Morphological analysis of erythrocytes of an Antarctic teleost under heat stress: Bias of the stabling effect

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ABSTRACT

The stenothermal Antarctic fish that live in the coastal waters of the Terra Nova Bay (Ross Sea) are rarely exposed to temperatures above zero during the year. We tested whether a slight temperature rise of 1.5 °C affects a sensitive biomarker such as erythrocytes morphology in sections of blood pellets of a small demersal notothen. The erythrocytes' shape descriptors showed significant or highly significant differences temporally from the capture of fish to the conclusion of the experiment. Surprisingly, the erythrocyte's morphology did not show significant differences between the two experimental conditions, returning similar results in control fish stabled at −0.9 °C and in the fish treated at +0.6 °C, although the values of the shape descriptors were often lower in the latter. This study demonstrates the critical issues of comparative physiology in the study of extremely sensitive organisms, such as the fish of the High Antarctic Zone. Moreover, the stabling effect inside the aquarium facilities appears to significantly obscure the effects of the experimental heat treatment.

1. Introduction

Ongoing global climate change will impact different regions of the globe unequally. The Antarctic is among those regions where the effects are expected to be faster and more intense, with possible irreparable damage to the rich and complex ecosystems that have evolved in this extreme and stable environment (Bassis, 2020; Peck et al., 2014; Schmidtko et al., 2014). The ichthyofauna from Antarctic waters is dominated by the endemic Perciformes' suborder Notothenioidei (Duhamel et al., 2014). The dominance by this single taxonomic group reaches unparalleled levels in the Ross Sea, where it constitutes 77% of fish biodiversity and 90% of fish biomass (La Mesa et al., 2004b). Antarctic notothenioids evolved a variety of physiological and biochemical adaptations in order to maintain their physiological functions in an extremely cold environment, such as high solute concentrations in blood, antifreeze proteins, hematocrit reduction, cold-stable tubulins and microtubule polymerization, enzymes with higher catalytic efficiencies, proliferation of cold-efficient mitochondria and homeoviscous adaptation of cell membranes (Beers and Jayasundara, 2015; Buckley and Somero, 2009; Peck, 2018). The thermal stability experienced by these fish has reduced selective pressure on maintaining costly functional traits useful in dealing with temperature fluctuations. Although they have generally lost inducible heat shock response, recent studies have demonstrated that they show a wide inter-specific variety of responses to thermal stress with some species showing acclimation or heat hardening to 4 °C and others being subjected to death at temperatures only a few degrees above their habitat temperature (Bilyk et al., 2018; Huth and Place, 2016a; Peck et al., 2014; Podrabsky and Somero, 2006). However, despite molecular and cellular buffering systems to acclimation, negative trade-offs were found on the ecologically relevant whole-organism level, indicating an overall insufficient capacity of compensation (Sandersfeld et al., 2015). Moreover, the simultaneous variations of multiple stressors (e.g. warming and acidification) may interact synergistically, having a greater effect than the sum of individual stressors and potentially reducing the organisms' ability to cope with it (Huth and Place, 2016b). To investigate the effects of heating stress on teleosts, it is often considered that temperature increase leads to respiratory stress and this can lead to erythrocytic and nuclear morphological abnormalities (Burgos-Aceves et al., 2019; Witeska, 2013; Zafalon-Silva et al., 2017). The introduction into the bloodstream

