Journal of Insect Physiology 150 (2023) 104559

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Oxygen and temperature affect cell sizes differently among tissues and between sexes of *Drosophila melanogaster*

Marcin Czarnoleski [a,1,*], Ewa Szlachcic [a,2], Valeriya Privalova [a,3], Anna Maria Labecka [a,4], Anna Sikorska [a,5], Łukasz Sobczyk [a,6], John VandenBrooks [b,7], Michael J. Angilletta Jr [b,8]

[a] *Life History Evolution Group, Institute of Environmental Sciences, Faculty of Biology, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland*
[b] *Arizona State University, Tempe, AZ, USA*

ARTICLE INFO

Keywords:
Cell size
Temperature
Oxygen
Temperature-size rule
TSR
Sexual dimorphism

ABSTRACT

Spatio-temporal gradients in thermal and oxygen conditions trigger evolutionary and developmental responses in ectotherms' body size and cell size, which are commonly interpreted as adaptive. However, the evidence for cell-size responses is fragmentary, as cell size is typically assessed in single tissues. In a laboratory experiment, we raised genotypes of *Drosophila melanogaster* at all combinations of two temperatures (16 °C or 25 °C) and two oxygen levels (10% or 22%) and measured body size and the sizes of cells in different tissues. For each sex, we measured epidermal cells in a wing and a leg and ommatidial cells of an eye. For males, we also measured epithelial cells of a Malpighian tubule and muscle cells of a flight muscle. On average, females emerged at a larger body size than did males, having larger cells in all tissues. Flies of either sex emerged at a smaller body size when raised under warm or hypoxic conditions. Development at 25 °C resulted in smaller cells in most tissues. Development under hypoxia resulted in smaller cells in some tissues, especially among females. Altogether, our results show thermal and oxygen conditions trigger shifts in adult size, coupled with the systemic orchestration of cell sizes throughout the body of a fly. The nature of these patterns supports a model in which an ectotherm adjusts its life-history traits and cellular composition to prevent severe hypoxia at the cellular level. However, our results revealed some inconsistencies linked to sex, cell type, and environmental parameters, which suggest caution in translating information obtained for single type of cells to the organism as a whole.

1. Introduction

Environments represent a wide range of abiotic variables, including radiation, water content, temperature and oxygen availability (Angilletta, 2009; Harrison et al., 2018; Huang et al., 2021; Kellermann et al., 2012). Over large time scales, changes in these parameters on a global scale have impacted the ecology and evolution of organisms (Harrison et al., 2010; Hunt and Roy, 2006; Poulsen et al., 2015; Scotese et al., 2021). Human impacts have accelerated these effects on an unprecedented scale (Jaureguiberry et al., 2022; Pörtner et al., 2022). For ectotherms, changes in thermal and oxygen conditions impose immediate physiological responses of cells and tissues by influencing their metabolic demand and supply, and initiate cascading responses that alter ecological interactions, such as competition, predation, and parasitism

* Corresponding author.
E-mail addresses: marcin.czarnoleski@uj.edu.pl (M. Czarnoleski), ewa.szlachcic@uj.edu.pl (E. Szlachcic), valeriya.privalova@doctoral.uj.edu.pl (V. Privalova), anna.labecka@uj.edu.pl (A. Maria Labecka), anna.sikorska@uj.edu.pl (A. Sikorska), lukasz.sobczyk@uj.edu.pl (Ł. Sobczyk), john.vandenbrooks@asu.edu (J. VandenBrooks), ma@asu.edu (M.J. Angilletta Jr).
[1] MC: 0000-0003-2645-0360.
[2] ES: 0000-0002-4179-4068.
[3] VP: 0000-0002-8496-7352.
[4] AML: 0000-0002-8810-7093.
[5] AS: 0000-0003-1668-2264.
[6] ŁS: 0000-0002-4804-3419.
[7] JV: 0000-0001-6357-1415.
[8] MA: 0000-0002-3181-8361.

https://doi.org/10.1016/j.jinsphys.2023.104559
Received 6 July 2023; Received in revised form 24 August 2023; Accepted 25 August 2023
Available online 26 August 2023

(Angilletta, 2009; Woods et al., 2022). The ability to survive and reproduce in the face of a changing environment depends on existing physiological tolerance and the ability to respond adaptively through phenotypic plasticity. In the long term, local populations may adapt to these changes through microevolutionary processes. Abundant evidence shows that life-history traits vary in ectotherms along environmental gradients. For example, comparisons of different populations of the rotifer *Keratella cochlearis* reported that body size decreased under hypoxic and warmer conditions (Czarnoleski et al., 2015b). At large geographic scales, many ectotherms show a systematic increase in body size at higher latitudes (Ashton, 2002; Ashton and Feldman, 2003; De Jong and Bochdanovits, 2003; Sears and Angilletta, 2004; Zwaan et al., 2000), reminiscent of Bergmann's rule, which was originally formulated for endotherms under the assumption that a larger animal can thermoregulate better in a cold climate (Meiri, 2011). Ectothermic animals are also strikingly consistent in their developmental responses to thermal and oxygen conditions. According to the temperature-size rule (TSR), ectotherms tend to develop more slowly under cold conditions, but ultimately achieve larger body sizes at maturity compared to their warm counterparts (Atkinson, 1994; Horne et al., 2015; Klok and Harrison, 2013). Experiments also commonly show slower growth and smaller adult size in ectotherms exposed to low-oxygen conditions during development (Harrison et al., 2018; Harrison et al., 2015; Heinrich et al., 2011; Peck and Maddrell, 2005). Life-history traits are strong determinants of Darwinian fitness (Stearns, 1992), so there is little doubt that their response to environmental factors manifests adaptive phenomena rather than fundamental physiological constraints (Angilletta and Dunham, 2003; Kozlowski et al., 2004). However, while this expectation may seem well-founded on conceptual grounds, the adaptive mechanism underlying these patterns of plasticity remain unknown.

