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RESEARCH

# Evaluating the hypoxic tolerance of two maturity stages of Antarctic krill (*Euphausia superba)* at its range edge

**Nelly Tremblay[1,4] · Kévin Cascella[2] · Jean-Yves Toullec[2] · Christoph Held[1] · Sophie Fielding[3] · Geraint A. Tarling[3] · Doris Abele[1]**

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## Abstract

The South Georgia region of the Southern Ocean represents the northernmost range edge for Antarctic krill. Of concern is the extent to which rapid warming of surface water temperatures and reduced oxygen contents around this region might challenge the physiological tolerance of krill, particularly the later maturity stages. Hypoxia is generally considered to be less than 30 to 20% of air saturation, hereafter as threshold hypoxia, while less than 10% of air saturation would qualify as severe hypoxia. These levels are unlikely to occur in the Southern Ocean but might happen in the middle of dense krill swarms. We investigated gene expression and biochemical markers related to aerobic metabolism, antioxidant defence, and heat-shock response under 6-h threshold (4 kPa; TH) and 1-h severe (0.6 kPa; SH) hypoxia exposure, to understand how hypoxia might alter respiratory and biochemical pathways in adult and subadult krill. After 6-h TH, subadults induced expression of citrate synthase (*CS*), and mitochondrial superoxide dismutase (also after 1-h SH) over normoxic expression levels. The maturity stages responded differently in glutathione peroxidase (1-h SH; lower in subadults and higher in adults), and CS (6-h TH; higher in subadults and lower in adults) activities as for the oxidative damage marker to lipids (6-h TH; lower in subadults and higher in adults). Subadults had a greater capacity than adults to deal with hypoxic conditions. This may be a strategy allowing them to exist in larger swarms to reduce predation pressure before reaching reproductive condition.

**Keywords** South Georgia · Oxygen consumption · Heat-shock proteins · Superoxide dismutase · Citrate synthase · Oxidative stress

## Introduction

The South Georgia region (54°17'S; 36°30'W) at the northern border of the Antarctic Polar Frontal Zone is a highly productive area supporting large krill biomass, mainly Antarctic krill *Euphausia superba*, which links the primary production to higher trophic levels, including fish, penguins, seals, and whales. Important krill fishery in the area harvested over 2.5 million tonnes between 1995 and 2010 (Grant et al. 2013). Total krill biomass is composed of subadult and adult stages entrained in the Antarctic Circumpolar Current from nursery grounds at the Western Antarctic Peninsula and transported across the Scotia Sea to South Georgia (Murphy et al. 2007). This region is currently one of the fastest warming waters in the world (Whitehouse et al. 2008) and is considered to be at the thermal tolerance limit for the distribution of Antarctic ectotherms (Morley et al. 2010) and specifically for Antarctic krill (Tarling 2020). This may in part explain the proximity of the region

Doris Abele—Deceased.

✉ Nelly Tremblay
nelly_tremblay@uqar.ca

1 Functional Ecology, Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany

2 Sorbonne Universités, CNRS, UMR 7144, Station Biologique de Roscoff, 29680 Roscoff, France

3 British Antarctic Survey, Natural Environment Research Council, High Cross, Cambridge CB3 0ET, UK

4 Present Address: Département de Biologie, Chimie et Géographie, Université du Québec à Rimouski, Rimouski, Canada

to the northernmost geographical limit of Antarctic krill (Atkinson et al. 2008). The decline of sea-ice extent as a consequence of sea surface water warming (Meredith et al. 2008; Pritchard et al. 2012; Rignot et al. 2013) has negative effects for the abundance, distribution, and life cycle of this species (Flores et al. 2012). Ocean acidification resulting from rapid uptake of atmospheric $CO_2$ in ice free surface waters is expected to have important consequences for their life history, specifically embryonic and larval development (Kawaguchi et al. 2010, 2013), for adult feeding and excretion rates (Saba et al. 2012), and on metabolic key enzyme activities (Saba et al. 2012).

Another factor impinging on marine life in a warming Antarctic oceans is the decreasing oxygen concentration. Polar waters do not display severe hypoxic conditions, but mild-hypoxia (50% $O_2$ saturation) has been reported in the Indian sector of the Southern Ocean at depth greater than 500 m (Dehairs et al. 1990). Deoxygenation in the Southern Ocean is currently taking place in this sector at 200–400 m depth between 50 and 60° of latitude, tied to both thermal buoyancy and changes in circumpolar wind patterns (Matear et al. 2000; Aoki 2005). As an "obligatory schooling species" (Hamner and Hamner 2000), it is believed that krill experience frequent short periods (hrs) of hypoxia, while swimming in the centre region of a swarm for safety (Brierley and Cox 2010). The actual level of hypoxia individuals are exposed to in the swarm centre depends primarily on swarm density, which can reach beyond 25,000 ind $m^{-3}$ (Hamner and Hamner 2000), and size, which can be greater than 100 $km^{-2}$ (Nowacek et al. 2011). According to Brierley and Cox (2010), the oxygen concentration in a median packed *E. superba* swarm (40 m diameter, 111 ind $m^{-3}$) can fall from 6.8 to 5.8 mL $O_2$ $L^{-1}$ (76 to 65% air saturation or 16 to 14 kPa in South Georgia) after approx. 3 min spent in the middle of it. Johnson et al. (1984) detected a decrease of 0.15 mL $O_2$ $L^{-1}$ in the middle of a krill swarm in 1981 (170 ind $m^{-3}$) at the north shelf slope of Elephant Island. Oxygen depletion can reduce greatly the routine (aerobic) metabolism and swimming speed of the individuals (Rakuza-Suszczewski and Opalinski 1978). The physiological challenge swarms present to the individuals has been little investigated since krill within swarms are difficult to track in situ and the position of individuals within swarms changes dynamically. Indeed, krill are known to avoid instruments deployed in the water column like nets (Mackintosh 1934) and gliders (Guihen et al. 2014), so it is difficult to measure physical parameters within and outside of small krill swarms.

