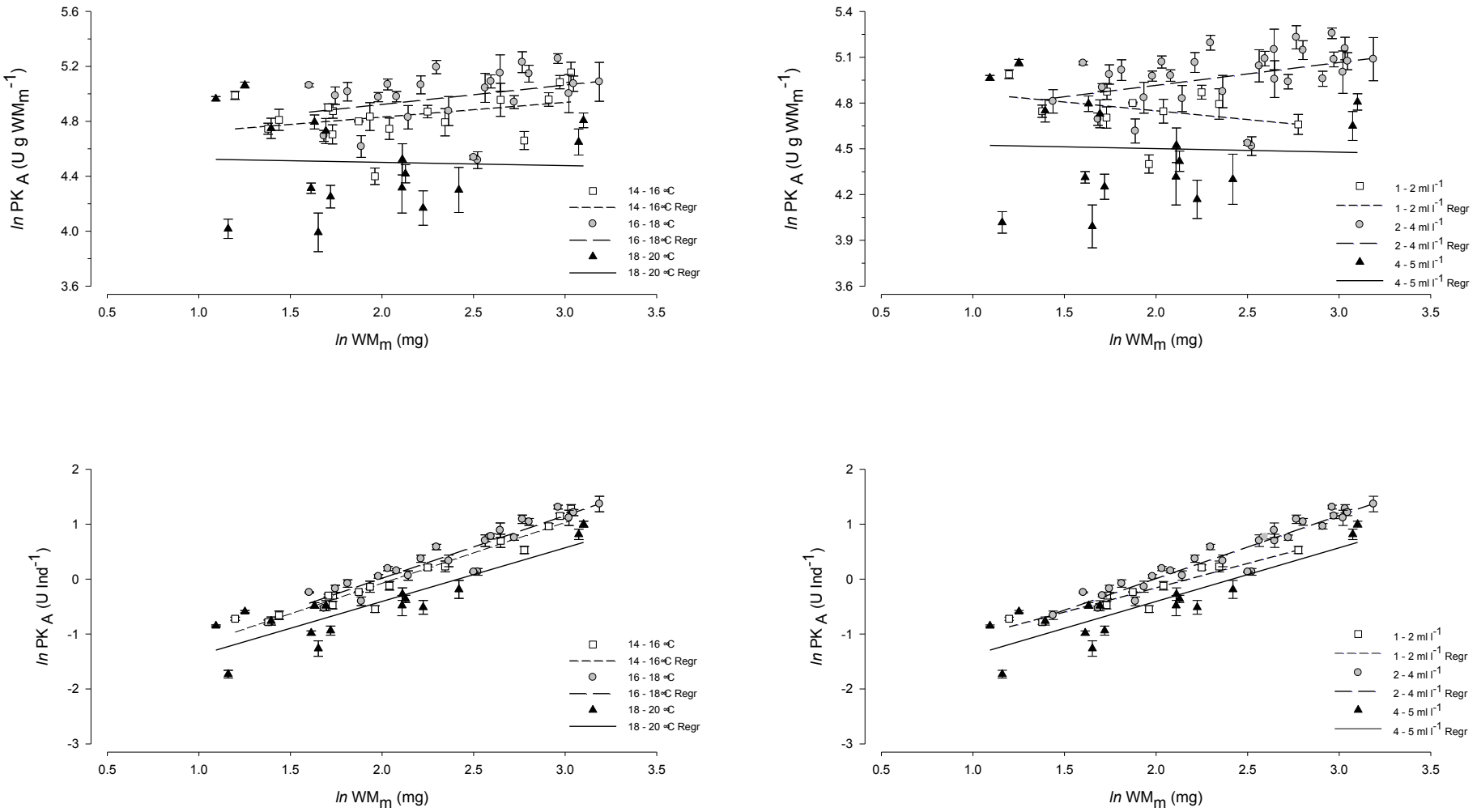

Fig 13: Relationship between pyruvate kinase activity (PK_A) and wet mass of the muscle tissue (WM_m) at different temperatures (left) and dissolved oxygen concentrations (right). Sample specific T and DO groups are explained by symbols beneath the graphs. Allometric relations and regression statistics are shown in Tab 6 and 7.

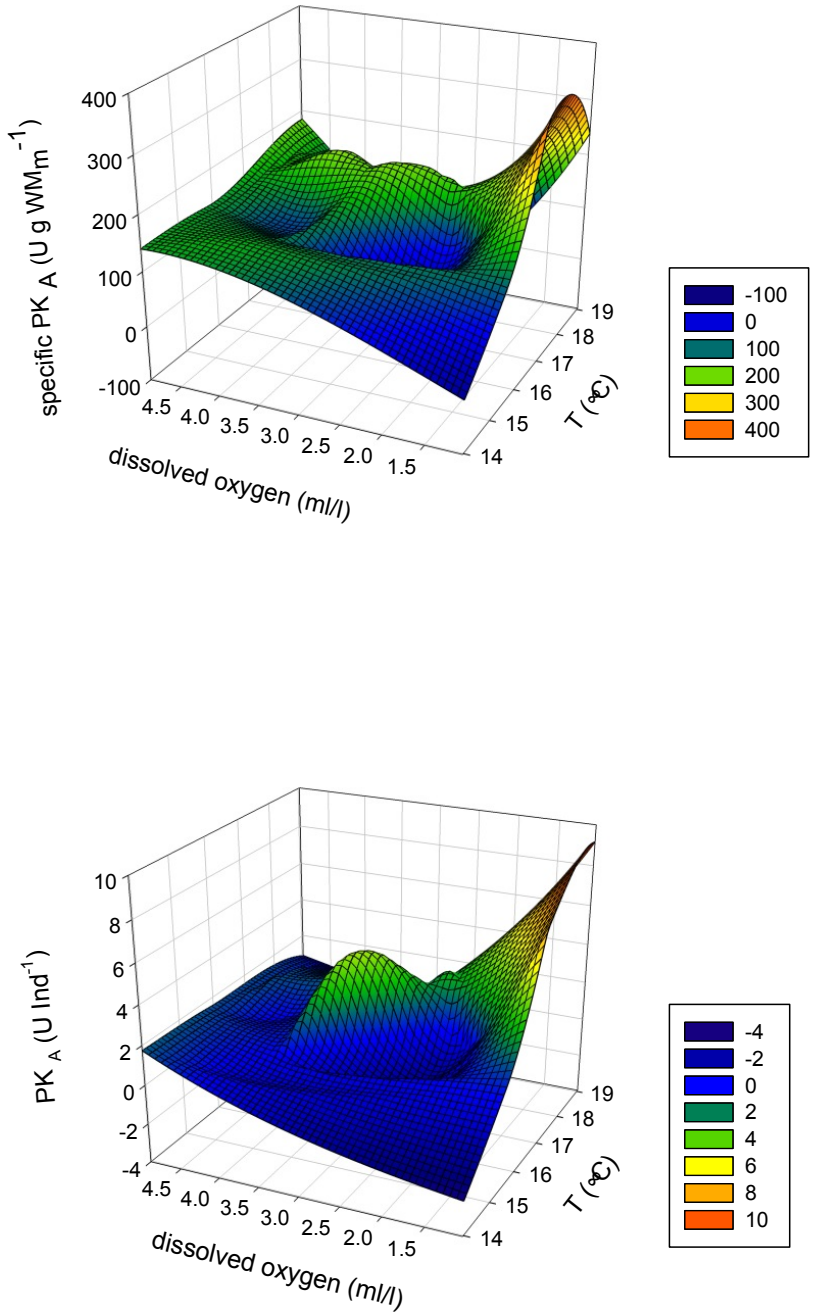

Fig 14: 3D mesh plots showing correlations between pyruvate kinase activity (**top**: specific; **bottom**: individual activity), temperature (°C) and dissolved oxygen (ml l^{-1}) levels. Different activity values are indicated by coloration as explained in the box beneath the graph.