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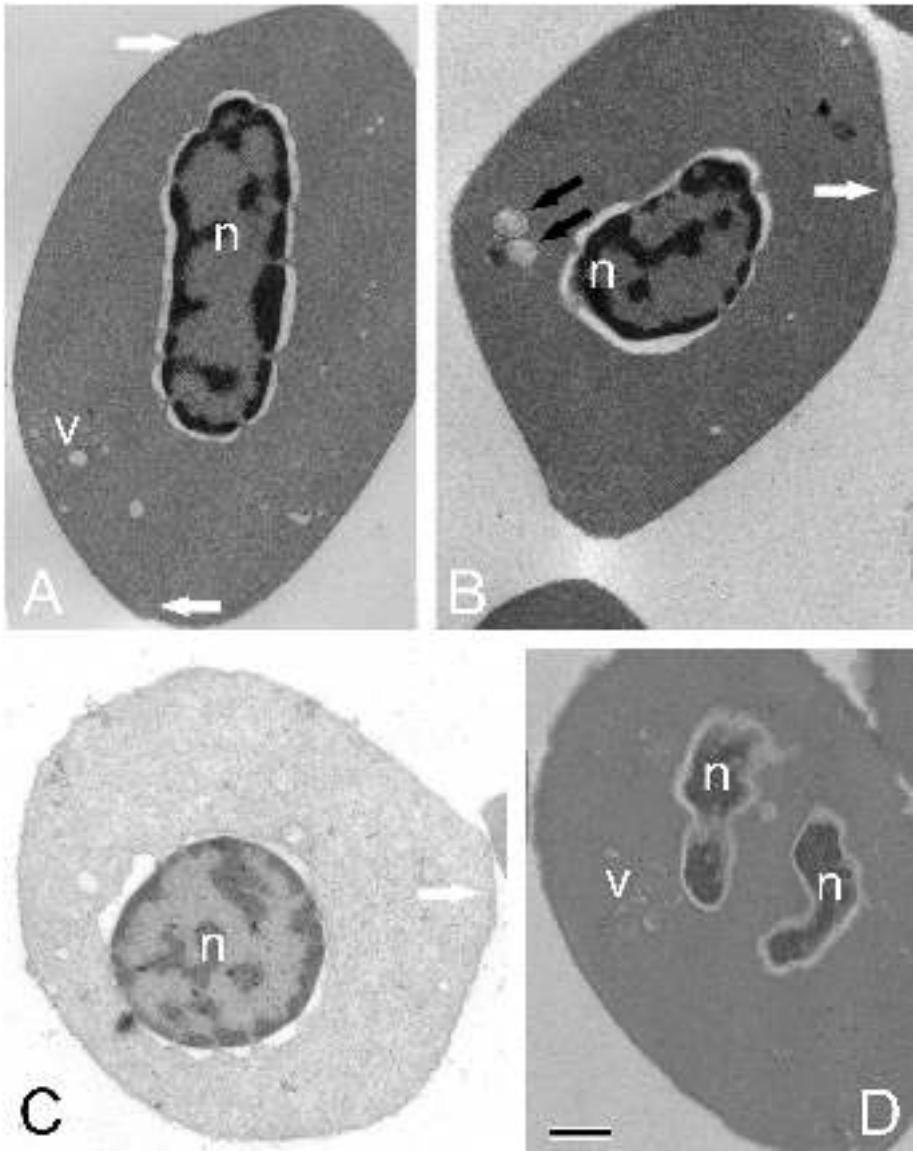

Fig. 1. Transmission electron microscopy of *T. bernacchii* blood pellet sections embedded in Embed812-Araldite. (A and B) Erythrocytes from "environmental control" fish. Note the bundle of microtubules running just beneath the plasma membrane (white arrows) and the multivesicular bodies (black arrows). (C) Immature erythrocyte from "environmental control" fish. (D) Erythrocyte from specimen stabled for 7 days showing a bilobed nucleus in section. n: nucleus; v: electron lucent vesicles. Scale bar 1 μm.

of new cells from the spleen (reserve) and head kidney (erythropoiesis) in response to hostile environments leads to an increase in the percentage of circulating immature or newly mature red cells (Houston and Murad, 1991). Changes in the relative abundance of the different stages of development are reflected in changes of morphological characteristics. The analysis of erythrocytes morphology can therefore be used as a more sensitive stress indicator than quantitative parameters (Spasić et al., 2020). However, it is difficult to recognize the exact stage of development of an erythrocyte (Spasić et al., 2020). Their maturation is a continuum without clear and evident distinctions between consequential development phases. In general, high percentages of large, rounded cells in a blood smear are the result of an abnormal increase in erythropoiesis, and may thus indicate a stressful condition (Rahman and Baek, 2019). Moreover, in the specific case of thermal stress, homeoviscosal adaptation systems can intervene by regulating the fatty acid composition of the membranes (Islam et al., 2019). Depending on how a certain organism responds, cellular abnormalities can occur such as: anisocytosis, echinocytosis, hemolysis, hypochromasia, poikilocytosis (e.g. elongated, teardrop-shaped or dracocytosis, sickle-shaped, contracted cells), wrinkling and other cell deformation (Islam et al., 2019; Witeska, 2013). Nuclear abnormalities are also widely used as biomarker with rapid response and long persistence to study toxic agents and thermal stress. Increases in temperature can induce DNA damage through the release of DNase from lysosomes and the denaturation or thermal inactivation of enzymes involved in DNA repair (Zafalon-Silva et al., 2017). Nucleus deformation involves crucial elements of the cell such as the endoplasmic reticulum, nuclear lamina, chromosomal organization and gene expression. Erythrocytes nuclear abnormalities include micronuclei, nuclear invaginations, binuclei, notched nuclei, blebbed nuclei, chromatin alteration and other. These alterations are an effective tool for detecting early signs of physiological stress, providing complementary information to studies on the erythrocytic composition through morphological characterization (Ashaf-Ud-Doulah et al., 2019; Braham et al., 2017; Zafalon-Silva et al., 2017). The specimens studied in this work belong to *Trematomus bernacchii*, a small demersal notothen with benthic lifestyle and common representative of shallow-water fish communities in the High Antarctic coasts, particularly in Terra Nova Bay (Ross Sea). Its wide geographical distribution, the extent of its trophic niche, its importance as prey for Adélie penguins (*Pygoscelis adeliae*) and occasionally for Weddell seals (*Leptonychotes weddellii*), and its stenothermy have placed *T. bernacchii* in the spotlight of Antarctic research, especially with regard to global warming and anthropogenic impact (Parkinson, 2019). It is therefore the subject of several physiological, ecological and genomic studies and is also used as bioindicator

