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RESEARCH ARTICLE

Are acute and acclimated thermal effects on metabolic rate modulated by cell size? A comparison between diploid and triploid zebrafish larvae

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ABSTRACT

Being composed of small cells may carry energetic costs related to maintaining ionic gradients across cell membranes as well as benefits related to diffusive oxygen uptake. Here, we test the hypothesis that these costs and benefits of cell size in ectotherms are temperature dependent. To study the consequences of cell size for whole-organism metabolic rate, we compared diploid and triploid zebrafish larvae differing in cell size. A fully factorial design was applied combining three different rearing and test temperatures that allowed us to distinguish acute from acclimated thermal effects. Individual oxygen consumption rates of diploid and triploid larvae across declining levels of oxygen availability were measured. We found that both acute and acclimated thermal effects affected the metabolic response. In comparison with triploids, diploids responded more strongly to acute temperatures, especially when reared at the highest temperature. These observations support the hypothesis that animals composed of smaller cells (i.e. diploids) are less vulnerable to oxygen limitation in warm aquatic habitats. Furthermore, we found slightly improved hypoxia tolerance in diploids. By contrast, warm-reared triploids had higher metabolic rates when they were tested at acute cold temperature, suggesting that being composed of larger cells may provide metabolic advantages in the cold. We offer two mechanisms as a potential explanation of this result, related to homeoviscous adaptation of membrane function and the mitigation of developmental noise. Our results suggest that being composed of larger cells provides metabolic advantages in cold water, while being composed of smaller cells provides metabolic advantages in warm water.

KEY WORDS: Cell size, Metabolic rates, Oxygen limitation, Polyploidy, Temperature

INTRODUCTION

The substantial variation in cell size observed among extant animal species is largely mirrored by variation in genome size (Gregory, 2001; Dufresne and Jeffery, 2011). Although cell size and genome size are strongly and positively correlated, the causality is not fully resolved (Gregory, 2005; Czarnoleski et al., 2018). While cell size and genome size may seem esoteric aspects of an organism, there is evidence that it could have important life-history ramifications owing to their influence on body mass, metabolic rate and the mass scaling of metabolic rate (Gregory et al., 2000; Kozłowski et al., 2003; Czarnoleski et al., 2018; Kozłowski et al., 2020). Smaller cells have a higher ratio of membrane surface area to cell volume. Therefore, they will require more energy for phospholipid turnover and to maintain ionic gradients between the cytoplasm and the cell's surroundings (Szarski, 1983; Rolfe and Brown, 1997; Czarnoleski et al., 2013, 2015a). Studies on freshly isolated tissues and cultured cells have indeed found that smaller cells exhibit higher mass-specific metabolism compared with larger cells (Goniakowska, 1970; Monnickendam and Balls, 1973). In addition to higher demand for energy, smaller cells also have a greater capacity for oxygen uptake, as intracellular diffusion distances are shorter (Woods, 1999; Miettinen et al., 2017) and oxygen diffuses more rapidly in lipid membranes than in aqueous cytosol (Subczynski et al., 1989). As a result, risks of oxygen limitation are hypothesized to be greater for tissues composed of larger cells (Atkinson et al., 2006; Czarnoleski et al., 2013; Verberk et al., 2020). Thus, there are distinct metabolic consequences to cell size.

Previous studies investigating the link between metabolic rate and cell size have exploited natural variation in genome size and cell size within species and complexes of closely related species (reviewed by Hermaniuk et al., 2017) or focused on interspecific comparisons (Vinogradov, 1995; Gregory, 2002, 2003; Starostová et al., 2009; Czarnoleski et al., 2018; Gardner et al., 2020). Comparing species with different evolutionary histories is problematic as it is difficult to isolate the effect of cell size (or its proxy genome size) on metabolic rate. Support for a connection between cell size and whole-body metabolic rate was not consistently found in different animal groups. Polyploidy originates almost exclusively in ectothermic animals as a consequence of duplication of entire chromosome sets (Otto, 2007; Choleva and Janko, 2013) and the increase in genome size is associated with increases in cell size across a range of tissues (Fankhauser, 1945; Suresh and Sheehan, 1998; Hermaniuk et al., 2016). Despite the advantages of using variation in ploidy within species, research on the relationship between cell size and whole-body metabolic rate in animals of different ploidy also provided inconclusive results.

A way forward in clarifying the relationship between cell size and whole-body metabolic rate could be the need to include temperature. In ectotherms, warming is known to reduce cell size (Arendt, 2007; Hessen et al., 2013; Walczyńska et al., 2015) for most, but not all, tissue types (Czarnoleski et al., 2016, 2017). Such changes in cell size could be adaptive to reduce risks of oxygen limitation (Szarski, 1983; Walczyńska et al., 2015; Verberk et al., 2020), as warming increases metabolic oxygen demand (Clarke and Fraser, 2004), possibly resulting in a mismatch between oxygen demand and (the capacity for) oxygen supply (Pörtner, 2010; Verberk et al., 2011). Indeed, the concept of understanding thermal

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responses from an oxygen perspective is gaining traction (e.g. Pörtner, 2010; Czarnoleski et al., 2015b; Hoefnagel and Verberk, 2015; Verberk et al., 2016b), especially for aquatic ectotherms, as oxygen uptake is more challenging in water than in air due to the much lower diffusion rate of oxygen (Woods, 1999; Verberk et al., 2011). Thus, metabolic consequences of cell size in aquatic ectotherms could depend on temperature, with smaller cells being more advantageous in warmer conditions.

A second key ingredient could be to include test conditions that are pushing animals to their limits. Previous work comparing metabolic rate in animals of different ploidy focused on either standard or routine metabolic rate, which relates to energetic costs of maintaining basic life function. Under these conditions, the large cells of polyploids are less likely to constrain metabolic rate – a pattern observed in many studies (invertebrates: Ellenby, 1953; Shpigel et al., 1992; fish: Sezaki et al., 1991; Parsons, 1993; Hyndman et al., 2003; amphibians: Licht and Bogart, 1990; Lukose and Reinert, 1998). However, capacity limitations for oxygen uptake are more likely to be manifested under challenging conditions, such as high temperatures, strenuous activity or low oxygen conditions (Hyndman et al., 2003; Atkins and Benfey, 2008; van de Pol et al., 2020; Rubalcaba et al., 2021).

Given the thermal sensitivity of aquatic ectotherms, we designed a full factorial experiment that investigates the link between cell size and whole-body metabolic rate in fish larvae combining different rearing (developmental) and test temperatures and different levels of oxygen availability. This approach allows us to distinguish between the two temperature effects (acute and acclimated) that are regulated by different mechanisms (Havird et al., 2020). Acute test temperature induces passive responses shaped by the thermodynamics of molecular interactions and subsequent immediate energetic feedbacks while acclimation (developmental) temperature induces active responses through molecular mechanisms including gene expression, membrane composition and enzyme concentrations (Angilletta, 2009; Havird et al., 2020). As a model system we used diploid and artificially induced triploid zebrafish larvae [*Danio rerio* (Hamilton 1822)], which have previously been shown to differ in genome size and cell size; the 50% increase in DNA content results in a 1.5-fold increase in cell size (erythrocytes were directly measured) in triploids, while their cell number was reduced by the same factor of 1.5 (van de Pol et al., 2020). Hence, triploids were of similar body size, consisting of larger but fewer cells compared with their diploid counterparts. A similar correlation between erythrocyte size as a proxy of cell size of other tissues has been reported for amphibians (Kozłowski et al., 2010). A previous study showed that under non-demanding conditions, diploid and triploid zebrafish larvae were highly similar in terms of gene expression, growth and development (van de Pol et al., 2020). Furthermore, zebrafish larvae rely on cutaneous oxygen uptake, as their gills are not fully developed until 14 days after fertilization (Kimmel et al., 1995; Rombough, 2002). Therefore, they cannot increase their capacity to deliver oxygen by increasing gill ventilation rates. Taken together, this makes zebrafish larvae an excellent model for studying the consequences of cell size variation on thermal physiology of ectotherms.

Although some studies have reported triploid individuals to have reduced aerobic metabolism at elevated developmental temperatures (Stillwell and Benfey, 1996; Atkins and Benfey, 2008), none has looked at the interacting effects of developmental and test temperatures combined with lowering P_{O_2} on metabolic rate in diploid and triploid animals. We hypothesized that triploids composed of larger cells have a lower capacity for oxygen supply and that this would be manifested as a lower metabolic rate at higher water temperatures and in hypoxic water, when compared with diploid counterparts. We expected that developmental temperature would modulate the effect of test temperature such that animals reared at high temperatures would be better able to meet their elevated oxygen demand at high test temperatures. We also assessed hypoxia tolerance in diploid and triploid fish at different temperatures, by means of determining P_{crit}, which is the value of P_{O_2} below which the animal can no longer maintain a stable rate of oxygen consumption. Wood (2018) recently questioned the usefulness of using a single P_{crit} value as an indicator of hypoxia tolerance and suggested that instead one should consider the entire metabolic response to declining oxygen tensions. Here we compared different methodological approaches to assess the ability of the animal to regulate its metabolic rate across progressively lowering P_{O_2}.