Lactate Dehydrogenase

LDH activity related to the wet mass of the muscle tissue did not differ significantly with temperature ($p = 0.205$, ANOVA) (Fig 15, left). Overall highest activities were found at lowest T when related to a standard muscle mass, but the goodness of the linear fit was low ($r^2 = 0.025$). Comparably to PK, highest LDH activities were found at low dissolved oxygen concentrations (Fig 15, right; Tab 7). Glycolytic activities increased with decreasing DO concentrations while oxidative activities (CS) decreased.

From multiple regression analysis, the following equations of the model were derived:

$$\text{specific LDH}_A = 280.287 - 3.866 \times T - 9.285 \times DO$$

$$\text{individual LDH}_A = 5.925 - 0.169 \times T - 0.328 \times DO$$

Either 11.6 % or 15.3 % of the variance in LDH activities could be explained by the effect of temperature and dissolved oxygen concentrations. In low temperature regimes, specific LDH activities did not change with changing oxygen concentrations, but at higher temperatures an increase could be observed (Fig 16).

Overall, enzyme activity values were not evenly distributed within the three dimensional plots but interrupted by elevations and depressions. This can be related to the character of upwelling system, as the correlation between T and DO concentrations is not uniform. In general, the solubility of oxygen increases with decreasing temperatures. In upwelling systems, cold water masses can be low in oxygen due to strong oxygen depletions, causing unequally distributed correlations between T, DO and enzyme activities.

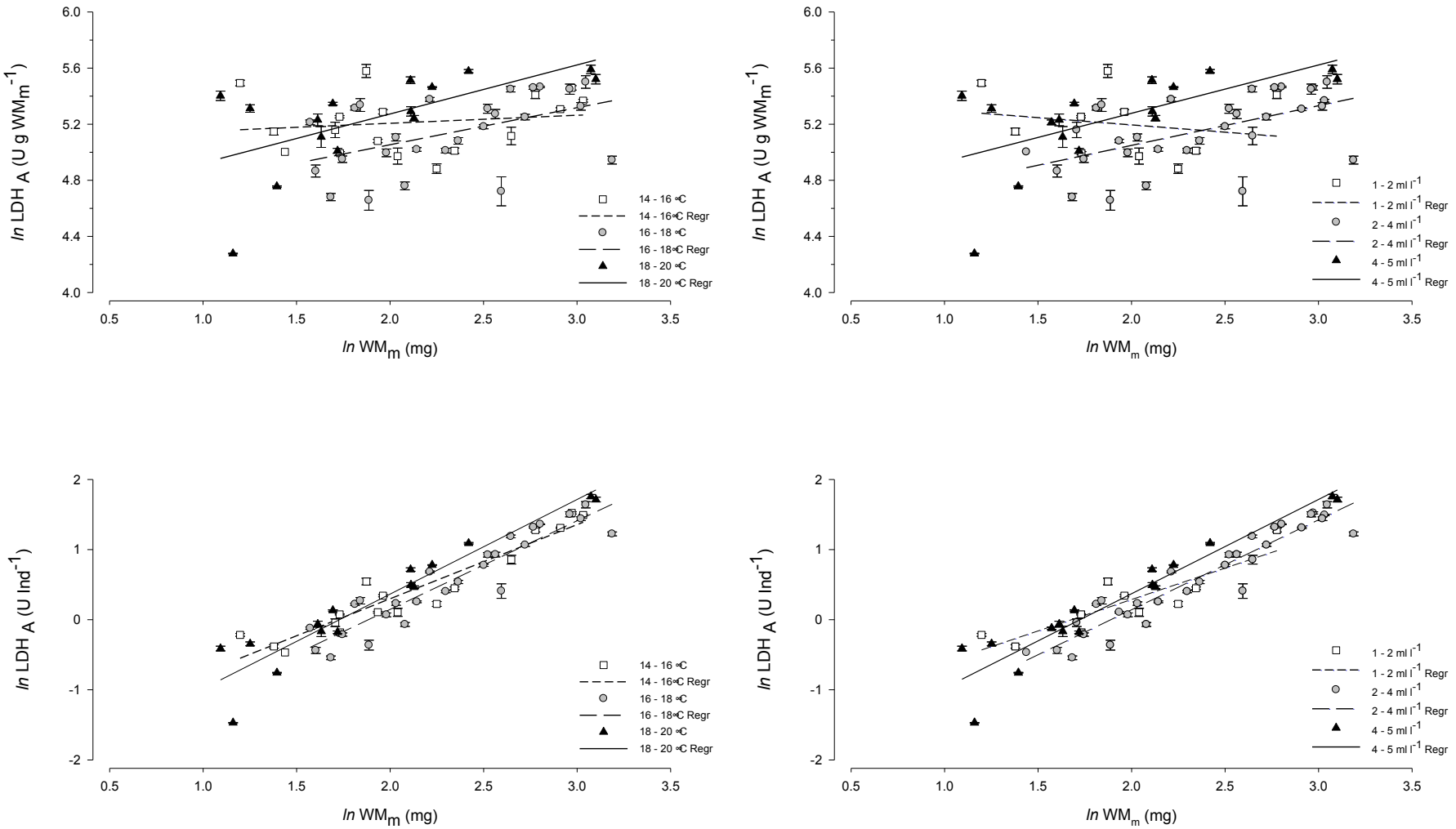

Fig 15: Relationship between lactate dehydrogenase activity (LDH $_A$) and wet mass of the muscle tissue (WM_m) at different temperatures (**left**) and dissolved oxygen levels (**right**). Sample specific T and DO groups are explained by symbols beneath the graphs. Allometric relations and regression statistics are shown in Tab 6 and 7.