Like most aerobic organisms, krill rely on $O_2$ for their energy production. When oxygen partial pressure ($pO_2$) decreases, krill can adopt two oxystrategies, oxyconformity or oxyregulation. Oxyconformity is observed when the respiration rate is decreasing as a function of ambient $pO_2$, while oxyregulation describes the maintenance of constant respiration rates against decreasing $pO_2$ (Bishop 1973). The $pO_2$ where oxyregulators fail to compensate oxygen uptake (mostly through enhanced ventilation) and respiration becomes oxyconforming marks the critical $pO_2$ ($pc$). Many organisms with larval stages change oxystrategies in response to decreasing $pO_2$ throughout their life cycle. The best account of $pO_2$ ontogenetic strategies in Antarctic krill from egg to post-larvae is given by Quetin and Ross (1989): (1) During the embryonic stage until shortly before the egg hatches, oxygen uptake is mainly by diffusion so that embryonic respiration is $pO_2$ dependent or 100% oxyconforming. As embryos rely on lipid reserves, they use < 5% of the metabolic costs of the other non-feeding stages. Eggs are generally released in mid-water layers and sink to deeper layers where temperature is often colder and sometimes less oxygenated. (2) After hatching, the larvae still breathe by diffusion, but their metabolic rate is significantly less $O_2$ dependent. (3) In post-larval (feeding) stages, diffusive oxygen uptake decreases as the larvae grow and their exoskeleton becomes thicker. At this stage, euphausiids have higher energy requirements and possess external gills (from furcilia I stage) to increase the respiratory surface for oxygen uptake. In different larval stages of Northern krill *Meganyctiphanes norvegica*, Spicer and Strömberg (2003) identified a better ability to regulate $O_2$ uptake from furcilia V stage, when gills are still not fully developed compared with adults.

In addition to environmental $pO_2$, the respiration rates of euphausiids depend on temperature (Small and Hebard 1967; Gilfillan 1972) and salinity (Gilfillan 1972). When oxyregulators reach $pc$ and switch to oxyconformity, anaerobic glycolysis from pyruvate to lactate is initiated to fulfil the essential metabolic requirements for survival. Anaerobic glycolysis with lactate as a final product is, however, not very energy efficient, so that prolonged exposure to hypoxia quickly exhausts energy reserves (glycogen), which eventually leads to mortality (Taylor and Spicer 1987). In North Pacific krill *Euphausia pacifica*, $pc$ was detected at 20% oxygen saturation (4 kPa) and exposure below this limit causes dramatic reduction of swimming speed and high mortality (Childress 1975; Ikeda 1977; $pc$ of 18 mm Hg at 10 °C and 20% $O_2$ saturation at 13 °C, respectively). Insufficient anaerobic capacity to survive were observed in northern Atlantic krill *M. norvegica* exposed to unusual natural hypoxic deep-water intrusion of 6.1 kPa at 70 m depth (6.5 °C) into the Swedish Gullmarsfjord (Spicer et al. 1999; Spicer and Strömberg 2003). In South Georgia, Tarling (2020) found that both subadult and adult Antarctic krill had already reached their aerobic capacity by 5.5 °C and would likely revert to anaerobic metabolism when experiencing temperatures that were any warmer.

In the present study, we investigated the physiological performance of Antarctic krill fished in wild swarms around South Georgia among maturity stages. We designed experiments testing Southern krill performance during oxygen depletion to simulate transient hypoxia episodes, including its oxystrategies, stress gene transcription, and the biochemical response to hypoxic stress in terms of a key aerobic marker (citrate synthase), antioxidant enzyme activities, and oxidative damage markers. The respiration measurements and lactate accumulation were already published in Tremblay and Abele (2016), but reanalysed here to understand whether the physiological specific response to hypoxia is related to life-history stage of the individuals (subadult vs. adult).

In terms of gene expression, we tested citrate synthase, manganese superoxide dismutase isoform in both mitochondria and cytosol and five isoforms of 70 kilodalton heat-shock proteins (Hsp70). The Hsp70 isoforms were previously characterized by Cascella et al. (2015) in common Antarctic krill and Antarctic neritic krill *Euphausia crystallorophias* from the eastern part of Antarctica. Hsp70 isoforms A, B, and E with a carboxy-terminal tetrapeptide repeat (glycine-glycine-methionine-proline, GGMP) pattern belong to the Hsp70 subfamily assumed to be constitutively expressed; form C with high sequence similarity to inducible Hsp70 of decapods; and form D which is a mitochondrial heat-shock protein. Because of logistical limitation, gene expression was analysed only in subadult krill.

For further insight into the cellular stress response to hypoxia, antioxidant enzyme activities (superoxide dismutase, catalase, glutathione-*S*-transferase, and glutathione peroxidase) were analysed in the cephalothorax of the experimentally exposed krill (subadult vs. adult). As transition to severe hypoxia can also be accompanied by a release of ROS from oxygen-limited mitochondria in hypoxia-sensitive species (Welker et al. 2013), we tested malondialdehyde (MDA) and protein carbonyl levels as markers for oxidative damage to the lipid and the protein fraction, respectively (subadult vs. adult). Citrate synthase activity was also measured in the same tissue to complement gene expression of this mitochondria density marker (subadult vs. adult).

## Materials and methods

### Krill collection

Sampling and experiments were undertaken aboard *RRS James Clark Ross* (cruise JR260B) between January 1st and 10th 2012 northwest of South Georgia (53–55°S/37–41°W). Surface temperatures were around 3.5 °C, decreased steadily near 0 °C at 120 m water depth, and increased to approximately 2 °C between 120 and 300 m. Salinity decreased between 0 and 10 m water depth, from 35.0 to 33.8 PSU, and returned to 35 PSU at 400 m water depth. Oxygen was fully saturated between 0 and 400 m water depth (21 kPa). Krill swarms were acoustically detected with a SIMRAD EK60 echo sounder (38, 120 and 200 kHz) and collected during night time using a remotely operated opening/closing Rectangular Midwater Trawl (RMT8; 8 $m^2$ mouth area) targeted at the acoustically located krill swarms. On retrieval, the RMT8 cod-end content was immediately transferred to 20 L buckets filled with surface seawater. Live krill showing a lot of movement (fast swimming and no visible damage) were manually sorted in two 100-L cylindrical tanks filled with filtered seawater (particles larger than 2 µm were removed) in a 4 °C cold room. The catch of 8 trawls were used for respirometry and/or experiments and only the larger individuals were selected (NT personal observation). The animals were left to recover for at least 6 h in the cold room before respirometry and hypoxia experiments were started. This short acclimation was a good compromise to avoid starvation, alterations of the routine metabolism, or natural mobility (Ikeda et al. 2000).

