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Low hypoxia tolerance in larvae of the sardine *Sardinops sagax* and anchovy *Engraulis encrasicolus* may limit their stock recovery in the northern Benguela upwelling system

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Physiological traits of five key fish species (Cape hake *Merluccius* spp., pelagic goby *Sufflogobius bibarbatus*, Cape horse mackerel *Trachurus capensis*, European anchovy *Engraulis encrasicolus* and sardine *Sardinops sagax*) from the northern Benguela upwelling system (NBUS) were compared during their larval stage by measurements of enzymatic activities of key metabolic enzymes (citrate synthase [CS] and pyruvate kinase [PK]). Two distinct age classes (early larvae: 8–14 days old; late larvae: 22–29 days old) for each species and from two areas were compared: Terrace Bay (20° S), the main spawning ground of Cape horse mackerel and anchovy, and Kunene (17° S), near the Angola–Benguela frontal zone, where warm and hypoxic water masses intrude into the NBUS. The results revealed significantly higher CS activity in both larval age classes in Cape horse mackerel, anchovy and sardine compared with Cape hake and pelagic goby. Pelagic goby and Cape horse mackerel had significantly higher PK activity compared with Cape hake, anchovy and sardine, apparent in both larval age classes and both areas. Results for anaerobic metabolism indicate higher capacity for pelagic goby and Cape horse mackerel to recover from oxygen debt built up in prey capture and predator escape behaviour and a higher potential for hypoxia tolerance when compared with Cape hake, anchovy and sardine. These results suggest higher survival probability for pelagic goby and Cape horse mackerel compared with the other species under conditions of a shoaling oxygen minimum zone and may explain their current dominance in the NBUS.

Keywords: hypoxic environment, *Merluccius* spp., metabolic enzymes, oxygen minimum zone, *Sufflogobius bibarbatus*, southeastern Atlantic, survival probability, *Trachurus capensis*

Introduction

Upwelling ecosystems are highly productive areas of seas that include some of the world's most valuable fishing grounds, including small and medium pelagic species as important economic resources. In particular, high productivity in eastern boundary upwelling systems (Benguela, Humboldt, Canary and California systems) is driven by upwelling of cold, nutrient-rich water from deeper layers that is transported upwards into the euphotic zone and provides the basis for increased primary production (Auel et al. 2009; Lachkar and Gruber 2012).

The Benguela upwelling system is located off the southwest coast of Africa in the southeastern Atlantic Ocean, along the coasts of Namibia and South Africa, and extends from the Cape of Good Hope in the south (34° S) to the Angola–Benguela frontal zone in the north (15° S). This wind-driven coastal upwelling system can be divided into northern and southern subsystems created by a relatively powerful perennial upwelling cell off Lüderitz, Namibia (26° S) (Summerhayes et al. 1995; Boyer et al. 2000; Cury and Shannon 2004; Hutchings et al. 2009). The northern subsystem constitutes the northern Benguela upwelling system (NBUS) and extends from ~15° S to 26° S (Cury and Shannon 2004; Heymans et al. 2004).

The dominant fish species in the NBUS have changed over time. The sardine *Sardinops sagax* was the most abundant fish in the northern Benguela from 1950 to 1975, but the stock collapsed in the mid-1970s and was replaced by the Cape horse mackerel *Trachurus capensis* and pelagic goby *Sufflogobius bibarbatus* in the late 1970s and early 1980s (Cury and Shannon 2004; Hutchings et al. 2009; Heymans and Tomczak 2016). Catch trends show a steady decline in the European anchovy *Engraulis encrasicolus* over three decades (1970s to 1990s), and a sharp decline in demersal fishes (particularly Cape hake *Merluccius* spp.), large pelagic species and the chub mackerel *Scomber japonicus* from the 1980s to the 1990s (Heymans et al. 2004). Lack of recovery by sardine and a decline in anchovy led to a steep decline in pelagic catches from 1.3 million tonnes to <100 000 tonnes through the 1980s and early 1990s (Hutchings et al. 2009). Fish stocks have not recovered to levels present in the 1980s and catches remain low.

High fishing pressure and changes in environmental conditions, including increased water temperatures and the expansion of hypoxic zones, have been identified as possible drivers of the decline in the abundance

of multiple fish species and as triggers for observed changes in species composition in the NBUS (Ekau and Verheye 2005; Ekau et al. 2010, 2018). Intensification of coastal upwelling contributes to the expansion of oxygen minimum zones in large parts of the eastern Pacific Ocean, in the southeastern Atlantic off West Africa, and in the northern Indian Ocean (Helly and Levin 2004). In particular, the spread of hypoxic zones in the NBUS has been shown to have four main causes: (i) the southerly influx of low oxygen waters into the NBUS from the Angola Gyre (Mohrholz et al. 2008); (ii) intensification of coastal upwelling and its subsequent effect of increased primary production leading to oxygen depletion as unutilised phytoplankton biomass decays (Bakun 1990; Bakun and Weeks 2004); (iii) stratification of the shelf waters (Hutchings et al. 2009); and (iv) extraordinary conditions as described by Weeks et al. (2002). Those authors describe a phenomenon called sudden hydrogen sulphide (H_2S) eruptions, whereby massive eruptions of this toxic gas strip the water column of dissolved oxygen, contributing to hypoxia and anoxia in the NBUS. Hypoxic zones in the NBUS were also demonstrated by a 10-year composite monthly depth-time series in Walvis Bay (23° S) between 1994 and 2004 that showed that, at 50-m depth, oxygen levels of <2 mg l^{-1} were already encountered (Monteiro et al. 2006).