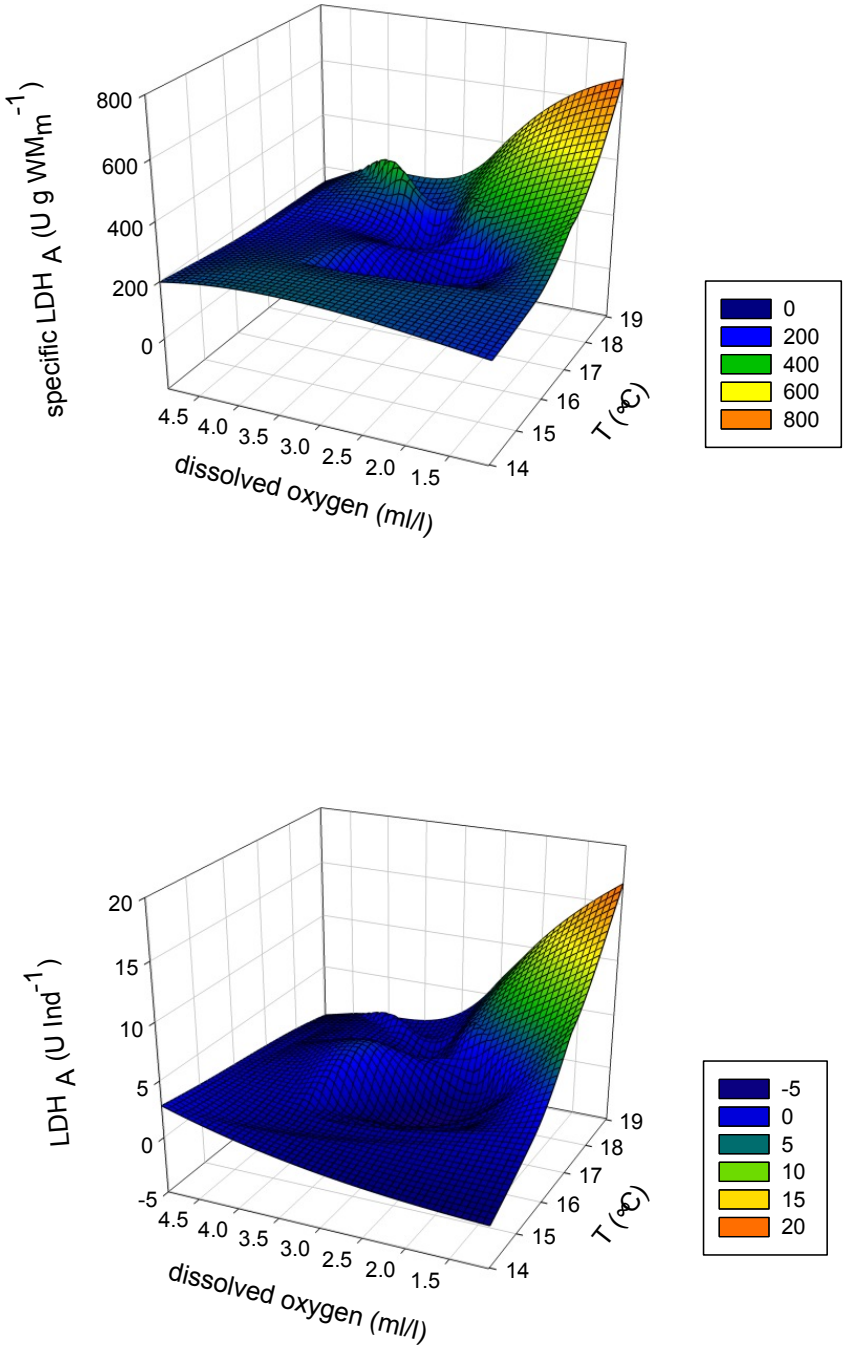

Fig 16: 3D mesh plots showing correlations between lactate dehydrogenase activity (**top**: specificl; **bottom**: individual activity), temperature (°C) and dissolved oxygen (ml l^{-1}) levels. Different activity values are indicated by coloration as explained in the box beneath the graph.

Tab 6: Relationship between enzyme activities (CS, PK, LDH) and muscle tissue wet mass as a function of temperature. Allometric relations are given with b = metabolic coefficient and a = intercept for specific and individual activities for three temperature groups. Activities were calculated for two standard muscle weights (10 mg and 20 mg) for each enzyme respectively. Sample sizes (n) and regression coefficients (r^2) are shown.

						specific Act. (U g^{-1} WM_m)					individual Act. (U Ind^{-1})	
			Specific Act. = a x ln(WM_m)^b			WM Muscle		**Individual Act. = a x ln(WM_m)^b**			WM Muscle	
	T (°C)	n	a	b	r^2	10 mg	20 mg	a	b	r^2	10 mg	20 mg
CS	**14 - 16**	*15*	6.129	0.089	*0.040*	**6.601**	**6.757**	0.006	1.089	*0.861*	0.015	0.020
	16 - 18	*35*	6.987	-0.028	*0.007*	**6.825**	**6.775**	0.007	0.972	*0.894*	0.016	0.020
	18 - 20	*12*	6.032	0.037	*0.041*	**6.221**	**6.281**	0.006	1.037	*0.971*	0.014	0.019
PK	**14 - 16**	*17*	101.190	0.107	*0.117*	**110.636**	**113.795**	0.101	1.107	*0.934*	0.255	0.341
	16 - 18	*33*	103.544	0.141	*0.095*	**116.466**	**120.869**	0.104	1.141	*0.872*	0.268	0.362
	18 - 20	*16*	94.538	-0.024	*0.002*	**92.664**	**92.081**	0.095	0.976	*0.752*	0.213	0.276
LDH	**14 - 16**	*17*	162.715	0.057	*0.025*	**170.637**	**173.216**	0.163	1.057	*0.897*	0.393	0.519
	16 - 18	*26*	92.296	0.264	*0.237*	**115.029**	**123.305**	0.092	1.264	*0.877*	0.265	0.370
	18 - 20	*15*	96.834	0.350	*0.385*	**129.659**	**142.169**	0.097	1.350	*0.903*	0.299	0.426

Tab 7: Relationship between enzyme activities (CS, PK, LDH) and muscle tissue wet mass as a function of dissolved oxygen (DO) concentrations. Allometric relations are given with b = metabolic coefficient and a = intercept for specific and individual activities for three DO groups. Activities were calculated for two standard muscle weights (10 mg and 20 mg) for each enzyme respectively. Sample sizes (n) and regression coefficients (r^2) are shown.

						specific Act. (U g^{-1} WM_m)					individual Act. (U Ind^{-1})	
			Specific Act. = a x ln(WM_m)^b			WM Muscle		**Individual Act. = a x ln(WM_m)^b**			WM Muscle	
	DO (ml l^{-1})	n	a	b	r^2	10 mg	20 mg	a	b	r^2	10 mg	20 mg
CS	**1 - 2**	*15*	2.983	0.297	0.531	**3.822**	**4.132**	0.003	1.297	0.956	0.009	0.012
	2 - 4	*35*	6.567	-0.016	0.003	**6.480**	**6.452**	0.007	0.984	0.922	0.015	0.019
	4 - 5	*12*	6.821	0.075	0.109	**7.261**	**7.406**	0.007	1.075	0.962	0.017	0.022
PK	**1 - 2**	*17*	145.329	-0.115	0.104	**132.038**	**128.102**	0.145	0.885	0.873	0.304	0.384
	2 - 4	*33*	101.190	0.149	0.145	**114.580**	**119.162**	0.101	1.149	0.909	0.264	0.357
	4 - 5	*16*	94.538	-0.024	0.002	**92.664**	**92.081**	0.095	0.976	0.752	0.213	0.276
LDH	**1 - 2**	*17*	221.850	-0.104	0.039	**203.418**	**197.926**	0.222	0.896	0.753	0.469	0.593
	2 - 4	*26*	89.121	0.283	0.309	**112.847**	**121.572**	0.088	1.283	0.902	0.258	0.362
	4 - 5	*15*	98.396	0.345	0.381	**131.202**	**143.672**	0.098	1.345	0.904	0.302	0.430

4.3 Activity of the Electron Transport System

4.3.1 Correlation of ETS and CS Activity

The two oxidative enzymes CS and ETS were linearly correlated (Fig 17). Activities of both enzymes were measured in subsamples of 14 HMC, allowing for a direct comparison. ETS activities were positively correlated with the measured CS activities in each sample ($p < 0.001$, ANOVA), showing a distinct relationship between both ($r = 0.782$, $r^2 = 0.612$). In summary, with increasing potential metabolic rates the activity of oxidative enzymes increases.