### Krill total length and maturity stage

To avoid overstressing specimens used in respirometry and experiments, we removed a random subsample from the 100-L cylindrical tanks into less congested containers containing ambient filtered seawater. A further random subsample of 100 to 200 krill from each trawl was used to record growth parameters including total body length (TL; in mm) from the anterior margin of the eye to the tip of the telson (Morris et al. 1988). Sex and maturity stage were determined in each trawl following a scheme based on Makarov and Denys (1980). Total length measurements of krill from the random sampling are presented in Fig. 1. Detailed information on the proportion of each life stages within the trawls is summarized in Table 1. Trawls 1 and 4 showed a bimodal length distribution (Fig. 1), indicating these swarms to represent a mixture of adults and subadults. Trawls 2, 3, 5, and 6 had a more homogenous distribution of mostly larger krill, whereas trawls 7 and 8 were mainly composed of smaller individuals (Fig. 1). These observations were corroborated by the ratios of adults vs. subadults per trawl found (Table 1): adults dominated trawls 2, 3, 5, and 6 (respectively, 98, 79, 94, and 72%) but were seldom or absent in trawls 7 (5%) and 8 (0%). A considerable number of juveniles were counted in trawls 4 and 8 (29 and 44%, respectively). Based on the length frequency distribution among swarms (Fig. 1) and the stage ratio information (Table 1), data for respirometry and oxidative stress parameters from trawls 1, 2, 3, 4, 5, and 6 (adults) were jointly compared to the ones from trawls 7 and 8 (subadults).

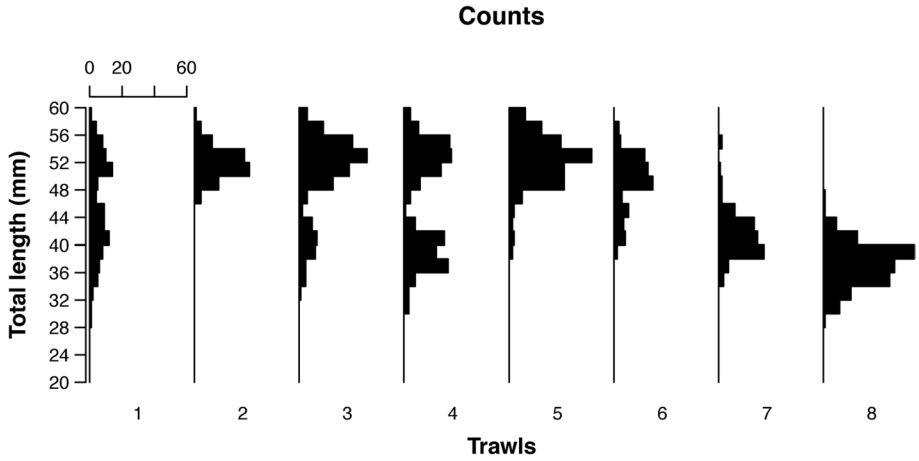


**Fig. 1** Krill total length (mm) frequencies measured from net sub-samples when sampling for experiments aboard *RRS James Clark Ross* between January 1st and 10th 2012 northwest of South Georgia (53–55°S/37–41°W)

**Table 1** Descriptive statistics of krill total length and maturity stage of each net sub-samples

| Trawl | Lat | Lon | Max depth (m) | Cruise event | Max | Min | Mean ± SD | *n* | % Subadults | % Adults | % Juveniles |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | −53.558 | −38.488 | 69 | 19 | 60 | 29 | 46 ± 7 | 100 | 55 | 38 | 6 |
| 2 | −53.798 | −37.953 | 150 | 28 | 59 | 47 | 52 ± 2 | 100 | 2 | 98 | 0 |
| 3 | −53.492 | −39.255 | 209 | 37 | 60 | 34 | 51 ± 6 | 192 | 20 | 79 | 1 |
| 4 | −53.678 | −38.581 | 65 | 38 | 60 | 32 | 47 ± 8 | 200 | 18 | 53 | 29 |
| 5 | −53.608 | −37.724 | 62 | 49 | 60 | 39 | 53 ± 4 | 199 | 6 | 94 | 0 |
| 6 | −53.654 | −37.732 | 60 | 70 | 58 | 39 | 50 ± 4 | 100 | 28 | 72 | 0 |
| 7 | −53.633 | −37.789 | 50 | 82 | 56 | 35 | 42 ± 4 | 100 | 90 | 5 | 5 |
| 8 | −53.798 | −38.089 | 47 | 95 | 47 | 30 | 38 ± 3 | 200 | 56 | 0 | 44 |

Maximum (max), minimum (min), and mean total length (± SD; in mm) of krill (*n* = number measured) contained in 8 trawls targeted for respirometry and hypoxia experiments. The percentage (%) of subadults, adults, and juveniles for each trawl is provided from the sex and maturity stage determination

Gene expression analyses were carried out exclusively with individuals from trawls 7 and 8, used for hypoxia exposure experiments. Therefore, the gene expression data represent only the response of subadults to hypoxia.

## Respirometry

Respirometry was conducted following the short acclimation phase after trawls 1 (adults; $n = 13$), 3 (adults, $n = 3$), and 7 (subadults; $n = 12$). Oxygen consumption was measured in individual krill, in the dark at 4 °C (cold room), using an OXY-4 channels PreSens Oxygen Ingress Measurement system (Germany). The system was equipped with 4 chambers (250 mL) for simultaneous measurement of three animals and a blank recording bacterial oxygen consumption throughout the experiments. All chambers were filled with filtered local seawater at 100% air saturation (21 kPa), and the oxygen concentration in each chamber was measured every 15 s in mbar (or hPa). Chambers were equipped with a magnetic stirrer (bottom) to achieve homogeneity of the oxygen concentration, and a 5 mm mesh separated the stirrer from the euphausiids and also served as substrate for the krill to settle down. The duration of the measurements varied between 7 and 20 h during which $pO_2$ decreased to 20% air saturation (4.2 kPa). The bacterial $O_2$ demand measured in the blank chamber was used to correct the $O_2$ consumption in the chambers containing krill individually for each run. The measurement stopped when the oxygen concentration in two of the three chambers was not decreasing, or when the krill died. The haemolymphatic lactate concentration in mmol/L was recorded in each individual, using an Accutrend R Lactate system (Roche Diagnostics, Germany) following respirometry as a measure of anaerobic metabolism. To obtain the haemolymph, krill were blotted dry on tissue paper and cut open below the cephalothorax using a scalpel. Drops of haemolymph from the abdominal section were directly applied to the testing strip, making sure the strip was completely covered with haemolymph (approximately 15 µL). Subsequently, both parts of the krill were frozen at −80 °C. Dry mass (DM) of each krill from the respiration experiments was measured after drying specimens for 48 h at 50 °C.

## Hypoxia exposure

Since work safety protocols of the *RRS James Clark Ross* did not allow gaseous $N_2$ handling in the cold room, hypoxia experiments took place on deck. The experimental incubations were conducted in the dark inside a Zarges box after each trawl. Two parallel experimental systems were set up in closed Zarges boxes of 81 L inner volume with cooling water from the deck hose (SST between 3 and 3.5 °C) entering at the bottom and exiting below the seawater level of the aquaria to prevent mixing of the cooling and incubation water inside the experimental aquaria. Hypoxia is generally considered to be less than 30 to 20% air saturation, hereafter as threshold hypoxia, while less than 10% air saturation would qualify as severe hypoxia. Each Zarges box contained three aquaria (10 L) filled with freshly filtered seawater for the incubation treatments: control (normoxia; C; 100% air saturation or 21 kPa), threshold hypoxia (TH; 20% air saturation or 4 kPa), and severe hypoxia (SH; 2.5% air saturation or 0.6 kPa). Krill was randomly divided among the aquaria and were allowed to acclimatize for 1 h before air or gas injection in the experimental units started. The number of krill per replicate varied according to the size of the krill, from 10 (trawls 2 to 6) to 20 (trawls 7 and 8) per aquarium.