MATERIALS AND METHODS

Fish stock and production of diploid and triploid progeny

The sources of parental zebrafish and the induction of triploidy have been described in detail elsewhere (van de Pol et al., 2020). Briefly, parental zebrafish [wild-type strain supplied by Zebrafish International Resource Center (ZIRC), ZFIN ID: ZDB-GENO-960809-7] were kept in tanks (4 liters volume) with recirculating tap water (temperature 27°C, pH 7.5–8) containing about 30 individuals under 14 h:10 h light:dark photoperiod. Once a week, one tank was chosen to perform *in vitro* fertilization (Westerfield, 2000; see van de Pol et al., 2020 for an in-depth description of the procedure) using randomly selected males and females. To ensure good quality eggs, zebrafish females were given about 20 days rest after spawning; therefore, care was taken not to re-use the same tank within 3 weeks. The procedure was repeated for eight consecutive weeks (i.e. eight rounds of fertilization). To induce triploidy, we used a cold shock treatment by immersing eggs 3 min after fertilization in a tank with 4°C E2 medium (standard medium for zebrafish care containing 5 mmol l^{-1} NaCl, 0.17 mol l^{-1} KCl and 0.33 mmol l^{-1} $MgSO_4$) for 20 min. For each round of fertilization, eggs were obtained from multiple females: three to four females were used to produce diploid progeny, and three to five females were used to produce triploid progeny, except for round eight, where we used only two females to produce diploids and triploids, one in each group. To secure the heterogeneity of the offspring, all eggs were fertilized using the pooled sperm suspension of eight to 12 males. Egg quality was visually assessed by checking their colour, transparency and shape. We only used eggs that appeared yellowish and translucent, with a regular round shape. Eggs that did not meet the accepted criteria were removed. Before transferring the fertilized eggs to the rearing tanks (see below), the progeny of different females of a given ploidy level were pooled.

Rearing conditions and experimental design

Within 2 h after fertilization, diploid and triploid embryos were divided over separate 48-well plates (three embryos per well, approximately 1 cm^3 volume) with a mesh bottom and placed in a rearing tank with E3 medium (E2 medium with addition of 10^{-5}% Methylene Blue). During development, these rearing tanks were kept in three water baths at constant temperatures of 23.5, 26.5 and 29.5°C (±0.5°C), each water bath holding one rearing tank with diploids and one with triploids. The E3 medium in the rearing tanks was constantly aerated to ensure full oxygen saturation. Larvae were reared until metabolic rate was measured, which was on the fifth day post-fertilization for larvae reared at 26.5°C. For larvae reared at

23.5 and 29.5°C, measurements were conducted 1 day later and earlier, respectively. This ensured that these larvae had reached the same developmental stage in physiological time, when their yolk sack was almost fully resorbed and larvae were able to produce a swift escape response. Survival and hatching rates were scored two times a day, and dead embryos or larvae were removed. A mixed effects model showed that average survival rate at 5 days post-fertilization for eight rounds of fertilization did not depend on rearing temperature (P=0.6611), but differed between diploids (33.8% mortality) and triploids (48.4% mortality) (P<0.0001).

A full factorial design was employed in this study combining two ploidy levels (diploid and triploid), three rearing temperatures (23.5, 26.5 and 29.5°C) and three test temperatures during metabolic rate measurements (23.5, 26.5 and 29.5°C). This allowed us to separate between developmental plasticity and acute thermal effects. For any given combination of rearing and test temperature (nine conditions in total), larvae originated from two different rounds of fertilization. Before each set of metabolic rate measurements, all experimental larvae were photographed with a dissection microscope (Leica MZ FLIII; Leica Microsystems, Germany) to assess body length in millimeters. To avoid effects of handling stress from photography during the metabolic rate experiments, the pictures were taken about 3 h before each respirometry trial. The length measurements were made using the segmented line tool of the Fiji image processing package (ImageJ open-source software: https://imagej.net/ImageJ).

This study was conducted at the Institute of Water and Wetland Research (IWWR) of the Radboud University, Nijmegen (Netherlands), in accordance with the Dutch Animals Act (https://wetten.overheid.nl/BWBR0003081/2019-01-01), the European guidelines for animal experiments (Directive 2010/63/EU; https://eur-lex.europa.eu) and institutional regulations. Because the experiments were performed with larvae up to a developmental stage of 5 days post-fertilization, a stage where larvae are not yet dependent on external feeding, no ethical approval was required.

Metabolic measurements

Oxygen consumption was measured in a closed respirometry system using a 24-well glass microplate equipped with oxygen sensor spots glued onto the bottom of 200 µl wells (Loligo Systems, Viborg, Denmark) integrated with a 24-channel fluorescence-based oxygen reading device (SDR SensorDish Reader; PreSens, Regensburg, Germany). The sensor spots measure the partial pressure of oxygen (in kPa), which we combined with the temperature-dependent solubility to calculate the oxygen concentrations at different temperatures. Depletion of oxygen over time was used to calculate oxygen consumption rates per individual (in nmoles O_2 per hour) as well as the critical value in P_{O_2} (in kPa).

In each run, diploid and triploid larvae without morphological abnormalities were transferred into the 24-well microplate with E2 medium, and these wells were then sealed using an adhesive optical PCR sealing film (Microseal 'B' PCR Plate Sealing Film; Bio-Rad, Hercules, CA, USA), making sure to avoid air bubbles inside the wells. In most cases, 10 diploids and 10 triploids were tested in one run, leaving four wells of our 24-well microplate empty, which were used as a control. The microplate with animals was placed in a flow-through water bath connected to a cooling/heating circulating bath (Grant LT ecocool 150) which allowed for temperature stabilization at 23.5, 26.5 and 29.5°C (±0.1°C), respectively. Oxygen concentrations inside the wells were recorded every 30 s with automatic temperature and pressure correction using MicroResp version 1.0.4 software (https://www.loligosystems.com/). All measurements on metabolic rate were carried out in darkness due to sensitivity of the sensor spots to ultraviolet light. Spontaneous activity of larvae was possible during measurements, so their metabolic rate in normoxia was defined as the routine metabolic rate. In each run, at least four randomly selected wells with E2 medium but without animals were used the assess background respiration. The entire trial lasted at least 16 h (between 16:00 and 08:00 h), during which all larvae completely depleted the available oxygen from the wells (Fig. S1). After each experimental run the dead larvae were removed from the wells. To prevent accumulation of bacteria in the wells, the microplate was bleached using a 35% H_2O_2 solution and rinsed with demineralized water. We conducted a total of 24 runs (8 rounds×3 temperatures), measuring oxygen levels in the wells until they were fully depleted in 422 larvae (208 diploids and 214 triploids). The first 20 min of each run was discarded from further analysis as the temperature equilibrated during this period and the larvae adjusted to being transferred to the microplate. Rates of oxygen consumption were calculated from the declines in oxygen levels over time as the slope of a linear regression using a moving time window of 5 min. We also evaluated in separate trials the extent of oxygen ingress for each test temperature by incubating the microplate sensors under hypoxic conditions and measuring how fast oxygen levels increased over time. The larval oxygen consumption rates were adjusted for both background respiration and oxygen ingress although both influences were negligible compared with the respiration rates of the larvae (e.g. background respiration rates as assessed with blanks never exceed 3% of the measured respiration rates in larvae).

Ploidy determination

Triploidy induction was verified by measuring the amount of DNA in cell nuclei of homogenized larvae using flow cytometry. This procedure was performed according to van de Pol et al. (2020) with minor changes. For each round of fertilization, triploidy induction efficiency was calculated from three pooled samples (one for each of the three rearing temperatures) of cold shocked larvae with one internal diploid control. Larvae without morphological abnormalities were selected for the metabolic rate measurements. The total number of larvae measured for each round ranged between 20 and 30, depending on how many high-quality larvae were available. The amount of lysis buffer added was adjusted to the number of larvae in the pool. The three pooled samples for each batch were processed simultaneously, by homogenizing the larvae and consequently staining the DNA with Propidium Iodide (Sigma-Aldrich). All samples were analysed with a Beckman Coulter FC500 5-color flow cytometer, and triploidy induction efficiency was calculated using the R package 'flowPloidy' (Smith et al., 2018). For each round of fertilization, we obtained the following percentages of triploidy induction efficiency: (1) 95.8, (2) 96.3, (3) 100, (4) 63.3, (5) 100, (6) 100, (7) 100 and (8) 100.