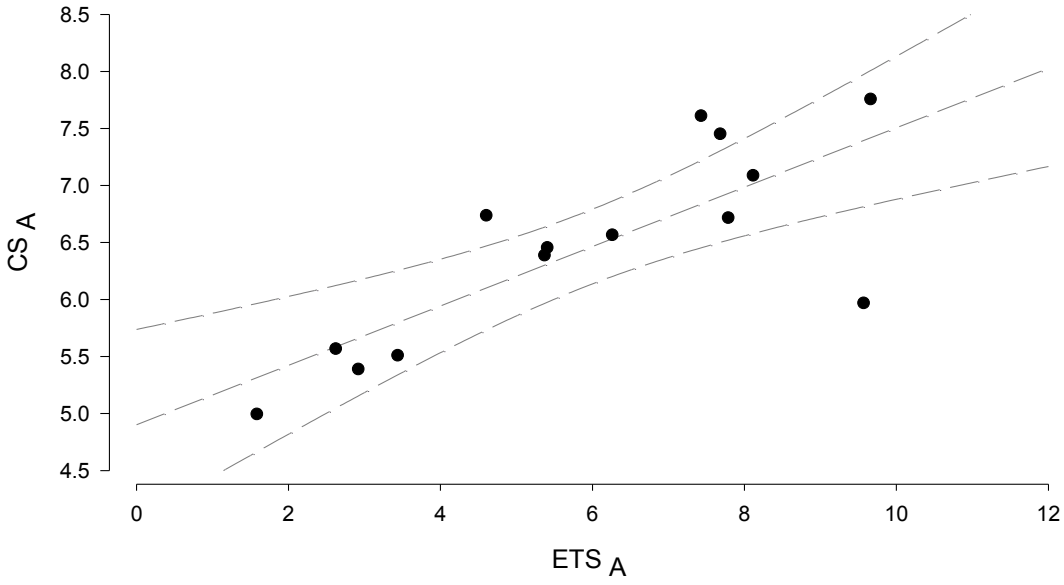

Fig 17: Citrate synthase activity as a function of electron transport system activity. Linear regression and 95% CI are given (n = 14). Equation of linear regression: $CS_A = 4.902 + 0.260\ ETS_A$.

4.3.2 RMR : ETS Ratio

Specific ETS activities (µmol min^{-1} g WM_m^{-1}) were converted to oxygen consumption equivalents (µmol O_2 min^{-1} g WM_m^{-1}), as described in chapter 3.4.4, and related to unit hour (µmol O_2 h^{-1} g WM_m^{-1}). Mass-specific ETS activity was calculated with 9.715 µmol O_2 h^{-1} g WM^{-1} for an averaged wet mass of 0.0708 g over the studied size range (n = 31). Routine metabolic rates derived from Geist *et al.* (2012, submitted for publication) were calculated

with 2.1935 µmol O_2 h^{-1} g WM^{-1} for this size. Accordingly, an averaged RMR : ETS ratio of 0.225 could be calculated, giving the factor by which measured ETS activities have to be multiplied to receive the respiration rate.

4.4 Adaptation to Hypoxic Conditions

4.4.1 Interspecific Comparison of Enzyme Activities

Activities of CS and LDH of Cape horse mackerels (HMC), anchovies (ANC) and sardines (SAR) were compared. Specific CS activities were much lower in anchovy and sardine than in Cape horse mackerel larvae (Fig 18). Comparison of the median values among the groups resulted in significant differences ($p < 0.001$, Kruskal-Wallis ANOVA). Both ANC and SAR did not differ significantly, but profound differences could be detected when compared to HMC ($p < 0.05$, Dunn`s method). Averaged specific CS activities in HMC larvae were 6.617 ± 1.336 U g WM_m^{-1} (n = 66) compared to significantly lower activities of 3.012 ± 1.076 U g WM_m^{-1} (n = 4) found in ANC and 2.894 ± 0.524 U g WM_m^{-1} (n = 4) in SAR larvae.

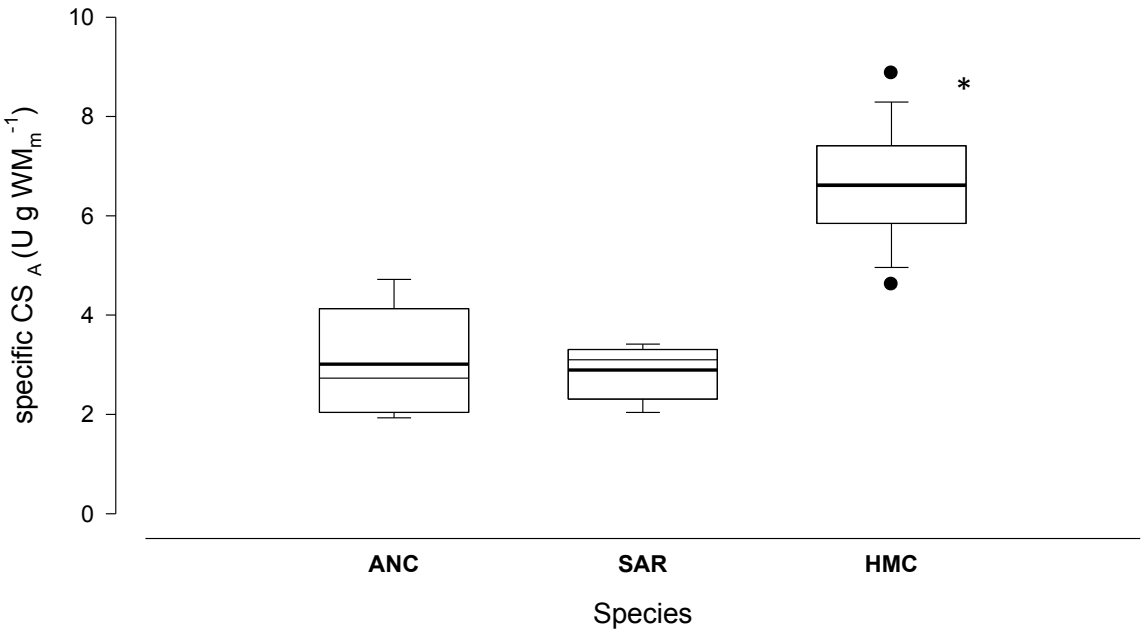

Fig 18: Comparison of specific citrate synthase activities in three pelagic larval fish (ANC = anchovy; SAR = sardine; HMC = Cape horse mackerel). Thick black lines show the mean and thin black lines the median, next to upper and lower quartiles and minimum and maximum values respectively. Significantly different groups were marked with an asterisk.