Shoaling of hypoxic zones since 1950 has been documented for the NBUS (Ekau et al. 2010), although this was not confirmed in a global review of coastal and open ocean oxygen depletion by Pitcher et al. (2021), who reported on known trends in oxygen concentrations over time for a variety of marine ecosystems, including the NBUS. The vertical distribution of larval stages of different species in relation to the oxycline depth has been shown (Ekau and Verheye 2005; Ekau et al. 2018). Hence, information on physiological traits and responses of early life stages of the fish species in the NBUS to changing environmental conditions, and in particular the potential spread of hypoxic zones, will contribute to our understanding of the observed changes in species composition and abundance in this system. Lower survival of eggs, larvae and juveniles of fish have been proposed to be responsible for the declining stocks in the NBUS (Ekau et al. 2010). A general sensitivity of early life stages of fish to low oxygen levels is well known. According to Miller et al. (2002), pelagic larvae living near surface waters are the most sensitive. Therefore, larval survival and recruitment will depend on the ability of early life stages to cope with potentially decreasing oxygen levels in the surface layer of the NBUS (Ekau et al. 2010; Geist 2013; Geist et al. 2013, 2015).

Unfortunately, high mortality of field-caught larval stages limits the application of respirometric techniques to determine critical oxygen levels among larvae of key fish species in the NBUS. This has led to the use of metabolic enzymes as indirect estimates of aerobic and anaerobic capacities to identify the physiological traits needed to cope with an increasingly hypoxic environment. Generally, organisms employ two initial metabolic strategies when exposed to hypoxic conditions: (i) an overall decrease in metabolic rate (Richards 2011), and (ii) a shift in the aerobic and anaerobic contributions to total metabolism (Cooper et al. 2002; Wu 2002). Measurement of the activity of metabolic enzymes can be used to provide rough estimates of the metabolic rates of aquatic animals (Bass et al. 1969; Sullivan and Somero 1980; Ombres et al. 2011). Two metabolic enzymes—citrate synthase (CS) and pyruvate kinase (PK)—were selected as proxies for estimation of the aerobic and anaerobic capacities of larvae of five key fish species from the NBUS. Assessment of CS activity, which catalyses the rate-limiting reaction in the tricarboxylic acid cycle (TCA), provides an estimate of an organism's aerobic metabolic rate (Berges et al. 1990; Childress and Thuesen 1992). Maximal potential flux through glycolysis can be estimated by determination of PK activity, which catalyses the last reaction in the glycolytic pathway (Newsholme and Crabtree 1986; Berges et al. 1990; Cooper et al. 2002). The amount of energy produced through glycolysis is critical when energy production via oxidative pathways is limited by low oxygen concentrations (Lushchak et al. 1998; Berg et al. 2002).

From previous studies by Sullivan and Somero (1980) and Childress and Thuesen (1992), it is known that the activities of enzymes involved in intermediary metabolism can be used to approximate the metabolic rates of aquatic animals.

The aim of this study was to compare aerobic and anaerobic capacities (as proxy indicators of hypoxia tolerance) in larval stages of five key fish species from the NBUS: Cape horse mackerel *Trachurus capensis*, pelagic goby *Sufflogobius bibarbatus*, Cape hake *Merluccius* spp., European anchovy *Engraulis encrasicolus* and sardine *Sardinops sagax*, to understand the potential effect of expanding hypoxic zones in the NBUS on the recruitment success of these species. In particular, we were interested whether we would find evidence for the hypothesis that low hypoxia tolerance in the larvae of small pelagics (i.e. sardine and anchovy) is one process limiting the rebuilding of these formerly abundant fish stocks.

This study was part of the broader research project, GENUS (Geochemistry and Ecology of the Namibian Upwelling System), which aimed to elucidate processes in the plankton community and the oceanographic and biogeochemical drivers that affect productivity of the highly dynamic NBUS ecosystem and the recruitment success of its major fish stocks.

Materials and methods

Study location

The study was carried out in two areas in the NBUS (Figure 1) off the coast of Namibia. The region around Terrace Bay (20° S) is located in the Central Namibian region (19–24° S) and is characterised by moderate upwelling, moderate winds and a wide, shallow continental shelf, which prevents the upwelling of water from below 150–200 m (Boyer et al. 2000); furthermore, Terrace Bay is one of the nursery grounds for *T. capensis* and *S. sagax* (Kreiner et al. 2011, 2014). The region around Kunene (17° S) is at the extreme of the distribution range of the species studied here, being close to the Angola–Benguela front, where the waters of the Benguela upwelling system meet tropical waters of the Angola Current, which