After 1 h, aeration was started by bubbling air into the control tank with certified $O_2/N_2$ mixtures (Air Products, Hersham, United-Kingdom). The same experiment was conducted after each trawl (2–8), amounting to a total of fourteen replicates for each treatment (C, TH, and SH). Both TH and SH exposures were intended to last for 6 h, but krill from SH treatment (2.5% air saturation) did not show movement of the pleopods after 30–45 min of exposure. Therefore, the animals were retrieved from the experiment 1 h after the beginning together with half of the control animals. From the total number of sampled specimens, half were frozen at −80 °C for biochemical analysis, while the abdominal muscle from the other half was dissected and preserved in RNAlater® at 4 °C for 12 h, and then transferred to −80 °C. The TH treatment (20% air saturation) lasted for 6 h, and the preservation of samples was conducted as described for SH treatment and the second half of the control group.

## Real-time quantitative polymerase chain reaction (qPCR)

Due to logistical limitations, gene expression was analysed only in RNAlater-preserved samples of krill from the two last experiments (corresponding to trawls 7 and 8), subadults. Primers were designed from the transcriptomes of two Antarctic krill species, *E. superba* (SRA023520; Clark et al. 2011) and its closely related species the Antarctic neritic krill *E. crystallorophias* (EMBL-EBI: ERP002510; Toullec et al. 2013). CLC Main Workbench (Version 7.6.4., USA) and the PerlPrimer software (Marshall 2004) were used to double-check the suitability of primer designs (Table 2). Primers were synthesized by Sigma-Aldrich (Germany). Three of the five isoforms of heat-shock protein 70 (*Hsp70*)

**Table 2** Primer used in the real-time quantitative polymerase chain reaction: sequences, size, and efficiency

| Genes | Forward (5'-3') | Reverse (5'-3') | Size (bp) | Primer efficiency |
|---|---|---|---|---|
| *Reference* | | | | |
| EuC-*18S* | TTCCGTCAATTCCTTTAAGTTTCAGC | CCCTAGTTCTAACCATAAACGATGC | 94 | 2.04 (102%) |
| EuC-*EF1α* | GTACAGGTAAGGAA CTTGAATCT | TCTTACATACACCTTGATAACA | 140 | 2.04 (102%) |
| EuC-*GAPDH* | GGAGTAACCAAATT CGTTGTCAT | GATGTTGTTTCTCAGACTTTGT | 80 | 2.05 (103%) |
| EuC-*RPL8* | CTGAAGGTACCATTGTTTGTAACCTC | CTTCAAGATGGGCTTATCAATACGTC | 169 | 1.86 (93%) |
| *Target* | | | | |
| EuC-*CS* | GGCAGATCCAACCAAGTGG | GCAGCAACAATCTACCGAAATC | 207 | 2.09 (105%) |
| EuS-*SODMn-m* | CCACCGTGACCCTAAGTAACC | GGTGATGTTTGGAGTGATGC | 188 | 2.08 (104%) |
| EuS-*SODMn-c* | GGCAGACTCAAAGGACGC | GGCAGCTATCTGTGGATCAAC | 211 | 2.10 (105%) |
| EuC-*Hsp70-A* | AATCATTACCAAGATGTACCAGGC | CTGGGGCACTTGCGTC | 148 | 2.19 (110%) |
| EuC-*Hsp70-C* | AAGAAGAGAAAGGATCAATCAGTA GTCAG | AGTTATGAATCTTAGCAGCAAGTGG | 147 | 2.16 (108%) |
| EuC-*Hsp70-D* | TCTGCAACAATCTTCACTGAAACTC | AACTAAATGCCAATTTATTGTTTGCCAC | 159 | 2.17 (109%) |

Reference gene candidates: 18S ribosomal RNA (*18S*), elongation factor 1-alpha (*EF1α*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and the ribosomal protein L8 (*RPL8*). Genes of interest (target): citrate synthase (*CS*), mitochondrial and cytosolic manganese superoxide dismutase (*SODMn-m* and *SODMn-c*), and *Hsp70* isoforms (*-A*, *-C*, and *-D*)

The prefixes EuS and EuC used in the gene names mean that the primers were designed on the species *Euphausia superba* or *Euphausia crystallorophias* from the transcriptomic information published by Clark et al. (2011) and Toullec et al. (2013), respectively

The primer efficiency refers to the rate at which an amplicon is generated; if the amplicon doubles in quantity, then the assay is said to have 100% efficiency. Primer efficiency between 95 and 105% is usually privileged

identified by Cascella et al. (2015) were analysed (*Hsp70-A*, *-C*, *and -D*), whereas the expression of *Hsp70-B* and *Hsp70-E* cannot be reported as the standard curve indicated unspecific binding of these primers (*-B*) or expression was too low (*-E*). Sequence comparisons ascertained the identity of the amplified sequences *Hsp70* in the present study by comparison with reported sequences of De Pittà et al. (2013) and Meyer et al. (2015). In addition, gene expressions of citrate synthase (*CS*) and of manganese superoxide dismutase isoform in both mitochondria and cytosol (*SODMn*) were analysed. Expression of the copper–zinc isoform (*SODCu,Zn*) was not measured in the study as this isoform is undetectable in many haemocyanin-carrying Malacostraca crustaceans like euphausiids (Brouwer et al. 1997). Samples were analysed in 2012 and 2013 with the molecular information and techniques of that time.