Compared to CS, LDH activities (Fig 19) varied stronger showing again highest activity values for HMC, 185.704 ± 46.277 U g WM_m^{-1} (mean ± SD, n = 58) followed by ANC, 112.962 ± 54.365 U g WM_m^{-1} (n = 5) and SAR showing a comparatively low LDH activity of 37.276 ± 15.353 U g WM_m^{-1} (n = 4). Here the differences in the median values of the three groups were even more pronounced than described for citrate synthase, and were highly statistically significant ($p < 0.001$, Kruskal-Wallis ANOVA). Again no significant difference could be found comparing ANC and SAR, but each of them was significantly different to HMC ($p < 0.05$).

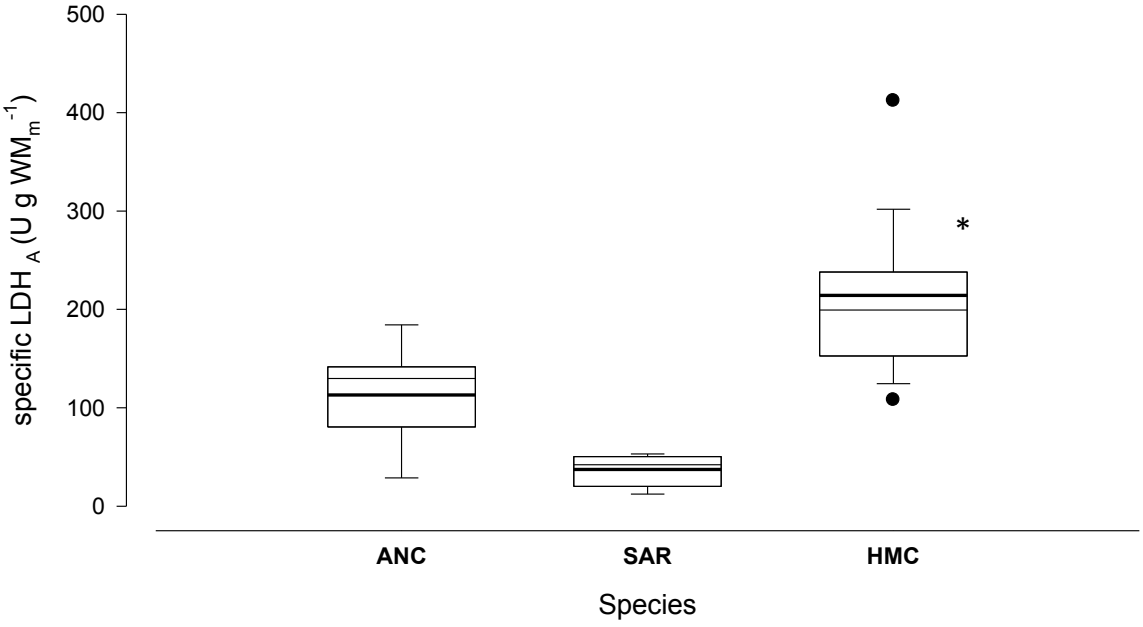

Fig 19: Comparison of specific lactate dehydrogenase activities in three pelagic larval fish (ANC = anchovy; SAR = sardine; HMC = Cape horse mackerel). Thick black lines show the mean and thin black lines the median, next to upper and lower quartiles and minimum and maximum values respectively. Significantly different groups were marked with an asterisk.

4.4.2 Normoxia vs. Hypoxia

Figure 20 shows averaged specific CS, LDH and PK activities (mean ± SD) among the different treatment groups (explained in chapter 3.5.3) indicated by varying bar colorations. Highest CS and LDH activities were reached for samples which died under oxygen depletion (treatment group: Lactate), while lowest values were found in samples reared at 30 or 50% oxygen saturation (treatment groups: stress I and II; Tab 8). As variances in CS activities were not equal, Kruskal-Wallis ANOVA on ranks was adopted showing no significant differences in median values among the treatment groups (df: 5, $p = 0.164$). Contrary, variances between groups were significantly different for LDH activities ($p = 0.004$, ANOVA), showing significant differences between the batch and lactate group (Holm-Sidak, $p = 0.025$). Pyruvate kinase activities were slightly elevated in standard and stressed samples, but overall no significant difference in the mean values could be found between treatment groups (df: 4, $p = 0.054$, ANOVA).