Data analysis

From our measurement of oxygen consumption rates ($\dot{M}_{O_2}$) across a range of oxygen tensions (as we allowed the animals to deplete the oxygen in the respirometer), we derived an index for the oxyregulatory capacity (the O_2 tension below which oxygen becomes limiting) and the average oxygen consumption rate when oxygen is not limiting. There are different ways to do this, as highlighted in recent papers (Marshall et al., 2013; Wood, 2018;

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Reemeyer and Rees, 2019). We employed two approaches. First, we fitted a modified Michaelis–Menten equation:

$$\dot{M}_{O_2} = \frac{\dot{M}_{O_2,routine} \times P_{O_2}^{h}}{P_{50}^{h} + P_{O_2}^{h}}, \quad (1)$$

where $\dot{M}_{O_2,routine}$ is the routine $\dot{M}_{O_2}$ under normoxia, P_{50} is the P_{O_2} at which $\dot{M}_{O_2}$ is 50% of $\dot{M}_{O_2,routine}$ – this represents the affinity of the organism for O_2 (the lower the P_{50}, the greater the affinity and therefore a greater hypoxia tolerance) – and h is Hill number (the slope of the relationship on double logged axes) (see Wood, 2018 for details). Under h=1, the equation defaults to the standard Michaelis–Menten equation, whereas higher numbers allow for an S-curve relationship to be fitted. In our analysis, we fitted this equation for h=1, h=2 and h=3. We compared these results with a second commonly employed method, which is piece-wise linear regression, whereby a breakpoint (termed P_{crit}, somewhat similar to the P_{50} above) is estimated from fitting two linear regressions. For this we used the R package 'segmented' (Muggeo, 2008). In essence, both approaches fit a curve to the entire metabolic rate profile of each larva and derive constants that describe the oxyregulatory capacity of the larva and its capacity for O_2 uptake.

All statistical analyses were conducted in R version 3.5.3 (https://cran.r-project.org/). We assessed the congruence between the P_{crit} derived from the piece-wise linear regressions and the P_{50} derived from the Michaelis–Menten equation (under h=1, h=2 and h=3). This analysis showed that both approaches yielded similar values when h=2. P_{crit} values were sometimes over-estimates when initial oxygen consumption rates were very high. At lower h values (h=1), P_{50} values tended to be under-estimated, whereas higher h values (h=3) resulted in over-estimates. Estimates for the $\dot{M}_{O_2,routine}$ under normoxia varied in tandem with P_{50} values, being higher for the higher P_{50}values associated with h=1 and lower for the lower P_{50} values associated with h=3. Estimates for the $\dot{M}_{O_2,routine}$ based on the Michaelis–Menten equation under h=2 also corresponded to the mean oxygen consumption rate at oxygen levels above 60% saturation (i.e. well above the oxygen level below which oxygen reduces respiration rates) (Fig. 1).

We performed a linear mixed effects analysis of the relationship between oxygen consumption rates ($\dot{M}_{O_2}$) of diploid and triploid larvae and different rearing and test temperatures using R package 'lme4' (Bates et al., 2015). Both rearing and test temperatures were treated as continuous variables to describe the relationship between temperature and $\dot{M}_{O_2}$. To account for differences in the efficiency of triploidy induction, ploidy level for each individual was coded as the

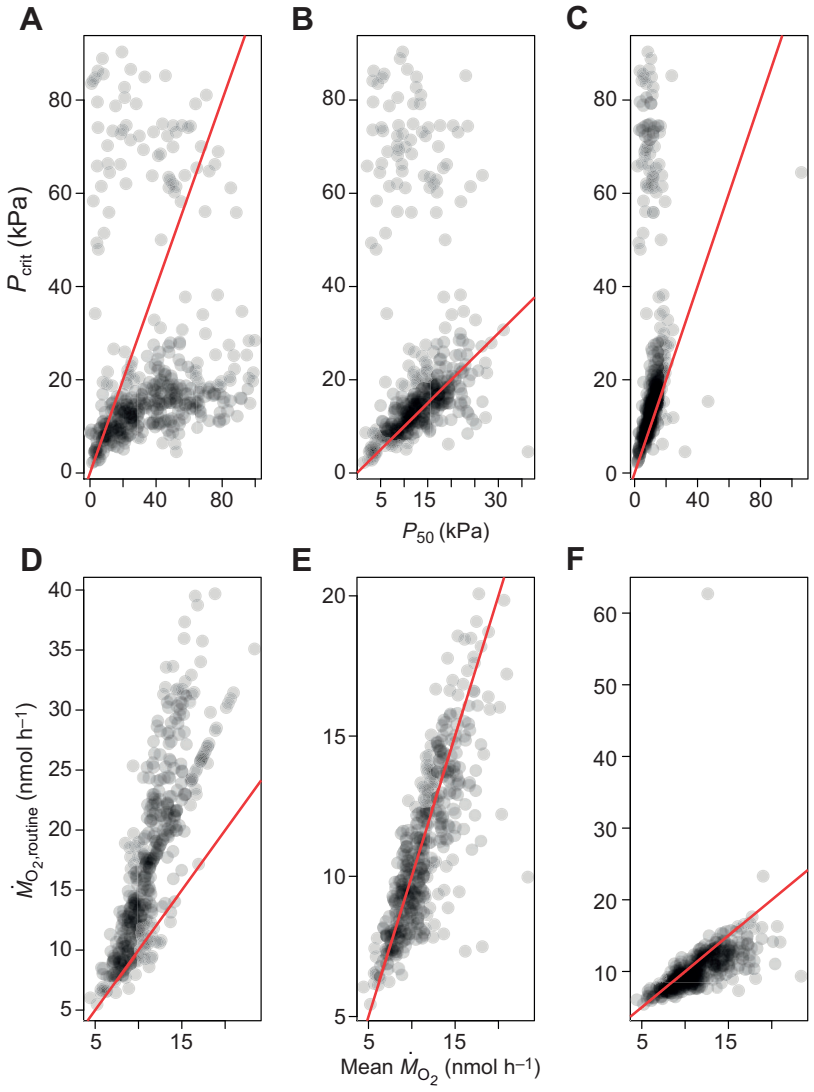

Fig. 1. Comparison of two commonly employed methods assessing the critical value of P_{O_2} and the average oxygen consumption rate by individuals of *Danio rerio* larvae in normoxia. P_{crit} and mean oxygen consumption rate (mean $\dot{M}_{O_2}$) are estimated from piece-wise linear regressions; P_{50} and $\dot{M}_{O_2,routine}$ are estimated from the Michaelis–Menten equation under different Hill numbers: h=1 (A,D), h=2 (B,E) and h=3 (C,F). The red line is the reference x=y. See 'Data analysis' section for further details.

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Table 1. ANOVA table (type III sum of squares) of the mixed effect model on the effects of rearing temperature, test temperature, ploidy and their interactions on routine oxygen consumption rates in *Danio rerio* larvae

Factor	χ^2	d.f.	*P*
(Intercept)	2.7978	1	0.0944
T_{test}	0.9833	1	0.3214
T_{rear}	4.5315	1	0.0333
Ploidy	5.1782	1	0.0229
T_{test}×T_{rear}	3.7926	1	0.0515
T_{test}×ploidy	5.0648	1	0.0244
T_{rear}×ploidy	6.2966	1	0.0121
T_{test}×T_{rear}×ploidy	6.1505	1	0.0131

T_{test}, test temperature; T_{rear}, rearing temperature. Round of fertilization is included as a random factor. Ploidy is expressed as per cent induction of 3n (see 'Ploidy determination' section for further details).
Note: estimates based on 422 observations.

induction efficiency obtained for a batch of that individual. The induction efficiency ranged between 63.3 and 100% across batches (see also above). Diploid larvae were encoded as having a 0% efficiency. As random effect, we used round of fertilization. In preliminary analyses we examined the larvae body length among experimental treatments. Body length differed with rearing temperature (F=27.650, P<0.0001) but not with ploidy level (F=0.155, P=0.6937). Body length was highest at 26.5°C, and lowest at 29.5°C (Fig. S2). Although the smaller length at high temperatures is consistent with the temperature–size rule, the difference was very small and temperature explained 9.7% of the variation in measured body size. We also tested for an effect of body mass (estimated as length cubed) on $\dot{M}_{O_2,routine}$ and P_{50}. We found no consistent effect of body mass. Instead, body mass appeared as a significant factor in diploids and effects of body mass were especially pronounced in larvae reared at 29.5°C. While a positive relationship between $\dot{M}_{O_2,routine}$ and body mass was observed for diploid larvae, no such relationship was found for triploids. Given a possible body size influence on the modelled relationships on $\dot{M}_{O_2,routine}$, we included body mass as a covariate. We found no qualitative differences when comparing the model on these $\dot{M}_{O_2,routine}$ values corrected for body mass with a model based on uncorrected values and which did not include body length as a factor (Tables S1 versus S2). Hence, we chose to present the model based on uncorrected values in our paper. In our analysis of the P_{50} values we similarly ran preliminary analyses but here we did not find any influence of body mass on the P_{50} value. All tests were based on the restricted maximum likelihood (REML) approach. The Akaike information criterion (AICc) and its variants (ΔAICc, AICcWt) were used to find best-fitting model for observed data.