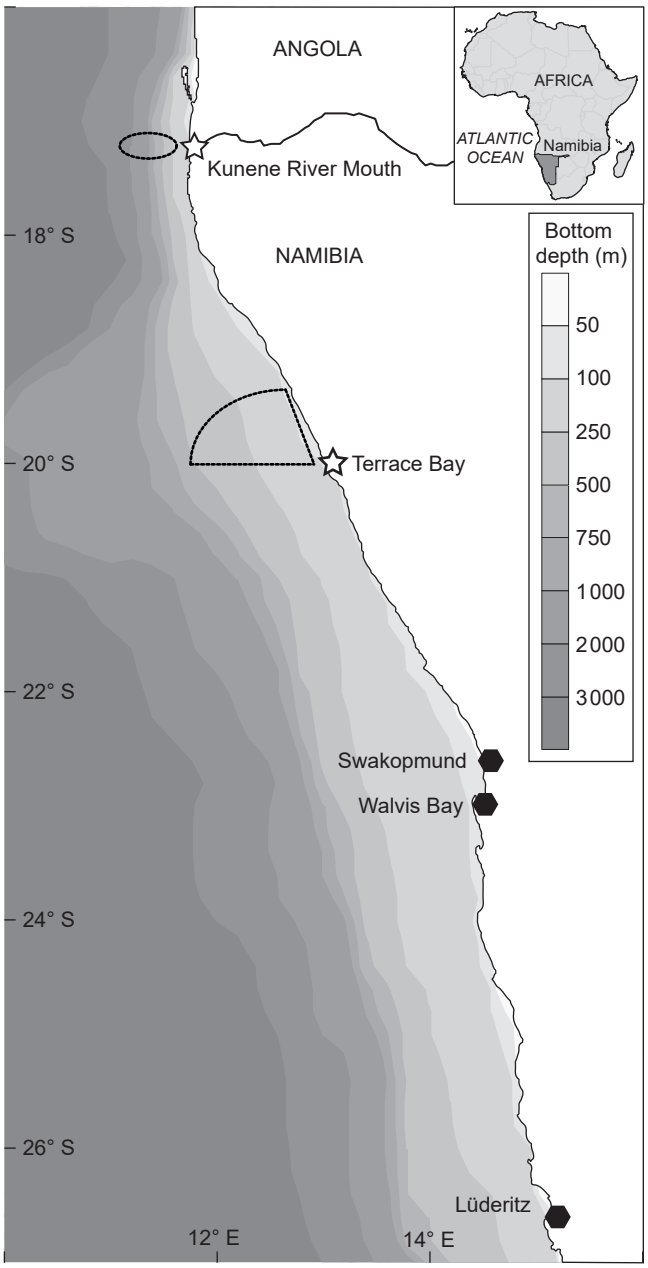

Figure 1: Map of the northern Benguela Current region. Stars indicate the two sampling areas off Namibia, around Kunene (17° S) and Terrace Bay (20° S). The Kunene River forms the border between Angola and Namibia. Dotted lines indicate areas of operation of the research vessel

are warmer and more oxygen deficient (Boyer et al. 2000; Hutchings et al. 2009). The two locations were ideal study sites, with the Terrace Bay area having conditions of moderate upwelling, and the Kunene area being affected by ocean warming and enhanced hypoxia.

Sample preparation

Sampling was conducted between 10 January and 6 February 2014, within the GENUS research cruises M103/1 and M103/2 of the RV *Meteor*. A total of 17 stations were sampled across the two areas off the coast of Namibia: eight stations in the Kunene area (~17° S), and nine stations in the Terrace Bay area (~20° S). Fish larvae were taken from nets that sampled the upper 50 m of the water column, with two types of plankton nets having different mouth areas and mesh sizes (multinet: 0.25 m^2 and 500 µm; ringtrawl: 2 m^2 and 1 000 µm, respectively), at a towing speed of 2 m s^{-1}. Owing to the shallow tow (fishing) depth, the tow times for the nets were short, always <20 min. The fish larvae used in this study did not survive being caught. Following retrieval of the nets, larvae were immediately sorted (i.e. within 5 min), placed on ice, identified to species and measured for length, before being rinsed with distilled water and frozen at −80 °C. After the cruise, the larvae samples were shipped on dry ice to the biolab at the Leibniz Centre for Tropical Marine Research (ZMT), Bremen, Germany, for subsequent metabolic enzymes analysis.

Since larvae with different morphologies were being compared (e.g. slender sardine and anchovy larvae versus stout horse mackerel and hake larvae), we grouped the larvae in age classes rather than size classes to reduce the effect of morphology on comparisons between species. Two age classes were established: early larvae (8–14 days old) and late larvae (22–29 days old). Respective lengths at age for the larvae (Appendix 1) were derived from previous works on larval growth-rate functions of the five species (*T. capensis* – Geist 2013; Simon 2014; Geist et al. 2015; *E. encrasicolus* and *S. sagax* – Geist 2013; *Merluccius* spp. – Grote et al. 2012; *S. bibarbatus* – Michalowski 2010). Growth equations and respective water temperatures used for calculating the growth rates are given in Appendix 1. A total of 134 larvae samples from the two study areas were used for the enzyme analyses.

In the biolab at ZMT, larvae were taken from the −80 °C ULT freezer, dissected on ice, and then the digestive tract and entire gut contents were removed. Gutted wet mass (mg) was determined for each larva, after which the head and tail were cut off and the wet muscle tissue from the posterior trunk was weighed on a microbalance (Mettler Toledo, MX-5). Owing to the very small size of early larvae, we only removed their gut contents and then used the whole larvae for the enzyme analyses.

Enzyme extraction was performed in a 50-fold volume (weight for volume) of homogenisation buffer (containing 75 mM tris-HCl and 1 mM EDTA at pH 7.5). The tissues were homogenised on ice for 1 min, using a plastic pestle (for very soft tissue, with homogenisation performed for 30 s). The homogenised tissues were ultrasonicated for 1 min to lyse the cells (Bandelin®, Sonopuls® ultrasonicator set at amplitude 20%, pulse on 0.1 s, and pulse off 1.0 s). The homogenates were centrifuged at 8 500 rpm for 10 min in a centrifuge (5804 R, Eppendorf®), and then pre-cooled at 2 °C. The supernatants (below the fatty layer) were pipetted into Eppendorf® tubes that were pre-cooled in dry ice. The extracts were immediately stored at −80 °C until the enzyme analyses. In preceding experiments we compared frozen and fresh enzyme preparations and did not find significant changes in the enzyme activities.

Enzyme assays

Assay buffer calibration

Assay buffer calibration was conducted for each enzyme to find a species-specific optimal assay buffer pH level (Appendix 2), as long as they were within the probable intracellular pH values of the species, following Hochachka et al. (1975). Enzyme activities were determined using a UV/Vis spectrophotometer (Lambda 35, PerkinElmer®) at 18 °C, based on average *in situ* water temperatures at catch. For PK activity, reaction rates were determined by monitoring the decrease in absorbance of nicotinamide adenine dinucleotide (NADH) at λ = 340 nm over 5 min. For CS activity, the reaction rate was determined by monitoring increase in absorbance of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at λ = 412 nm over 5 min. Protein content was determined following the assay method of Bradford (1976) using bovine serum albumin (BSA) as the standard. Protein content was determined to account for potential differences in the body masses caused by non-metabolically active tissues. Protein-specific enzyme activities were expressed as milliunits per microgramme (mU μg^{-1}) of protein.

Citrate synthase (CS), EC 4.1.3.7

CS activity was measured following the method of Sidell et al. (1987), modified and optimised for fish larvae. The final concentrations in the assay mixture were 75 mM of Tris-HCl buffer (pH: species-specific; see Appendix 2), 0.25 mM of DTNB, 0.2 mM of acetyl-CoA and 0.2 mM of oxaloacetate (OAA) (omitted in the control).