Variation in body size is the most conspicuous feature of organisms, but it cannot be fully understood without considering its rarely studied component: variation in the cellular composition of organs or tissues. The emergence of new life forms on Earth and the diversification of organisms has been closely linked to changes in their cellular composition (Grosberg and Strathmann, 2007; Valentine et al., 1994). Such evolution in turn has occurred in close association with changes in the molecular pathways controlling the cell cycle during development (Roustan et al., 2016; van Dam et al., 2011). Close links between variation in life history traits and cellularity have been supported by comparisons of populations within species. For example, when the subtropical African population of *Drosophila melanogaster* expanded to occupy different continents and latitudes, they evolved latitudinal clines in body size, cell size and the activity of cell-cycle regulators (De Jong and Bochdanovits, 2003; Fabian et al., 2012). Large size confers clear fitness consequences, such as protection from predators, high potential for fertility, and attractiveness to mates (Schmidt-Nielsen, 1984; Stearns, 1992). The sizes and shapes of cells are strongly associated with the functions of tissues and organs (Ginzberg et al., 2015). Therefore, the evidence that body size, cell phenotypes, and cell-cycle controllers vary in unison among organisms provides a strong argument for the adaptive nature of this covariation. Following a theoretical framework, which we refer to as the theory of optimal cell size (TOCS), a change in cell size should bring physiological costs and benefits that balance differently with shifts in the metabolic demand and supply of resources and oxygen (Antoł et al., 2020; Atkinson et al., 2006; Czarnoleski et al., 2015b; Czarnoleski et al., 2018; Davison, 1955, 1956; Glazier, 2022; Hermaniuk et al., 2017; Kozlowski et al., 2020; Kozlowski et al., 2003; Liu et al., 2022; Miettinen et al., 2017; Szarski, 1983; Szlachcic and Czarnoleski, 2021; Szlachcic et al., 2023a; Verspagen et al., 2020; Walczynska et al., 2015). Smaller cells offer larger cell surface areas for transport and communication, but at the same time require more molecular work to maintain plasma membranes and electro-chemical potentials. For an ectotherm, a balance between metabolic demand and supply changes rapidly along a gradient of temperature and oxygen supply. TOCS predicts that warm and hypoxic environments should favour ectotherms with smaller cells, which would help to deliver oxygen to tissue and ultimately to mitochondria. However, the fitness consequences of variation in cell size remains incompletely understood, because the study of cell size and its effects on organismal performance have been extremely rare. This situation is not surprising, since studying such cell size in an ecological-evolutionary context is logistically challenging, requiring the comparison of cell sizes among organisms, and development of histological techniques for each tissue. The shortage of information on cell size poses the risk of building an oversimplified view that obscures the complex nature of the links among cell size, life history, and Darwinian fitness. Most of the evidence for variation in cell size among organisms comes from measurements of a single type of cells (e.g., Arendt, 2007; Calboli et al., 2003; Czarnoleski et al., 2015a; Verberk et al., 2022), with the assumption that cell sizes are systemically orchestrated throughout the organism. However, emerging works has shown that cell size can vary either consistently (Czarnoleski et al., 2018; Privalova et al., 2023; Szlachcic et al., 2023b) or inconsistently (Antoł et al., 2020; Czarnoleski et al., 2016; Czarnoleski et al., 2017; Kozlowski et al., 2010; Savage et al., 2007) among tissues, suggesting that broader generalisations cannot be made. To address the need to understand how cell sizes in diverse tissues contribute to the plasticity of body size, we performed a laboratory experiment with *D. melanogaster*, raising flies of distinct genotypes in four combinations of thermal and oxygen conditions, focusing on the relationship between body size and cell size in five tissues. We also considered sex-related differences in body size and cell sizes. Our main goal was to explore whether variation in cell size, caused by developmental mechanisms related to either abiotic factors or sex, occur in a systemic way throughout the body. Ultimately, we integrate information about patterns at the levels of organismal size and cell size into one phenomenon, which is discussed in light of its potential impact on fitness.

2. Materials and methods

2.1. Flies

We used ten isofemale lines which were created in 2017 by sibmating over 29 generations from a wild population of *D. melanogaster* near Kraków, Poland (49°58′00.8″ N, 20°29′54.1″ E). Lines are maintained in a small fly stock at the Institute of Environmental Sciences (Jagiellonian University, Kraków) as a source of genotypes for studies (Szlachcic and Czarnoleski, 2021; Szlachcic et al., 2023a; Szlachcic et al., 2023b). The flies were kept in polyurethane 40-ml vials with foam plugs and cornmeal yeast medium (Bloomington Drosophila Stock Center, Bloomington, USA) in thermal cabinets (POL-EKO, Wodzisław Śląski, Poland), in 20.5 °C, 70% relative humidity and a 12 h:12 h L:D photoperiod. Transfers were performed every three weeks to prevent generational overlap.

2.2. Experimental design

Following a previous approach (e.g., Szlachcic et al., 2023b), we sampled stock flies to produce two consecutive generations of each line under conditions of controlled larval density. Upon each transfer, we placed 10 females with 5 males from each line in a 68-ml vial (20 ml of food medium) for 48 h for oviposition. The first generation was created under stock conditions. The second generation was placed for development in four environments, with each line represented in each environment, by placing parental flies for oviposition in our four developmental environments (Fig. 1). Adult flies originating from the second generation were used for body size and cell size measurements (2.1.3). Enclosing flies were monitored daily and the emergence of the first flies was noted to control for age in the cell size measurements (1–17 days after eclosion). Flies were reared under a combination of two oxygen levels (10% or 22%) and two temperatures (16 °C and 25 °C). The conditions were generated in two thermal cabinets (POL-EKO) with

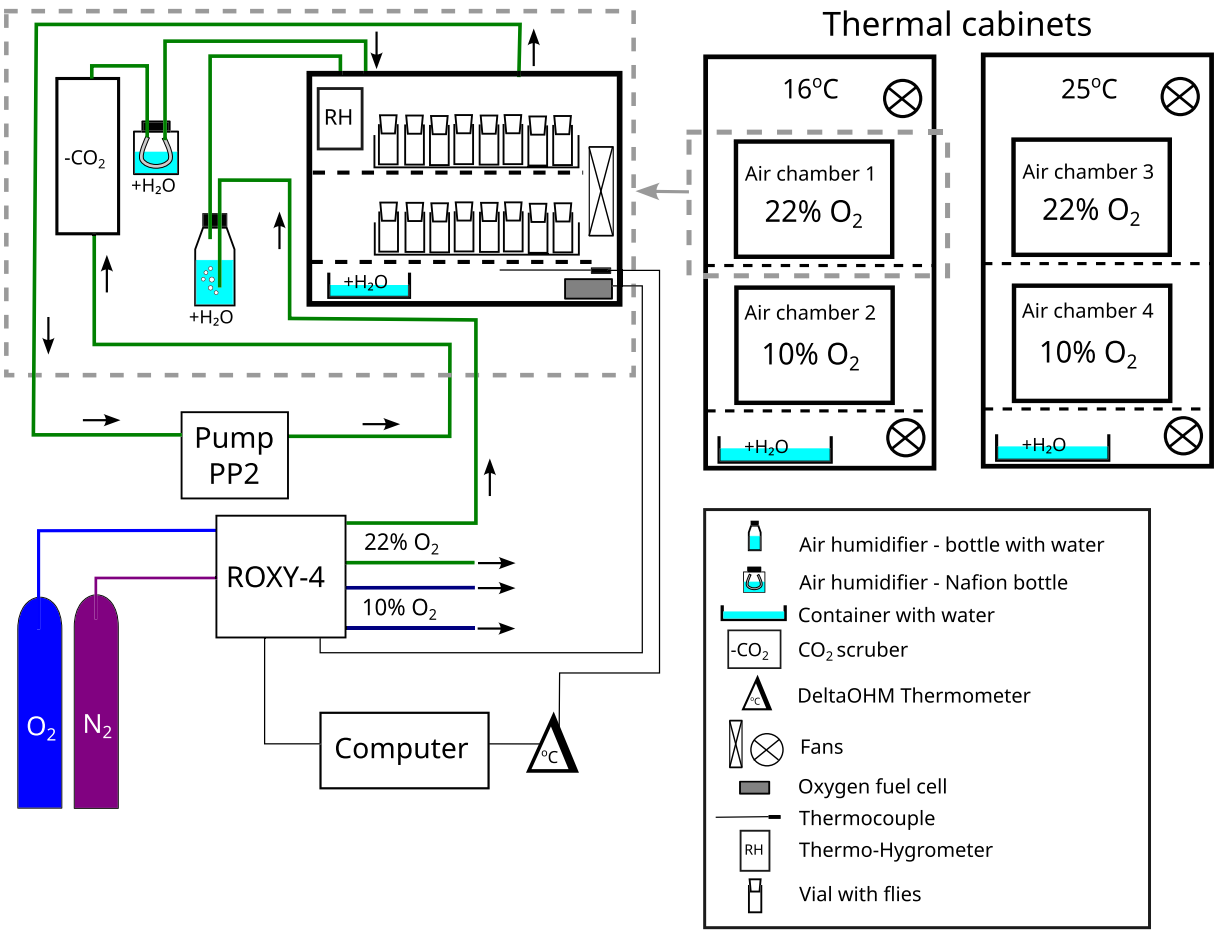

Fig. 1. Each of ten genotypes (isolines) of *Drosophila melanogaster* was raised in two thermal environments crossed with two oxygen conditions to study correlated phenotypic responses in the body size and cell sizes of adult flies. Thermal environments (16 °C and 25 °C) were generated by two thermal cabinets. Oxygen conditions (normoxia with 22% O_2 and hypoxia with 10% O_2) were generated by two air chambers placed to in each cabinet. Oxygen level was controlled by oxygen fuel cells placed to each air chamber and a four-channel gas regulator ROXY-4 connected to nitrogen and oxygen gas tanks. As the atmospheric air (21% O_2) was slowly leaking into the air chambers, the regulatory system was adding nitrogen to hypoxic chambers and oxygen to normoxic chamber. Air inside chambers was circulated by fans and constantly pushed by the PP2 pump through a close system with CO_2 scrubber and a humidifier. Gases supplied to air chambers by the ROXY system passed through another humidifier.