RESULTS

Comparison of methodological approaches

A comparison of two models determining the critical value of P_{O_2} during oxygen consumption measurements showed that P_{50} (derived from the Michaelis–Menten equation) correlates best to P_{crit} (breakpoint in piece-wise linear regression) under h=2 (Hill number) (Fig. 1B). Similarly, the fitted $\dot{M}_{O_2,routine}$ based on the Michaelis–Menten equation under h=2 also corresponded best to the mean oxygen consumption rate displayed by the larvae at oxygen levels above 60% saturation (Fig. 1E). This indicates that the $\dot{M}_{O_2}$ versus P_{O_2} profile in zebrafish larvae approximates a sigmoidal relationship. As we found both methodological approaches highly comparable, in further analyses we used the constants (P_{50} and $\dot{M}_{O_2,routine}$) derived from the Michaelis–Menten equation with h=2 for each of the 422 larvae.

Routine oxygen consumption rates in normoxia

As expected, there was a general increase in $\dot{M}_{O_2,routine}$ with test temperature for both diploid and triploid larvae. Nevertheless, diploid larvae revealed a different response to test temperature, showing a larger increase in metabolic rate at 29.5°C and a larger decrease at 23.5°C compared with triploids. This difference between ploidies in their thermal response of $\dot{M}_{O_2,routine}$ increased with rearing temperature and was most pronounced in larvae reared at 29.5°C (three-way interaction between ploidy, test temperature and rearing temperature: P=0.0131; Table 1; Fig. 2). The thermal sensitivity of oxygen consumption rates expressed as activation energy (E_a) or Q_{10} value (Table 2) decreased with rearing temperature in triploids, indicating an increasingly lower thermal

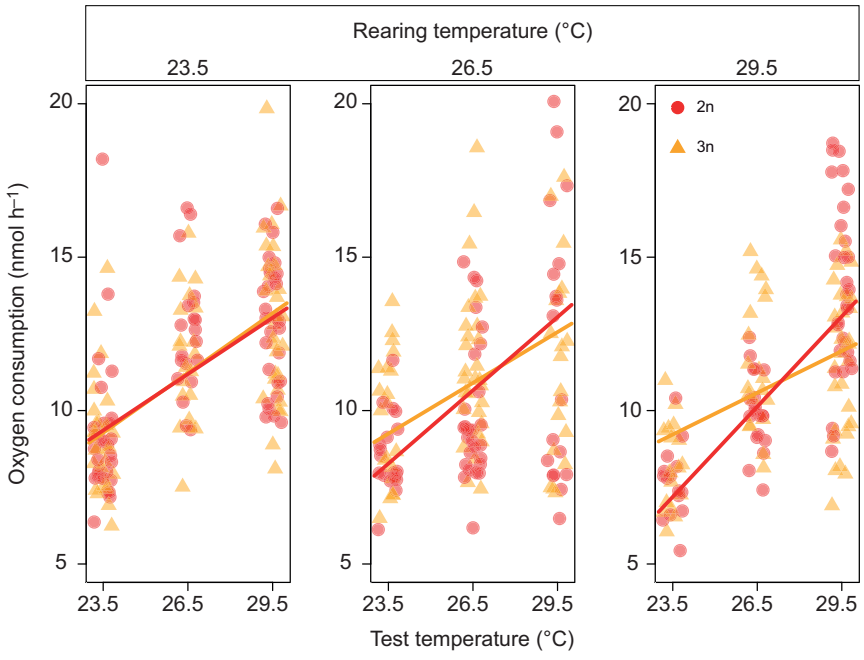

Fig. 2. Routine oxygen consumption rate per individual estimated from the Michaelis–Menten equation in diploid and triploid *Danio rerio* larvae under different rearing and test temperatures. Fitted lines are based on a mixed effects model (Table 1, Table S1). Separate lines were fitted for diploid (2n; i.e. 0% ploidy induction efficiency) and triploid (3n) larvae (i.e. 100% ploidy induction efficiency). Note that test temperature on the *x*-axis was horizontally jittered to prevent overlapping of individual data points.

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Table 2. Temperature sensitivity expressed as activation energy and Q_{10} value for routine oxygen consumption rates and critical value of P_{O_2} measured in diploid (2n) and triploid (3n) *Danio rerio* larvae at different rearing temperatures

	Ploidy	Rearing temperature	E_a (eV)	Q_{10}
Routine oxygen consumption rates	2n	23.5	0.4406	1.7402
		26.5	0.5620	2.1392
		29.5	0.6835	2.7061
	3n	23.5	0.4570	1.8008
		26.5	0.4016	1.6667
		29.5	0.3461	1.5362
Critical value of P_{O_2}	2n	23.5	1.0243	2.9522
		26.5	1.0243	3.1011
		29.5	1.0243	3.2734
	3n	23.5	1.0243	2.6543
		26.5	1.0243	2.7613
		29.5	1.0243	2.8827

E_a, activation energy. Note that for the critical value of P_{O_2}, the effect of test temperature is the same across rearing temperatures and ploidy levels (see Table 3) and therefore a single value for E_a is given.

sensitivity. In contrast, thermal sensitivity in diploids increased with rearing temperature (Table 2).

Hypoxia tolerance

We found opposite effects of rearing and test temperature: values of P_{50} increased significantly with test temperature (P<0.0001; Table 3; Fig. 3), while there was a trend for them to decrease with rearing temperature (P=0.0644). As expected, P_{50} values were significantly higher for the triploids, but the difference was small: on average, triploids had P_{50} values which were 1.3 kPa greater (Table S3; Fig. 3). We did not find significant interactions between ploidy level and temperature, suggesting that effects of rearing and testing temperature on P_{50} values were similar for triploids and diploids.

DISCUSSION

Comparison of methodological approaches

In this study we compared the thermal sensitivity of metabolism between diploid and triploid zebrafish larvae. We compared both the metabolic rate under normoxia as well as the critical values of P_{O_2} (P_{crit}) below which oxygen consumption becomes dependent upon the ambient oxygen partial pressure. A common approach is to establish the P_{crit} by means of fitting two lines to the $\dot{M}_{O_2}$ versus P_{O_2} relationships using a piece-wise linear regression model (Marshall et al., 2013; Reemeyer and Rees, 2019). Wood (2018) recently recommended fitting a Michaelis–Menten equation to the $\dot{M}_{O_2}$ versus P_{O_2} profile. We compared both the Michaelis–Menten approach and the piece-wise regression approach to delineate P_{crit}.

Table 3. ANOVA table (type III sum of squares) of the mixed effects model on the effects of rearing temperature, test temperature and ploidy on critical value of P_{O_2} in *Danio rerio* larvae

Factor	χ^2	d.f.	P
(Intercept)	30.3855	1	<0.0001
T_{test}	210.1365	1	<0.0001
T_{rear}	3.4194	1	0.0644
Ploidy	7.6425	1	0.0057

T_{test}, test temperature; T_{rear}, rearing temperature. Round of fertilization is included as a random factor. Ploidy is expressed as per cent induction of 3n (see 'Ploidy determination' section for further details). Note that the best fitting model did not include interactions; estimates are based on 422 observations.

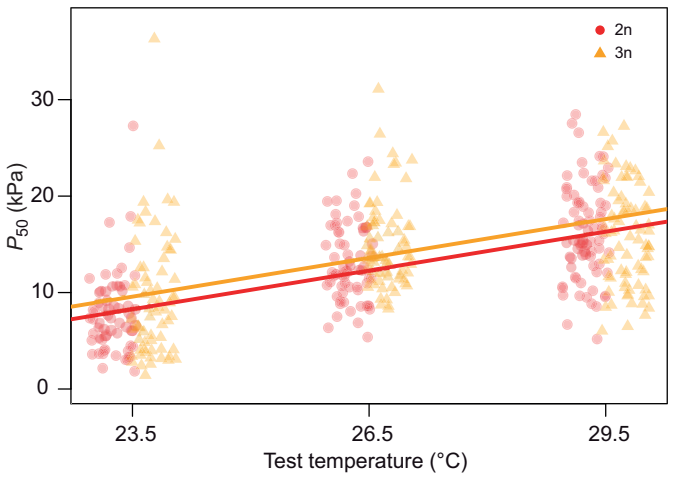

Fig. 3. P_{50} estimated from the Michaelis–Menten equation in diploid and triploid *Danio rerio* larvae under different test temperatures. Fitted lines are based on a mixed effects model (Table 3, Table S3). Separate lines were fitted for diploid (2n; i.e. 0% ploidy induction efficiency) and triploid (3n) larvae (i.e. 100% ploidy induction efficiency). Note that test temperature on the *x*-axis was horizontally jittered to prevent overlapping of individual data points.

