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RESEARCH ARTICLE

Aerobic scope does matter in the temperature–size rule, but only under optimal conditions

Aleksandra Walczyńska* and Mateusz Sobczyk

ABSTRACT

We united theoretical predictions of the factors responsible for the evolutionary significance of the temperature–size rule (TSR). We assumed that (i) the TSR is a response to temperature-dependent oxic conditions, (ii) body size decrease is a consequence of cell shrinkage in response to hypoxia, (iii) this response enables organisms to maintain a wide scope for aerobic performance, and (iv) it prevents a decrease in fitness. We examined three clones of the rotifer *Lecane inermis* exposed to three experimental regimes: mild hypoxia, severe hypoxia driven by too high of a temperature, and severe hypoxia driven by an inadequate oxygen concentration. We compared the following traits in normoxia- and hypoxia-exposed rotifers: nuclear size (a proxy for cell size), body size, specific dynamic action (SDA, a proxy of aerobic metabolism) and two fitness measures, the population growth rate and eggs/female ratio. The results showed that (i) under mildly hypoxic conditions, our causative reasoning was correct, except that one of the clones decreased in body size without a decrease in nuclear size, and (ii) in more stressful environments, rotifers exhibited clone- and condition-specific responses, which were equally successful in terms of fitness levels. Our results indicate the importance of the testing conditions. The important conclusions were that (i) a body size decrease at higher temperatures enabled the maintenance of a wide aerobic scope under clone-specific, thermally optimal conditions, and (ii) this response was not the only option to prevent fitness reduction under hypoxia.

KEY WORDS: Aerobic scope, Body size, Fitness, Rotifers, SDA, Temperature–size rule

INTRODUCTION

A decrease in phenotypic body size with increasing temperatures (the temperature–size rule, TSR; Atkinson, 1994) has created a major puzzle for evolutionary ecologists (Berrigan and Charnov, 1994) because of its counterintuitive pattern. The reason for this confusion is that according to the theory of optimal energy allocation, under advantageous conditions, organisms should grow faster and to larger sizes at maturity to maximize their own fitness, whereas those growing in harsh conditions are expected to grow slower, with shorter development to smaller sizes to avoid postponing maturity because of the risk of premature death. However, when temperature is the examined factor, a crossing of growth trajectories is observed, with organisms growing faster but to smaller sizes in hot conditions and growing slower, longer and to larger sizes in cold conditions (Arendt, 2007).

Different hypotheses regarding why the size response to temperature contradicts the general pattern have been proposed. Among them, the most promising is the hypothesis of an adaptive size response under oxygen deficiency (but see also Audzijonyte et al., 2019). The relative oxygen concentration decreases with increasing temperatures (see Wetzel, 2001 for aquatic systems); thus, a steeper increase in the oxygen demand compared with the oxygen supply causes oxic stress in organisms (Atkinson et al., 2006; Verberk et al., 2011; Woods, 1999). It is important to distinguish between physiological hypoxia, resulting from an elevated metabolic rate, and environmental hypoxia, when the environmental oxygen concentration is not sufficient to meet the current metabolic demands (Somero et al., 2017). In any case, a decrease in cell size has been suggested to increase the efficiency of oxygen transport to the mitochondria (Woods, 1999), and as a consequence, the body size also shrinks (Verberk et al., 2021).

The adaptive hypothesis proposed that a size decrease under hypoxia prevents narrowing of the organismal ability to undergo flexible aerobic performance. Temperature defines the window of the aerobic scope, namely, the range of aerobic metabolism that can be performed under given conditions, and maintaining flexible efficiency in aerobic respiration prevents a possible decrease in fitness (Poertner, 2010). This mechanism was linked to body size adjustment relative to temperature by Atkinson et al. (2006).

Existing studies have indirectly or directly confirmed the oxygen limitation of body size on an evolutionary scale. Low oxygen availability causes smaller body sizes in insects (Harrison et al., 2010; Verberk and Atkinson, 2013) and ectothermic vertebrates (Rollinson and Rowe, 2018; Santilli and Rollinson, 2018). Additionally, on an intraspecific scale, organisms such as flies (Frazier et al., 2001), crustaceans (Hoefnagel and Verberk, 2015) and rotifers (Czarnoleski et al., 2015) are reported to grow smaller under lower oxic availability. However, results directly relating this pattern to organismal fitness to enable conclusions on the possible evolutionary meaning of smaller sizes at higher temperatures (Arendt, 2011) are scarce. Prokosch et al. (2019) found that lighter wagtails survived better at higher temperatures, and heavier individuals survived better at lower temperatures, and Walczyńska et al. (2015a) showed that smaller rotifers were more fecund than larger rotifers under the combination of high temperature and low oxygen availability.

In this study, we linked the hypothesized proximate and ultimate mechanisms underlying the phenotypically driven size decrease with increasing temperatures in an aquatic organism, the rotifer *Lecane inermis*. We aimed to thoroughly test the theoretical reasoning behind hypoxia driving body size shrinkage to prevent a reduction in fitness, as presented in the literature. To achieve this, we compared the examined traits between rotifers exposed to normoxia and those exposed to hypoxia in three experimental stages differing

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in thermo-oxic conditions. The TSR has been empirically confirmed to be condition-sensitive and followed only within an optimal thermal range (Walczyńska et al., 2016). This prompted us to compare the responses to optimal (temperature of 30°C) versus suboptimal conditions in our study. We further divided the non-optimal conditions into two hypoxia treatments: a stress-inducing high temperature (38°C) and a stress-inducing low oxygen level (20% of the relative O_2 concentration). This distinction between mild versus harsh conditions enabled inference of the possible thresholds for TSR performance. Additionally, our aim was to understand the evolutionary meaning of the response and therefore the results were referred to fitness.

We examined the aerobic scope, which was our hypothesized ultimate factor, by estimating the specific dynamic action (SDA). SDA measures increasing metabolic expenditures in animals and is associated with the ingestion, digestion, assimilation and absorption of food (McCue, 2006; Secor, 2009), whereas increasing oxygen consumption is mostly associated with the biochemical transformation of food and the synthesis of new proteins (Jordan and Steffensen, 2007). SDA was estimated as the difference in respiration rates between fed and hungry individuals that could not be maintained at rest.

The hypothesized link between the aerobic scope and SDA is that the higher the oxygen consumption associated with the SDA is, the wider the capacity for aerobic metabolism (aerobic scope) (Auer et al., 2015; Jutfelt et al., 2020). This assumption was previously used for *Daphnia magna* (Chopelet et al., 2008). Our approach was to compare oxygen consumption in hypoxia between animals previously exposed to normoxic and hypoxic conditions. We expected that hypoxia would lower the SDA amplitude in hypoxia-naïve animals compared with hypoxia-experienced animals. This hypothesis was based on Fry (1947), who wrote that dissolved oxygen acts as a limiting factor for the metabolic rate; thus, its reduction decreases the aerobic scope and, consequently, the energy budget of aquatic breathers. Such an arresting effect of hypoxia on the SDA width was previously reported for cod fish (Jordan and Steffensen, 2007) and carabid beetles (Gudowska and Bauchinger, 2018).

We hypothesized that hypoxia leads to small cell sizes, followed by small body sizes, to maintain high effectiveness in aerobic metabolism and maximize fitness (Fig. 1A). According to empirical results showing that the TSR is restricted to optimal conditions (Walczyńska et al., 2016), we expected the abovementioned pattern to differ across hypoxia treatments, with a more direct response under mild hypoxia. Therefore, our main goal was to compare all examined traits in rotifers exposed to normoxia and those exposed to hypoxia across three experimental treatments, predicting that the causal relationships tested would be valid only or mainly under mildly hypoxic conditions.

We used three clones of the rotifer *L. inermis* as replicates. This species has previously been found to follow the TSR in the laboratory and in the field (Kiełbasa et al., 2014), to show adaptive body size responses to thermally driven oxygen conditions based on cell size adjustment (Walczyńska et al., 2015a), and to perform simple two-point TSR control within its life cycle via the mother and during egg development (Walczyńska et al., 2015b). It is important to mention that rotifers are eutelic, meaning that their constant number of somatic cells forces them to adjust to thermally mediated oxygen levels only through cell size manipulation.