two Plexiglass chambers for oxygen control (40 × 50 × 55 cm) placed in each cabinet (air chambers; Fig. 1). Temperature was constantly monitored to the nearest 0.1 °C with fast-response thermocouple thermometers (Delta OHM, Padova, Italy). The air inside thermal cabinets was circulated by two fans, one at the top and one at the bottom of each cabinet. Oxygen conditions inside air chambers were controlled by a four-channel gas regulator ROXY-4 (Sable Systems International (SSI), Las Vegas, NV, USA) connected to nitrogen and oxygen gas tanks (Air Products Sp. z o.o., Kraków, Poland). As the atmospheric air was slowly leaking into the air chambers, the regulatory system was adding nitrogen to hypoxic chambers and oxygen to normoxic chamber. Note that such control was possible in both oxygen treatments because we used 22% O_2 for normoxia, which is slightly above the oxygen concentration in the atmospheric air. Inside the chambers, air circulation was maintained by fans, and additionally by the airflow created by external PP2 pumps (SSI). The primary aim of the pumps was to prevent desiccation and CO_2 accumulation inside air chambers. The air from each chamber was constantly taken by the pump through a CO_2 scrubber and then pushed through a Nafion based humidifier bottle (SSI) before returning to the chamber. To further ensure that the relative air humidity inside the chambers stayed stably above 95% (monitored by a TFA 30.5015.02 thermo-hygrometer, TFA Dostmaann GmbH & Co, Wertheim, Germany), gases supplied to air chambers by the ROXY system passed through a glass bottle with distilled water. Also, we placed plastic containers with water on bottoms of air chambers and thermal cabinets to provide a baseline source of water vapour in the whole experimental space.

2.3. Measurements of traits

To measure body size and cell size, we followed methods established for *D. melanogaster* (Privalova et al., 2023; Szlachcic et al., 2023b), which are detailed in Supplementary material (S 1.1). We used a stereomicroscope with a calibrated ocular (Olympus SZX12, Olympus, Tokyo, Japan) for dissections and measurements of thorax length, and a light microscope (Eclipse 80i, Nikon, Tokyo, Japan), camera (Axio Cam MRc5, Zeiss, Oberkochen, Germany) and ZEN software (ver. 2011, ZEISS, Oberkochen, Germany) to image the wings, legs, ommatidia, flight muscles (with bright-field microscopy) and Malpighian tubules (with phase contrast objectives).

Flies from the second generation of controlled density were anaesthetized with CO_2 and dissected with a microtome knife and forceps to obtain organs for cell size measurements (Fig. 2). For each fly, we measured the distance from the neck edge to the tip of the scutellum (thorax length, mm). We took the left wing, left middle leg, and a head from two males and two females per line per treatment. We dissected Malpighian tubules from each male, but fat bodies prevented us from obtaining Malpighian tubules from females. From another two males per

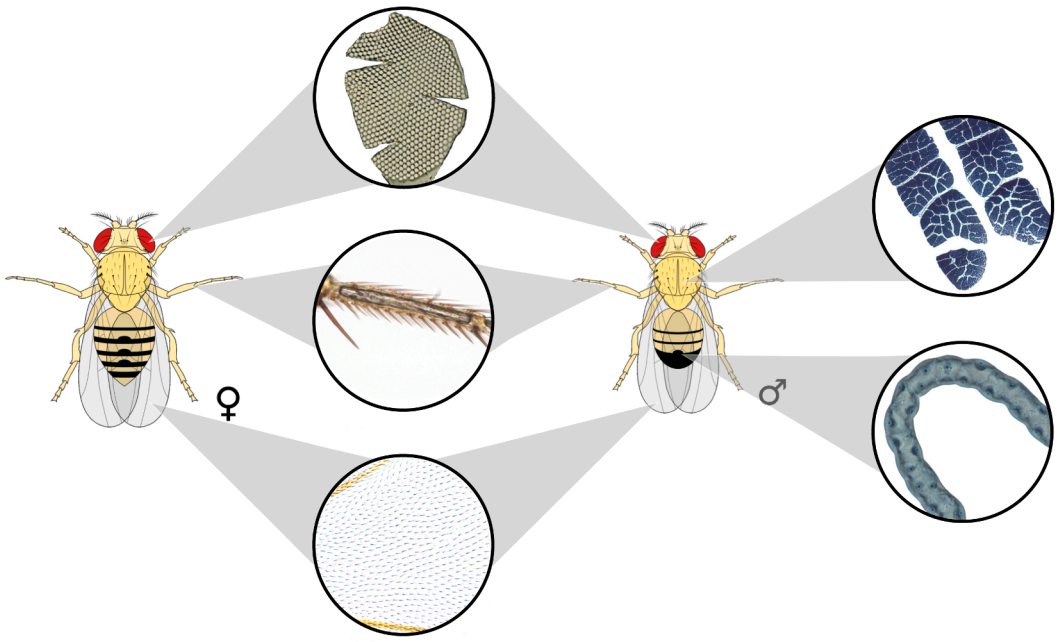

Fig. 2. Adult *Drosophila melanogaster* originating from the experiment (Fig. 1) were dissected to obtain organs for measuring body size (thorax length) and cell size in five cell types. Thorax: indirect flight muscles (mean cross-sectional area of fibres); wings: epidermal cells (from trichome number per area unit); ommatidia: ommatidial cells (mean area of ommatidia); legs: epidermal cells (from bristle number per length unit); Malpighian tubules: epithelial cells (from nuclei/nucleoli number per area unit). See Supplementary material for methodology.

line per treatment, we dissected thoraxes with flight muscles; because of time constraints, we did not obtain flight muscles for females. The legs, wings, heads and thoraxes were fixed and preserved for further analysis (see S 1.1 in Supplementary material for histological procedures); Malpighian tubules were imaged immediately without fixing. Using either direct images of cells or cellular structures (see S 1.1 in Supplementary material for measuring details), we estimated the size (μm^2) of epidermal cells in the wing and leg, ommatidial cells in the eye, epithelial cells in the Malpighian tubule, and muscles cells in the dorsal longitudinal indirect flight muscle. Overall, each fly was characterized by thorax length and the mean cell size in each tissue type. Each mean was calculated from the following number of cellular structures: 20 to 65 muscle cells of the thorax; 124 to 270 trichomes of the wing; 27 to 717 ommatidia of the eye; 9 to 17 bristles of the leg; 53 to 211 nuclei/nucleoli of the Malpighian tubule. Because histological techniques can alter the absolute size of a cell, our techniques were applied consistently among experimental groups, such that we preserved the relative cell size among flies. We also assumed that our indicators of cell size (ommatidia, wing trichomes, leg bristles) retained the same association with cell size over the range of environmental conditions in our experiment.