Total ribonucleic acid (RNA) from the abdominal samples were extracted using the QIAGEN RNeasy® Kit, and 1 µg of total RNA was reverse transcribed into single-stranded complementary deoxyribonucleic acid (cDNA) using oligo dT and RT-MMLV reverse transcriptase kits (Promega, USA), according to the manufacturer's instructions. Real-time PCR was performed in a Rotor-Gene Q (QIAGEN, Hilden, Germany) using Eva Green Type-it HRM PCR kit (QIAGEN, Germany) following the protocol: 5 min at 95 °C, 40 cycles of 10 s at 95 °C, and 30 s at 55 °C. Each sample was quantified in duplicate and treatment groups were distributed evenly between runs. To confirm the specificity of the amplification, melt step was recorded directly after the cycling by increasing temperature from 65 °C to 90 °C in increments of 0.5 °C for 2 s each. A standard curve for each primer was determined with a pool of all cDNA samples (serial dilution) to assess primer efficiency and linear range of the assay (Table 2). Sequencing of real-time PCR products was conducted to confirm the targeted amplification. Expression levels were normalized using the elongation factor 1-alpha *EF1α*, which was selected as the best applicable (most constitutive) reference gene out of the four candidates (the other candidates were 18S ribosomal RNA or *18S*, glyceraldehyde 3-phosphate dehydrogenase or *GAPDH*, and ribosomal protein L8 or *RPL8*) using the Normfinder (Andersen et al. 2004) and gNorm (Vandesompele et al. 2002) algorithms. Mean normalized expression (Muller et al. 2002) was calculated with the software qgene (Joehanes and Nelson 2008).

### Biochemistry

To understand which enzyme activities were up-regulated in support of damage prevention and ROS detoxification during hypoxia, the activities of the antioxidant enzymes (superoxide dismutase, catalase, glutathione-*S*-transferase, and glutathione peroxidase) were analysed in the cephalothorax of the experimental organisms as described in Tremblay and Abele (2016). CS activity, the detection of malondialdehyde (MDA) formation (lipid peroxidation), and protein carbonyl content (protein oxidative damages) were analysed in the abdominal part of the euphausiids as described in Tremblay and Abele (2016).

### Statistical analysis

All statistical analyses and figures were realized in R (R Core Team 2024). Smoothers (LOESS) were adjusted to oxygen consumption subsets (subadult vs. adult) using a span = 0.4, which was the best automatic smoothing parameter selection via Akaike information criterion. The package "fANCOVA" (Wang 2020) was used to test the equality of both curves based on an ANOVA-type statistic and a wide-bootstrap algorithm to obtain the null distribution. For all gene expression and biochemical comparisons, data were tested for outliers (box-whiskers plot, with coefficient of 1.5 for outliers and extremes), normality (Shapiro test), and variance homogeneity (Bartlett test). Data were transformed ($\log(x)$, $x^{-1}$, $x^{1/2}$) if the criteria of normal distribution and homogeneity of variance were not met. For the gene expression data, one-way analysis of variance (ANOVA) was used for each target gene among treatment (control vs. hypoxia) considering each experiment separately (TH and SH). For the biochemical data, a two-way ANOVA was used for each indicator considering "treatment" (control vs. hypoxia) and "stage" (subadults vs. adults) as factors for each experiment separately (TH and SH). The experiments were tested separately because both hypoxia intensity and length of exposure differed. If no transformation of data allowed the use of ANOVA, a non-parametric test was conducted. Significance level of all comparisons was fixed at 95% ($p = 0.05$). A detailed data set is provided at https://doi.org/10.1594/PANGAEA.834807.

## Results

### Respirometry

Mean dry mass comparison of the specimens used in respiration measurements confirms that krill collected from trawls 1 and 3 were significantly heavier than the ones from trawl 7 (Fig. 2a; Wilcoxon, $W = 192$, $p < 0.000$). Adult krill (trawls 1 and 3) had higher lactate values following the respiration measurement compared to subadults (trawl 7; Fig. 2b; Wilcoxon, $W = 94.5$, $p = 0.009$). The time spent in the respiration chamber was $639 \pm 212$ min for adults and $938 \pm 170$ min for subadults. Respiratory path adjusted with LOESS smoothening of adults (trawls 1 and 3) was significantly different compared to subadults from trawl 7 (Fig. 2c and d; fANCOVA:t.

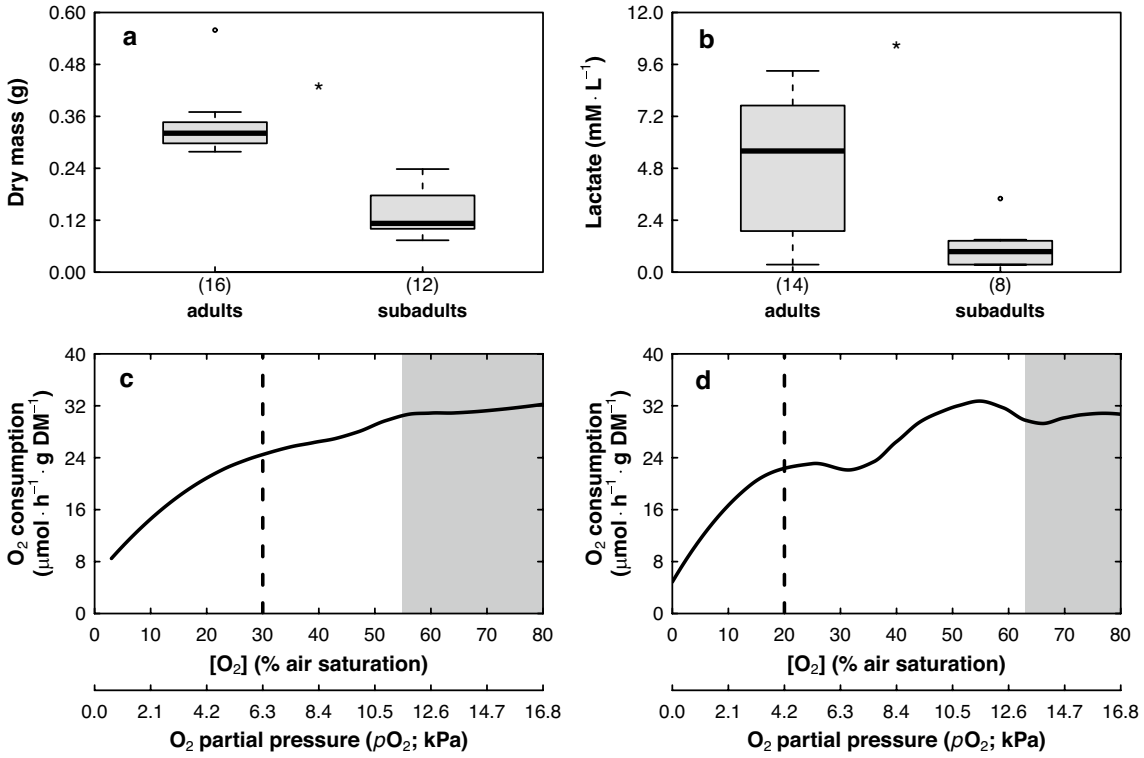


**Fig. 2** Dry mass (g; **a**), lactate concentration (mM $L^{-1}$; **b**), and LOESS adjusted oxygen ($O_2$) consumption (µmol $h^{-1}$ $gDM^{-1}$) as function of oxygen concentration (% air saturation) and oxygen partial pressure ($pO_2$; kPa) in the respiration chamber of adults (**c**) and subadults (**d**). Box plots: Horizontal bars indicate the median, upper, and lower edges of the rectangles show the first/third quartiles, and vertical error bars extend to the lowest and highest values in a 1.5-fold inter-quartile range; number in brackets = number of samples analysed; asterisk indicates significant difference between subadults and adults. For the LOESS model, smoothers were adjusted to oxygen consumption using a span = 0.4, which was the best automatic smoothing parameter selection via Akaike information criterion. Threshold of constant respiration rate (grey region) and critical oxygen partial pressure (dash lines) are shown for both ontogenetic stages