In addition to conducting the study under optimal and stressful conditions, we also examined possible differences in the responses of animals that preferred different thermal conditions: cold, intermediate and warm. This distinction addresses an issue regarding the possible linkage between thermal preferences and thermally induced body size adjustment. To our knowledge, this is the first attempt to comprehensively study the proximate and ultimate mechanisms behind the TSR.

MATERIALS AND METHODS

Clones studied

The rotifer species *Lecane inermis* (Bryce 1892) has previously been studied for different aspects of the size response to thermal and oxic conditions. Its thermal performance curve was also examined, and the optimal temperature was estimated as 30°C (Walczyńska et al., 2016). This species is very convenient for studies on changes in phenotypic responses between maternal and offspring generations because of its short generation time, which was previously estimated as 2 days at temperatures between 15°C and 25°C (Walczyńska et al., 2015b). We chose three *L. inermis* clones founded from one individual each, which were isolated from activated sludge from small wastewater treatment plants in southern Poland. The clones differed in their thermal preferences according to a previous study conducted using six clones (Stuczyńska et al., 2020 preprint). The three clones used are clones Cold, Int2 and Warm2 from the previous study and are herein named Cold for cold preference, Int for intermediate temperature preference and Warm for heat preference, respectively. When tested in the range from 8°C to 30°C (including supplementary data from the previous study), all three clones displayed relatively high population growth rates at 25–30°C, but the Cold and Warm clones were the best and poorest performers, respectively, at the lowest temperature. Preceding the experiment, all clones were kept in Petri dishes at 25°C in the dark in Żywiec spring water (Poland) and fed patented Novo powder (Pajdak-Stós et al., 2017).

Experimental setup

Our main goal was to compare the responses of rotifers exposed to normoxia versus hypoxia in a three-stage experiment under different thermo-oxic regimes, with different rotifers in each of the following conditions (Fig. 1B): mild hypoxia (MH), severe hypoxia at a temperature above the optimum (SHT) and severe hypoxia with a very low oxygen level (SHO2). Experimental conditions in the subsequent stages were set as follows: (i) MH treatment: optimal temperature of 30°C and differentiated oxic conditions between normoxia at 80% and hypoxia at 40% (relative O_2 concentration); (ii) SHT treatment: temperature elevated above the optimum (38°C, previously found to cause reduced but still positive population growth) (Walczyńska et al., 2016), with normoxia and hypoxia set to 80% and 40% O_2, respectively; and (iii) SHO2 treatment: optimal temperature of 30°C, with normoxia at 80% and hypoxia lowered to 20% of relative O_2 concentration (Fig. 1B).

The choice of 80% normoxia instead of 100% was because it is much easier to maintain stable 80% than 100% oxygen concentrations, and with this percentage, we ensured that the rotifers were not exposed to fluctuating oxygen conditions that could act as an uncontrolled factor. The relative experimental oxygen concentrations of 80%, 40% and 20% O_2 were equivalent to approximately 17%, 8% and 4% of the absolute oxygen concentration, respectively. As calculated by the equation for temperature-dependent oxygen solubility in water (Wetzel, 2001), 80% O_2 (relative) was 6.0 mg l^{-1} at 30°C (in stages MH and SHO2) and 5.3 mg l^{-1} at 38°C (in stage SHT); 40% O_2 was 3.0 mg l^{-1} at 30°C (in stage MH) and 2.6 mg l^{-1} at 38°C (in stage SHT); and 20% O_2 was 1.5 mg l^{-1} at 30°C (in stage SHO2). The 80% relative oxygen concentration was close to the optimal value for this species

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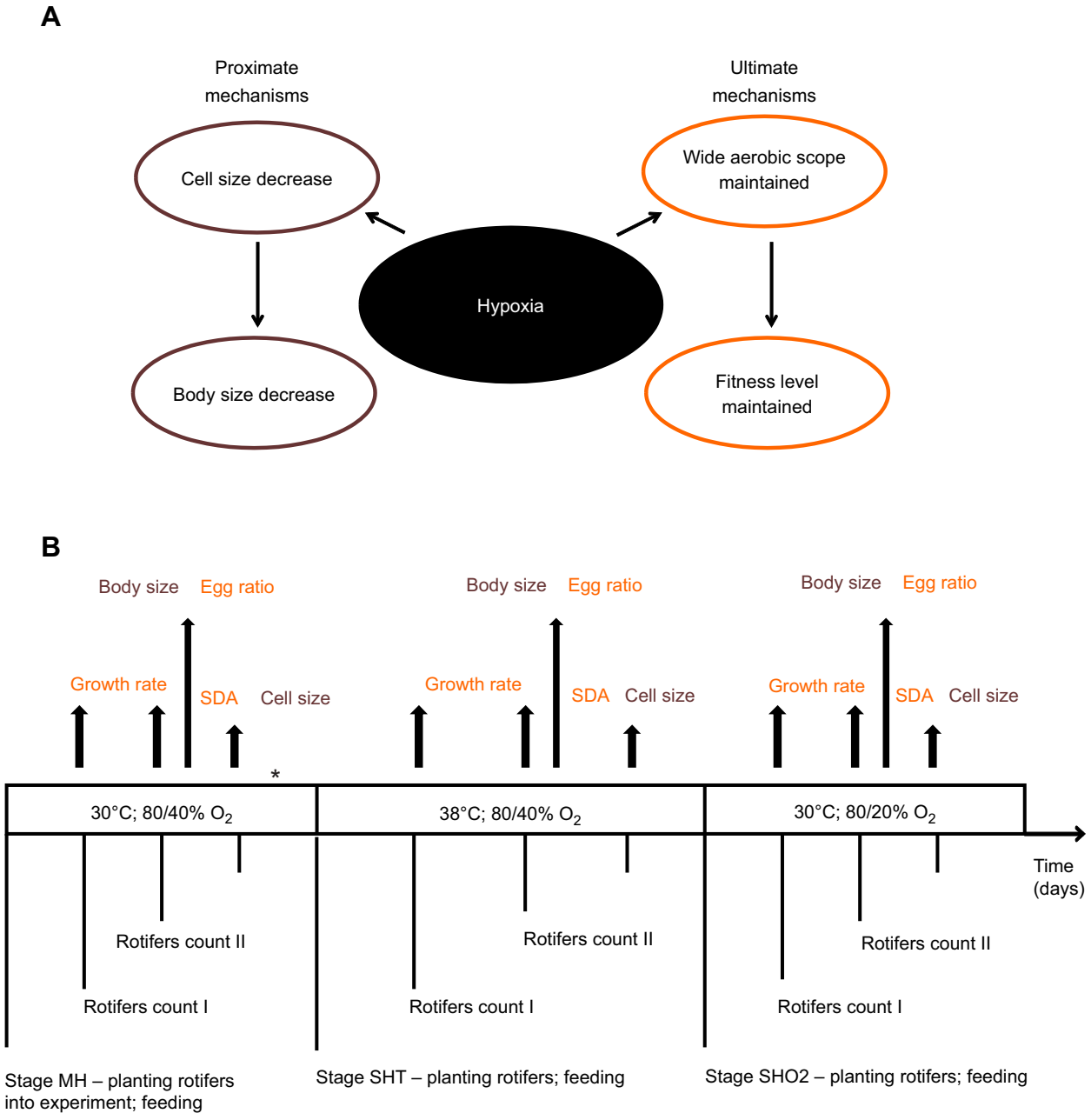

Fig. 1. Concept of the study. (A) Hypothetical effect of hypoxia on the traits estimated: hypoxia causes cell size and body size decreases (proximate mechanisms) to maintain a wide aerobic scope and prevent a possible fitness decline (ultimate mechanisms). (B) Experimental setup: the study was divided into three subsequent stages. In each stage, two simultaneous regimes differentiated by hypoxic conditions were conducted as indicated by the red slash, with both at the same temperature. Five traits were investigated within 3 days in each stage (description in Materials and Methods). SDA, specific dynamic action (a proxy for aerobic scope); MH stage, hypoxia type=mild hypoxia; SHT stage, hypoxia type=severe hypoxia with high temperature; SHO2 stage, hypoxia type=severe hypoxia with low oxygen concentration. *This stage was repeated after completion of the three stages to collect the lacking data on SDA for Cold clone.

living in nature (7.5 mg l^{-1} O_2) (Berzins and Pejler, 1989) and much higher than the minimal oxygen concentration under the activated sludge conditions (2 mg l^{-1} O_2) (Kiełbasa et al., 2014) from which all studied clones were established. The value of 2–3 mg l^{-1} O_2 was also provided as a standard threshold for hypoxia (reviewed in Hrycik et al., 2017). The experimental hypoxia conditions were slightly above (40%) and below (20%) this arbitrary threshold for hypoxia.