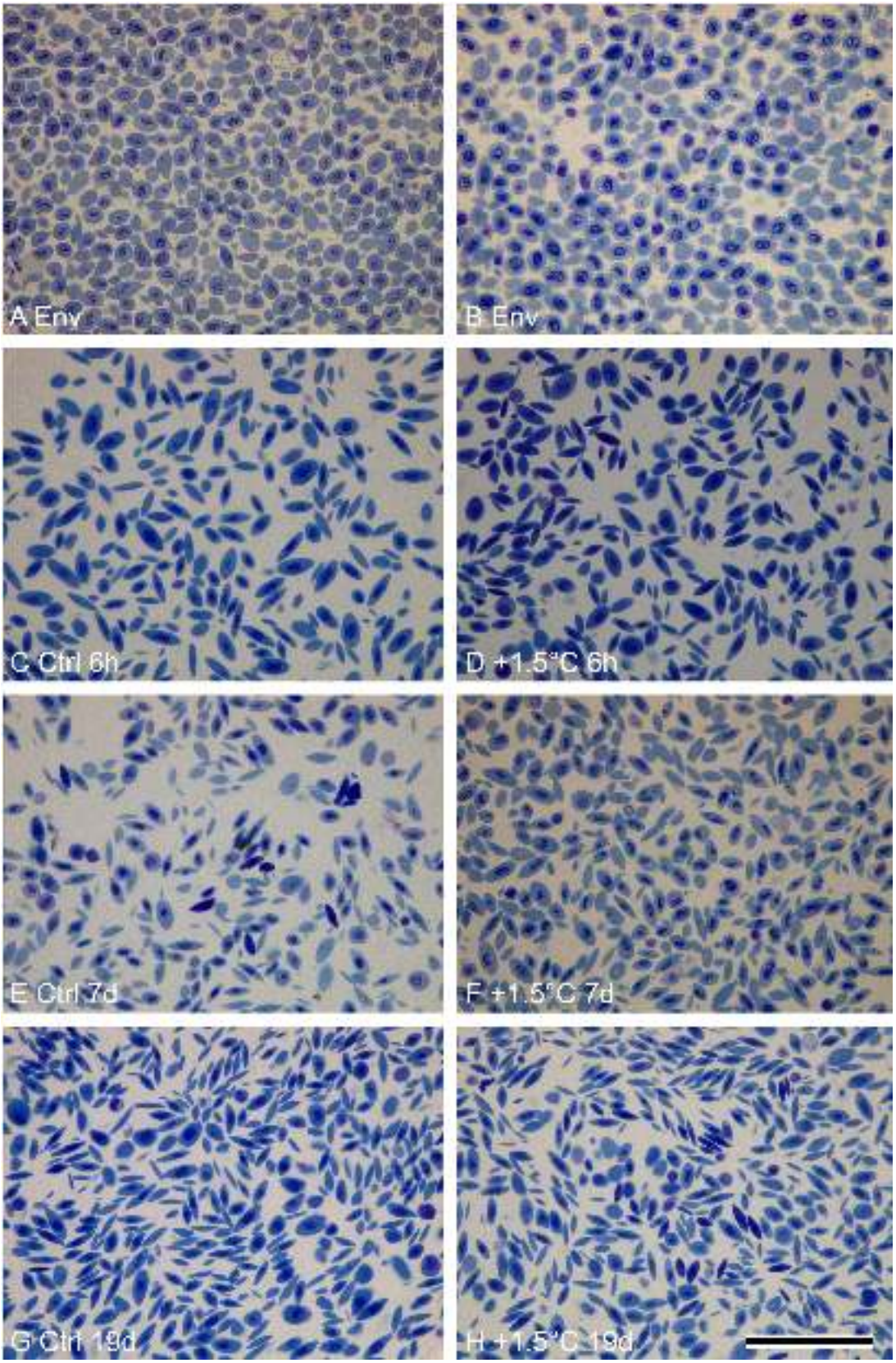

Fig. 2. Light microscopy of semithin sections of *T. bernacchii* blood pellets embedded in Embed812-Araldite from "environmental control" fish (A and B), after 6 h (C and D), 7 d (E and F) and 19 d (G and H)from the beginning of the thermal treatment. In C, E and G the sections from control fish and in D, F and H the section from heat stresses fish are shown. Scale bar 50 μm.

Table 1
Mean values of erythrocytes' area, circularity and roundness during the experiment. The data are shown for sections from pellet embedded in Embed 812-Araldite and in LR White.