aov, $T = 1.669$, $p = 0.005$). Despite the declining $pO_2$ in the respiration chambers, oxygen consumption of adult krill remained constant until approximately 11.6 kPa (55% air saturation) and decreased progressively below 6.3 kPa (30% air saturation), the latter representing the critical $pO_2$ ($pc$) of the adults (Fig. 2c). By contrast, oxygen consumption of subadults increased between approximately 13.5 and 11.6 kPa (63 to 55% air saturation), was less pronounced between 6.5 and 5.6 kPa (31 to 27% air saturation), and declined progressively below 4.2 kPa (20% air saturation), which represents the $pc$ (Fig. 2d).

## Gene expression and biochemical indicators

Subadult krill induced citrate synthase (*CS*) transcription following 6 h of threshold hypoxia (TH) treatment compared to the normoxic control (ANOVA; $F = 5.50$; $p = 0.041$; Table 3). This increase was coherent with increased activity in the TH compared to controls, whereas CS activity of adults was lower after 6 h of TH treatment (ANOVA; see Table 4). Overall, CS activity was generally higher in subadults compared to adults for control and 6 h of TH treatment (ANOVA; see Table 4; Online resource 1).

Mitochondrial superoxide dismutase manganese (*SODMn-m*) gene expression in subadults increased in both 1-h SH (ANOVA; $F = 9.99$; $p = 0.008$; Table 3) and 6-h TH (ANOVA; $F = 13.76$; $p = 0.004$; Table 3), whereas cytosolic superoxide dismutase manganese (*SODMn-c*) was similarly expressed across treatments (Table 3). SOD activity was generally lower in subadults compared to adults for control and 6-h threshold hypoxia treatment (ANOVA; see Table 4; Online resource 1), and was reduced by 6-h threshold hypoxia exposure in both stages (ANOVA; see Table 4). The only significant interaction between life stage and 1-h severe treatment was for the glutathione peroxidase (GPX) activity levels (ANOVA; see Table 4). GPX activity was reduced in subadults by 1-h SH exposure compared to control, while GPX activity was significantly higher in adult krill by 1-h SH exposure compared to control; Table 4; Online resource 1). Neither SH nor TH exposure had any significant effect on catalase (CAT) or glutathione S-transferase (GST) activities (Table 4).

**Table 3** Stress gene mean normalized expression (MNE; mean ± standard deviation) of subadult *Euphausia superba* in normoxia (control) and experimentally transferred to severe (SH; 2.5% air saturation) and threshold hypoxia (TH; 20% air saturation)

| Treatment | Control ($n=8$) | SH ($n=8$) | Control ($n=7$) | TH ($n=6$) |
|---|---|---|---|---|
| Duration (h) | 1 | 1 | 6 | 6 |
| *CS* | 0.012 ± 0.008 | 0.018 ± 0.008 | 0.011 ± 0.007 | **0.022 ± 0.008** * |
| *SODMn-m* | 0.021 ± 0.012 | **0.043 ± 0.014** ** | 0.032 ± 0.011 | **0.062 ± 0.015** ** |
| *SODMn-c* | 0.031 ± 0.007 | 0.037 ± 0.017 | 0.027 ± 0.014 | 0.042 ± 0.013 |
| *Hsp70-A* | 0.0040 ± 0.0017 | 0.0037 ± 0.0020 | 0.0030 ± 0.0005 | 0.0031 ± 0.0008 |
| *Hsp70-C* | 0.0012 ± 0.0007 | 0.0014 ± 0.0008 | 0.0012 ± 0.0010 | 0.0005 ± 0.0003 |
| *Hsp70-D* | 0.022 ± 0.012 | 0.019 ± 0.009 | 0.027 ± 0.013 | 0.046 ± 0.030 |

*CS* citrate synthase; *SODMn-m* and *SODMn-c* superoxide dismutase manganese mitochondrial and cytosolic; *Hsp70-A* heat-shock proteins 70 constitutive isoform; *Hsp70-C* inducible isoform; *Hsp70-D* mitochondrial isoform

Significant differences to the respective normoxia treatment (± 95% confidence limits) are marked in bold with $p < 0.05$ *; < 0.01 **

**Table 4** Compilation of the effects of treatment (severe or threshold hypoxia vs. respective control) and stage (subadults vs. adults) and their combined effects on selected biochemical indicators of *Euphausia superba*

| Indicators | ANOVA Values | Severe hypoxia (SH) | | | Threshold hypoxia (TH) | | |
|---|---|---|---|---|---|---|---|
| | | Treatment | Stage | Treatment * Stage | Treatment | Stage | Treatment * Stage |
| CS | *F* | 0.149 | 2.634 | 0.086 | 0.436 | **5.860** | **4.951** |
| | *p* | 0.701 | 0.109 | 0.770 | 0.512 | **0.019** | **0.031** |
| SOD | *F* | 0.612 | 0.060 | 0.187 | **7.203** | **5.178** | 3.559 |
| | *p* | 0.437 | 0.807 | 0.666 | **0.009** | **0.026** | 0.064 |
| CAT | *F* | 0.004 | 1.308 | 2.065 | 1.187 | 1.678 | 1.431 |
| | *p* | 0.952 | 0.257 | 0.156 | 0.280 | 0.200 | 0.236 |
| GPX | *F* | 0.410 | 0.058 | **6.647** | 0.041 | 0.444 | 1.674 |
| | *p* | 0.524 | 0.810 | **0.012** | 0.841 | 0.508 | 0.201 |
| GST | *F* | 0.216 | 2.282 | 0.091 | 0.034 | 3.113 | 0.953 |
| | *p* | 0.643 | 0.136 | 0.763 | 0.855 | 0.082 | 0.332 |
| MDA | *F* | 0.459 | 1.017 | 0.029 | 0.062 | **4.484** | **8.715** |
| | *p* | 0.500 | 0.317 | 0.865 | 0.804 | **0.038** | **0.004** |
| CO | *F* | 0.036 | **20.435** | 0.036 | 2.041 | **8.663** | 1.850 |
| | *p* | 0.850 | **0.000** | 0.851 | 0.163 | **0.006** | 0.183 |

*CS* citrate synthase activity; *SOD* superoxide dismutase activity; *CAT* catalase activity; *GPX* glutathione peroxidase activity; *GST* glutathione S-transferase activity; *MDA* [malondialdehyde]; *CO* [carbonyls];

Significant differences to the respective control (normoxia) treatment (± 95% confidence limits) are marked in bold

During hypoxic exposure, expression levels of the *Hsp70* isoforms were unchanged in subadults. In the control group of young krill, the mitochondrial isoform *Hsp70-D* was the most highly expressed of all *Hsp70* forms, with a tenfold higher expression than the constitutive isoform *Hsp70-A* (Table 3).