The experimental conditions were established simultaneously in four temperature-regulated water baths (Memmert, Germany) connected to a four-channel OxyReg O_2 regulation system (Loligo Systems, Denmark) calibrated to 0% and 100% air-saturated water. Stable hypoxia levels were maintained by adding pure (comestible) nitrogen to the baths. Two baths were set to normoxia and two were set to hypoxia, all with the same temperature. Six 100 ml cages made of 5 µm mesh enclosed in slide frames sealed with silicone were placed in each water bath (two cage replicates per clone in each bath; Fig. S1). This type of cage has been successfully used previously (Walczyńska et al., 2015a).

Each water bath was filled with Żywiec spring water as the medium. The initial densities of each of the three rotifer clones taken directly from the main culture at 25°C and added simultaneously at

the beginning of the experimental stages were 110, 120 and 250 individuals ml^{-1} for the MH, SHT and SHO2 stages, respectively. Our aim was to start with the highest possible number of rotifers that was even across the clones, which was dictated by the clone that was the least numerous at the moment of counting. These initial densities of rotifers were far below the population carrying capacity because *L. inermis* rotifers may reach densities as high as tens of thousands per milliliter (45,000 individuals ml^{-1} was reported by Walczyńska et al., 2017). We added the rotifers at the beginning of each stage in the experiment to avoid equipment calibration between the stages because after each stage, virtually no rotifers remained. Thus, each stage began with new rotifers taken from the 25°C stock in 4 day intervals.

The rotifers in each cage were fed 200 µl of the commercial bioproduct Bio-Trakt®, which has been successfully used in *L. inermis* mass culture previously (Fiałkowska et al., 2019). During or just after the 3 days of each experimental stage, we collected samples to estimate the physiological (SDA, as a proxy for the aerobic scope, and nucleus size, as a proxy for cell size) and life history (body size, population growth rate and eggs/female ratio) traits (Fig. 1B).

Oxygen consumption

The hypothesis regarding the decrease in size under hypoxic conditions to maintain a wide aerobic scope was tested by estimating the SDA. This physiological difference in respiration rates between fed and hungry animals was previously used as a proxy for aerobic scope in *Dapnia magna* (Chopelet et al., 2008). We estimated the SDA using spectrophotometry (Orion™ AquaMate 8000 UV-VIS, Thermo Fisher Scientific, USA) with a modified Winkler titration (Carpenter, 1965; Chopelet et al., 2008; Roland et al., 1999), which has been utilized as a highly accurate method for determining dissolved oxygen (Helm et al., 2012). In this method, the absorbance is proportional to the amount of oxygen dissolved in a sample owing to the chemical reaction chain. In each experimental stage, we sampled rotifers from the normoxia and hypoxia treatments and measured the aerobic scope of both groups when exposed to hypoxic conditions in the given stage. We expected that rotifers exposed to experimental hypoxia in the preceding 3 days would have wider SDAs in the hypoxia test than normoxia-exposed rotifers. Two clones, Cold and Warm, were examined for the SDA because the Int clone did not proliferate well enough to achieve reasonable sample sizes. On the third day of each experimental stage, we collected 10 ml samples from each cage and pooled the two replicates within the clone×oxic regime combination. For logistical reasons, the clones were analyzed separately but within the same day for the SHT stage and SHO2 stage. Because we did not analyze both clones after the MH stage, we repeated this stage at the end of the experiment to collect the missing SDA data for the Cold clone.

Each clone was divided into two 50 ml glass containers with a 5 ml rotifer sample and 15 ml of hypoxic medium taken directly from the corresponding water bath (outside the cages), representing the experimental hypoxic conditions. By this procedure, we diluted the nutritional particles sampled along with the rotifers. The rotifers in one glass container were then fed 10 µl of Bio-Trakt® per 1 ml of sample (treatment: fed). The rotifers in the second glass container were not fed (treatment: hungry). The random division of clone samples into the fed and hungry treatments caused the average rotifer body size to be similar in both regimes. Therefore, we could assume that the possible differences in oxygen consumption were not related to size differences.

Both samples were incubated for 2 h at the same temperature that the rotifers experienced during the respective experimental stage. The relatively short exposure to starving conditions was dictated by the fact that rotifers become starved relatively quickly and that severe starvation has a very profound effect on metabolism (reviewed by Galkovskaya, 1995). After this period, under both treatments, we removed the swimming, healthy-looking rotifers in groups of 30 and transferred them to the wells of 24-well tissue plates filled with a parafilm membrane, with four or five replicates (=rotifer bunches) per treatment. The rotifers from each well were then quantitatively transferred into individual 2 ml autosampler vials (Agilent Technologies, USA) containing hypoxic medium taken directly from one experimental water bath under hypoxic conditions. The vials (five replicates each for the fed and hungry treatments) were filled according to the following procedure: 1 ml of medium, the rotifer sample from a well, and another 1 ml of medium until a convex meniscus was achieved. The vials were then closed with dedicated caps previously punctured with a sterile needle to remove excess fluid and air. The control tubes were prepared following the same procedure but without the animals. The samples were incubated for another 2 h (38°C) or 3 h (30°C) in hypoxic conditions, with the level of hypoxia set to 40%, 40% and 20% O_2 for the experimental MH, SHT and SHO2 stages, respectively. The shorter incubation time at a higher temperature was dictated by the faster rates of all energetic processes.

After incubation, the Winkler chemicals were added following the procedure adopted from Carpenter (1965): 16 µl of $MnCl_2\,4H_2O$ (3 mol l^{-1}), 16 µl of NaI (4 mol l^{-1}) in NaOH (8 mol l^{-1}), and, after vigorous shaking to cause precipitation, 16 µl of H_2SO_4 (5 mol l^{-1}). The vials were then shaken to dissolve the precipitate, and 800 µl subsamples were added directly to spectrophotometric microcuvettes (Brand, Germany). The absorbance was measured at a wavelength of 440 nm within the first 10 min to prevent iodine evaporation (Roland et al., 1999). The sequence of measurements was as follows: sample number one from each of the five treatments (normoxic, hypoxic and fed or hungry rotifers plus control), sample number two, etc. The calibration curve for absorbance was estimated for 0%, 15%, 35% and 50% O_2 (relative), with five replicates per oxygen level. In the final estimation, the extreme values were excluded, and the average was calculated for the remaining three values per oxygen level. The calibration curve fitting was estimated at R^2=0.9953.

The control samples were separately checked for reliability for each combination of hypoxia type and clone. In all but two cases, the controls showed similar and reliable patterns. In the case of the MH treatment with the Warm clone, the control samples showed a pattern indicating a methodological error (Fig. S2), and only one value attained for the first sample was taken into consideration. In the SHT treatment with the Cold clone, one control sample had a very low value (Fig. S2), which made it unreliable. The final control value was calculated as the mean of the remaining two samples. The final data used in the formal statistical analyses were oxygen consumption (in %), as calculated from the calibration curve and subtracted from the control, which acted as the background. The control value was different for each combination of experimental stage and clone.

The SDA was estimated both as the difference in raw absorbance between the fed and hungry rotifers (absolute SDA) and as their division product (factorial SDA). The comparison of these two approaches for aerobic scope calculation was suggested by Clark et al. (2013).