Pyruvate kinase (PK), EC 2.7.1.40

PK activity was determined following the method of Hickey and Clements (2003) and optimised for fish larvae. The final concentrations in the assay mixture were 50 mM of Tris-HCl buffer (pH: species-specific; see Appendix 2), with 50 mM of KCl and 5 mM of $MgSO_4.7H_2O$ (Epsom salt), 0.25 mM of NADH, 0.5 mM of PEP, 1.1 U LDH and 1 mM of ADP (omitted in the control).

Relative enzyme activities

Enzyme activity PK/CS ratios were calculated from the obtained measurements of enzyme activity to assess the relative index of anaerobic versus aerobic metabolic capacities to differentiate distinct types of metabolism in the studied species (Bass et al. 1969; Hochachka et al. 1982; Miller et al. 2014).

Statistical analyses

All statistical analyses were performed using the software JMP Pro 10.0.0. Before data analysis, normality assumptions and variance homogeneity were checked using the Shapiro–Wilk test and Levene's test, respectively. Analysis of variance (ANOVA) was performed to test for significant differences where the data were parametric, and a Kruskal–Wallis test was used when the data were not parametric.

Differences in enzyme activities among species and the effect of the study area were tested by two-way ANOVA (α = 0.05), using species as one effect and sampling area (Kunene or Terrace Bay) as the other. A Tukey HSD *post hoc* test was used to analyse pairwise comparisons of significant results, with significance at α = 0.05. The generalised logarithm (glog) was used to transform the data to meet statistical assumptions of the homogeneity of variances, where the data were non-normal or variances unequal. Unless indicated otherwise, all quantitative data are presented as mean ± standard error (SE).

Results

Hydrographic conditions

Hydrographic conditions (water temperature and dissolved oxygen [DO]) of the two areas during the cruise are presented in Table 1. The hydrographic data show slightly warmer conditions in the Kunene area compared with the Terrace Bay area, while the differences in DO between the two areas were not significant (Table 1), at least during the time the conditions were measured. However, studies have generally shown that the region around Terrace Bay (20° S) in central Namibian waters is characterised by moderate upwelling and moderate winds over a wide, shallow continental shelf, which prevents the upwelling of water from below 150–200 m (Boyer et al. 2000). In contrast, the region around Kunene (17° S) is close to the Angola–Benguela front, where the waters of the Benguela upwelling system meet tropical waters of the Angola Current, which are warmer and more oxygen deficient (Boyer et al. 2000; Hutchings et al. 2009).

Enzyme activities

Enzyme activities were analysed separately for the two larval age classes, since different sources of tissues were used (i.e. the whole organism of early larvae versus the muscle tissue of late larvae), and enzyme concentrations have been shown to vary depending on the body tissue (Sullivan and Somero 1980). The enzyme activities in larvae of five fish species from the two study areas (Kunene and Terrace Bay) are compared below.

Citrate synthase (CS) activity

Differences in CS activity between areas were apparent only in early larvae, with two species showing significant differences in CS activity between the two areas of study (Figure 2a; Appendix 3): *S. bibarbatus* early larvae had higher CS activity in the Terrace Bay area compared with in the Kunene area, while *T. capensis* early larvae showed higher CS activity in the Kunene area than in the Terrace Bay area. There were no significant differences in CS activity between areas within species in late larvae (Figure 2b; Appendix 3). Overall, CS activity was more variable in early larvae than in late larvae. In Terrace Bay, the early larvae of *S. bibarbatus*, *E. encrasicolus* and *S. sagax* had significantly higher CS activity than early larvae of *Merluccius* spp.; at Kunene, early larvae of all species had significantly higher CS activity than early larvae of *Merluccius* spp. The data suggest a similar pattern for the late larvae age class, where, with the exception of *S. bibarbatus*, CS activity was significantly lower in *Merluccius* spp. and similar among the rest of the species.

Pyruvate kinase (PK) activity

There were significant interspecific differences in PK activity in both the early larvae (Figure 3a; Appendix 4) and

Table 1: Seawater temperatures and dissolved oxygen (DO) (mean [SD]) in the two study areas in the northern Benguela upwelling system off the coast of Namibia, sampled between 10 January and 6 February 2014

Depth (m)	Kunene		Terrace Bay	
	Temp. (°C)	DO (ml l^{-1})	Temp. (°C)	DO (ml l^{-1})
Surface	20.0 (0.5)	5.8 (0.4)	19.4 (0.8)	5.6 (0.4)
10	19.5 (0.9)	5.3 (0.7)	18.7 (1.4)	4.8 (0.9)
20	18.1 (1.4)	3.6 (1.3)	17.3 (1.7)	3.6 (1.1)
50	16.1 (0.8)	1.9 (1.2)	15.4 (1.1)	2.0 (1.3)
100	14.8 (0.8)	1.2 (0.4)	14.3 (0.4)	0.9 (0.8)

late larvae (Figure 3b; Appendix 4) age classes, revealing similar trends in both age classes: significantly higher PK activity in *S. bibarbatus* and *T. capensis* as compared with the other three species.

In early larvae, only *S. sagax* showed significant differences between the two areas of study, with higher PK activity in the Terrace Bay sample than in the Kunene sample (Figure 3a; Appendix 4). In late larvae, PK activity in *S. bibarbatus* was significantly higher in the Kunene sample than in the Terrace Bay sample (Figure 3b; Appendix 4).

Relative enzyme activities

Relative enzyme activities are summarised in Table 2.