2.4. Statistical analysis

Thorax lengths and cell sizes were analysed with general linear mixed models (GLMMs) in R software (v4.0.3) (R Core Team, 2020) with the *lme4* (Bates et al., 2015), lmerTest (Kuznetsova et al., 2017) and *car* (Fox and Weisberg, 2019) packages. Figures were generated with the *ggplot2* (Wickham, 2016) and *emmeans* (Lenth, 2020) packages, as well as Inkscape (Harrington et al., 2004–2005). To improve the assumptions of parametric methods, thorax lengths were cube transformed (mm^3). Each GLMM included temperature (16 °C or 25 °C), oxygen supply (10% or 22%), and sex as fixed factors (except for analysis of cell sizes in Malpighian tubules and muscles, which were obtained for males only). Isofemale line was treated as a random factor. We used dredge function of *MuMIn* package to identify the model with the lowest value of *AICc* (Bartoń, 2022). This most likely model was used to estimate coefficients.

3. Results

The most likely models of thorax length and the sizes of wing epidermal cells, leg epidermal cells, and ommatidial cells included an interaction between sex and oxygen supply (see Table 1). The most likely model of the sizes of muscle cells and Malpighian tubule cells contained no interaction terms; these models also omitted the main effect of sex, because no data were collected from female flies.

Looking at sex differences (Table 1), independently of developmental environments, females were characterised by larger thoraxes and consistently larger cells in wings, legs and ommatidia (Fig. 3a-d). Moreover, models for all of these cell types retained an interaction between sex and oxygen supply during development (Table 1), indicating that males were generally less responsive to hypoxia than were females (Fig. 3a-d). For thorax length and the size of epidermal cells in wings, males responded to oxygen supply in the same fashion as females, but their response was less pronounced (Fig. 3a,b). For the sizes of leg epidermal cells and ommatidial cells, males did not respond, while females responded to oxygen conditions, as seen for (Fig. 3c,d). At the same time, thermal conditions did not interact with either sex or oxygen conditions, as indicated by the removal of interaction terms for these effects from all models (Table 1).

Looking further at the effects of developmental conditions (Table 1), temperature and oxygen conditions both affected phenotypes of eclosing flies, but generally the effects of temperature were more pronounced than the effects of oxygen (Fig. 3). Moreover, as noted earlier, the effects of oxygen were more pronounced in females than in males. Focusing on temperature (Table 1), the only trait whose value increased with developmental temperature was the size of leg epidermal cells (Fig. 3c), with males and females eclosing with larger cells at the higher temperature. The value of all other traits decreased in response to a higher temperature; specifically, flies that developed at 25 °C had smaller thoraxes and smaller wing epidermal cells (Fig. 3a,b; effects studied in males and females), smaller muscle cells, and smaller epithelial cells of the Malpighian tubule (Fig. 3e,f; effects studied in males only). Additionally, ommatidial cells were smaller in flies that developed at the higher temperature (Fig. 3d), but this trend was not statistically

Table 1
GLMM comparisons of *Drosophila melanogaster* originating from four developmental environments: low or high temperature (16 °C and 25 °C) crossed with normal or hypoxic air (22% O_2 and 10% O_2). Representatives of each genotype (isoline) were raised in each of four environments (total ten isolines). Adult flies originating from the experiment (Fig. 1) were dissected to obtain organs for measuring body size and cell size in five cell types (Fig. 2).

Fixed effects	Body size: thorax length (mm^3) N = 481		Cell size: wings (μm^2) N = 158		Cell size: legs (μm^2) N = 159		Cell size: ommatidia (μm^2) N = 154		Cell size: thorax muscle (μm^2) N = 81		Cell size: Malpighian tubules (μm^2) N = 80	
	t	p	t	p	t	p	t	p	t	p	t	p
Intercept (female, 16 °C, 10% O_2)	56.687	0.001	48.837	0.001	28.147	0.001	92.559	0.001	27.889	0.001	20.825	0.001
Temperature (25 °C)	−19.756	0.001	−30.473	0.001	3.458	0.001	−1.150	0.252	−6.174	0.001	−7.861	0.001
Oxygen (22% O_2)	5.438	0.001	4.854	0.001	2.557	0.012	2.982	0.003	−0.141	0.889	0.862	0.391
Sex (male)	−26.487	0.001	−11.066	0.001	−1.948	0.053	−3.81	0.001				
sex × oxygen (male, 22% O_2)	−2.518	0.012	−1.757	0.081	−2.022	0.045	−2.177	0.031				
Random effects variance estimates												
isoline (intercept)		0.002915		144.59		1089		43.76		1044		18,250
residual		0.008564		79.12		2650		95.19		1971		85,146

significant (Table 1; $p = 0.252$). Focusing on oxygen, development at 10% oxygen resulted in flies with smaller thoraxes and smaller wing epidermal cells than development at 22% oxygen (Fig. 3a,b); this response was stronger in females than in males (see the sex-by- oxygen interaction in the models; Table 1). Leg epidermal cells and ommatidial cells followed the same response, becoming smaller under hypoxia (Fig. 3c,d), but this effect was observed only in females; in males, these types of cells were similar between the two oxygen treatments (see sex-by-oxygen interaction in the models; Table 1). Consistent with the lower responsiveness of males to hypoxia, two other cell types that were studied only in males—muscle cells and Malpighian tubule epithelial cells—remained unaltered by oxygen supply (Table 1; Fig. 3e,f).

4. Discussion

Throughout the body of *D. melanogaster*, different tissues synchronized their plasticity of cell size, though not all cell types that we examined did so. The systemic synchronisation of cell size resulted in differences in adult body size and variation in adult size between sexes when genotypes were raised under different thermal and oxygen conditions. Across all four treatments in our experiment, females eclosed with a consistently larger mean body size and larger mean cell sizes in all tissues studied (i.e., ommatidia and epidermal cells of wings and legs). This indicates that a systemic increase in cell size contributes mechanistically to the attainment of larger body size by *D. melanogaster* females. Also, the coupling between body size and cell size was involved in the phenotypic response of flies to developmental conditions, but overall these responses were more pronounced for the effects of thermal than oxygen conditions. In particular, a higher developmental temperature resulted in adult flies with smaller body size (regardless of sex) and smaller cells in wing epidermis, flight muscle, and Malpighian tubules. Consistent with these patterns, ommatidial cells also tended to be smaller when flies developed at a higher temperature, but our models estimated low statistical confidence in this effect. At the same time, epidermal cells of the leg showed the opposite response to temperature, becoming larger in flies that developed at a higher temperature, even though the body size of these flies was smaller. Consistently among thermal treatments, decreased oxygen supply resulted in smaller adult flies with generally smaller cells. However, this effect predominantly was observed in females, as indicated by (i) the nature of interactions between oxygen supply and sex in our statistical models of cell size in wings, legs and ommatidia, and by (ii) an undetectable effect of oxygen supply on cells of the flight muscle and Malpighian tubule (the tissues studied only in males). Interestingly, consistent with the sex-related responses of cell size to oxygen conditions, we also observed a weaker response of male body size to oxygen conditions compared to female body size.