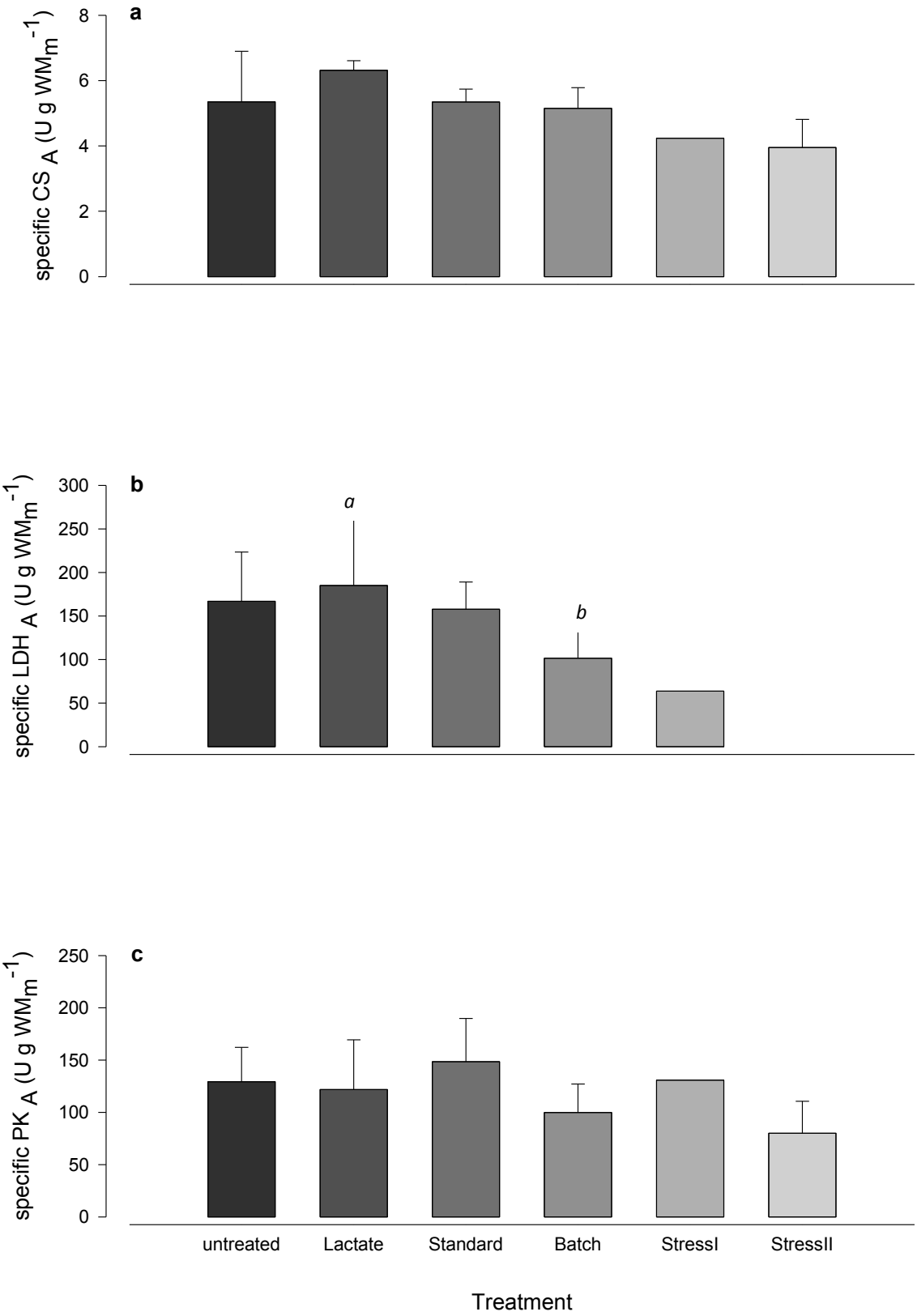

Fig 20: Comparison of specific CS (a), LDH (b) and PK (c) activities of samples from different treatments (see chapter 3.5.3, page 19 for explanation) indicated by bar coloration. Significant different groups are highlighted with a letter above the bar.

Tab 8: Overview of activities of CS, LDH and PK in samples from different normoxic and hypoxic treatment groups (Mean ± SD). Given are sample sizes (n), standard length ranges and body mass values (WM_m and WM_g; mean ± SD) for each group.

Treatment	standard length	muscle tissue wet mass	gutted wet mass	PK Activity		LDH Activity		CS Activity	
	(L_s) in mm	(WM_m) in mg	(WM_g) in mg	Mean ± SD	*n*	Mean ± SD	*n*	Mean ± SD	*n*
untreated	6.3 - 10.0	3.728 ± 0.724	12.887 ± 1.570	122.278 ± 33.841	*7*	170.624 ± 60.068	*7*	5.354 ± 2.271	*2*
	21.0 - 32.5	19.707 ± 6.386	257.036 ± 198.039	154.124 ± 12.687	*2*	140.413 ± n. d.	*1*	5.344 ± 1.422	*2*
Lactate	6.5 - 9.0	2.158 ± 1.051	7.675 ± 4.053	96.468 ± 33.666	*4*	162.114 ± 82.209	*4*		
	13.0 - 15.5	16.743 ± 3.139	41.556 ± 10.385	172.560 ± 13.997	*2*	231.032 ± 39.392	*2*	6.317 ± 0.292	*2*
Batch	7.5 - 9.0	2.014 ± 0.579	6.631 ± 0.935	85.592 ± 19.290	*7*	100.767 ± 38.063	*7*	4.706 ± n. d.	*1*
	10.0 - 15.0	8.743 ± 6.058	24.238 ± 13.474	114.077 ± 27.862	*7*	102.270 ± 41.276	*7*	5.213 ± 0.658	*7*
Standard	12.0 - 20.5	17.340 ± 6.600	58.814 ± 37.831	148.502 ± 41.342	*4*	157.807 ± 31.213	*3*	5.345 ± 0.393	*4*
StressI	19.5	21.026 ± n. d.	77.308 ± n. d.	130.847 ± n. d.	*1*	63.799 ± n. d.	*1*	4.238 ± n. d.	*1*
StressII	34.5 - 51.0	18.992 ± 13.341	708.550 ± 491.482	79.993 ± 30.713	*2*			3.951 ± 0.862	*2*

5 Discussion

This study was aimed to establish a set of basic data of metabolic key enzyme activities in larvae and juveniles of Cape horse mackerels, *Trachurus trachurus capensis*, from the Northern Benguela Upwelling System. The study covered aerobic and anaerobic metabolic pathways, investigating enzymes from oxidative, CS and ETS, and glycolytic pathways, PK and LDH. Standardized to a unit mass, they serve as biochemical condition indices, specifying general condition in fish larvae and investigating physiological and biochemical characteristics to understand how key species of the Northern Benguela cope with changing environmental conditions.

5.1 Methodological Discussion

In general, enzyme activities differ among species (Hickey and Clements, 2003), developmental stages (Overnell and Batty, 2000) and body tissues (Childress and Somero, 1979). Concerning the latter the choice of suitable samples is essential to achieve highly reproducible and practicable enzymatic measurements (Saborowski and Buchholz, 2002). The skeletal muscle of fish represents the majority of the body mass and provides energy through protein degradation under unfavorable conditions. CS and PK activities varied between whole larvae and muscle tissue samples. They were significantly higher in the muscle samples, due to the high locomotive activity implemented by fast-swimming performances (Pelletier *et al.*, 1993). The choice of suitable samples also varies in literature as it depends on the size of the study object and the focus of the research.

For the purposes of this study samples of muscle tissue gave reliable and reproducible results. Modifications of the extraction procedure and the enzyme assays allowed for rapid handling. Despite the small sizes muscle tissue samples were easy to handle and the assays were not affected by other compounds such as digestive enzymes which can have an effect when analyzing whole organisms.

Protocols for extraction and enzyme assays vary according to the purpose of the study. The purification of the crude homogenate via centrifugation is important as it can either result in insufficient sedimentation of cellular debris or in a loss of heavy

enzyme complexes (e.g. NADH dehydrogenase with 850 kDa; Boyle 2005) depending on the applied centrifugation speed. Moderate speed was shown to be appropriate for our purpose, resulting in significantly higher ETS activities. Furthermore, enzymatic extracts were shown to be stable to freezing for CS, PK and LDH, but not for ETS assays. Most studies assayed enzymes in freshly-prepared supernatant fractions as enzyme activity was found to react highly sensitive to freezing (Childress and Somero, 1979; Clarke *et al.*, 1992) and elongated storage periods (e.g. Lannig et al., 2003). Others found prepared homogenates being stable to freezing (e.g. Pelletier *et al.*, 1993; Catalán *et al.*, 2007), allowing for separated working procedures and an optimized time management as applied in this study.

Modifications of metabolic enzyme assays conducted in this study optimized the measurement of aerobic and anaerobic enzymes in Cape horse mackerel larvae and juveniles and it is assumed that this also applies to early life stages of other fish species. Especially as modifications of the ETS assay for fish larvae are limited in the literature (Gopalan *et al.*, 1996; Ikeda, 1989; Yamashita and Bailey, 1990) methodological adjustments are important allowing for comparative analysis.

5.2 Specific Activity

Specific activities were low for CS (6.62 ± 1.34 U g^{-1} WM_m) and high for PK and LDH (128.6 ± 37.1 and 185.7 ± 46.3 U g^{-1} WM_m) over the studied size range of 4 – 62 mm, and 0.0018 – 1.0561 g gutted wet mass, respectively.