		Time 0		6 h		7 days		19 days	
	Environmental	Control	+1.5 °C	Control	+1.5 °C	Control	+1.5 °C	Control	+1.5 °C
Area									
Embed812-Araldite	48.89 ± 5.08	44.57 ± 7.44	47.33 ± 2.55	37.75 ± 5.94	36.62 ± 4.79	38.54 ± 12.13	37.00 ± 6.38	37.37 ± 6.42	31.58 ± 4.49
LR White	73.23 ± 9.31	66.43 ± 10.99	71.09 ± 13.25	53.71 ± 2.16	56.99 ± 9.94	65.43 ± 16.46	54.91 ± 16.34	63.41 ± 16.54	53.48 ± 16.47
Circularity									
Embed812-Araldite	0.911 ± 0.018	0.810 ± 0.034	0.820 ± 0.030	0.752 ± 0.023	0.732 ± 0.014	0.732 ± 0.065	0.691 ± .050	0.672 ± 0.079	0.626 ± 0.051
LR White	0.902 ± .0.013	0.079 ± 0.039	0.800 ± 0.027	0.746 ± 0.039	0.715 ± 0.012	0.706 ± 0.034	0.682 ± 0.049	0.681 ± 0.056	0.644 ± 0.029
Roundness									
Embed812-Araldite	0.738 ± 0.044	0.055 ± 0.044	0.566 ± 0.037	0.473 ± 0.020	0.457 ± 0.016	0.463 ± 0.060	0.414 ± 0.046	0.402 ± 0.072	0.356 ± 0.040
LR White	0.727 ± 0.029	0.538 ± 0.057	0.543 ± 0.034	0.471 ± 0.034	0.440 ± 0.019	0.445 ± 0.031	0.415 ± 0.050	0.412 ± 0.056	0.376 ± 0.029

(Cincinelli et al., 2016; Ghosh et al., 2013; Huth and Place, 2013, 2016a; La Mesa et al., 2004a). This study presents the impact of a medium-term exposure to a temperature increase of 1.5 °C on the morphology of erythrocytes of *T. bernacchii*. This kind of study is pivotal in understanding Antarctic ecosystems responses to climate changes, especially considering that coastal communities will likely suffer the greatest negative consequences of the climate change (Rogers et al., 2020).

2. Materials and methods

2.1. Animal collection

The specimens were sampled in the antarctic summer of 2017 during the 33rd Italian Antarctic Expedition (Programma Nazionale di Ricerche in Antartide, PNRA). Forty-five *T. bernacchii* (255 ± 28 mm of total length) were sampled in the first week of November 2017 in the Terra Nova Bay near the Italian Station Mario Zucchelli (74.694206°S, 164.113869°E). The fish were collected by fish traps placed between 20 and 25 m deep, primed with frozen Argentine red shrimps.

2.2. Heat stress

Five fish were immediately transferred to the base in an aerated bin and lethally anesthetized with 1 mg × mL^{-1} tricaine methanesulfonate (Sigma, Saint Louis, MO, USA) to be used as environmental control. Forty fish were taken to the Mario Zucchelli Station aquarium facility and acclimated for 1 week at −0.9 °C in a fiberglass tank of about 1500 L of capacity. During the experiment, 20 fish used as control group and 20 fish for thermal challenge were placed in 2 glass aquaria of 180 L (100 × 40 × 45 cm) at −0.9 °C and +0.6 °C, respectively. Temperatures were measured every 15 min with a Tinytag Aquatic 2 data logger placed in each aquarium tank. Five specimens from each tank were sacrificed at the beginning of the experiment (time 0), and then after 6 h, 7 days and 19 days. Fish were photographed to measure their length later on. During the experiment the fish were fed once a week with pieces of Antarctic scallop (*Adamussium colbecki*).

2.3. Light and electron microscopy

About 200 μL of blood were withdrawn from the heart of each specimen and fixed in 0.5 mL of 1% paraformaldehyde, 2.5% glutaraldehyde in 0.1 mol L^{-1} phosphate buffer saline, pH 7.4 (PGB). The blood was immediately centrifuged at 890×*g* for 1 min with Costar Mini centrifuge. After 4 h the supernatant was discarded and 1 mL of fresh PGB was added. The samples were stored at 4 °C and sent to the Department of Life Sciences at the University of Trieste for subsequent analysis. The blood pellets were then post-fixed in 1% osmium tetroxide in the same phosphate buffer saline, dehydrated in ethanol (50%, 70%, 95% and absolute) and embedded in LR White (Sigma-Aldrich) or via propylene oxide, in Embed 812-Araldite (Electron Microscopy Sciences). A Pabisch TOP Ultra 150 was used to cut semi-thin resin sections (1 μm) which were stained with toluidine blue and examined with an Olympus BX50; images were acquired with an Olympus PEN E-P1 camera (Olympus Corporation, Shinjuku, Tokyo, Japan) at 100× magnification. For transmission electron microscopy, ultra-thin sections (120 nm) were stained with uranyl acetate and lead citrate, and examined with a Philips EM 208 electron microscope at 100 kV; images were acquired with a Quemesa bottom-mounted TEM CCD Camera (Olympus, Germany) provided with an iTEM imaging platform and saved in TIF format.