The amount of O_2 consumed was converted from a percentage to mg l^{-1} using the equation describing the relationship between these two variables provided by the OxyReg system. The atmospheric pressure and temperature were manually set throughout the experiment to enable proper recalculation by the system. This was possible only at 30°C owing to the relatively wide range of O_2 (%; from approximately 19 to 95%), as the much shorter range at 38°C made this estimation less reliable. The equation for calculating the amount of consumed oxygen at 30°C was O_2 (mg l^{-1})=0.0752×O_2 (%).

Cell size

We measured the nucleus size, as a proxy for the cell size, according to the method developed and used previously for the same species (Walczyńska et al., 2015a). We adopted this procedure because (i) the nucleus size is a good proxy of the cell size at the interspecific level because a eukaryotic cell needs to maintain the optimal nuclear/cytoplasmic ratio (karyoplasmic ratio) for balanced growth (reviewed by Cavalier-Smith, 2005), (ii) the cell size response to temperature is realized through changes in noncoding DNA (Hessen, 2015; Hessen et al., 2013), which is a mechanism that requires and indicates the proportional nucleus/cell size ratio, and (iii) indirect evidence shows the importance of matching either the nucleus size or cell size with the body size under stressful thermo-oxic conditions (Walczyńska et al., 2018).

The rotifers were sampled on the third day of each experimental stage. Approximately 50 swimming rotifers were collected and placed in 1 ml Eppendorf tubes, and the samples were fixed with 10% buffered neutral formalin (Avantor Performance Materials Poland S.A., Poland). The staining procedure was based on Nuclear Red (Carl Roth, Germany), as described in Walczyńska et al. (2015a). We measured two perpendicular diameters of 10 nuclei per rotifer using ImageJ (National Institutes of Health, Bethesda, MD, USA), as described in Walczyńska et al. (2015a), and calculated their product. We chose the most visible nuclei for measurements, which were scattered throughout the rotifer body to represent different animal organs. Of note, rotifers do not possess any specialized respiratory cells, and they respire through the whole body surface (Galkovskaya, 1995).

Body size

We estimated the strength of the response to hypoxia in comparison to normoxia by measuring the body size. For this purpose, we sampled the rotifers on the second day of the experiment and fixed them with Lugol's solution. The rotifers were photographed using an Eclipse 80i microscope (Nikon, Japan) equipped with a DS-Ri2 camera assisted by NIS-Elements D software (Nikon). The length and width were measured in ImageJ, and their product acted as a proxy for the body area, as previously applied to the rotifers *Brachionus* sp. (Walczyńska and Serra, 2014) and *L. inermis* (Walczyńska et al., 2015a).

Fitness measures

Two measures of fitness were compared: population growth rate and the eggs/female ratio, which is the number of eggs laid by an average female. To estimate the former, we sampled 1 ml from each cage (two cages×two water baths for four replicates per clone×oxic regime combination), fixed the rotifers with Lugol's solution and counted them. These procedures were used twice, 1 day (count I) and 2 days (count II) after the onset of the stage. During count II, both the egg and female numbers were noted for the eggs/female ratio estimation.

Statistical analyses

All analyses were conducted in SAS 9 (SAS Institute, Cary, NC, USA) in PROC MIXED with the REML (restricted maximum likelihood) method. In each case, the significance level was P=0.05. For the SDA, ANOVA included four fixed factors – hypoxia type (experimental stage), oxic regime of rotifer origin (normoxia versus hypoxia), clone and food regime (fed versus hungry) – along with their interactions. Non-significant interactions in the model were excluded using backward elimination.

Possible differences in nuclear size and body size were tested separately by ANOVA including three fixed factors – hypoxia type, clone and oxic regime – and their interactions. Non-significant interactions were excluded using backward elimination. For the body size, differences in size among the hypoxia types caused a considerable lack of homogeneity of variance, which prevented statistical analysis. Therefore, the data were standardized by recalculation to units of standard deviation for each experimental stage (=hypoxia type), separately.

Both fitness measures were analyzed with ANCOVA (PROC MIXED) instead of as ratios, which has been cited as more appropriate (Raubenheimer, 1995; Raubenheimer and Simpson, 1992). The models included three fixed factors – hypoxia type, clone and oxic regime – and their interactions. For the population growth rate r, the dependent variable was the rotifer number from count II, while that from count I was a covariate. For the eggs/female ratio, the dependent variable was the number of eggs, while the covariate was the number of rotifer females in count II. The dependent variables and covariates were natural logarithm-transformed. In the initial stages, we included all possible first-level interactions of the covariates with the main factors to test whether they were appropriately used in the models. In both models, the covariates significantly affected the dependent variables and did not interact with the main factors. In the next step, we constructed the models of all main factors and possible interactions plus a covariate. Non-significant interactions were removed using backward elimination.

RESULTS

Oxygen consumption

The results for absolute oxygen consumption were compared with other published data for different rotifer species obtained from the literature (Table 1). Generally, the oxygen consumption rate for the units provided in Table 1 was approximately four times higher in fed rotifers than in hungry rotifers. This estimated difference was previously shown to be 2- to 3-fold (Galkovskaya, 1995), although the units and starvation times for this comparison were unclear.

The O_2 consumption of the hungry and fed rotifers differed between the clones and hypoxia types, according to the significant hypoxia type×clone×food interaction. It was also differentially affected by the hypoxia type for normoxic and hypoxic rotifers (significant hypoxia type×oxic regime×food interaction; Table 2). In each case, the fed rotifers consumed more oxygen than the hungry rotifers owing to the metabolic costs associated with the SDA. The ceiling of these costs, namely, the highest oxygen consumption by the rotifers, was relatively constant across hypoxia types in the Warm clone and elevated in Cold rotifers exposed to hypoxia driven by stress-inducing high temperatures (Fig. 2A). The SDA-related costs were generally higher in the rotifers that had experienced hypoxia than in hypoxia-naïve rotifers. The negative value for the Warm clone under normoxia in the MH treatment was caused by two elevated points (see Fig. S2), but outlier analysis showed that there was no objective reason for their removal from the

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Table 1. Comparison of the absolute oxygen consumption measured under different conditions in different rotifer species

Rotifer species	O_2 consumption (mg individual^{-1} h^{-1})	O_2 consumption (ml individual^{-1} h^{-1})	Measurement conditions	Reference
Asplanchna priodonta		0.48×10^{-5} and 0.71×10^{-5}	20°C, well-fed rotifers and after 28 h of starvation, respectively	Kirk et al. (1999)
Asplanchna silvestrii		1.65×10^{-5} and 1.54×10^{-5}	20°C, well-fed rotifers and after 57 h of starvation, respectively	Kirk et al. (1999)
Brachionus calyciflorus		$0.16–0.62\times10^{-5}$	Large numbers, unknown age distribution, different conditions	Pourriot and Deluzarches (1970), after Doohan (1973)
Brachionus calyciflorus		0.22×10^{-5}	–	Belyatskaya (1959), after Doohan (1973)
Brachionus calyciflorus		0.5×10^{-5}	–	Galkovskaya (1963) after Doohan (1973)
Brachionus calyciflorus		0.31×10^{-5} and 0.13×10^{-5}	20°C, well-fed rotifers and after 53 h of starvation, respectively	Kirk et al. (1999)
Brachionus plicatilis		0.27×10^{-5}	Female without eggs; 20°C, seawater with no food	Doohan (1973)
Brachionus plicatilis		$0.1–0.7\times10^{-5}$	20‰ salinity, 20°C	Hirata and Yamasaki (1987)
Keratella quadrata	0.08×10^{-5} and 0.11×10^{-5}		14.5–18°C and 20°C, respectively	Vinberg (1937), after Galkovskaya (1995)
Lecane inermis, two clones	0.38×10^{-5} and 0.09×10^{-5}	0.29×10^{-5} and 0.07×10^{-5}	30°C, fed and 2 h starved, respectively	This study
Polyarthra dolichoptera		0.09×10^{-5} and 0.027×10^{-5}	2 and 5°C, respectively	Galkovskaya (1995)
Synchaeta pectinata		0.21×10^{-5} and 0.22×10^{-5}	20°C, well-fed rotifers and after 18 h of starvation, respectively	Kirk et al. (1999)

dataset. The pattern of the SDA was similar regardless of the measure (whether absolute or factorial; Fig. 2B,C). Generally, the width of the SDA, namely, the difference in oxygen consumption between fed and hungry rotifers, was similar for the Cold clone across the hypoxia types, while in the Warm clone, the values were much higher under mild hypoxia than under the two severe hypoxia treatments. Under mildly hypoxic conditions, both clones showed wider SDAs when previously exposed to hypoxia during the experiment than those that were hypoxia-naïve. This pattern was not repeated in the following severe hypoxia treatment. The Cold clone maintained relatively high and similar SDA values, regardless of previous hypoxia experience, while the Warm clone displayed a clearly narrower SDA in rotifers that experienced hypoxia than in those taken from the normoxia treatment (Fig. 2).