Both approaches were highly comparable when we used a modified Michaelis–Menten equation with a Hill number of 2, which approximates a sigmoidal relationship for $\dot{M}_{O_2}$ versus P_{O_2}. Hence, when one uses a Michaelis–Menten equation to fit a curve to the entire $\dot{M}_{O_2}$ versus P_{O_2} profile, we recommend checking whether a hyperbolic or sigmoidal relationship is more appropriate, and use the corresponding Hill number.

Metabolic rate and hypoxia tolerance

We found that the routine metabolic rate of diploid zebrafish larvae reared under the highest temperature (29.5°C) showed a different response to the test temperature than that of triploid larvae. At a test temperature of 29.5°C, the metabolic rate of diploids was higher than that of triploids, but at 23.5°C we found the opposite, with diploids having lower metabolic rates than triploids. Previous studies investigating metabolic rates at relatively high temperatures also reported higher aerobic metabolism in diploids compared with polyploids (Stillwell and Benfey, 1996; Atkins and Benfey, 2008; Maciak et al., 2011; Hermaniuk et al., 2017). The lower metabolic rate of triploids could indicate that for them, oxygen becomes limiting at high temperatures. With increasing temperature, maximum rates of oxygen diffusion can be slightly enhanced as the diffusion coefficient of oxygen in water increases with temperature more so than the concomitant decrease in the solubility of oxygen in water (Dejours, 1981; Verberk et al., 2011). However, because oxygen consumption increases more than rates of oxygen diffusion, boundary layers may become oxygen depleted (Verberk and Atkinson, 2013), and consequently aquatic organisms may nevertheless experience a shortage of oxygen in their environment (Pörtner, 2010; Verberk et al., 2016b; Kielland et al., 2019). Although this situation would be similar for triploids and diploids, diploids may be able to maintain higher metabolic rates for several reasons. First, being composed of more, but smaller cells, they also have more, smaller erythrocytes which may confer them with higher oxygen transport capacities. Second, being composed of smaller cells results in shorter diffusion distances which may help individual cells to take up more oxygen and thus generate a larger pressure gradient between the tissues and their environment, promoting diffusive oxygen uptake (Woods, 1999;

Atkinson et al., 2006; Czarnoleski et al., 2013). While such effects are probably small, our study showed that hypoxia tolerance in zebrafish was slightly different between triploids and diploids, with diploids having lower P_{50} values. We also found that P_{50} values increased with test temperature. In their meta-analysis, Rogers et al. (2016) found a similar effect of temperature and these findings fit well with the idea of a mismatch between oxygen supply and oxygen demand in warmer waters. A recent study on water fleas (*Daphnia magna*) with a similar experimental set-up to ours likewise found a higher P_{crit} in warmer water and concluded that phenotypic plasticity in oxygen supply was insufficient to fully compensate for the increase in oxygen demand (Kielland et al., 2019). Their study focused on the combined effect of developmental and test temperature, but in our study design these could be disentangled. While our results indicate that test temperature had a larger effect (increasing P_{50} and $\dot{M}_{O_2,routine}$), developmental temperature modulated the effects of test temperature ($\dot{M}_{O_2,routine}$) or had opposite effects (P_{50}). The opposing effects of test temperature and development temperature on P_{50} suggest that there was some developmental acclimation, such that warm-reared larvae were more tolerant to hypoxia. The between-ploidy difference in hypoxia tolerance was not prominent although, as expected, triploids displayed a lower P_{50} in oxygen-limited conditions. Hypoxia tolerance has recently been argued to be linked to maximum metabolic rate and aerobic scope (Seibel and Deutsch, 2020). A lower hypoxia tolerance could thus indicate a lower athleticism, which could explain the reduced swimming performance in triploids compared with diploids that has been reported previously (van de Pol et al., 2020). Along the same lines, hypoxia decreased thermal performance in triploid Atlantic salmon, manifested in negative effects on survival, growth and feed intake, as well as changes in swimming behavior (Hansen et al., 2015; Sambraus et al., 2017). Other studies, similarly to our results, revealed only small effects of ploidy on hypoxia tolerance above their thermal optima (Benfey and Devlin, 2018; Sambraus et al., 2018). Nevertheless, these results confirm our hypothesis of greater sensitivity to hypoxia in triploids. Consistent with this notion, previous work on triploid frogs has shown that triploids may compensate and increase their capacity to deliver oxygen by developing larger hearts (Hermaniuk et al., 2017). Other work has shown increased gill ventilation rates in triploid trouts (Lahnsteiner et al., 2019), although this would not be applicable to our zebrafish larvae as they do not yet use their gills for gas exchange (Rombough, 2002).

While oxygen limitation mediated by cell size could explain the higher metabolism of diploids in warmer temperatures, it does not explain why warm-reared diploids had lower metabolic rates at the lower test temperature of 23.5°C. Atkins and Benfey (2008) also demonstrated that the routine metabolic rate in triploid fish (*Salmo salar* and *Salvelinus fontinalis*) is higher at low temperatures and lower at high temperatures when compared with diploids. A higher metabolic rate can be interpreted both as a burden (high maintenance costs) and as a boon (high energy budget to fuel performance) (see Verberk et al., 2016a; Clarke et al., 2003). Given that we measured routine metabolic rate, rather than standard metabolic rate, and that energetic costs related to maintenance of ionic gradients across membranes are expected to be greater in diploids than in triploids, we tentatively interpret the higher metabolic rate of triploids in the cold as beneficial, reflecting a larger energy budget. Similarly, in a comparison between diploid and polyploid cladocerans (Dufresne and Hebert, 1998; Van Geest et al., 2010) and amphibian larvae (Hermaniuk et al., 2016), faster development was observed in polyploids, but only in the colder test temperatures. Thus, the difference in thermal sensitivity between different ploidies goes beyond oxygen limitation in the warm: there appears to be a genuine advantage to polyploidy in the cold. What could this be? Here, we offer two potential mechanisms, related to homeoviscous adaptation of membrane function (Hazel, 1995) and developmental noise (Woods, 2014).

Membrane fluidity is strongly tied to the capacity for transport of oxygen and substrates (Subczynski et al., 1989; Govers-Riemslag et al., 1992; Sidell, 1998; Möller et al., 2016), the activity of many membrane-associated enzymes (Houslay and Gordon, 1982; Ushio and Watabe, 1993), and the permeability of the membrane to cations and water (Singer, 1981). Temperature affects membrane fluidity, but organisms maintain membrane fluidity within some range, by remodeling their cell membranes (e.g. changes in lipid head group composition, acyl chain length and saturation, as well as changes in the cholesterol content of membranes; see Hazel, 1995). Interestingly, ectotherms inhabiting environments characterized by large and rapid thermal fluctuations possess the capacity to rapidly modify (within hours) the lipid composition of their cellular membranes in response to diurnal variations in water temperature (Carey and Hazel, 1989; Williams and Somero, 1996). In our experiments, larvae took 3–5 h to deplete oxygen down to their P_{50}, which should allow for at least some membrane remodeling. While this time period was shorter for animals tested at higher temperatures (see Fig. S3), the higher activity in warmer conditions compensates for this; the rate at which they depleted oxygen down to their P_{50} had a thermal sensitivity that is comparable to most physiological rates (Q_{10} of approximately 2). Thus, in thermally unstable environments the larger membrane surface area associated with smaller cells could create limits to membrane remodeling (for review see Czarnoleski et al., 2013; Czarnoleski et al., 2015a). This mechanism would put warm-reared diploids at a disadvantage relative to triploids when tested at a lower temperature; their lower membrane fluidity and lower activity of membrane-associated enzymes could explain their lower metabolic rates. In principle, cold-reared diploids tested at a high temperature would be expected to have higher metabolic rates compared with triploids, as diploids will face the opposite problem of not being able to reduce their membrane fluidity as rapidly as triploids. However, we found no difference in metabolic rates between ploidy levels for cold-reared larvae tested at the highest temperature. Perhaps the speed at which animals can remodel their membrane is lower when faced with colder temperatures (requiring an increase in fluidity) than when faced with warmer temperatures (requiring a decrease in fluidity), but this will require further study. At least under relatively cold conditions, we found that triploids are able to maintain a higher metabolic rate compared with diploids. Other studies have likewise found that larger cells are disproportionately more productive compared with smaller cells in tissues with active transcription and translation machinery (Ginzberg et al., 2015). Both a larger volume of cytoplasm and a larger genome may increase expression of specific genes and increase rates of protein synthesis (Marguerat and Bahler, 2012; Hessen et al., 2013; Doyle and Coate, 2019); during exposure to low temperatures, such effects may boost overall cellular activity as well as the production of membrane-associated enzymes that may additionally shorten the time of membrane remodeling and therefore improve metabolic efficiency. A second mechanism also draws on the importance of gene translation and transcription but focuses on developmental noise that arises from stochasticity in developmental pathways whose regulation arising from random interactions of molecules becomes increasingly unpredictable and variable with