Another strikingly different response of the two ontogenetic stages to the 6-h TH treatment was demonstrated for the malondialdehyde (MDA) levels (ANOVA; see Table 4): MDA concentrations were reduced in subadults after 6-h TH compared to the control, while it was higher in adults after 6-h TH compared to the control (Online resource 1). Overall, higher MDA concentrations occurred in subadults compared to adult animals for control and 6-h threshold hypoxia treatment (ANOVA; see Table 4; Online resource 1). Finally, protein oxidation levels (carbonyls) were unaltered by either severe or threshold hypoxia in both life stages, but were generally higher in adults than younger animals reflecting increasing ROS damage to proteins with age (ANOVA; see Table 4; Online resource 1).

## Discussion

This study demonstrated that physiological adjustments of Antarctic krill during oxygen depletion depend on the developmental maturity stage. Under decreasing $pO_2$, the respirometry results showed two different patterns

depending on the maturity stage (subadults vs. adults). Adults were able to regulate their oxygen consumption down to 30% air saturation (*pc*) similar to data reported by Clarke and Morris (1983; *pc* to 30% air saturation at unknown experimental temperature and handling) in South Georgia and by Torres et al. (1994; *pc* between 30 and 52 mm Hg corresponding to 19–33% air saturation and 4–7 kPa at 0.5 °C) for the Scotia Sea. We can now assume that the upward shift of *pc* (11.6 kPa; 55% air saturation) reported by Tremblay and Abele (2016), which included subadults and adults measurements, is reflecting life-stage variability in the capacity of Antarctic krill to oxyregulate at low oxygen saturation levels. Indeed, subadults showed compensatory efforts towards hypoxic stress. The increasing oxygen consumption, which was measured from 13.5 to 11.6 kPa (63 to 55% air saturation) and 6.5 to 5.6 kPa (31 to 27% air saturation) could be related to increase swimming activity (NT personal observation) in the attempt of escaping the hypoxic condition. In this way, the animals accumulate an oxygen debt, which leads to a depression of the metabolic rate at *pc* (4.2 kPa; 20% air saturation). Subadults did not involve the anaerobic pathway, as lactate concentration was lower than in adults at the end of the incubation. Accordingly, subadults show a higher tolerance than adults in terms of *pc* and use of the anaerobic pathway to obtain energy at threshold levels of hypoxia. The cost of swimming could account for up to 73% of total daily metabolic expenditure during early summer for this species (Swadling et al. 2005). Larger adults may have a higher cost of swimming and so cannot avoid involving the anaerobic pathway to the same extent as subadults. Avoidance of lactate accumulation in subadults, which exhausts energy reserves and increases blood acidosis, seems pertinent to maintain active swimming, as krill face a high risk of predation once they fall behind the school (Brierley and Cox 2010). This might even be more critical in polar species, in which lactate removal by reconversion to pyruvate or through gluconeogenesis is curtailed by the low temperatures (Bushnell et al. 1994). Lactate accumulation has been studied as proxy of anaerobic pathway in the Nordic krill *Meganyctiphanes norvegica* during its diel vertical migration in the hypoxic waters of the Gullmarsfjord (Sweden; Spicer et al. 1999).

At least in subadult Antarctic krill (gene expression analysis considered only experiments with subadults), the difference in the respiration pattern was reflected in the alteration of gene expression of citrate synthase (*CS*), a mitochondrial marker. Increased transcription of *CS* in subadults was induced during 6 h of threshold hypoxia (and also enhanced within 1 h of severe hypoxic exposure), indicating an attempt to sustain CS activities and mitochondrial aerobic capacities during short-term oxygen deficiency. This is a surprising finding, as new protein synthesis is a costly process under hypoxic conditions as shown in the shore crab *Carcinus maenas* (Mente et al. 2003). Transcriptional induction could serve to maintain CS activity until krill can return to normoxic conditions. The higher CS activity in abdomen muscle of subadults after 6-h threshold hypoxia treatment supports the escaping behaviour hypothesis under hypoxic conditions. As we did not measure CS protein concentration, we can only speculate that transcriptional induction (much less energetic expenditure than protein synthesis) serves to support CS activity in this highly energetic swimmer (Zhou and Dorland 2004) once the animals return to normoxia and to a fully and unrepressed aerobic metabolism. In adults, the lower CS activity after 6-h threshold hypoxia exposure could correspond to the decrease of the respiration rate identified in respirometry when reaching *pc* and the use of the anaerobic pathway, which was confirmed with the higher lactate concentration measured at the end of the respirometry.

Transcription of the mitochondrial isoform of superoxide dismutase (*SODMn-m*) was significantly increased in both hypoxia treatments, which indicates that the subadults were preparing to resume synthesis of the protein after hypoxia exposure. Induction of SOD mRNA may also indicate that antioxidant capacities are kept high during transient hypoxic episodes in preparation for oxidative stress during post-reoxygenation. Up-regulation of the mitochondrial *SODMn-m* gene was observed in a similar way after several days of experimental cycling hypoxia (simulating natural conditions) in the blue crab *Callinectes sapidus* (Brown-Peterson et al. 2005) and the grass shrimp *Palaemonetes pugio* (Brown-Peterson et al. 2008)—both organisms are tolerant to hypoxia (Stickle et al. 1989) as they are exposed to it on a daily basis. Activities of SOD were stable (1-h severe) and lower (6-h threshold) in hypoxic subadult krill in spite of the upregulation of the mRNA, which shows that protein translation had not happened, while the krill was experiencing hypoxia. SOD activity was also lower in adults exposed to the same 6-h threshold hypoxia condition. However, as malondialdehyde (MDA) concentrations were lower in subadults after 6-h threshold hypoxia exposure and higher in adults, it seems that no other mechanisms were activated in adults to counterbalance the lower SOD activity. The link between SOD activity and MDA concentrations is further explained by the significant difference among stages measured after the 6-h experiment (combining control and hypoxia samples) as subadults had generally low SOD and high MDA levels while adults had the opposite. High SOD activity under normoxic conditions seems thus essential to maintain low MDA levels in adult krill. Adult krill had slightly higher carbonyl levels, which may be related to the age of the individuals, as no difference among hypoxia

treatments were observed. Accumulation of carbonyls with age was shown during the embryonic development of the berried Norway lobsters (*Nephrops norvegicus*; Styf et al. 2013).