Cell size

In total, we measured 1386 nuclei in 143 animals. In the SHO2 experimental stage, we were only able to collect measurements for the Warm clone. The samples of the other two clones were lost, most likely because of an unknown error with fixing. The mean±s.d. nuclear size of 1.99±0.62 μm^2 was close to the 1.62 μm^2 achieved previously at 32°C for different *L. inermis* clones (Walczyńska et al., 2015a). The clones differed in cell size, and their diverse responses to oxic conditions varied with hypoxia type (significant hypoxia type×clone×oxic regime interaction; Table 3). In general, the Cold clone had the largest nuclei, followed by the Int clone, and those of the Warm clone were the smallest. The response to hypoxia was clone-specific – a decreased cell size under hypoxic conditions was shown by the Cold clone (and less apparently by the Warm clone) in the MH stage and by Int and Warm clones in the SHT stage (Fig. 3A).

Table 2. Final results of ANOVA for oxygen consumption in two *Lecane inermis* clones

Effect	*F*-value (d.f. num, d.f. den)	*P*-value
Hypoxia type	(2, 91) 38.81	<0.0001
Clone	(1, 91) 7.69	0.0067
Oxic regime	(1, 91) 100.88	<0.0001
Food regime	(1, 91) 643.58	<0.0001
Hypoxia type×clone	(2, 91) 20.15	<0.0001
Hypoxia type×oxic regime	(2, 91) 0.63	0.5371
Clone×oxic regime	(1, 91) 0.81	0.3698
Hypoxia type×food regime	(2, 91) 4.81	0.0104
Clone×food regime	(1, 91) 2.59	0.1108
Oxic regime×food regime	(1, 91) 0.01	0.9034
Hypoxia type×clone×food regime	(2, 91) 8.21	0.0005
Hypoxia type×oxic regime×food regime	(2, 91) 3.88	0.0240

Hypoxia type: subsequent experimental stages, standardized within each stage to the SD units. Oxic regime: the origin of the animals (rotifers originated from normoxic or hypoxic experimental conditions, but their respiration was always measured under hypoxia). Food regime: fed or hungry rotifers.

Body size

In total, we measured 1676 individuals. The mean±s.d. body size of 2679±354 μm^2 was similar to the mean of 2411 μm^2 for a different *L. inermis* clone across four thermal regimes (Walczyńska et al., 2015a). We did not find a difference between the experimental series, which was expected because of the preceding standardization. The body size of each clone was differentially affected by the hypoxia type and differed across oxic regimes (significant second-level interactions), and the difference between

Table 3. Final results of ANOVA for nuclear size (a proxy for cell size) in two *L. inermis* clones

Effect	*F*-value (d.f. num, d.f. den)	*P*-value
Hypoxia type	(2, 126) 4.39	0.0144
Clone	(2, 126) 27.04	<0.0001
Oxic regime	(1, 126) 1.98	0.1615
Hypoxia type×clone	(2, 126) 7.98	0.0005
Hypoxia type×oxic regime	(2, 126) 7.03	0.0013
Clone×oxic regime	(2, 126) 1.29	0.2789
Hypoxia type×clone×oxic regime	(4, 126) 14.72	<0.0001

Hypoxia type: subsequent experimental stages. Oxic regime: the origin of the animals (rotifers originated from normoxic or hypoxic experimental conditions, but their respiration was always measured under hypoxia).

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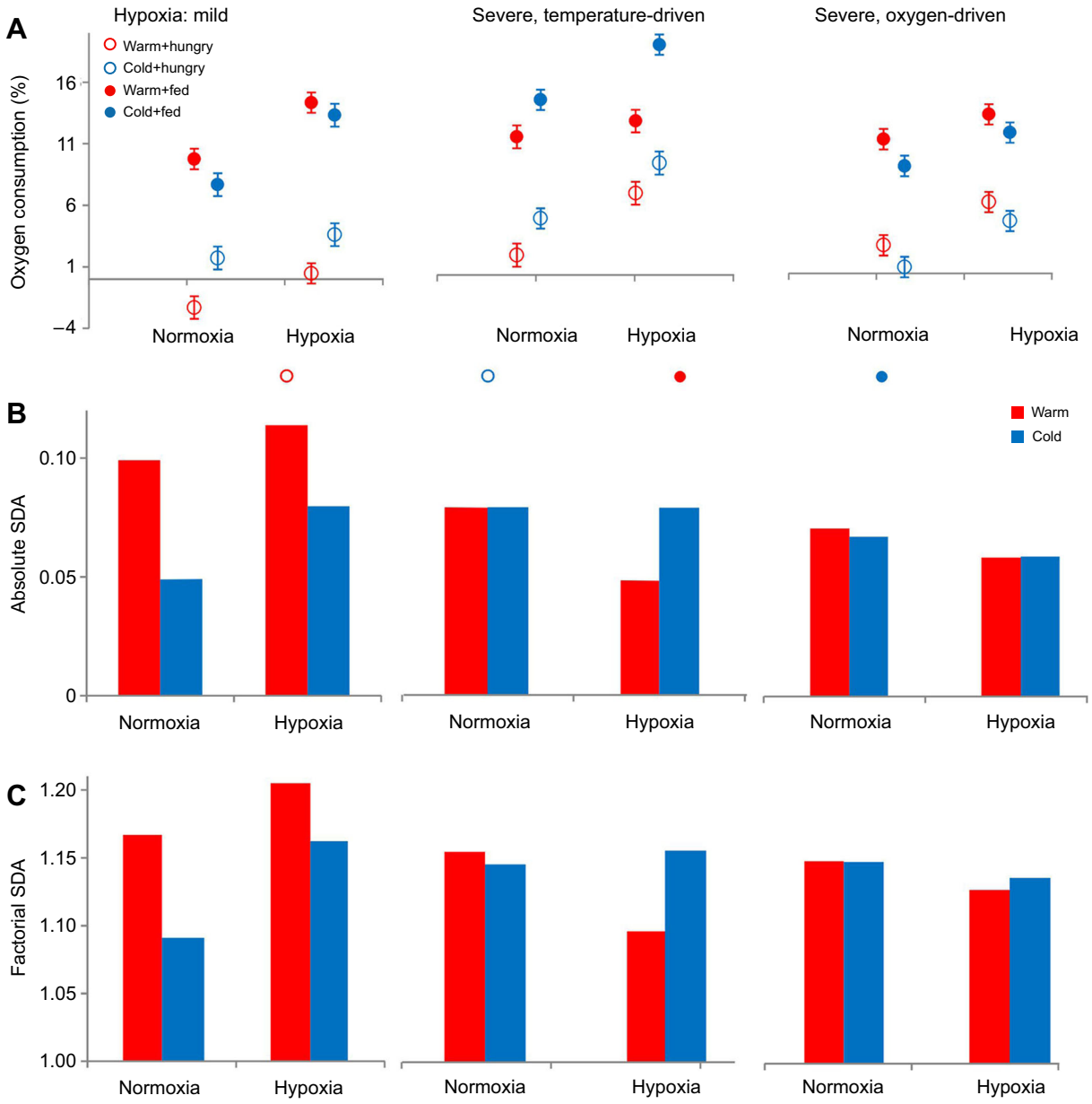

Fig. 2. Results for oxygen consumption (%), absolute aerobic scope and factorial aerobic scope for Warm and Cold clones across three regimes of different hypoxia types. (A) Oxygen consumption by rotifers exposed to experimental hypoxia or normoxia, divided into groups of fed and hungry and tested for respiration rates, all under hypoxic conditions (4 or 5 replicates per group tested). Data are least square means±s.e.m. (B) Absolute scope of aerobic metabolism as the difference between the oxygen consumption by fed and hungry rotifers; absolute SDA. (C) Factorial scope of aerobic metabolism from the division of the amount of oxygen consumed by fed rotifers by that consumed by hungry rotifers; factorial SDA. 'Normoxia' and 'hypoxia' describe the experimental conditions of rotifer maintenance that preceded the metabolism measurements.

normoxic and hypoxic rotifers also interacted significantly with the hypoxia type (Table 4, Fig. 3B).