reduced absolute numbers of molecules (see Woods, 2014). Such stochasticity potentially depresses performance (Blomberg, 2006; Woods, 2014) and increases if the number of molecules that participate in a reaction are lower or if the reaction rates are slower. Thus, having larger cells with higher absolute numbers of molecules can mitigate the effect of slower reaction rates in the cold. Under cold conditions, a large cell size would therefore provide better control of cell-level stochasticity (Verberk et al., 2020), which may also help explain the generally lower thermal sensitivity of the metabolic rate of the triploid larvae observed in our study.

Conclusions

The results of our study show that metabolic consequences of cell size are dependent on temperature. Both developmental temperature and the temperature to which larvae were acutely exposed affected the metabolic response, and differences between ploidy levels were strongest at the highest rearing temperature. At high temperatures, diploids could maintain higher levels of oxygen consumption rates than triploids, which is consistent with the hypothesis that animals composed of larger cells are more susceptible to oxygen limitation in warm waters. This also fits with our finding of a lower hypoxia tolerance in triploids. In contrast, at low temperatures, triploids could maintain higher levels of oxygen consumption rates, which could be related to membrane remodeling and/or molecular stochastic noise. Our results suggest that being composed of larger cells provides metabolic advantages in cold water, while being composed of smaller cells provides metabolic advantages in warm water.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.H., I.L.E.v.d.P., W.C.E.P.V.; Methodology: A.H., I.L.E.v.d.P.; Formal analysis: A.H., W.C.E.P.V.; Investigation: A.H., I.L.E.v.d.P.; Data curation: W.C.E.P.V.; Writing - original draft: A.H.; Writing - review & editing: I.L.E.v.d.P., W.C.E.P.V.; Visualization: A.H., W.C.E.P.V.; Funding acquisition: A.H., W.C.E.P.V.

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Data availability

Raw data from this study are available in the Dryad Digital Repository (Hermaniuk et al., 2020): https://doi.org/10.5061/dryad.2280gb5qw

Supplementary information

Supplementary information available online at
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References

Angilletta, M. J. (2009). *Thermal Adaptation: A Theoretical and Empirical Synthesis*. Oxford: Oxford University Press.

Arendt, J. (2007). Ecological correlates of body size in relation to cell size and cell number: patterns in flies, fish, fruits and foliage. *Biol. Rev.* **82**, 241-256. doi:10.1111/j.1469-185X.2007.00013.x

Atkins, M. E. and Benfey, T. J. (2008). Effect of acclimation temperature on routine metabolic rate in triploid salmonids. *Comp. Biochem. Physiol.* **149A**, 157-161. doi:10.1016/j.cbpa.2007.11.004

Atkinson, D., Morley, S. A. and Hughes, R. N. (2006). From cells to colonies: at what levels of body organization does the 'temperature–size rule' apply? *Evol. Dev.* **8**, 202-214. doi:10.1111/j.1525-142X.2006.00090.x

Bates, D., Maechler, M., Bolker, B. and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67**, 1-48. doi:10.18637/jss.v067.i01

Benfey, T. J. and Devlin, R. H. (2018). Ploidy has minimal effect on hypoxia tolerance at high temperature in rainbow trout (*Oncorhynchus mykiss*). *Physiol. Biochem. Zool.* **91**, 1091-1101. doi:10.1086/700218

Blomberg, C. (2006). Fluctuations for good and bad: the role of noise in living systems. *Phys. Life Rev.* **3**, 133-161. doi:10.1016/j.plrev.2006.06.001

Carey, C. and Hazel, J. R. (1989). Diurnal variation in membrane lipid composition of Sonoran Desert teleosts. *J. Exp. Biol.* **147**, 375-391.

Choleva, L. and Janko, K. (2013). Rise and persistence of animal polyploidy: evolutionary constraints and potential. *Cytogenet. Genome Res.* **140**, 151-170. doi:10.1159/000353464

Clarke, A. (2003). Costs and consequences of evolutionary temperature adaptation. *Trends Ecol. Evol.* **18**, 573-581. doi:10.1016/j.tree.2003.08.007

Clarke, A. and Fraser, K. P. P. (2004). Why does metabolism scale with temperature? *Funct. Ecol.* **18**, 243-251. doi:10.1111/j.0269-8463.2004.00841.x

Czarnoleski, M., Cooper, B. S., Kierat, J. and Angilletta, M. J. (2013). Flies developed small bodies and small cells in warm and thermally fluctuating environments. *J. Exp. Biol.* **216**, 2896-2901. doi:10.1242/jeb.083535

Czarnoleski, M., Dragosz-Kluska, D. and Angilletta, M. J. (2015a). Flies developed smaller cells when temperature fluctuated more frequently. *J. Therm. Biol.* **54**, 106-110. doi:10.1016/j.jtherbio.2014.09.010

Czarnoleski, M., Ejsmont-Karabin, J., Angilletta, M. J. and Kozłowski, J. (2015b). Colder rotifers grow larger but only in oxygenated waters. *Ecosphere* **6**, 164. doi:10.1890/ES15-00024.1

Czarnoleski, M., Labecka, A. M. and Kozłowski, J. (2016). Thermal plasticity of body size and cell size in snails from two subspecies of *Cornu aspersum*. *J. Molluscan. Stud.* **82**, 235-243. doi:10.1093/mollus/eyv059

Czarnoleski, M., Labecka, A. M., Dragosz-Kluska, D., Pis, T., Pawlik, K., Kapustka, F., Kilarski, W. M. and Kozłowski, J. (2018). Concerted evolution of body mass and cell size: similar patterns among species of birds (Galliformes) and mammals (Rodentia). *Biol. Open* **7**, bio029603. doi:10.1242/bio.029603

Czarnoleski, M., Labecka, A. M., Starostová, Z., Sikorska, A., Bonda-Ostaszewska, E., Woch, K., Kubička, L., Kratochvíl, L. and Kozlowski, J. (2017). Not all cells are equal: effects of temperature and sex on the size of different cell types in the Madagascar ground gecko *Paroedura picta*. *Biol. Open* **6**, 1149-1154. doi:10.1242/bio.025817

Dejours, P. (1981). *Principles of Comparative Respiratory Physiology*. Amsterdam, New York: Elsevier/North-Holland Biomedical Press.

Doyle, J. J. and Coate, J. E. (2019). Polyploidy, the nucleotype, and novelty: the impact of genome doubling on the biology of the cell. *Int. J. Plant Sci.* **180**, 1-52. doi:10.1086/700636

Dufresne, F. and Hebert, P. D. N. (1998). Temperature-related differences in life-history characteristics between diploid and polyploid clones of the *Daphnia pulex* complex. *Ecoscience* **5**, 433-437. doi:10.1080/11956860.1998.11682481

Dufresne, F. and Jeffery, N. (2011). A guided tour of large genome size in animals: what we know and where we are heading. *Chromosome Res.* **19**, 925-938. doi:10.1007/s10577-011-9248-x

Ellenby, C. (1953). Oxygen consumption and cell size. A comparison of the rate of oxygen consumption of diploid and triploid prepupae of *Drosophila melanogaster* Meigen. *J. Exp. Biol.* **30**, 475-491.

Fankhauser, G. (1945). The effects of changes in chromosome number on amphibian development. *Q. Rev. Biol.* **20**, 20-78. doi:10.1086/394703

Gardner, J. D., Laurin, M. and Organ, C. L. (2020). The relationship between genome size and metabolic rate in extant vertebrates. *Philos. T. Roy. Soc. B.* **375**, 20190146. doi:10.1098/rstb.2019.0146

Ginzberg, M. B., Kafri, R. and Kirschner, M. (2015). Cell biology. On being the right (cell) size. *Science* **348**, 1245075. doi:10.1126/science.1245075

Goniakowska, L. (1970). The respiration of erythrocytes of some amphibians *in vitro*. *Bull. Acad. Pol. Sci., Ser. Biol.* **18**, 793-797.