The 1-h severe hypoxia treatment only caused a different biochemical response in the glutathione peroxidase (GPX) activity of subadults (lower activity) and adults (higher activity). The severity of the hypoxia level was probably lethal if prolonged more than 1 h, as all the krill were lying on the floor of the aquaria quickly after the beginning of the exposure. Significant MDA accumulation and ROS production only happened after reoxygenation in *Neohelice granulata* crabs collected from the salt marshes around Rio Grande City (Brazil), not during the 4- and 10-h severe hypoxia exposure (1.2 kPa or 6% air saturation at 20 °C), while no effect was shown either during hypoxia exposure or during reoxygenation after 1-h hypoxia exposure (Geihs et al. 2014). Unlike *N. granulata*, krill has an extremely energy-consuming lifestyle and might not have the option to "turn off the engine" in times of high stress like in the above study, where the level used was almost anoxic. Therefore, in the present study, the short time of the hypoxia exposure, its intensity, and the lack of reoxygenation procedure post-hypoxia exposure do not allow a clear analysis of the mechanisms involved in both stages when exposed to this short term and severe hypoxia level.

In higher eukaryotes, the Hsp70 family comprises constitutive and inducible isoforms (Tavaria et al. 1996). The unchanged expression of all three isoforms reported, constitutive, and inducible, detonate from downregulation responses observed in hypoxia tolerant grass shrimp *P. pugio* as it enters a reduced metabolic state in hypoxia in which protein synthesis slows down (Brouwer et al. 2007; Brown-Peterson et al. 2008). Of all *Hsp* isoforms, the mitochondrial *Hsp70-D* was most highly expressed in line with the high expression of both *SODMn* enzymes. Mitochondrial Hsp70s are located in the mitochondrial matrix to stabilize polypeptide chains, subunits of mitochondrial enzymes, synthesized in the cytosol and entering the mitochondrion through the inner membrane (Kang et al. 1990; Chacinska et al. 2009). The Hsps also remove denatured proteins through the membrane into the matrix for proteolysis (Lee et al. 2004; Doyle and Wickner 2009). The fact that this mitochondrial *Hsp70-D* was not up-regulated during hypoxic exposure is another indication of protein synthesis having come to a halt as a result of insufficient oxygenation. Interestingly, compared to basal expression levels of *Hsp* in Antarctic krill at Terre Adélie (Cascella et al. 2015), where the habitat temperature is significantly colder all year round (~ 0 °C), basal expression levels in the present study were tenfold lower for *Hsp70-A* and 20-fold higher for *Hsp70-D*. The drastic quantitative decrease observed in the expression of constitutive isoform such as *Hsp70-A* confirmed the observation previously made by Toullec et al. (2020) that this isoform was particularly well represented in samples originating from the eastern part of Antarctica and was undetectable by western blot in samples fished north of the Antarctic Peninsula, where temperatures are much higher overall. This suggests that this isoform is expressed and translated preferentially in coldest waters and would act as a cold-shock protein. Similarly, the higher expression of the mitochondrial form, *Hsp70-D*, in western southern waters could be a response to higher water temperatures. This already substantial and locally constitutive increase could have masked a potential response linked to hypoxic shock. As temperature stress seems to outweigh hypoxia stress, it would have been interesting to apply the same experimental design to krill living in colder waters to assess if *Hsp* isoforms can play a role in hypoxia tolerance within colder waters.

Thus, the major patterns of *Hsp70* isoform expression might be indicative of the adaptation of Antarctic krill swarms to different regional environmental conditions in the Southern Ocean, especially with respect to the interlinked factors of water temperature and oxygenation. This is interesting with respect to the apparent lack of phylogeographic structure in krill (see e.g., Bortolotto et al. 2011) as it indicates that the underlying process may not be a regional accumulation of alleles coding for one or the other type of response, but a phenotypic plasticity of the reaction norm (an array of phenotypes that will be developed by a genotype over an array of environments). Such regional acclimation is consistent with the findings of Tarling (2020) who showed that the respiration response of Antarctic krill at South Georgia to experimental temperature gradients differed to populations from elsewhere (principally those further south). Specifically, whereas the respiration rate of South Georgia krill at ambient temperature (2 to 5.5 °C) was as expected according to a globally fitted Arrhenius function for all Antarctic krill, it was much higher than predicted at lower temperatures (< 2 °C). Tarling (2020) considered that regional acclimation may have been achieved through decreasing the density and capacity of mitochondria (Pörtner 2002). Accordingly, the present study further suggests that the functioning, recruitment, and regulation of the mitochondrial Hsp70 may be part of this same process.

It is widely accepted that the individuals of a swarm make a trade-off between greater protection in the centre of the swarm and greater availability of oxygen (and food) in its periphery. However, the physiological challenge that oxygen gradients in swarms pose to individual krill and how it influences their hypoxia tolerance has hardly been addressed in science. It should be noted that our results only refer to acute responses. Nevertheless, in relation to the warming of the South Georgia region, our results suggest that adults may need to revert even more to the peripheries of swarms

or else to smaller and/or less dense swarms (Brierley and Cox 2010) to reduce levels of short-term hypoxic exposure. An alternative is to adopt deeper positions below the warmer surface layers (Hill et al. 2013), although this will have implications on their capability to feed. The greater effort required to exploit more dispersed local stocks of krill will in turn have large impacts on the viability of krill-dependent higher predators in the region.

## Conclusion

In subadult krill, we found that enhanced oxygen consumption with decreasing oxygen partial pressure, which is less reliant on an anaerobic pathway, along with higher gene expression (*CS* and mitochondrial *SODMn*) and higher CS activity during in vitro hypoxia exposures, suggests a need to reoxygenate at the swarm periphery after enduring periods of hypoxia in the swarm interior. These physiological responses could prepare them for the stress of reoxygenation when moving to the periphery, thus preventing harmful cellular damage from both hypoxia and reoxygenation. In comparison, adults are less capable of coping with hypoxia and must therefore resign themselves to remaining in the more oxygenated periphery of the swarm, which entails greater risk to predation. This may be an adequate trade-off in terms of life-time fitness since adults may have already reproduced and will maximize future fitness through investing in further reproduction than hypoxia tolerance. Subadults, by contrast, must stay alive to fulfil any reproductive potential.

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**Data availability** A detailed data set is provided at https://doi.org/10.1594/PANGAEA.834807.

## Declarations

**Competing interests** The authors declare no competing interests.



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