Fitness measures

The mean±s.d. population growth rate r was 0.64±0.08 individuals day^{-1}, which was similar to the value of 0.5 individuals day^{-1} previously shown at 30°C for a different *L. inermis* clone (Walczyńska et al., 2016). The mean eggs/female ratio was estimated as 0.16±0.02 eggs/female. Both measures of fitness showed similar patterns across experimental regimes. Fitness differed (or tended to differ, in the case of the eggs/female ratio) across hypoxia types and clones (Table 5), with the lowest values in the SHT stage and the Warm clone generally showing the highest values (Fig. 4). Measures were strongly similar between rotifers from normoxia or hypoxia (Table 5). The highest average population growth rate was under mild hypoxia (approximately 15% higher than in the SHT treatment and approximately 4% higher than in the SHO2 treatment), followed by the SHO2 treatment (approximately 1% higher than in the SHT treatment).

DISCUSSION

The results of our comprehensive study show two important issues. First, the linkage of plastic body size changes with the cell response to hypoxia to maintain a wide aerobic scope is correct. Second, this

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Table 4. Final results of ANOVA for body size standardized to SD units among the hypoxia types

Effect	*F*-value (d.f. num, d.f. den)	*P*-value
Hypoxia type	(2, 1662) 1.09	0.3370
Clone	(2, 1662) 30.23	<0.0001
Oxic regime	(1, 1662) 4.76	0.0292
Hypoxia type×clone	(4, 1662) 18.26	<0.0001
Hypoxia type×oxic regime	(2, 1662) 9.00	0.0001
Clone×oxic regime	(2, 1662) 6.37	0.0018

Hypoxia type: subsequent experimental stages. Oxic regime: the origin of the animals (rotifers originated from normoxic or hypoxic experimental conditions, but their respiration was always measured under hypoxia).

Table 5. Final results of ANCOVA for the two fitness measures, population growth rate *r* and eggs/female ratio

Effect	*F*-value (d.f. num, d.f. den)	*P*-value
Population growth rate *r*: rotifer number as a dependent variable		
Hypoxia type	(2, 65) 9.13	0.0003
Clone	(2, 65) 12.98	<0.0001
Oxic regime	(1, 65) 0.62	0.4340
Initial number	(1, 65) 11.30	0.0013
Eggs/female ratio: egg number as a dependent variable		
Hypoxia type	(2, 65) 3.12	0.0507
Clone	(2, 65) 2.99	0.0572
Oxic regime	(1, 65) 0.20	0.6587
Final number	(1, 65) 50.81	<0.0001

Hypoxia type: subsequent experimental stages. Oxic regime: the origin of the animals (rotifers originated from normoxic or hypoxic experimental conditions, but their respiration was always measured under hypoxia). Initial and final numbers: rotifers counted in count I and count II, respectively.

pattern is valid only under optimal conditions, when phenotypic plasticity is probably less costly than alternative physiological mechanisms. In contrast, under stressful conditions, these alternative mechanisms (not controlled here) prevent hypoxia-driven metabolic limitations, and responses vary with conditions

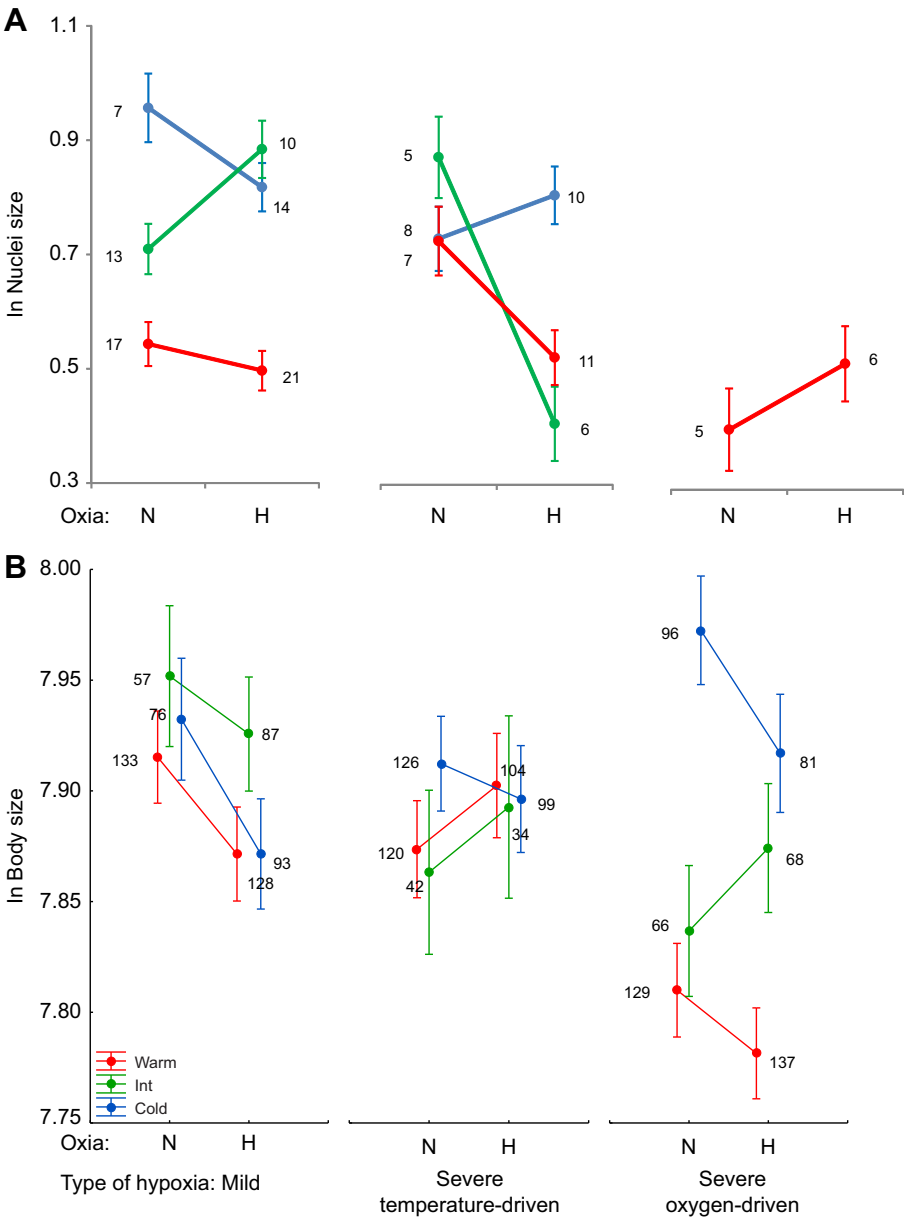

Fig. 3. Results for cell- and body-size response. The interactions among hypoxia type, oxic regime (N, normoxia; H, hypoxia) and clone in the ANOVA for (A) nuclear size (a proxy for cell size) and (B) body size. The numbers denote the sample size of rotifers of which the nuclei were measured (A) or the number of measured individuals (B). Data are least square means±95% confidence intervals.