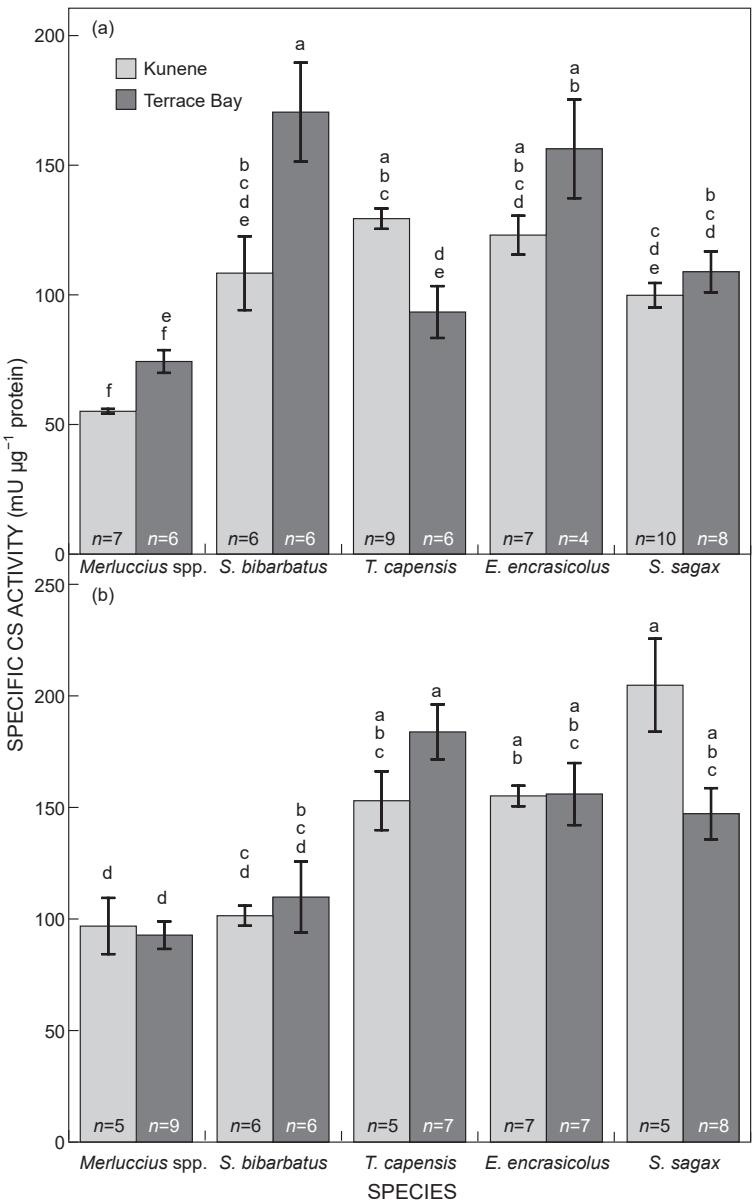

Figure 2: Protein-specific citrate synthase (CS) activity (mean ± SE) in (a) early-larvae and (b) late-larvae age classes sampled from two areas (Kunene and Terrace Bay) in the northern Benguela ecosystem; the whole organism of early larvae and the muscle tissue of late larvae were analysed. Tukey HSD *post hoc* test results comparing five species within and between the two study areas are shown as letters above columns; means not sharing the same letter are significantly different at the 0.05 α-level. See Table 2 for full species names