Metabolic key enzyme activities have been intensively investigated in fish growth studies (Tab 9), showing their species- and stage-specific variability. Glycolytic activities differ by up to two orders of magnitude according to species-specific parameters, including depth of occurrence, locomotory mode, feeding habits and body size (Pelletier *et al.*, 1993). In this study comparatively high activities of glycolytic enzymes were found for young *T. trachurus capensis* in contrast to larvae and juveniles of other marine teleosts. As the activity of these enzymes in white skeletal muscle is known to reflect the burst swimming capacity in fish, elevated activities in HMC may reflect their pronounced active swimming ability (Shulman and

Love 1999). This is supported by Somero and Childress (1990) who found lower LDH and CS activities in the white muscle of benthic compared to pelagic species, assuming that these differences reflect locomotory differences between the species. Large differences in absolute activity among skeletal muscle enzymes have already been found in pelagic (Childress and Somero, 1979) as well as demersal fish species (Hickey & Clements, 2003), explaining higher activities of glycolytic enzymes with a generally higher anaerobic metabolic character of fish white muscle fibers. Johnston *et al.* (1977) studied the recruitment of red, pink and white muscle fibers of mirror carp, *Cyprinus carpio carpio*, showing high oxidative enzyme activities in red muscle compared to low activities in white muscle, respectively. Investigations of muscle-specific CS activity in vertebrates and invertebrates support these findings and suggest that either low energetic requirements or a higher reliability on anaerobic glycolysis is decisive for low oxidative activities in fish white muscles (Alp *et al.*, 1976).

Estimated specific activities reflect highest possible activities, giving an estimate of maximum enzymatic potentials of the muscle tissue. However, in situ activities might be lower since they depend on physiological conditions such as intracellular concentrations of substrates or allosteric modulators, being well below saturation level in the cell (Childress and Somero, 1990; Johnston *et al.*, 1977).

5.3 Metabolic Scaling

Distinct allometric relationships were found referring metabolic enzyme activities to body weight. In white muscle of Cape horse mackerel larvae and juveniles, specific activities of PK and LDH are positively correlated with body size (b_{PK} = 0.11 and b_{LDH} = 0.15; Fig 9 and 10). The relationship between glycolytic enzyme activities and body size is much stronger than that found for CS (b = 0.003). ETS activities decrease while CS activities show no correlations to increasing body sizes, when activities were expressed per gram muscle mass. Regarding individual activities, normalized to the whole muscle weight of the samples, even stronger correlations were found showing overall positive allometric relations in all enzymes analyzed (b_{CS} = 0.80 , b_{PK} = 0.81, b

$_{LDH}$ = 0.78; Tab 5). Geist *et al.* (2012; submitted manuscript) already investigated that metabolic activity of young Cape horse mackerels follows an allometric relationship, deriving comparable metabolic scaling coefficients (b_{RMR} = 0.76, b_{SMR} = 0.80).

This metabolic scaling was subject to numerous studies and intensively investigated in fish (e.g. Childress and Somero, 1990; Somero and Childress, 1980). Overall strong positive correlations between growth rate and glycolytic enzyme activities were found in larval and early juvenile fish, such as herring and plaice (Overnell and Batty, 2000) or sea bass (Catalán *et al.*, 2007) but also in late juvenile fish, e.g. cod (Pelletier *et al.*, 1993a). Unlike absent relations of specific CS activity to body size in Cape horse mackerels, a negative allometric scaling correlation was found in numerous fish species (Overnell and Batty, 2000; Pelletier *et al.*, 1993b; Childress and Somero, 1979) showing a decrease in aerobic enzyme activities along ontogenetic development.

As metabolic activities are known to be size- and stage-dependent, effects of metabolic scaling can be explained by developmental variability in myotomal architecture over the studied size range. The primary myotome in teleosts consists of dorsal and ventral bulks of fast-contracting white fibers, a horizontal septum and a superficial lateral layer of slow-contracting red fibers, summarized by Stoiber *et al.* (1999). With larval growth, muscle tissue increases exponentially, still dominated by white muscle fibers containing less mitochondria and therefore lower mitochondrial enzyme activities than red fibers, but higher glycolytic potentials (Crabtree and Newsholme, 1972; Stoiber *et al.*, 1999). Consequently, with progressing growth, the relative proportion of glycolytic enzymes, PK and LDH, becomes larger as the span between mitochondrial and glycolytic activities does. This pattern was clearly shown in larval and early juvenile Cape horse mackerels, as PK and LDH activities increased and ETS activities decreased with body size. A possible explanation for the absence of an inverse relation in CS, hence, unchanged aerobic potentials with ontogenetic development could be a more continuous metamorphosis of Cape horse mackerels (Geist *et al.*, 2012; submitted manuscript). In addition, citric acid cycle activity is the primary supplier of ATP for steady-state, aerobic swimming performance and

therefore CS is highly important for basic metabolic processes in the cell (Catalán *et al.,* 2007; Johnston *et al.,* 1977).

5.4 Reaction to Temperature

Ambient temperature is responsible for differences in metabolism, which develop between fish of the same species at different seasons and latitudes (Love and Shulman, 1999). In fish aerobic metabolism normally predominates over anaerobic holding over a wide temperature range but their early life stages are very sensitive to changing temperature regimes. In this study differences in metabolic enzyme activities were found depending on the ambient temperature. Standardized to a representative muscle tissue weight, specific glycolytic and oxidative enzyme activities were highest at lower temperatures (Tab 6). Although the tested temperature range was narrow (14 - 20 °C) specific activities differed considerably for samples with comparable body size. No significant differences in enzyme activities could be detected for the different temperature groups. However, the high scatter of the activity values restricted estimations of a potential statistically significant temperature effect and lowered the power of the performed test.

The correlation between ambient temperature and enzymatic activity in the muscle tissue is not governed exclusively by the law of van`t Hoff and Arrhenius (Q_{10} - rule). Poikilothermic animals can show considerably varying internal temperatures as a consequence of changing environmental temperatures. This temperature compensation is partially realized by the heterogeneous character of enzymes where each isoenzyme displays a characteristic optimum temperature (Love and Shulman, 1999). Enzyme activity may change because of increasing amounts of enzymes present, changes in their conformation state or modifications of their relation with various physico-chemical factors (Hochachka and Somero, 1984).

Tab 9: Literature comparison of muscle specific CS, PK and LDH activity from different species and developmental stages