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(physiological versus environmental hypoxia) and organismal thermo-oxic preferences.

We generally confirmed the hypotheses that (i) body size decreases in response to hypoxia, (ii) this decrease is a consequence of cell size decrease, (iii) this response prevents the narrowing of aerobic metabolism and (iv) it also prevents a subsequent decrease in fitness. Strikingly, we observed this causative pattern only for the mildly hypoxic conditions and not the two severe hypoxia treatments (Fig. 5). In each case, rotifers originating from hypoxic conditions displayed a similar level of fitness to those from normoxic conditions (Fig. 4), which could indicate alternative mechanisms to the plastic size response that effectively prevented the decrease in fitness under stress-inducing hypoxic conditions. We therefore confirmed the importance of the 'optimal thermal range' within which the TSR operates (Atkinson et al., 2003; Walczyńska et al., 2016), but we extended this issue by relating the optimality of the conditions to temperature acting closely with oxygen. Additionally, the patterns obtained concurred with a meta-analysis of 52 species of aquatic invertebrates by Galic et al. (2019), who found that (i) the lower the oxygen concentration, the larger the variation in the measured response traits, and that (ii) respiration was more sensitive than growth to hypoxic conditions.

Proximate mechanisms

Because of methodological problems, we did not manage to collect a complete dataset for the nuclear size response. Our partial results show that in the MH treatment, the consequent body size decrease in hypoxia compared with normoxia was associated with a mechanism other than cell shrinkage in the case of one clone. In contrast, the nuclei and body size differences between normoxic and hypoxic rotifers showed completely opposite patterns in all clones exposed to severe temperature-driven hypoxia. A similar opposing pattern was also found for the Warm clone in the SHO2 treatment.

We were not able to determine the cause of the observed variability in the nuclear size/body size response, but this is not the first time that variability in the match between nuclear size and body

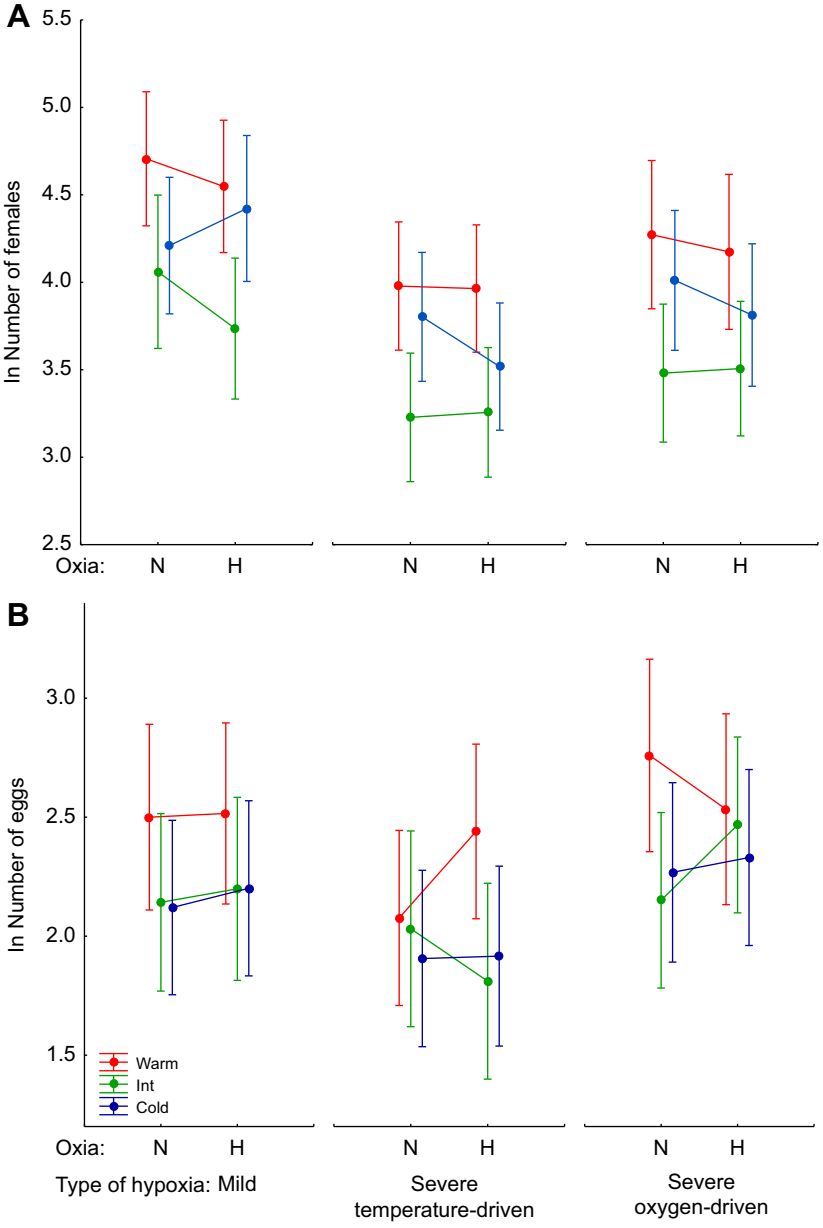

Fig. 4. Results for the fitness measures. The interactions among the hypoxia types, oxic regime (N, normoxia; H, hypoxia) and clone in the ANCOVA analyses for two fitness measures: (A) number of females, with count II as a dependent variable and count I as a covariate, and (B) number of eggs, with the egg number as a dependent variable and count II as a covariate. *N*=4 replicates. Data are least square means±95% confidence intervals.

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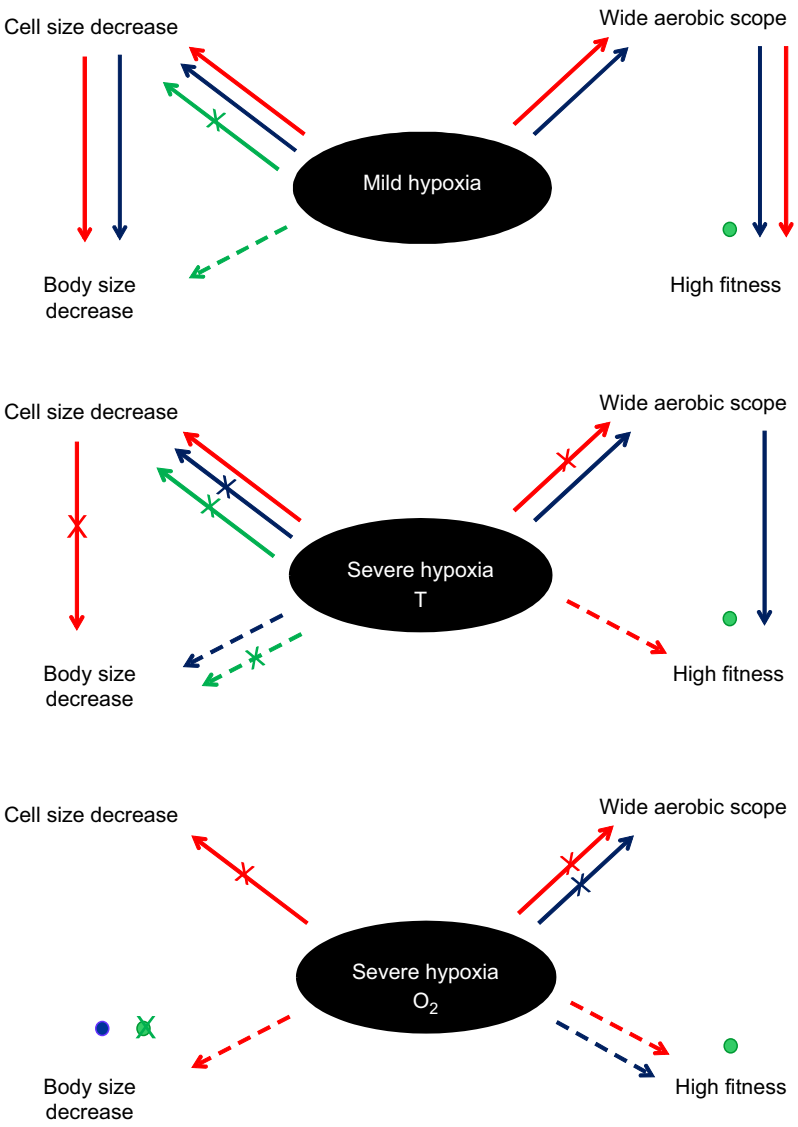