2.4. Image analysis

Erythrocytes' measurements were made from 1 μm semi-thin transverse sections of the full pellet thickness stained with toluidine blue. A total of 220–650 cells were analysed from at least two slides per pellet for each embedding resin. The images of the hematological samples obtained were uploaded, scaled and measured on ImageJ (Rasband, 2018). Erythrocytes were randomly sampled in each picture with the "Polygon Selection" tool. Area, circularity and roundness (found in "Shape descriptors") of each cell were calculated and used as indicators of the morphological alteration of erythrocytes (Houston and Murad, 1991). The morphology of the nuclei was investigated only by pictures of pellets embedded in Embed812-Araldite, as the staining in this resin showed a greater contrast between nucleus and cytoplasm. Area, circularity, roundness and solidity (found in "Shape descriptors") of each nucleus were calculated and used as measures of nuclear abnormalities. In particular, "solidity" calculates the convexity of the selected shape.

2.5. Statistical analysis

Statistical analyses were performed using R version 4.0.0 software (R Core Team, 2020). The values of erythrocytes' area, circularity and roundness were organized in a dataset counting approximately 30,600 observations. The means of each of the three measured variables were calculated for each fish and were aggregated in a new dataset. To assess differences in the response variables (area, circularity and roundness) during the experiment, the analysis of covariance (ANCOVA) with time as continuous explanatory variable and temperature as categorial explanatory variable was performed. Complete models comprising the response variable together with the continuous variable and the categorial variable were performed. Final simplified models were obtained