To date, a coupling between variation in body size and systemic variation in cell size changes has been rarely studied. In fact, most information about variation in cell size comes from comparing cells of a single tissue among populations or species. For example, in *Drosophila*, wing epidermal cells have been routinely used as a proxy of organismal patterns of cell size to suggest the coupling between cell size and body size in cases of developmental responses to thermal environments, latitudinal differentiation of populations, and sexual size dimorphism (Arendt, 2007; Czarnoleski et al., 2013; French et al., 1998; Partridge et al., 1994; Zwaan et al., 2000). In vertebrates, erythrocyte size has been commonly used as a proxy of other cell types, suggesting that changes in cell size have been associated with the evolutionary differentiation of body size among species (Promislow, 1991; Starostova et al., 2009). Correlated changes in the size of multiple cell types were shown to be involved in the divergence of body size among different species of Hawaiian *Drosophila* (Stevenson et al., 1995), carabid beetles (Schramm et al., 2021), Galliformes birds, and rodents (Czarnoleski et al., 2018). Although intraspecific comparisons of cell size in multiple tissues are less frequent, they seem to indicate a more complex picture,

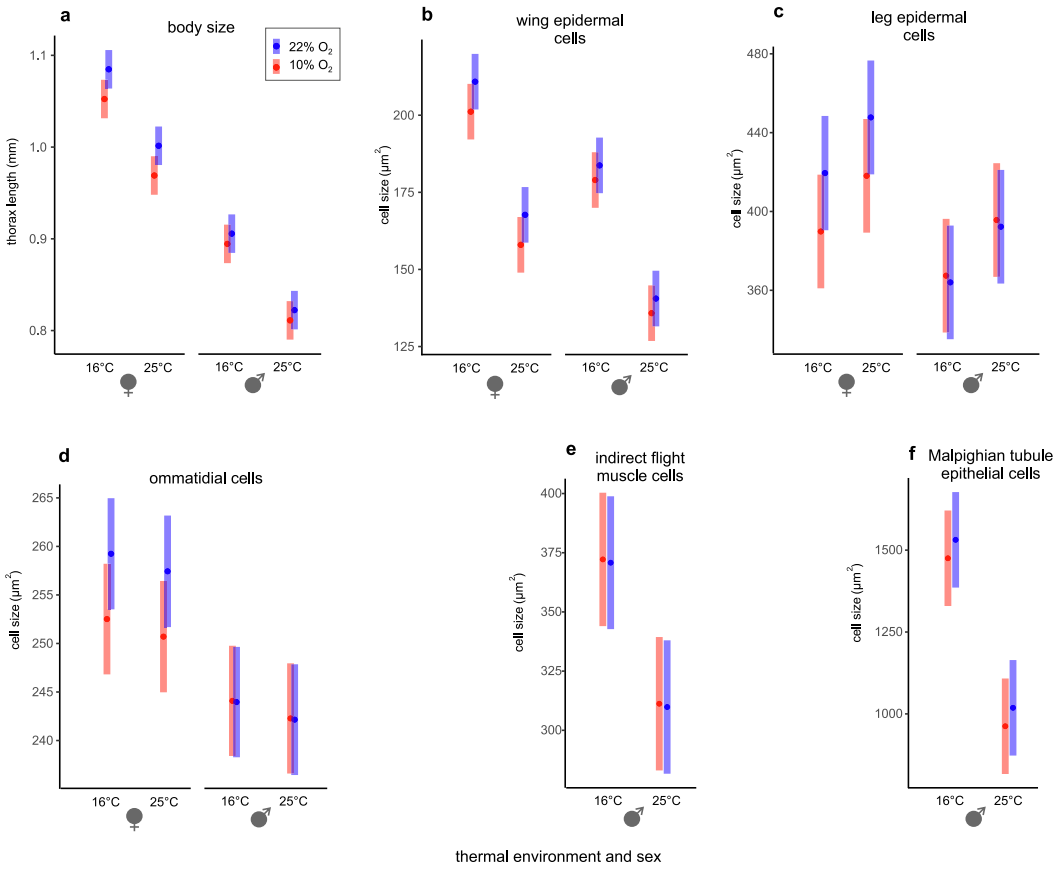

Fig. 3. Body size and cell size in multiple organs of adult *Drosophila melanogaster* was differentiated by thermal and oxygen conditions during development. The experiment (Fig. 1) crossed low and high temperatures (16 °C and 25 °C) with normal and hypoxic air (22% O_2 and 10% O_2). Modelled means and 95% confidence intervals (Table 1). Thorax length was back-transformed for display.

with only some tissues showing consistent variation in cell size that aligns with the variation in body size. For example, such complexity has been reported for geckos and snails raised at different temperatures (Czarnoleski et al., 2016; Czarnoleski et al., 2017), and for woodlice raised at different temperatures and oxygen concentrations (Antoł et al., 2020). Our results for developmental plasticity of *D. melanogaster* also indicate that cell sizes respond differently among tissues. In sharp contrast, Szlachcic et al. (2023b) demonstrated that larvae of *D. melanogaster* raised on a rapamycin-enriched diet developed a smaller adult size and smaller cells in five tissues. At the molecular level, rapamycin blocks a protein kinase called target of rapamycin (TOR), reducing its activity in TOR complex 1, a key part of the TOR/insulin signalling pathways that control cellular processes (Bjedov et al., 2010). The activities of TOR/insulin pathways appear to be involved in how environmental factors affect the body size and cell size of *Drosophila* (Harrison et al., 2018; Mirth and Shingleton, 2012). Therefore, the inconsistent patterns of cell size in response to thermal and oxygen treatments in our experiment suggest that changes in TOR activity may not be the only molecular mechanism involved. Interestingly, Privalova et al. (2023) demonstrated that mutations permanently downregulating the activity of two molecular elements in the TOR/insulin pathways also resulted in systemic changes in cell size throughout the body of adults of *D. melanogaster*; one type of mutation resulted in smaller cells and the other type resulted in larger cells. However, compared to other cell types, the sizes of cells in indirect flight muscles (also studied here) were unaffected by mutations.

Emerging evidence suggests that the cellular composition of tissues and organs in *Drosophila* is mediated by systemic control mechanisms, as well as cell-autonomous ones (Hudry et al., 2016; Mirth and Shingleton, 2012; Rideout et al., 2015). It would be interesting to explore how these two types of regulatory mechanisms produce the complexity of developmental responses of cell size to environmental conditions, such as ones revealed by our study. Interestingly, Szlachcic et al. (2023b) showed that, with or without rapamycin in the larval diet, females eclosed with larger body size and larger cells in various tissues than did males. Also, Privalova et al. (2023) showed that, despite mutations altering the activity of TOR/insulin pathways, females had larger cells than males in all tissues studied. Similar sex differences and its consistency across different cell types characterised flies emerging in four different environmental conditions in our experiment. Recent findings show that developmental programmes that affect the sexually dimorphic phenotypes of *D. melanogaster* rely on autonomous mechanisms that regulate cell size, which in females but not in males, are also linked to a systemic signalling network via TOR/insulin pathways (Deng and Jasper, 2016; Hudry et al., 2016; Rideout et al., 2015). Interestingly, a simultaneous inhibition of the activity of TOR and insulin pathways in larvae blurred sex differences in body size of *D. melanogaster*, but the inhibition of TOR activity alone did not do so (Rideout et al., 2015). In light of our results, this sex-dependent regulatory system seems to show tolerance to thermal and oxygen conditions during development, maintaining sex differences at the level of body size and the cellular composition of organs. We speculate that, at a molecular level, a change in thermal and oxygen conditions may not influence the TOR and insulin pathways strongly enough to blur the sexual dimorphism in body size and cell size, but this hypothesis needs to be tested.