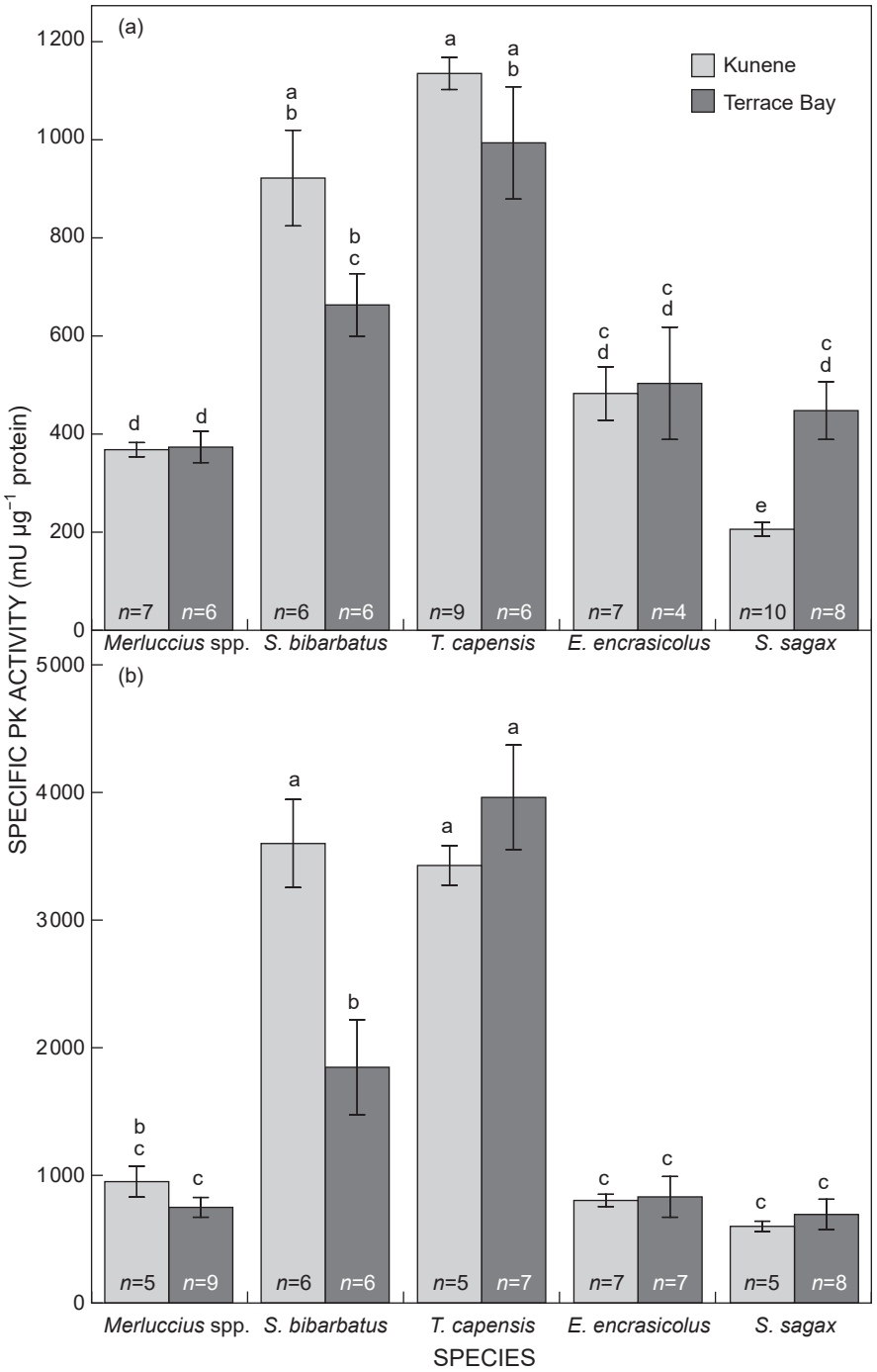

Figure 3: Protein-specific pyruvate kinase (PK) activity (mean ± SE) in (a) early-larvae and (b) late-larvae age classes sampled from two areas (Kunene and Terrace Bay) in the northern Benguela ecosystem; the whole organism of early larvae and the muscle tissue of late larvae were analysed. Tukey HSD *post hoc* test results comparing five species within and between the two study areas are shown as letters above columns; means not sharing the same letter are significantly different at the 0.05 α-level. See Table 2 for full species names

Enzyme activity PK/CS ratios were calculated from the measured enzyme activities.

PK/CS activity ratios were significantly higher in *S. bibarbatus* and *T. capensis* in the late larvae age class in both areas, differing by up to one order of magnitude as compared with in the other species (Table 2; Appendix 5). With the exception of *T. capensis* from Terrace Bay, which had a significantly higher ratio compared with the other species, the data suggest a similar pattern in PK/CS activity ratios in the early larvae age class (Table 2; Appendix 5). In particular, PK/CS activity ratios were generally lower in *E. encrasicolus* and *S. sagax* compared with in the other species.

Table 2: Pyruvate kinase/citrate synthase (PK/CS) ratios of enzyme activities (mean [SE]) in fish larvae in the northern Benguela upwelling system (see Figure 2a,b for sample sizes). Tukey HSD *post hoc* test results within and between the two study areas are shown as letters; means not sharing the same letter are significantly different at the 0.05 α-level

	Merluccius spp.	*Sufflogobius bibarbatus*	*Trachurus capensis*	*Engraulis encrasicolus*	*Sardinops sagax*
			Late larvae		
Kunene	9.96 (0.87)^{cd}	35.52 (2.80)^{a}	22.95 (1.97)^{ab}	5.23 (0.39)^{ef}	2.98 (0.16)^{f}
Terrace Bay	7.97 (0.62)^{de}	18.95 (4.83)^{bc}	21.50 (2.02)^{ab}	5.34 (0.75)^{ef}	4.56 (0.72)^{f}
			Early larvae		
Kunene	6.71 (0.31)^{abc}	9.55 (2.04)^{ab}	8.80 (0.17)^{ab}	3.95 (0.44)^{cd}	2.10 (0.16)^{e}
Terrace Bay	5.16 (0.55)^{bcd}	4.33 (0.84)^{cd}	10.66 (0.51)^{a}	3.11 (0.27)^{de}	4.45 (0.65)^{cd}

Discussion

In this study, we found physiological differences in aerobic and anaerobic capacities in larvae of five species of fish studied in the NBUS, suggesting differences in hypoxia-tolerance capabilities among species, at least during their early life. In particular, the study found enhanced anaerobic capacity in larval *S. bibarbatus* and *T. capensis*, indicating superior hypoxia tolerance traits compared with the other species studied. These results provide additional evidence that improves our understanding of the changes in species composition in an NBUS environment that might become increasingly hypoxic.

Whole larvae were used for the enzyme analyses of the early-larvae age class, and only muscle tissue was used for the late-larvae age class. The very small sizes of early larvae present challenges for obtaining muscle tissue and reliably determining muscle weights. Sullivan and Somero (1980) showed the existence of differences in metabolic enzyme activities (CS and PK) between fish skeletal muscle and brain for various fish species. The difference in methods between studies strongly reflects the different results (patterns) obtained for the larval age classes in the present study.

Aerobic capacity

CS activities were variable in early larvae, with lower CS activity for *Merluccius* spp. compared with the rest of the species. In addition, area differences in CS activity were apparent in two species: *S. bibarbatus* (higher CS activity in Terrace Bay than in Kunene samples) and *T. capensis* (lower CS activity in Terrace Bay than in Kunene samples). There were no significant area differences in CS activity in late larvae. These differences in larvae between ocean regions are presumably attributable to responses to different environmental conditions, with the Kunene area being relatively warmer and with lower oxygen concentrations (owing to close proximity to the Angola–Benguela Front, with frequent intrusions of warm and hypoxic waters from the north) and because higher water temperatures will lower oxygen saturation, compared with at the Terrace Bay area (Hutchings et al. 2002). Thus, we hypothesise that there is potentially an environmental driver that is eliminating a subset of the larvae, here resulting in the late larvae being similar in the two areas. Therefore, in the long term, there may be area differences that select for different CS traits (thereby resulting in the observed differences in early larvae): in high-hypoxia years, the early larvae of some fish species may have a selective advantage, whereas in years with less hypoxic conditions all larval age classes will tend to survive.

Measurement of the activities of enzymes involved in intermediary metabolism can be used to provide rough estimates of the metabolic rates of aquatic animals (Sullivan and Somero 1980; Childress and Thuesen 1992). Furthermore, enhanced metabolism partly acts on digestive and assimilative performance, subsequently improving efficiency and growth (Pang et al. 2016, and references therein). High aerobic capacity in the late larvae of *T. capensis, E. encrasicolus* and *S. sagax* indicated by high CS activity suggests active larval life modes and possible higher growth rates when compared with the larvae of *Merluccius* spp. and *S. bibarbatus*, which showed low aerobic capacities.

Moreover, high metabolic rates have been demonstrated in larvae and juveniles of *T. capensis* (Geist et al. 2013). Relatively higher metabolic rates lead to higher energy demands that are met by higher aerobic capacity, a likely indication of higher growth rates for *T. capensis, E. encrasicolus* and *S. sagax* larvae when compared with *Merluccius* spp. and *S. bibarbatus* larvae. The growth–survival paradigm of Houde (1987) assumes that among other advantages, rapid growth of a larva lowers the risk of predation, thereby providing the larva with the ecological refuge of increased size.