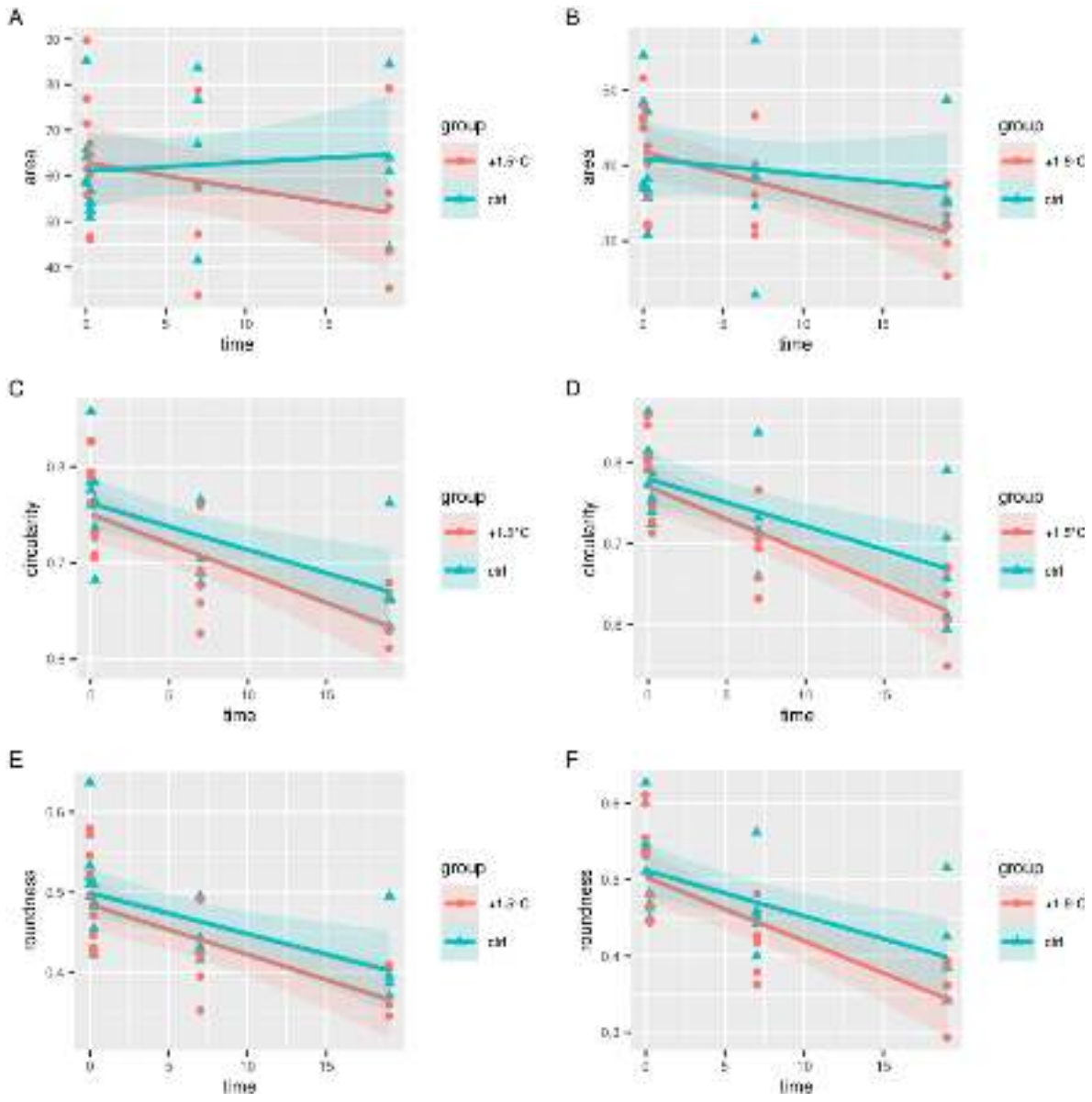

Fig. 3. Plot of means and linear models of measured area (A and B) and shape descriptors (C to F) of the erythrocytes of control fish (light blue) and heat stressed ones (light red). A, C, and E are values measured from pellets embedded in Embed812-Araldite whilst B, D and F from pellets embedded in LR-White. 95% confidence intervals of the linear models are shown in the same color as the lines.

by backward elimination of non-significant interaction term. The model simplification was assessed using the anova () function and it was justified for a non-significant reduction of the explanatory power ($p > 0.05$). Data were checked for normality (Shapiro test) and homoscedasticity (Levene test) prior to analysis for each response variable. The means obtained from the measurements of the same pellet embedded in the 2 resins (LR White and Embed 812-Araldite) were analysed separately in order to avoid pseudo-replicas. Data are shown as mean ± standard deviation.

3. Results

3.1. Erythrocytes' ultrastructure

Sections, obtained from the environmental controls prior to acclimation in tanks, show erythrocytes with an oval profile with a maximum diameter of about 10.5 μm and a minimum one of about 7 μm (Fig. 1A). The cytoplasm shows a homogeneous finely granular appearance with a medium to high electron density. It is poor in organelles with the exception of: rare small elongated mitochondria, few electron lucent vesicles, multivesicular bodies and numerous free ribosomes (Fig. 1A and B). Bundles of microtubules under the membrane are often visible in cross section at the opposite poles of the cell (Fig. 1A) and in the tangential ones (Fig. 1B), they form the so-called marginal microtubular ring important for erythrocyte morphology. The nucleoplasm is finely dispersed with some chromatin lumps mainly located beneath the nuclear envelope. Rare immature erythrocytes, with a round profile and a homogeneous but less packed and electron-dense cytoplasm are also observed (Fig. 1C). Sections, obtained from the fish during the experiment, show erythrocytes with indented and bilobed nuclei (Fig. 1D). These anomalies were quantitatively analysed in section 3.3 with the solidity parameter (Fig. 4D).