The available evidence for *D. melanogaster*, including the results of this study, indicates that molecular pathways controlling development of flies orchestrate cellular composition in various tissues throughout a body, while also shaping body size of adult flies. So, what are the evolutionary implications of these phenomena? Effects of oxygen supply

on the life-history traits of ectotherms have been less frequently studied than those of temperature, and most studies targeting oxygen effects have focused on aquatic species, because they are considered more oxygen-limited than terrestrial species (Verberk et al., 2021). Also, contrasting our approach here, most previous studies of ectotherms have focused on only one of the two variables (but see Atkinson et al., 2006; Hoefnagel and Verberk, 2015). In our experiment, *D. melanogaster* responded to either warm or hypoxic environments, eclosing at a smaller body size. These responses were very consistent in males and females, which agrees with the findings of Hirst et al. (2015) on the sex-dependent responses of body size to ambient temperatures in various arthropods, including Diptera. The body-size responses in our experiment support the temperature-size rule (TSR) and common effects of oxygen supply on growth (Atkinson, 1994; Harrison et al., 2018). These responses are often regarded as adaptive, because life-history theory identifies adult size as one of the key determinants of Darwinian fitness (Kozlowski et al., 2004). Larger adult size is often associated with greater fertility, sexual attractiveness, competitiveness, or protection from predators (Stearns, 1992). Nevertheless, low oxygen supply can favour maturation at small size, given that a small body shortens internal pathways for transporting nutrients, which should help to supply mitochondria with oxygen. The temperature-size rule seems to apply more commonly to aquatic ectotherms than to terrestrial ones (Forster et al., 2012; but see Klok and Harrison, 2013 for other generalizations), suggesting that this type of reaction norm evolved more frequently under restricted access to oxygen. According to Verberk et al. (2021), this pattern of plasticity may manifest adaptations that prevent severe hypoxia at the cellular level, which can be achieved by reducing body size under high oxygen demands (increased body temperature speeds metabolic rate and thus oxygen demand) and low oxygen supply (warm habitats are often more hypoxic restricting oxygen supply). Consistent with this view, the smallest flies in our experiment originated from the warm and hypoxic treatment, whereas the largest flies originated from the cold and normoxic treatment.

The patterns of body size that we observed are consistent with those documented for a species of rotifers (*K. cochlearis*) along natural gradients of thermal and oxygen conditions, studied at different depths in twenty European lakes (Czarnoleski et al., 2015b). In this study, the largest rotifers occupied cold, oxygen-rich water, while the smallest rotifers occupied warm, oxygen-poor water. Importantly, a laboratory experiment showed that large rotifers were less fertile than the small rotifers when exposed to warm and hypoxic water (Walczynska et al., 2015), despite a positive relationship between body size and fecundity in this species. These two case studies of rotifers draw attention to physiological consequences of plasticity in cell size, which is complementary to adaptive theories of life-history evolution along environmental gradients of temperature and oxygen. Rotifers are eutelic, which means that consist of a fixed number of cells; therefore a change in the body size of a rotifer inevitably results from a change in the mean size of its cells. Atkinson et al. (2006) hypothesized that oxygen limitation at higher temperatures is more likely to occur in larger ectotherms, suggesting the accompanying changes in physiological properties associated with oxygen transport and oxygen consumption are adaptive, including changes in cell size and mitochondrial characteristics. Although these mechanisms should be less relevant for terrestrial insects that ventilate convectively through a tracheal system (Angilletta and Dunham, 2003), our results for *D. melanogaster* showed that smaller body size in a warm, hypoxic environment was coupled with a systemic reduction in cell size among tissues. Indeed, the similarity between plastic and evolutionary effects of temperature on body size and cell size of *D. melanogaster* (Adrian et al., 2016; Partridge et al., 1994) indirectly supports the adaptive value of coupled responses of body size and cell size to developmental temperature.

According to the theory of optimal cell size (TOCS), smaller cells offer a greater capacity to transport nutrients through an increased area of plasma membranes, despite imposing higher metabolic costs to maintain membrane potentials. Moreover, oxygen diffuses better through lipid than through an aqueous medium, especially at higher temperatures, suggesting that the internal region of a phospholipid bilayer provides a convenient path for oxygen to penetrate a tissue (Subczynski et al., 1989). If so, a tissue composed of many small cells could help to deliver oxygen to mitochondria under warm hypoxic conditions. Indeed, the analysis of flight performance in flies with different cell sizes revealed that flies with smaller cells performed better under hypoxia (Szlachcic and Czarnoleski, 2021). Evidence suggests that small cells can increase tolerance of acute heat stress in *D. melanogaster* (Verspagen et al., 2020). Additionally, species of fish with smaller cells tolerate hypoxia in warm waters better than other species (Verberk et al., 2022). Therefore, if the temperature-size rule represents adaptive plasticity to meet an elevated metabolic demand under conditions of low oxygen supply, then coupling this response with a systemic reduction in cell size throughout the body should help to keep tissue at a positive oxygen balance. If so, we hypothesise an ectotherm living in warm and hypoxic environments would need an even smaller body size to deliver sufficient oxygen to tissues by reducing its number of cells, than it would by reducing the size of its cells.

TOCS predicts that an organism-wide coordination of the cellular composition of tissues saves energy and promotes transport of oxygen and resources. Nevertheless, highly specialized physiological functions can require specific surface-to-volume ratios or organelle contents, which could explain the variation in cell-size patterns among tissues observed in our experiment and in earlier studies. Among the five tissues of *D. melanogaster* that we studied, cells were smaller in four tissues when flies were raised at a higher temperature, while the opposite pattern was observed in leg epidermal cells. In females, all three types of cells were smaller in flies raised in a hypoxic treatment. In males, cells of only one tissue (wing epidermal cells) were smaller when flies were raised in hypoxia while cells of the other four tissues were similar in size between normoxia and hypoxia. The relative insensitivity of cell size in males accords with the idea that changes in body size and cell size influence the capacity to meet oxygen demand by supply. Females are the larger sex in *D. melanogaster*, and as shown here, they are also characterised by consistently larger cells in various tissues. These two traits combined, females should be more prone to oxygen limitation, displaying more pronounced responses in cell size to decreased oxygen supply compared to males. Supporting this scenario, Leiva et al. (2023) reported that large-celled genotypes of *D. melanogaster* expressed stronger thermal plasticity of body size compared to the small-celled genotypes.

5. Conclusions

Altogether, responses of *D. melanogaster* to temperature and oxygen supply involve shifts in body size coupled with a systemic orchestration of cell size throughout the body. At the same time, we showed some inconsistencies in this coupling, which can be attributed to sex, cell type, and abiotic variables. These inconsistencies suggest caution in translating the findings for single tissue to the level of the organism. Overall, our results are consistent with existing theories that invoke fitness consequences of cell size and view the temperature-size rule as an adaptation that prevents functional hypoxia within tissues. However, looking at these responses solely in terms of oxygen limitation ignores a basic principle of life-history evolution—adult body size is not 'given' to an organism at birth, and reaching a certain body size takes time and imposes a risk of mortality. Following these principles, allocating resources to growth instead of reproduction is an investment with delayed returns; if an organism lives, an increased body size may afford greater reproduction in the future (Czarnoleski and Kozlowski, 1998; Kozlowski et al., 2020; Stearns, 1992). Models of optimal resource allocation have demonstrated that warm, hypoxic environments can favour early maturation at a smaller body size if such environments impose a higher risk of mortality (predation, parasitism), even without an effect of

oxygen supply on physiological performance (Kozlowski et al., 2004; Audzijonyte et al., 2022). Higher death rates in warmer environments make investments in future reproduction less profitable, favouring an earlier switch from growth to reproduction at a smaller body size. Without invoking the thermal dependence of mortality, optimality models show also that the temperature-size rule can become adaptive if temperature determines the mass-scaling of the capacity to produce new tissue (Kozlowski et al., 2004; Audzijonyte et al., 2022). As Kozlowski et al. (2004) noted, this mechanism could indicate physiological effects of correlated changes in cell size and body size. Indeed, a coupling between body size and cell size shapes the mass-scaling of metabolic rates (Czarnoleski et al., 2018; Glazier, 2022; Kozlowski et al., 2020; Schramm et al., 2021), and this scaling depends on temperature in ectotherms (Glazier, 2005, 2020, 2022). That said, the two explanations for the evolution of the temperature-size rule, one relating to oxygen limitation and cell size and the other to optimisation of life strategies, should not be treated as mutually exclusive. On the contrary, all indications are that we should strive for a better integration of these theories of optimality, one focused on cell size and the other focused on resource allocation.