Differences in aerobic capacities among species observed in the larvae analysed in this study possibly resemble the lifestyle patterns of adults of the studied species. Adult anchovy and sardines have active schooling behaviour, with a high metabolic strategy that favours optimal growth rates, as demonstrated in *E. encrasicolus* larvae in the Bay of Biscay, northeastern Atlantic (Díaz et al. 2008). Adults of *T. capensis* also maintain an active lifestyle; in particular, they have a high swimming capacity and a high gastric evacuation rate to meet high energetic demands (Hunter 1971; Pillar and Barange 1998). All these traits in adults of *T. capensis, E. encrasicolus* and *S. sagax* require the mobilisation of energy, which can be best achieved through aerobic processes, as suggested by Beamish (1978). The low aerobic capacity exhibited by larvae of *Merluccius* spp. and *S. bibarbatus* likely reflects the less-active lifestyle of the adults as benthic and

bentho-pelagic species, as suggested by Huse et al. (1998) and Geist et al. (2013), respectively.

Anaerobic capacity

Enhanced glycolytic flux is advantageous to the organism to overcome oxygen debt as a result of excessive locomotion or hypoxia. The amount of ATP produced in the glycolytic pathway is critical to maintain cell functions when energy production via oxidative pathways is limited by low oxygen concentrations (Lushchak et al. 1998; Berg et al. 2002). The importance of the glycolytic pathway in the hypoxia-tolerance responses of aquatic organisms has been demonstrated by several studies.

In general, for aquatic organisms, when aerobic pathways are limited by low oxygen levels, the ATP demand is downregulated to maintain an energy balance, while anaerobic glycolytic fluxes are activated to make up the energy deficit (Jong and Davis 1983; Hochachka 1992; Cooper et al. 2002). Apparent in both larval age classes and sample areas, *S. bibarbatus* and *T. capensis* had significantly higher PK activity than the other species. Indications of higher glycolytic fluxes in larvae of *S. bibarbatus* and *T. capensis* than observed in the rest of the species studied here is advantageous to those species when hypoxic waters are encountered, allowing them to sustain activity and the cellular redox state. This is further affirmed by the PK/CS activity ratios, which were higher by up to one order of magnitude in *S. bibarbatus* and *T. capensis* when compared with the other three species in the late-larvae age class in both areas (Table 2). Miller et al. (2014) note that higher PK/CS activity ratios suggest a higher potential for anaerobic processes to exceed aerobic processes. These results indicate higher capacity for the larvae of *S. bibarbatus* and *T. capensis* to recover from an oxygen debt built up in prey capture and predator escape behaviour, and a higher potential for hypoxia tolerance, as compared with the other species studied here.

Studies of juveniles and adults of those two species found high tolerance to low oxygen concentrations at both life stages (Utne-Palm et al. 2010; Geist et al. 2013), and our results indicate that larval stages also are physiologically prepared to survive low oxygen conditions. In the Angola–Benguela front and NBUS, Ekau and Verheye (2005) examined the horizontal and vertical distribution of eggs and larvae of *S. sagax*, *E. encrasicolus* and *T. capensis* in relation to distribution patterns of temperature, salinity and DO; they hypothesised that, in the NBUS, recruitment of pelagic species relies more on the potential upward extension of the oxygen minimum layer. In Ekau et al. (2018), *S. bibarbatus* and *Merluccius* spp. larvae were added to the three mentioned species, with *S. bibarbatus* larvae found deepest in the water column and closest to the oxycline. With predicted decline in DO, which could influence the NBUS and the documented shoaling of the upper oxygen minimum zone boundaries in all major eastern boundary currents (Gilly et al. 2013; Bakun et al. 2015), tolerance to low DO levels will be key to the survival of marine organisms. Pitcher et al. (2021) refer to future scenarios of low-oxygen waters in the NBUS—but not all point to a general increase of low-oxygen water in the entire region.

In the NBUS, expansion of hypoxic zones is further compounded by massive submarine eruptions of toxic hydrogen sulphide gas (Weeks et al. 2002; Bakun and Weeks 2004). The higher anaerobic capacity demonstrated in *S. bibarbatus* and *T. capensis* larvae appears to suggest that, in the NBUS, expansion of hypoxic zones and predicted warming of the oceans, or a combination of both, will likely negatively impact recruitment of *Merluccius* spp., *E. encrasicolus* and *S. sagax* first. Kreiner et al. (2009) had already shown that, in the NBUS, *E. encrasicolus* and *S. sagax* larvae avoided regions with low DO. However, until this study, this phenomenon of the larvae of these two species had not been investigated at the cellular level in comparison to larvae of other dominant species in the NBUS.

Unsurprisingly, regional differences in enzymatic activities for some species were found in this study. In the early-larvae age class, *S. sagax* showed higher PK activity in the Terrace Bay area compared with the Kunene area, while in the late-larvae age class, only *S. bibabartus* showed higher PK activity in the Kunene area compared with the Terrace Bay area. A number of factors can contribute to regional variability in metabolic enzyme activities, including water temperatures and oxygen variations (Vetter and Lynn 1997; Zakhartsev et al. 2004), as well as differences in food availability (Dahlhoff 2004). Such influences cannot be ruled out in the present study and may explain the observed differences in PK activity in larvae between the two areas—where waters around Kunene are generally warmer than at Terrace Bay, which is located in the central Namibia nursery ground (Hutchings et al. 2002).