3.2. Erythrocyte's size and shape

Semi-thin sections of blood pellets stained with toluidine blue show a

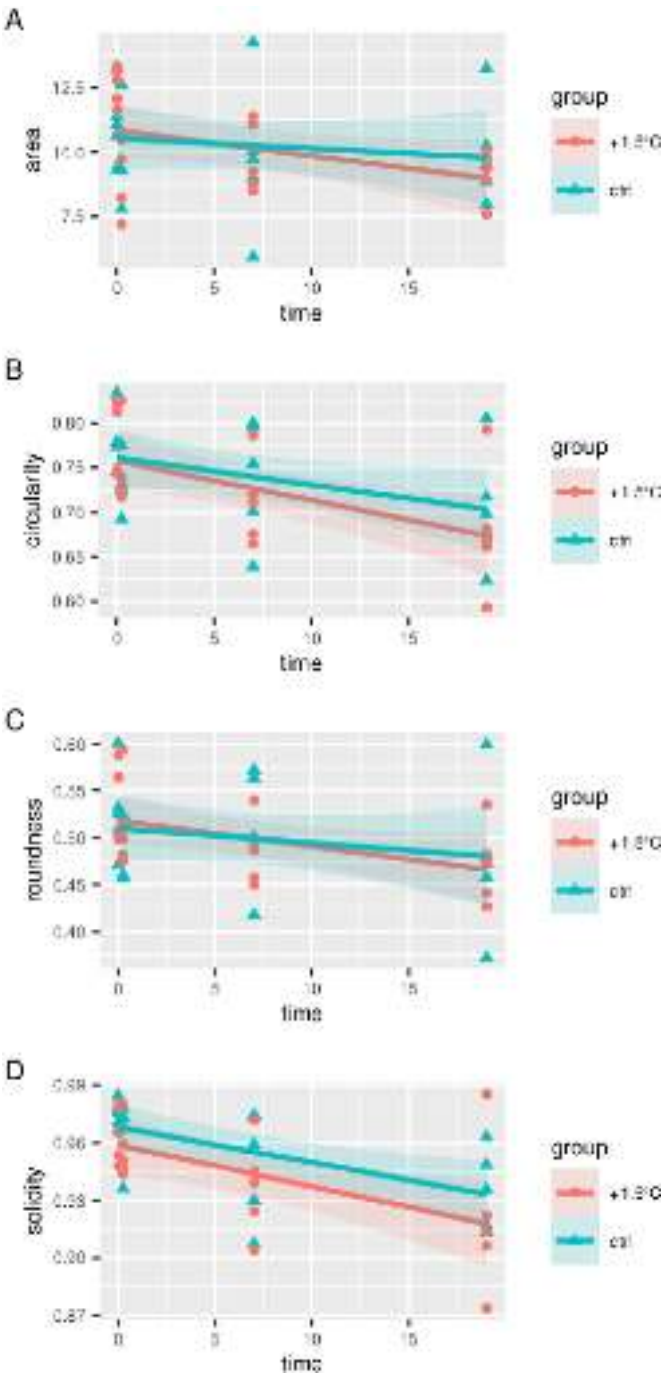

Fig. 4. Plot of means and linear models of measured area (A) and shape descriptors (B to D) of the erythrocytes' nuclei of control fish (light blue) and heat stressed ones (light red) from pellets embedded in Embed812-Araldite. 95% confidence intervals of the linear models are shown in the same color as the lines.

progressive change in the erythrocyte profile during the course of the experiment (Fig. 2). In environmental controls, the majority of erythrocytes present a circular to slightly oval profile and already after a week of acclimation in the large tanks of the aquarium facility, it is possible to observe the presence of erythrocytes that have an elongated profile in section, whose frequency increases over time in both the control and experimental groups with 1.5 °C raised temperature (Fig. 2).

The mean values of the area, circularity and roundness of the erythrocytes in section during the experiment are shown in Table 1. For all the shape descriptors, a decrease in values is observed during acclimation in the aquarium facility and this trend continues throughout the experiment (Table 1, Fig. 3).

The effect of time on the reduction of erythrocyte area is significant for the pellets embedded in Embed 812-Araldite ($p < 0.05$, Fig. 3B) but not for those embedded in LR White (Fig. 3A). No significant difference is recorded between the control group and the one with 1.5 °C increased temperature. For circularity and roundness, a highly significant effect of time is recorded for both resins (Fig. 3C–F, $p < 0.001$) but, even in this case, although the mean values of the fish at the highest temperature are always lower than the control ones, there are no significant differences between the 2 groups.

3.3. Nuclear size and shape

The mean values of the area, circularity, roundness and solidity of the erythrocyte's nuclei in section during the experiment are shown in Table 2.

All the measured parameters, area and shape descriptors, showed a decreasing trend with increasing stabling time. No significant difference is recorded between the control group and the one with 1.5 °C increased temperature for all the measured parameters. The area of erythrocyte's nuclei did not show a significant effect of time during the experiment whilst for circularity ($p < 0.01$), roundness ($p < 0.05$) and for solidity ($p < 0.001$) a significant or highly significant effect of the time during the experiment is recorded (Fig. 4A–D).

4. Discussion

The analyses of erythrocyte's morphology in sections showed no significant differences between the two experimental conditions and yielded similar results for control fish stabled at −0.9 °C and fish challenged at + 0.6 °C. On the contrary, significant or highly significant differences were observed in the time course from the beginning of the experiment to its conclusion. The values of the measured parameters also showed the same decreasing tendency during the 7-day acclimatisation, whereby the erythrocytes of the "environmental control" fish were larger than those of the experimental fish (Tables 1 and 2, "environmental" values versus "time 0"). These findings suggest the influence of the rearing system and stressors on erythrocyte morphology rather than a temperature effect.

For erythrocyte's parameters the two embedding resins showed different absolute values, but the trend is perfectly comparable (Fig. 3). The progressive loss of the area, circularity and roundness of erythrocyte's profile in section exclude an increase in erythropoiesis as a response to the stress suffered by the animals.

Table 2
Mean values area, circularity, roundness and solidity of erythrocytes' nuclei during the experiment. The data are shown for sections from pellet embedded in Embed 812-Araldite.

		Time 0		6 h		7 days		19 days	
	Environmental	Control	+1.5 °C	Control	+1.5 °C	Control	+1.5 °C	Control	+1.5 °C
Area	12.71 ± 2.21	11.14 ± 1.43	12.60 ± 0.71	10.21 ± 1.79	9.25 ± 1.48	9.79 ± 2.97	9.79 ± 1.34	9.37 ± 0.94	9.73 ± 2.11
Circularity	0.856 ± 0.023	0.783 ± 0.032	0.775 ± 0.040	0.740 ± 0.036	0.749 ± 0.044	0.738 ± 0.068	0.712 ± 0.0478	0.654 ± 0.048	0.730 ± 0.065
Roundness	0.640 ± 0.042	0.527 ± 0.047	0.532 ± 0.042	0.484 ± 0.036	0.512 ± 0.048	0.511 ± 0.062	0.485 ± 0.035	0.445 ± 0.046	0.503 ± 0.064
Solidity	0.983 ± 0.007	0.975 ± 0.007	0.965 ± 0.014	0.965 ± 0.016	0.956 ± 0.015	0.949 ± 0.029	0.937 ± 0.025	0.912 ± 0.027	0.944 ± 0.030