Govers-Riemslag, J. W. P., Janssen, M. P., Zwaal, R. F. A. and Rosing, J. (1992). Effect of membrane fluidity and fatty acid composition on the prothrombin-converting activity of phospholipid vesicles. *Biochemistry* **31**, 10000-10008. doi:10.1021/bi00156a020

Gregory, T., Hebert, P. and Kolasa, J. (2000). Evolutionary implications of the relationship between genome size and body size in flatworms and copepods. *Heredity* **84**, 201-208. doi:10.1046/j.1365-2540.2000.00661.x

Gregory, T. R. (2001). Coincidence, coevolution, or causation? DNA content, cell size, and the C-value enigma. *Biol. Rev.* **76**, 65-101. doi:10.1017/S1464793100005595

Gregory, T. R. (2002). A bird's-eye view of the C-value enigma: genome size, cell size, and metabolic rate in the class Aves. *Evolution* **56**, 121-130. doi:10.1111/j.0014-3820.2002.tb00854.x

Gregory, T. R. (2003). Variation across amphibian species in the size of the nuclear genome supports a pluralistic, hierarchical approach to the C-value enigma. *Biol. J. Linn. Soc.* **79**, 329-339. doi:10.1046/j.1095-8312.2003.00191.x

Gregory, T. R. (2005). The C-value enigma in plants and animals: a review of parallels and appeal for partnership. *Ann. Bot.-London* **95**, 133-146. doi:10.1093/aob/mci009

Hansen, T. J., Olsen, R. E., Stien, L., Oppedal, F., Torgersen, T., Breck, O., Remen, M., Vågseth, T. and Fjelldal, P. G. (2015). Effect of water oxygen level on

performance of diploid and triploid Atlantic salmon post-smolts reared at high temperature. *Aquaculture* **435**, 354-360. doi:10.1016/j.aquaculture.2014.10.017
Havird, J. C., Neuwald, J. L., Shah, A. A., Mauro, A., Marshall, C. A. and Ghalambor, C. K. (2020). Distinguishing between active plasticity due to thermal acclimation and passive plasticity due to Q_{10} effects: why methodology matters. *Funct. Ecol.* **34**, 1015-1028. doi:10.1111/1365-2435.13534
Hazel, J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annu. Rev. Physiol.* **57**, 19-42. doi:10.1146/annurev.ph.57.030195.000315
Hermaniuk, A., Rybacki, M. and Taylor, J. R. E. (2016). Low temperature and polyploidy result in larger cell and body size in an ectothermic vertebrate. *Physiol. Biochem. Zool.* **89**, 118-129. doi:10.1086/684974
Hermaniuk, A., Rybacki, M. and Taylor, J. R. E. (2017). Metabolic rate of diploid and triploid edible frog *Pelophylax esculentus* correlates inversely with cell size in tadpoles but not in frogs. *Physiol. Biochem. Zool.* **90**, 230-239. doi:10.1086/689408
Hermaniuk, A., van de Pol, I. L. E. and Verberk, W. (2020). Data from: Are acute and acclimated thermal effects on metabolic rate modulated by cell size? A comparison between diploid and triploid zebrafish larvae. *Dryad Dataset* doi:10.5061/dryad.2280gb5qw
Hessen, D. O., Daufresne, M. and Leinaas, H. (2013). Temperature–size relations from the cellular-genomic perspective. *Biol. Rev.* **88**, 476-489. doi:10.1111/brv.12006
Hoefnagel, K. N. and Verberk, W. C. E. P. (2015). Is the temperature–size rule mediated by oxygen in aquatic ectotherms? *J. Therm. Biol.* **54**, 56-65. doi:10.1016/j.jtherbio.2014.12.003
Houslay, M. D. and Gordon, L. M. (1982). The activity of adenylate cyclase is regulated by the nature of its lipid environment. *Curr. Top. Membr. Trans.* **18**, 179-231. doi:10.1016/S0070-2161(08)60531-6
Hyndman, C. A., Kieffer, J. D. and Benfey, T. J. (2003). Physiology and survival of triploid brook trout following exhaustive exercise in warm water. *Aquaculture* **221**, 629-643. doi:10.1016/S0044-8486(03)00119-4
Kielland, Ø. N., Bech, B. and Einum, S. (2019). Warm and out of breath: thermal phenotypic plasticity in oxygen supply. *Funct. Ecol.* **33**, 2142-2149. doi:10.1111/1365-2435.13449
Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310. doi:10.1002/aja.1002030302
Kozłowski, J., Czarnoleski, M., Francois-Krassowska, A., Maciak, S. and Pis, T. (2010). Cell size is positively correlated between different tissues in passerine birds and amphibians, but not necessarily in mammals. *Biol. Lett.* **6**, 792-796. doi:10.1098/rsbl.2010.0288
Kozłowski, J., Konarzewski, M. and Czarnoleski, M. (2020). Coevolution of body size and metabolic rate in vertebrates: a life-history perspective. *Biol. Rev.* **95**, 1393-1141. doi:10.1111/brv.12615
Kozłowski, J., Konarzewski, M. and Gawelczyk, A. T. (2003). Cell size as a link between noncoding DNA and metabolic rate scaling. *Proc. Natl. Acad. Sci. USA* **100**, 14080-14085. doi:10.1073/pnas.2334605100
Lahnsteiner, F., Lahnsteiner, E. and Kletzl, M. (2019). Differences in metabolism of triploid and diploid *Salmo trutta f. lacustris* under acclimation conditions and after exposure to stress situations. *Aquac. Res.* **50**, 2444-2459. doi:10.1111/are.14198
Licht, L. E. and Bogart, J. P. (1990). Comparative rates of oxygen consumption and water loss in diploid and polyploid salamander genus Ambystoma. *Comp. Biochem. Physiol.* **97A**, 569-572. doi:10.1016/0300-9629(90)90129-G
Lukose, R. L. and Reinert, H. K. (1998). Absence of cold acclimation response in gray treefrogs, *Hyla chrysoscelis* and *Hyla versicolor*. *J. Herpetol.* **32**, 283-285. doi:10.2307/1565311
Maciak, S., Janko, K., Kotusz, J., Choleva, L., Boroń, A., Juchno, D., Kujawa, R., Kozłowski, J. and Konarzewski, M. (2011). Standard metabolic rate (SMR) is inversely related to erythrocyte and genome size in allopolyploid fish of the *Cobitis taenia* hybrid complex. *Funct. Ecol.* **25**, 1072-1078. doi:10.1111/j.1365-2435.2011.01870.x
Marguerat, S. and Bahler, J. (2012). Coordinating genome expression with cell size. *Trends Genet.* **28**, 560-565. doi:10.1016/j.tig.2012.07.003
Marshall, D. J., Bode, M. and White, C. R. (2013). Estimating physiological tolerances – a comparison of traditional approaches to nonlinear regression techniques. *J. Exp. Biol.* **216**, 2176-2182. doi:10.1242/jeb.085712
Miettinen, T. P., Caldez, M. J., Kaldis, P. and Björklund, M. (2017). Cell size control – a mechanism for maintaining fitness and function. *BioEssays* **39**, 1700058. doi:10.1002/bies.201700058
Möller, M. N., Li, Q., Chinnaraj, M., Cheung, H. C., Lancaster, J. R. and Denicola, A. (2016). Solubility and diffusion of oxygen in phospholipid membranes. *BBA-Biomembranes* **1858**, 2923-2930. doi:10.1016/j.bbamem.2016.09.003
Monnickendam, M. A. and Balls, M. (1973). The relationship between cell sizes, respiration rates and survival of amphibian tissues in long-term organ cultures. *Comp. Biochem. Physiol.* **44**, 871-880. doi:10.1016/0300-9629(73)90150-3
Muggeo, V. M. R. (2008). Segmented: an R Package to fit regression models with broken-line relationships. *R News* **8**, 20-25.
Otto, S. P. (2007). The evolutionary consequences of polyploidy. *Cell* **131**, 452-462. doi:10.1016/j.cell.2007.10.022
Parsons, G. R. (1993). Comparisons of triploid and diploid white crappies. *Trans. Amer. Fish Soc.* **122**, 237-243. doi:10.1577/1548-8659(1993)122<0237:COTADW>2.3.CO;2
Pörtner, H.-O. (2010). Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. *J. Exp. Biol.* **213**, 881-893. doi:10.1242/jeb.037523
Reemeyer, J. E. and Rees, B. B. (2019). Standardizing the determination and interpretation of P_{crit} in fishes. *J. Exp. Biol.* **222**, jeb.210633. doi:10.1242/jeb.210633
Rogers, N. J., Urbina, M. A., Reardon, E. E., McKenzie, D. J. and Wilson, R. W. (2016). A new analysis of hypoxia tolerance in fishes using a database of critical oxygen level (P_{crit}). *Conserv. Physiol.* **4**, cow012. doi:10.1093/conphys/cow012
Rolfe, D. F. and Brown, G. C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol. Rev.* **77**, 731-758. doi:10.1152/physrev.1997.77.3.731
Rombough, P. (2002). Gills are needed for ionoregulation before they are needed for O_2 uptake in developing zebrafish, *Danio rerio*. *J. Exp. Biol.* **205**, 1787-1794.
Rubalcaba, J. G., Verberk, W. C. E. P., Hendriks, A J., Saris, B. and Woods, H. A. (2021). Oxygen limitation may affect the temperature and size dependence of metabolism in aquatic ectotherms. *Proc. Natl Acad. Sci. USA* (in press) doi:10.1073/pnas.2003292117
Sambraus, F., Olsen, R. E., Remen, M., Hansen, T. J., Torgersen, T. and Fjelldal, P. G. (2017). Water temperature and oxygen: the effect of triploidy on performance and metabolism in farmed Atlantic salmon (*Salmo salar* L.) post-smolts. *Aquaculture* **473**, 1-12. doi:10.1016/j.aquaculture.2017.01.024
Sambraus, F., Remen, M., Olsen, R. E., Hansen, T. J., Waagbø, R., Torgersen, T., Lock, E. J., Imsland, A., Fraser, T. W. K. and Fjelldal, P. G. (2018). Changes in water temperature and oxygen: the effect of triploidy on performance and metabolism in large farmed Atlantic salmon. *Aquacult. Environ. Interact.* **10**, 157-172. doi:10.3354/aei00260
Seibel, B. A. and Deutsch, C. (2020). Oxygen supply capacity in animals evolves to meet maximum demand at the current oxygen partial pressure regardless of size or temperature. *J. Exp. Biol.* **223**, jeb210492. doi:10.1242/jeb.210492
Sezaki, K., Watabe, S., Tsukamoto, K. and Hashimoto, K. (1991). Effects of increase in ploidy status on respiratory function of ginbuna, *Carassius auratus langsdorfi* (Cyprinidae). *Comp. Biochem. Physiol.* **99A**, 123-127. doi:10.1016/0300-9629(91)90246-9
Shpigel, M., Barber, B. J. and Mann, R. (1992). Effects of elevated temperature on growth, gametogenesis, physiology, and biochemical composition in diploid and triploid Pacific oysters, *Crassostrea gigas* Thunberg. *J. Exp. Mar. Biol. Ecol.* **161**, 15-25. doi:10.1016/0022-0981(92)90186-E
Sidell, B. D. (1998). Intracellular oxygen diffusion: the roles of myoglobin and lipid at cold body temperature. *J. Exp. Biol.* **201**, 1119-1128.
Singer, M. (1981). Permeability of phosphatidylcholine and phosphatidylethanolamine bilayers. *Chem. Phys. Lipids* **28**, 253-267. doi:10.1016/0009-3084(81)90012-8
Smith, T. W., Kron, P. and Martin, S. L. (2018). flowPloidy: an R package for genome size and ploidy assessment of flow cytometry data. *Appl. Plant Sci.* **6**, e01164. doi:10.1002/aps3.1164
Starostová, Z., Kubička, L., Kozłowski, J., Konarzewski, M. and Kratochvíl, L. (2009). Cell size but not genome size affects scaling of metabolic rate in eyelid geckos. *Am. Nat.* **174**, 100-105. doi:10.1086/603610
Stillwell, E. J. and Benfey, T. J. (1996). Hemoglobin level, metabolic rate and swimming efficiency in female triploid brook trout (*Salvelinus fontinalis*). *Fish. Physiol. Biochem.* **15**, 377-383. doi:10.1007/BF01875580
Subczynski, W. K., Hyde, J. S. and Kusumi, A. (1989). Oxygen permeability of phosphatidylcholine–cholesterol membranes. *Proc. Natl. Acad. Sci. USA* **86**, 4474-4478. doi:10.1073/pnas.86.12.4474
Suresh, A. V. and Sheehan, R. J. (1998). Muscle fibre growth dynamics in diploid and triploid rainbow trout. *J. Fish Biol.* **52**, 570-587. doi:10.1111/j.1095-8649.1998.tb02018.x
Szarski, H. (1983). Cell size and the concept of wasteful and frugal evolutionary strategies. *J. Theor. Biol.* **105**, 201-209. doi:10.1016/S0022-5193(83)80002-2
Ushio, H. and Watabe, S. (1993). Effects of temperature acclimation on Ca^{2+}-ATPase of the carp sarcoplasmic reticulum. *J. Exp. Zool.* **265**, 9-17. doi:10.1002/jez.1402650103
van de Pol, I. L. E., Flik, G. and Verberk, W. C. E. P. (2020). Triploidy in zebrafish larvae: effects on gene expression, cell size and cell number, growth, development and swimming performance. *PLoS ONE* **15**, e0229468. doi:10.1371/journal.pone.0229468
Van Geest, G. J., Sachse, R., Brehm, M., van Donk, E. and Hessen, D. O. (2010). Maximizing growth rate at low temperatures: RNA:DNA allocation strategies and life history traits of Arctic and temperate *Daphnia*. *Polar Biol.* **33**, 1255-1262. doi:10.1007/s00300-010-0814-z
Verberk, W. C. E. P. and Atkinson, D. (2013). Why polar gigantism and Palaeozoic gigantism are not equivalent: effects of oxygen and temperature on the body size of ectotherms. *Funct. Ecol.* **27**, 1275-1285. doi:10.1111/1365-2435.12152