Notably, we sampled only in the northern areas and not in the entire spawning areas of the different species. This might limit the generalisation of our results. Another notable aspect is that Terrace Bay is only one of the spawning grounds. Kreiner et al. (2014) clearly state that spawning of *S. sagax* is driven by environmental conditions: during cooler conditions the main spawning occurs north of 22° S, and during warmer conditions south of 22° S. Therefore, it is recommended that further studies consider other important spawning grounds, as well as other seasons. Furthermore, future studies should consider regional differences of low-oxygen waters within the NBUS. The origin and extent of low-oxygen waters in the central region (around 23° S) is quite different from that at 20° S or 17° S; for example, the localised production leading to low-oxygen waters is in the area around Walvis Bay—not necessarily in more northern areas (Monteiro et al. 2006; Mohrholz et al. 2008). In addition, it should be noted that increased upwelling also leads to increased proportions of well-aerated Cape Basin South Atlantic Central Water (Monteiro et al. 2006; Mohrholz et al. 2008). Also, seasonal variability might lead to different water masses and consequent sources of low-oxygen waters between the central (23° S) and northern (20° S and 17° S) areas (Pitcher et al. 2021).

Conclusions

This study found evidence (aerobic capacity) for more-active lifestyles in larvae of pelagic fish species in the NBUS, namely *T. capensis, E. encrasicolus* and *S. sagax*,

resembling the active lifestyles of their adult counterparts. The study also found enhanced anaerobic capacities in the larvae of *S. bibarbatus* and *T. capensis*, suggesting that, compared with the other species studied here, the larvae of these two species are better prepared when hypoxic waters are encountered and have a higher capacity to recover from oxygen debt built up in prey capture and predator escape responses, and this likely reflects the lifestyles of their adult counterparts, which are known to perform vertical migrations. While the findings of this study support the hypothesis that low hypoxia tolerance in the larvae of small pelagics (sardine and anchovy) could be limiting the recovery of these formerly abundant fish stocks in the NBUS, more-detailed studies on the physiological responses of the studied species to hypoxic waters are required to strengthen the evidence for this hypothesis. In particular, since the fish larvae analysed in this study were sampled from the upper 50 m of the water column, the extension of similar studies that consider the effects of depth of occurrence, nutrition and hydrographic conditions over different seasons (with varying temperatures, salinity and oxygen) is recommended.

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Appendix 1: Established age classes of the early and late larvae, lengths at age, and respective growth equations used in this study conducted in the northern Benguela upwelling system. L = standard length (mm); t = age (days); T_c = temperature at time of catch

	Merluccius spp.[a]	*Sufflogobius bibarbatus*[b]	*Trachurus capensis*[c]	*Engraulis encrasicolus*[d]	*Sardinops sagax*[d]
Length at age (mm) –					
Early larvae (8–14 days)	3–5	3–4	4–7	8–12	10–12
Late larvae (22–29 days)	8–12	5–7	10–14	17–21	16–19
Species growth equation	$L = 1.528e^{4.205\ (1-e^{\wedge}-0.023t)}$	$L = 2.12e^{2.75\ (1-e^{\wedge}-0.019t)}$	$L = -0.33 + 0.49t$	$L = 3.37 + 0.608t$	$L = 6.66 + 0.41t$
T_c (°C)	15–17	14.6–21.7	16.7–22.7	16–23	15–20
Size range (for the growth equations)	0–100 mm	3–30 mm	3–20 mm	5–24 mm	9–19 mm

[a]Grote et al. (2012)
[b]Michalowski (2010)
[c]Geist (2013); Simon (2014); Geist et al. (2015)
[d]Geist (2013)

Appendix 2: Optimal assay buffer pH levels used in the study of low hypoxia tolerance of fish larvae in the northern Benguela upwelling system

Species	Citrate synthase	Pyruvate kinase
Merluccius spp.	8.2	7.4
Sufflogobius bibarbatus	8.2	7.4
Trachurus capensis	8.2	7.6
Engraulis encrasicolus	8.2	7.4
Sardinops sagax	8.2	7.4

Appendix 3: Results of two-way ANOVA for citrate synthase activity in early-larvae and late-larvae age classes of five fish species (*Trachurus capensis, Sufflogobius bibarbatus*, *Merluccius* spp., *Engraulis encrasicolus* and *Sardinops sagax*) in the northern Benguela upwelling system. Bold font denotes significance at $\alpha = 0.05$

	Early larvae (n = 69)			Late larvae (n = 65)		
	df	F	p-value	df	F	p-value
Model	9,59	18.137	**<0.001**	9,55	10.525	**<0.001**
Species	4,59	30.013	**<0.001**	4,55	20.914	**<0.001**
Area	1,59	8.764	**0.004**	1,55	0.354	0.555
Species × Area	4,59	8.444	**<0.001**	4,55	2.180	0.083

Appendix 4: Results of two-way ANOVA for pyruvate kinase activity in early-larvae and late-larvae age classes of five fish species (*Trachurus capensis, Sufflogobius bibarbatus*, *Merluccius* spp., *Engraulis encrasicolus* and *Sardinops sagax*) in the northern Benguela upwelling system. Bold font denotes significance at $\alpha = 0.05$

	Early larvae (n = 69)			Late larvae (n = 65)		
	df	F	p-value	df	F	p-value
Model	9,59	29.880	**<0.001**	9,55	32.472	**<0.001**
Species	4,59	53.433	**<0.001**	4,55	65.648	**<0.001**
Area	1,59	0.478	0.492	1,55	4.218	**0.045**
Species × Area	4,59	8.152	**<0.001**	4,55	3.095	**0.023**

Appendix 5: Results of two-way ANOVA for pyruvate kinase/citrate synthase (PK/CS) activity ratios in early-larvae and late-larvae age classes of five fish species (*Trachurus capensis*, *Sufflogobius bibarbatus*, *Merluccius* spp., *Engraulis encrasicolus* and *Sardinops sagax*) in the northern Benguela upwelling system. Bold font denotes significance at $\alpha = 0.05$

	Early larvae (n = 69)			Late larvae (n = 65)		
	df	F	p-value	df	F	p-value
Model	9,59	17.598	**<0.001**	9,55	43.900	**<0.001**
Species	4,59	29.526	**<0.001**	4,55	93.022	**<0.001**
Area	1,59	1.216	0.275	1,55	3.381	0.071
Species × Area	4,59	8.722	**<0.001**	4,55	5.493	**<0.001**