Juvenile erythrocytes are characterized by a more rounded appearance with larger nuclei (Clauss et al., 2008). The increase in erythropoiesis should therefore have led to an opposite trend to that observed (Houston and Murad, 1991). The increased number of erythrocytes with more elongated profile in section, suggests an acceleration of the maturation and the aging of this circulating cells. The latter responses were observed in teleost fish underwent thermal stress (Shahjahan et al., 2018). However, also oxidative stress could cause aging and deformation of erythrocytes (Farag and Alagawany, 2018). Reactive oxygen species that damage cellular components at the molecular level, could change the lipid composition of the plasma membrane and affect membrane protein constituents and cytoskeleton.

The polymerization of microtubules is a rapid process that affects all newly formed cells. Clearly, the morphology of a cell is a direct consequence of the assembly of its cytoskeletal components including microtubules, contractile networks of actin filaments, intermediate filaments and other mechanical elements. For this reason, any damage to the microtubules of the marginal band can cause significant alterations and deformations such as teardrop-shaped erythrocytes (Joseph-Silverstein and Cohen, 1984). In mammals, the shape of erythrocytes depends mainly by two components: the marginal band (a ring of microtubules) and a protein cortex at the cell periphery (Dmitrieff et al., 2017). A recent study indicated that the marginal band destabilization could be due to ring extension (Diagouraga et al., 2014).

It is worth to note that the changed morphology of blood cells was observed without any mortality of fish, indicating that the synergic stresses provided (stabling and heat stress) were sub-lethal to *T. bernacchii* for at least 20 days.

The analysis of the morphology of erythrocyte's nuclei confirmed that there were no differences between the control and the thermal-challenged groups but, again, significant or highly significant differences were observed temporally from the beginning of the experiment to its conclusion (Fig. 4).

The difficulty of dealing with biases and sources of disturbance due to the experimental setting during the investigation with such sensitive organisms highlights the need to develop new approaches and methodologies suitable for ecophysiological studies (Peck, 2002). Complementary information can be provided by 'omics technologies' (Peck, 2018). The study of the genome and the transcriptomes can reveal more in depth on the molecular basis of adaptation to extreme marine environments. Interestingly, preliminary gene expression analyses carried out on samples from the gills of *T. bernacchii*, obtained from the same specimens of the present study, resulted in the consistent down-regulation dependent from stabling time of factors related to: cytoskeleton assembly, cell differentiation, glycogen biosynthetic processes and lipid transporter activity. The expression profiles do not change linearly with time, suggesting that different gene clusters respond to different stabling time, which in the end may not be the only disturbance, as more uncontrolled factors may come in play (i.e. tank density, fish interactions) (S. Greco, personal communication, April 2, 2021). Further 'omics' analyses can also reveal the mechanisms involved in adaptation to changes in temperature, how these have evolved in the past and what responses may emerge in the future (Turner et al., 2009).

It remains to be investigated why organs such as the spleen were not able to replace the pathological form of the erythrocytes, which lasted for almost 3 weeks, with young erythrocytes and what consequences this has for the blood flow. Also, additional analysis should be addressed to the long-term size distribution of erythrocytes to know the limit of compensation and the physiological effects of this condition for *T. bernacchii*.

The results of the present study confirm that hematological data, even in pellet sections, are extremely sensitive biomarkers. Paradoxically, in Antarctic stenothermic fish, which live in extremely stable conditions in a pristine environment with very low anthropogenic pressure, the stabling effect inside the aquarium facilities appears to significantly obscure the effects of the experimental heat treatment.

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Institutional review board statement

The sample collection and animal research conducted in this study comply with Italian Ministry of Education, University and Research regulations concerning activities and environmental protection in Antarctica and with the Protocol on Environmental Protection to the 137 Antarctic Treaty, Annex II, Art. 3. All the activities on animals performed during the Italian Antarctic Expedition were under the control of a PNRA Ethics Referent, which acts on behalf of the Italian Ministry of Foreign Affairs. In particular, the required data for the project identification code PNRA16_00099 are as follows. Name of the ethics committee or institutional review board: Italian Ministry of Foreign Affairs. Name of PNRA Ethics Referent: Dr. Carla Ubaldi, ENEA Antarctica, Technical Unit (UTA).

Authors contribution

Piero G. Giulianini and **Gianfranco Santovito**: Conceptualization, Methodology, Software **Damiano Rizzotti, Chiara Manfrin** and **Samuele Greco**: Data curation, Writing- Original draft preparation. **Piero G. Giulianini** and **Marco Gerdol**: Visualization, Investigation. **Piero G. Giulianini**: Supervision.: **Damiano Rizzotti**: Software, Validation.: **Chiara Manfrin** and **Piero G. Giulianini**: Writing- Reviewing and Editing.

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