CRediT authorship contribution statement

Marcin Czarnoleski: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Ewa Szlachcic:** Investigation, Methodology, Validation, Writing – review & editing. **Valeriya Privalova:** Investigation, Methodology, Visualization, Writing – review & editing. **Anna Maria Labecka:** Data curation, Investigation, Methodology, Resources, Supervision, Validation, Writing – review & editing. **Anna Sikorska:** Investigation, Methodology, Writing – review & editing. **Łukasz Sobczyk:** Methodology, Visualization, Writing – review & editing. **John VandenBrooks:** Conceptualization, Validation, Writing – original draft, Writing – review & editing. **Michael J. Angilletta Jr:** Conceptualization, Resources, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data and R code are available in on-line supplementary material

Acknowledgements

We thank reviewers for their valuable suggestions to earlier versions of the manuscript.

Funding

The research was funded by the National Science Centre, Poland (OPUS 2016/21/B/NZ8/00303 to M.C.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2023.104559.

References

Adrian, G.J., Czarnoleski, M., Angilletta, M.J., 2016. Flies evolved small bodies and cells at high or fluctuating temperatures. Ecology and Evolution 6, 7991–7996. https://doi.org/10.1002/ece3.2534.

Angilletta, M.J., 2009. Thermal adaptation: A theoretical and empirical synthesis. Oxford University Press, Oxford.

Angilletta, M.J., Dunham, A., 2003. The temperature-size rule in ectotherms: simple evolutionary explanations may not be general. American Naturalist 162 (3), 332–342.

Antoł, A., Labecka, A.M., Horváthová, T., Sikorska, A., Szabla, N., Bauchinger, U., Kozłowski, J., Czarnoleski, M., 2020. Effects of thermal and oxygen conditions during development on cell size in the common rough woodlice *Porcellio scaber*. Ecology and Evolution 10 (17), 9552–9566.

Arendt, J., 2007. Ecological correlates of body size in relation to cell size and cell number: patterns in flies, fish, fruits and foliage. Biological Reviews 82 (2), 241–256.

Ashton, K.G., 2002. Do amphibians follow Bergmann's rule? Canadian Journal of Zoology 80 (4), 708–716.

Ashton, K.G., Feldman, C.R., 2003. Bergmann's rule in nonavian reptiles: turtles follow it, lizards and snakes reverse it. Evolution 57 (5), 1151–1163.

Atkinson, D., 1994. Temperature and organism size: a biological law for ectotherms. Advances in Ecological Research 25, 1–58.

Atkinson, D., Morley, S.A., Hughes, R.N., 2006. From cells to colonies: at what levels of body organization does the 'temperature-size rule' apply? Evolution & Development 8 (2), 202–214.

Audzijonyte, A., Jakubavičiūtė, E., Lindmark, M., Richards, S.A., 2022. Mechanistic temperature-size rule explanation should reconcile physiological and mortality responses to temperature. Biological Bulletin 243 (2), 220–238.

Bartoń, K., 2022. MuMIn: Multi-Model Inference. R package version 1 (46). https://CRAN.R-project.org/package=MuMIn.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Usinglme4. Journal of Statistical Software 67 (1), 1–48.

Bjedov, I., Toivonen, J.M., Kerr, F., Slack, C., Jacobson, J., Foley, A., Partridge, L., 2010. Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila melanogaster*. Cell Metab 11 (1), 35–46.

Calboli, F.C.F., Gilchrist, G.W., Partridge, L., 2003. Different cell size and cell number contribution in two newly established and one ancient body size cline of *Drosophila subobscura*. Evolution 57 (3), 566–573.

Czarnoleski, M., Cooper, B.S., Kierat, J., Angilletta, M.J., 2013. Flies developed small bodies and small cells in warm and in thermally fluctuating environments. Journal of Experimental Biology 216, 2896–2901.

Czarnoleski, M., Dragosz-Kluska, D., Angilletta, M.J., 2015a. Flies developed smaller cells when temperature fluctuated more frequently. Journal of Thermal Biology 54, 106–110.

Czarnoleski, M., Ejsmont-Karabin, J., Angilletta, M.J., Kozlowski, J., 2015b. Colder rotifers grow larger but only in oxygenated waters. Ecosphere 6 (9), art164.

Czarnoleski, M., Labecka, A.M., Kozłowski, J., 2016. Thermal plasticity of body size and cell size in snails from two subspecies of *Cornu aspersum*. Journal of Molluscan Studies 82 (2), 235–243.

Czarnoleski, M., Labecka, A.M., Starostová, Z., Sikorska, A., Bonda-Ostaszewska, E., Woch, K., Kubička, L., Kratochvíl, L., 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*. Biology Open 6, 1149–1154.

Czarnoleski, M., Kozlowski, J., 1998. Do Bertalanffy's growth curves result from optimal resource allocation? Ecology Letters 1, 5–7.

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

Davison, JOHN, 1955. Body weight, cell surface, and metabolic rate in anuran Amphibia. Biological Bulletin 109 (3), 407–419.

Davison, JOHN, 1956. An analysis of cell growth and metabolism in the crayfish (Procambarus alleni). Biological Bulletin 110 (3), 264–273.

de Jong, G., Bochdanovits, Z., 2003. Latitudinal clines in *Drosophila melanogaster*: body size, allozyme frequencies, inversion frequencies, and the insulin-signalling pathway. Journal of Genetics 82 (3), 207–223.

Deng, H., Jasper, H., 2016. Sexual Dimorphism: How Female Cells Win the Race. Current Biology 26, R212-215.

Fabian, D.K., Kapun, M., Nolte, V., Kofler, R., Schmidt, P.S., Schlötterer, C., Flatt, T., 2012. Genome-wide patterns of latitudinal differentiation among populations of Drosophila melanogaster from North America. Molecular Ecology 21, 4748–4769.

Forster, J., Hirst, A.G., Atkinson, D., 2012. Warming-induced reductions in body size are greater in aquatic than terrestrial species. Proceedings of the National Academy of Sciences of the United States of America 109 (47), 19310–19314.

Fox, J., Weisberg, S., 2019. An R companion to applied regression. SAGE Publications Inc, Thousand Oaks, CA.

French, V., Feast, M., Partridge, L., 1998. Body size and cell size in *Drosophila*: the developmental response to temperature. Journal of Insect Physiology 44 (11), 1081–1089.

Ginzberg, M.B., Kafri, R., Kirschner, M., 2015. On being the right (cell) size. Science 348, 1245075.

Glazier, D.S., 2005. Beyond the '3/4-power law': variation in the intra- and interspecific scaling of metabolic rate in animals. Biological Reviews of the Cambridge Philosophical Society 80, 611–662.