Verberk, W. C. E. P., Atkinson, D., Hoefnagel, K. N., Hirst, A. G., Horne, C. R. and Siepel, H. (2020). Shrinking body sizes in response to warming: explanations for the temperature–size rule with special emphasis on the role of oxygen. *Biol. Rev.* doi:10.1111/brv.12653

Verberk, W. C. E. P., Bartolini, F., Marshall, D. J., Pörtner, H.-O., Terblanche, J. S., White, C. R. and Giomi, F. (2016a). Can respiratory physiology predict thermal niches? *Ann. N. Y. Acad. Sci.* **1365**, 73-88. doi:10.1111/nyas.12876

Verberk, W. C. E. P., Bilton, D. T., Calosi, P. and Spicer, J. I. (2011). Oxygen supply in aquatic ectotherms: partial pressure and solubility together explain biodiversity and size patterns. *Ecology* **92**, 1565-1572. doi:10.1890/10-2369.1

Verberk, W. C. E. P., Overgaard, J., Ern, R., Bayley, M., Wang, T., Boardman, L. and Terblanche, J. S. (2016b). Does oxygen limit thermal tolerance in arthropods? A critical review of current evidence. *Comp. Biochem. Physiol. A. Physiol.* **192**, 64-78. doi:10.1016/j.cbpa.2015.10.020

Vinogradov, A. E. (1995). Nucleotypic effect in homeotherms: body-mass-corrected basal metabolic rate of mammals is related to genome size. *Evolution* **49**, 1249-1259. doi:10.1111/j.1558-5646.1995.tb04451.x

Walczyńska, A., Labecka, A. M., Sobczyk, M., Czarnoleski, M. and Kozłowski, J. (2015). The temperature–size rule in *Lecane inermis* (Rotifera) is adaptive and driven by nuclei size adjustment to temperature and oxygen combinations. *J. Therm. Biol.* **54**, 78-85. doi:10.1016/j.jtherbio.2014.11.002

Westerfield, M. (2000). *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio)*. Eugene, OR: University of Oregon Press.

Williams, E. E. and Somero, G. N. (1996). Seasonal-, tidal cycle- and microhabitat-related variation in membrane order of phospholipid vesicles from gills of the mussel *Mytilus californianus*. *J. Exp. Biol.* **199**, 1587-1596.

Wood, C. M. (2018). The fallacy of the P_{crit} – are there more useful alternatives? *J. Exp. Biol.* **221**, jeb163717. doi:10.1242/jeb.163717

Woods, H. A. (1999). Egg-mass size and cell size: effects of temperature on oxygen distribution. *Am. Zool.* **39**, 244-252. doi:10.1093/icb/39.2.244

Woods, H. A. (2014). Mosaic physiology from developmental noise: within-organism physiological diversity as an alternative to phenotypic plasticity and phenotypic flexibility. *J. Exp. Biol.* **217**, 35-45. doi:10.1242/jeb.089698