Species		Length (mm)	Wet Mass (g)	T (°C)	Activities (U g^{-1} muscle WM) CS	PK	LDH	Source
Larvae & Juveniles								
Cape horse mackerel	*Trachurus trachurus capensis*	4 - 62	0.002 - 1.056	18	6.62 ± 1.34	128.6 ± 37.1	185.7 ± 46.3	this study
Atlantic herring	*Clupea harengus*		< 0.001	10	1.33 ± 0.18[a]	n.d.	11.61 ± 3.12[a]	Overnell and Batty, 2000
Red drum	*Sciaenops ocellatus*	2.9 - 7.5	0.02 - 0.04	25	n.d.	n.d.	7 - 43[b]	Brightman *et al.*, 1997
Spot croaker	*Leiostomus xanthurus*	66 - 137	0.5 - 8.2 (M)		0.11 - 0.22[d]	n.d.	6.5 - 10.7[d]	Cooper *et al.*, 2002
Adults								
Horse Mackerel	*T. mediterraneus ponticus*		25 - 40	25	n.d.	241 ± 24	140 ± 4	Lushchak *et al.*, 2001
Gulf killifish	*Fundulus grandis*	80 - 85	10 - 15	27	n.d.	103 ± 21	346 ± 54	Martinez *et al*., 2006
Atlantic cod	*Gadus morhua*	350 - 500	400 - 1000	10	0.66 - 1.63*	n.d.	n.d.	Pelletier *et al*., 1993
Sea scorpion	*Scorpaena porcus*		120 - 400	25	n.d.	124 ± 8.0	55.1 ± 9.7	Lushchak *et al*., 1998
Californian anchovy	*Engraulis mordax*				1.52	60	540	
Kelp bass	*Paralabrax clathrtus*				0.79	75	389	
White croaker	*Genyonemus lineatus*			10	0.67	88	267	Sullivan and Somero, 1980
Longspine thornyhead	*Sebastolobus altivelis*				0.19	4	25	
Marlin-spike grenadier	*Nezumia bairdii*				0.82	5	9	
Sablefish	*Anoplopoma fimbria*	40 - 80	430 - 3600	10	n.d.	24 ± 10	181 ± 46	Sullivan and Somero, 1983
Mirror carp	*Cyprinus carpio*	400		20	n.d.	299 ± 51[c]	440 ± 41[c]	Johnston *et al.*, 1977

* standardized for a 1.2 kg fish; [a] Activity given in nmol min^{-1} mg^{-1} wet mass; [b] Activity in units per g wet mass (2-3 larvae pooled); [c] Activity in units per gram dry weight; [d] Activity in units per mg protein in the muscle tissue

(M) muscle tissue wet mass

5.5 Reaction to Hypoxia

Responses to lowered dissolved oxygen (DO) levels can be either an increase or decrease in metabolic activity, starting with avoidance reactions until a critical threshold in DO level is reached and metabolic activities decrease. Immediate reactions include activation in anaerobic glycolysis when mitochondrial oxidative phosphorylation is limited by oxygen availability. With persistent exposure to oxygen-depleted environments metabolic depression can be initiated lowering overall energy demands and metabolic costs (Lushchak *et al.*, 1998) and changes on the gene expression and protein level can alter processes of anaerobic metabolism (Gamperl and Driedzic, 2009). Larvae and early juveniles of *T. trachurus capensis* of comparable size classes showed different glycolytic activities under normoxic and hypoxic conditions. Larvae reared in hypoxia without recovery periods died under oxygen depletion and showed highest specific CS and LDH activities. When allowing for repeated recovery time-periods in between, glycolytic enzyme activities were comparatively lower (Fig 20, Tab 8). Overall, individuals of comparable size classes showed higher glycolytic activities when continuously exposed to low oxygen levels than those which were repeatedly allowed to recover by oxygen supply. These results are supported by enzyme data derived from non-experimental samples which were used to establish a basic set of data for *T. capensis*. Standardized to a unit muscle mass, glycolytic enzyme activities for both PK and LDH increased and CS activities decreased with lower DO concentrations (Tab 7). High anaerobic activities have been reported in organisms that inhabit or pass through the oxygen minimum layer (Ludsin *et al.,* 2009). For example teleosts, which undergo daily vertical migration, through regions with pronounced oxygen minima are thought to possess enhanced anaerobic capabilities (Douglas *et al.*, 1976). In contrast Childress and Somero (1979) investigated an exponential decrease of mitochondrial and glycolytic enzyme activities as well as respiration rates with increasing minimum depth of occurrence in the white skeletal muscle of numerous pelagic fishes. They concluded that fishes having contact with the oxygen minimum layer do not show special adaptations in enzyme activities for enhanced anaerobic metabolism and that reduced metabolic rates could derive from lower enzyme concentrations and/or reductions in enzymes catalytic efficiencies.

The ambient oxygen level is a highly important factor governing modes of metabolism in aquatic animals. It is already known that glycolytic catabolism, including glycolysis and pentose phosphate way, plays an active role when dissolved oxygen concentrations decrease (Love and Shulman, 1999). Although the response of the glycolytic pathway to hypoxia has been extensively investigated (e.g. Lushchak *et al.*, 1998), the mechanisms initiating the metabolic adaption are not completely understood. Stolbov *et al.* (1995) conducted short-term hypoxia experiments on Black Sea horse mackerel, describing a continuous decrease in oxygen consumption with oxygen saturation until the latter becomes insufficient for aerobic metabolism. They also showed that at 20-40% saturation most proteins, playing an active role in maintaining energy metabolism during hypoxia, are already catabolized anaerobically.

Moreover, this study showed significantly higher aerobic and anaerobic enzyme activities for *T. trachurus capensis* compared to other small pelagic fishes, anchovies and sardines, from the Northern Benguela upwelling system (NBUS). Simultaneously, respiratory studies showed a comparatively high tolerance of *T. trachurus capensis* against lowered dissolved oxygen levels, down to 20 % saturation (Geist *et al.*, 2012; submitted manuscript) compared to anchovy with a reported tolerance threshold down to 60 % oxygen saturation (Weihs, 1980). One explanation for this high tolerance was assumed to be in the spawning behavior of Cape horse mackerels. The eggs are released at a depth where hypoxia can occur before rising towards the surface. This upward movement is slower than for sardine and anchovy eggs and might require a higher hypoxia tolerance. In this study another potential explanation evolved. Due to higher CS and LDH activities, Cape horse mackerel larvae seem to cope better with increasing hypoxic zones in the NBUS compared to anchovies and sardines. As citrate synthase activity is not completely restricted to the availability of oxygen, it provides energy by the oxidation of acetate as well as the production of NADH which is fed into the oxidative phosphorylation pathway. Hence, elevated CS activities are generally beneficial to oxygen limitations as they provide higher energy yields compared to enzymes from anaerobic metabolic pathways.

6 Conclusion

This study presents basic data of metabolic key enzyme activities for an important pelagic fish species of the Northern Benguela Upwelling System, *Trachurus trachurus capensis*. The results clearly show the expected allometric relationship between enzyme activities and body size over a broad size range of young Cape horse mackerels. Ambient water temperatures and dissolved oxygen concentrations are shown to affect both aerobic and anaerobic metabolic activities and extend our knowledge of biochemical responses to global climatic changes. Furthermore, inter-specific differences in enzymatic activities were observed in comparison with other small pelagic fishes from the NBUS, indicating elevated aerobic and anaerobic capacities in *T. trachurus capensis*. These metabolic properties are crucial to succeed within the changing environment off the Namibian coast.

Activities and characteristics of metabolic key enzymes gave reliable estimates of metabolic potentials and adaptive properties of fish´s early life stages. The influence of environmental factors on enzyme activities should be investigated in more detail to detect underlying biochemical mechanisms for adaptive traits in *T. trachurus capensis*. Further studies on species-specific molecular responses to increasing hypoxic zones in the NBUS are highly desirable for the dominating pelagic species.

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Declaration

I hereby confirm that I have independently composed this Master thesis and that no other than the indicated aid and sources have been used.

This work has not been presented to any other examination board.

No data can be taken out of this work without prior approval of the thesis promoter.

Bremen, _____ _____

Kati Michalek