Fig. 5. A schematic summary of patterns relative to the hypotheses displayed in Fig. 1 A. The arrows (red, warm-preferring clone; green, medium temperature-preferring clone; blue, cold-preferring clone) represent the positive or (when crossed) negative causative effects found in testing across the three experimental stages. Dashed arrows indicate effects without the intermediate impact that we expected. The lack of a specific arrow indicates that no result was achieved for a given relationship. The dots show the positive or (when crossed) negative ultimate results when the causative effect through the intermediate trait was not known. 'Severe hypoxia T' refers to the experimental stage in which hypoxia was driven by the too high temperature, while 'Severe hypoxia O_2' refers to the stage with hypoxia driven by very low oxygen concentration.

size has been observed under different conditions. In the same rotifer species, this match differed between lower and higher temperature treatments (fig. 7 in Walczyńska et al., 2015a), while Walczyńska et al. (2018) showed a stronger relationship between nuclear size and body size in the *L. inermis* rotifer and between cell size and body size in an annelid when exposed to stressful conditions. The canalization versus plasticity in body versus cell size was found to vary across *D. melanogaster* organs, as shown by McDonald et al. (2018). Finally, in a study in which different life stages of *D. melanogaster* were exposed to hypoxia, the cell size response was found to be stage-specific (Heinrich et al., 2011). All of these results showed that the issue of body and cell size adjustment was not trivial, and much work can be performed in this field.

The general characteristic of body size variations versus cell size variations was classified as fundamental by Leinaas et al. (2016). The novel contribution of the present study to this issue was showing that the body–cell size match was not only due to the conditions but was also clone-dependent and apparently varied with thermal preference. In a warm-preferring clone, the unsuitably high temperature in the SHT treatment caused a decrease in cell size under hypoxia, but this was not followed by an adequate body size adjustment, while in the cold-preferring clone, the response was reversed under the same conditions. The intermediate clone showed a similar pattern to the warm clone in this treatment, but its body size response was opposite (to both remaining clones) in the SHO2 treatment.

Ultimate mechanisms

Overall, hypoxia-naïve rotifers displayed a lower consumption rate than hypoxia-exposed rotifers (Fig. 2A). Lowered metabolic rates during exposure to hypoxic conditions are well known in ectothermic and endothermal vertebrates (Gu and Jun, 2018) and several invertebrate species (Gorr et al., 2010), and this is termed 'hypoxic hypometabolism'. We found that under mildly hypoxic conditions (MH treatment), rotifers were able to adjust their metabolism to oxic conditions within a relatively quick period of 3 days (approximately two generations), such that the aerobic scope of rotifers from hypoxic conditions was, on average, 30% wider than that of rotifers from normoxic conditions when exposed to acute hypoxia.

The comparison of the examined traits between normoxic and hypoxic rotifers indicated that the cue for sensing hypoxia was different for the organisms from the two severe hypoxia

experimental stages. In animals that were tolerant to oxic conditions (Galkovskaya, 1995), overly elevated temperatures seemed to be more stressful than too little oxygen. Previous research has shown that this group of animals is able to switch from the regular low efficiency and rapid metabolism of the lactate metabolic pathway under hypoxia to the more efficient but slower glucose–succinate metabolic pathway when exposed to severely hypoxic conditions (Esparcia et al., 1992).

The reliability of our results was confirmed by the Warm clone, which was the smallest clone and displayed the highest fitness under hot experimental conditions. An interesting finding resulted from the comparison between the two applied measures of fitness. In our short study, clones exposed to stressful conditions invested in either higher fecundity (higher eggs/female ratio) or accelerated development (higher population growth rate). On a longer time scale, these two mechanisms would probably produce similar results in overall fitness. The clearest pattern was shown by the Int clone, which had the slowest development (such that we did not even manage to examine its SDA), but its fecundity was comparable with that of the Cold clone across regimes. Differences in reproductive strategies in response to hypoxia were previously found across four rotifer species by Kirk et al. (1999).

The role of oxygen deficiency in the TSR

We concur with the conclusions from previous reports on the diverse response of ectotherms to high temperatures combined with hypoxia. However, we also showed flexibility of the TSR from this general perspective – plasticity in size (decrease under high temperature/hypoxia) occurs within relatively mild conditions, most likely as the primary mechanism to maintain a wide scope of aerobic metabolism. Above a certain threshold, which was previously designated by Walczyńska et al. (2016), the response changes. The plastic response is replaced by physiological mechanisms preventing or counteracting any possible physiological damage.

With the growing number of empirical studies confirming the role of oxygen behind the size-to-temperature response, there has been some criticism. These doubts are associated with ambiguous results obtained for other animals, mostly including fishes (reviewed in Audzijonyte et al., 2019). In a study on *Daphnia magna*, Kielland et al. (2019) confirmed the limiting role of oxygen availability for ectotherm performance at high temperatures but concluded that plasticity did not counteract the negative effects of temperature-dependent hypoxia at very high temperatures. In our opinion, our results show that oxygen is the limiting factor (*sensu* Fry, 1947), inducing a decrease in cell size and a consequent decrease in body size, but this causative relationship should be expected only under optimal conditions, which are dependent on many factors, especially organismal life strategies and thermal preferences.

Let us provide an example from everyday life. Imagine that one wants to buy a house. If enough funds are available, one simply buys the house, but if not, different potential strategies for gaining the necessary money exist, such as taking on an additional job, saving more from one's current salary, borrowing from family or friends, or obtaining a bank loan. In each case, the result is the same: there is enough money to buy the house. However, there are always various strategy-dependent costs, which, in each case, are higher than if there had been adequate funds at the beginning. The lowest-cost strategy for struggling with mild, ecologically relevant hypoxia is a plastic response. The possible differing physiological costs for organisms struggling with severe oxygen deficiency in their environment await discovery, although we already know that they are most likely associated with hypoxia sensing at the mitochondrial level (Sokolova, 2018; Sokolova et al., 2019). A comparison of physiological responses to mild or severe hypoxia in insects was reviewed by Harrison et al. (2018). The two ecological and physiological approaches should be matched to determine how costly the response to temperature-dependent oxygen concentrations is, with a distinction between mild and severe conditions.

Our results contribute to the discussion by Kingsolver and Huey (2008) on the contradiction among three rules: 'bigger is better', 'hotter is smaller' and 'hotter is better'. Tests of these rules may refer to the three respective comparison types in our study: interclonal, among experimental stages and linking experimental stages and fitness. These tests show that the response is complex rather than straightforward. In addition to the solution to this paradox provided by the authors, who stress that each rule operates at different levels, we speculate that the problem also results from the entanglement of oxygen with one of these rules. 'Bigger is better' derives from the eco-evolutionary fact that living organisms are limited by the energy that they may acquire and process, and 'hotter is better' is a physical phenomenon (although there is a hidden assumption regarding the physiologically relevant thermal range involved). In contrast, 'hotter is smaller' actually does not refer to temperature but to temperature-dependent oxygen availability, and according to this and a previous study (Walczyńska et al., 2016), it is realized only within the optimal thermal range and only under conditions where a negative relationship between temperature and oxygen occurs (Walczyńska and Serra, 2020 preprint; Walczyńska and Sobczyk, 2017).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.W., M.S.; Methodology: M.S.; Validation: A.W.; Formal analysis: A.W.; Investigation: A.W., M.S.; Data curation: A.W.; Writing - original draft: A.W.; Writing - review & editing: A.W.; Visualization: A.W.; Supervision: A.W.; Project administration: A.W.; Funding acquisition: A.W.

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Data availability

The data supporting the results are available from a public repository of Jagiellonian University: https://ruj.uj.edu.pl/xmlui/handle/item/278414.

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