Glazier, D.S., 2020. Activity alters how temperature influences intraspecific metabolic scaling: testing the metabolic-level boundaries hypothesis. Journal of Comparative Physiology B 190 (4), 445–454.
Glazier, D.S., 2022. How metabolic rate relates to cell size. Biology 11, 1106.
Grosberg, R.K., Strathmann, R.R., 2007. The evolution of multicellularity: a minor major transition? Annual Review of Ecology, Evolution, and Systematics 38 (1), 621–654.
Harrington, B., Gould, T., Hurst, N., MenTaLgu, Y., 2004–2005. Inkscape. https://www.inkscape.org.
Harrison, J.F., Kaiser, A., VandenBrooks, J.M., 2010. Atmospheric oxygen level and the evolution of insect body size. Proceedings of the Royal Society B-Biological Sciences 277 (1690), 1937–1946.
Harrison, J.F., Shingleton, A.W., Callier, V., 2015. Stunted by Developing in Hypoxia: Linking Comparative and Model Organism Studies. Physiological and Biochemical Zoology 88 (5), 455–470.
Harrison, J.F., Greenlee, K.J., Verberk, W.C.E.P., 2018. Functional Hypoxia in Insects: Definition, Assessment, and Consequences for Physiology, Ecology, and Evolution. Annual Review of Entomology 63 (1), 303–325.
Heinrich, E., Farzin, M., Klok, C., Harrison, J., 2011. The effect of developmental stage on the sensitivity of cell and body size to hypoxia in *Drosophila melanogaster*. Journal of Experimental Biology 214, 1419–1427.
Hermaniuk, A., Rybacki, M., 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. Physiological and Biochemical Zoology 90 (2), 230–239.
Hirst, A.G., Horne, C.R., Atkinson, D., 2015. Equal temperature–size responses of the sexes are widespread within arthropod species. Proc. R. Soc. B. 282 (1820), 20152475.
Hoefnagel, K.N., Verberk, W.C., 2015. Is the temperature-size rule mediated by oxygen in aquatic ectotherms? Journal of Thermal Biology 54, 56–65.
Horne, C.R., Hirst, A.G., Atkinson, D., Enquist, B., 2015. Temperature-size responses match latitudinal-size clines in arthropods, revealing critical differences between aquatic and terrestrial species. Ecology Letters 18 (4), 327–335.
Huang, E., Chen, Y., Fang, M., Zheng, Y.i., Yu, S., Bjorkman, A., 2021. Environmental drivers of plant distributions at global and regional scales. Global Ecology and Biogeography 30 (3), 697–709.
Hudry, B., Khadayate, S., Miguel-Aliaga, I., 2016. The sexual identity of adult intestinal stem cells controls organ size and plasticity. Nature 530 (7590), 344–348.
Hunt, G., Roy, K., 2006. Climate change, body size evolution, and Cope's Rule in deep-sea ostracodes. Proc. Natl. Acad. Sci. U.S.A. 103 (5), 1347–1352.
Jaureguiberry, P., Titeux, N., Wiemers, M., Bowler, D.E., Coscieme, L., Golden, A.S., Guerra, C.A., Jacob, U., Takahashi, Y., Settele, J., Díaz, S., Molnár, Z., Purvis, A., 2022. The direct drivers of recent global anthropogenic biodiversity loss. Science. Advances 8 (45).
Kellermann, V., Overgaard, J., Hoffmann, A.A., Fløjgaard, C., Svenning, J.-C., Loeschcke, V., 2012. Upper thermal limits of Drosophila are linked to species distributions and strongly constrained phylogenetically. Proc. Natl. Acad. Sci. U.S.A. 109 (40), 16228–16233.
Klok, C.J., Harrison, J.F., 2013. The temperature size rule in arthropods: independent of macro-environmental variables but size dependent. Integrative and Comparative Biology 53, 557–570.
Kozlowski, J., Czarnoleski, M., Danko, M., 2004. Can optimal resource allocation models explain why ectotherms grow larger in cold? Integrative and Comparative Biology 44, 480–493.
Kozlowski, J., Czarnoleski, M., François-Krassowska, A., Maciak, S., Pis, T., 2010. Cell size is positively correlated between different tissues in passerine birds and amphibians, but not necessarily in mammals. Biology Letters 6 (6), 792–796.
Kozlowski, J., Konarzewski, M., Czarnoleski, M., 2020. Coevolution of body size and metabolic rate in vertebrates: a life-history perspective. Biological Reviews 95 (5), 1393–1417.
Kozlowski, J., Konarzewski, M., Gawelczyk, A.T., 2003. Cell size as a link between noncoding DNA and metabolic rate scaling. Proc. Natl. Acad. Sci. U.S.A. 100 (24), 14080–14085.
Kuznetsova, A., Brockhoff, P.B., Christensen, R.H.B., 2017. lmerTest Package: Tests in Linear Mixed Effects Models. Journal of Statistical Software 82 (13), 1–26.
Leiva, F.P., Boerrigter, J.G.J., Verberk, W.C.E.P., 2023. The role of cell size in shaping responses to oxygen and temperature in fruit flies. Functional Ecology 37 (5), 1269–1279.
Lenth, R.V., 2020. emmeans: Estimated marginal means, aka least-squares means. R package version 1 (5), 3. https://CRAN.R-project.org/package=emmeans.
Liu, S., Tan, C., Tyers, M., Zetterberg, A., Kafri, R., 2022. What programs the size of animal cells? Frontiers in Cell and Developmental Biology 10, 949382.
Meiri, S., 2011. Bergmann's Rule – what's in a name? Global Ecology and Biogeography 20, 203–207.
Miettinen, T.P., Caldez, M.J., Kaldis, P., Björklund, M., 2017. Cell size control–a mechanism for maintaining fitness and function. Bioessays 39 (9), 1700058.
Mirth, C.K., Shingleton, A.W., 2012. Integrating body and organ size in *Drosophila*: recent advances and outstanding problems. Frontiers in Endocrinology (Lausanne) 3, 49.
Partridge, L., Barrie, B., Fowler, K., French, V., 1994. Evolution and development of body size and cell size in *Drosophila melanogaster* in response to temperature. Evolution 48 (4), 1269–1276.
Peck, L.S., Maddrell, S.H.P., 2005. Limitation of size by hypoxia in the fruit fly *Drosophila melanogaster*. Journal of Experimental Zoology Part A Comparative Experimental Biology 303A (11), 968–975.
Pörtner, H.O., Roberts, D.C., Poloczanska, E.S., Mintenbeck, K., Tignor, M., Alegría, A., Craig, M., Langsdorf, S., Löschke, S., Möller, V., Okem, A., 2022. Climate Change 2022: Impacts, Adaptation, and Vulnerability. Working Group II Contribution to the IPCC Sixth Assessment Report. Cambridge University Press. Cambridge University Press, Cambridge, UK and New York, NY, USA.
Poulsen, C.J., Tabor, C., White, J.D., 2015. Long-term climate forcing by atmospheric oxygen concentrations. Science 348 (6240), 1238–1241.
Privalova, V., Labecka, A.M., Szlachcic, E., Sikorska, A., Czarnoleski, M., 2023. Systemic changes in cell size throughout the body of *Drosophila melanogaster* associated with mutations in molecular cell cycle regulators. Scientific Repports 13, 7565.
Promislow, D.E.L., 1991. The evolution of mammalian blood parameters: patterns and their interpretation. Physiological Zoology 64 (2), 393–431.
R Core Team, 2020. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria https://www.r-project.org/833.
Rideout, E.J., Narsaiya, M.S., Grewal, S.S., Kopp, A., 2015. The Sex Determination Gene transformer Regulates Male-Female Differences in *Drosophila* Body Size. PLoS Genetics 11 (12), e1005683.
Roustan, V., Jain, A., Teige, M., Ebersberger, I., Weckwerth, W., 2016. An evolutionary perspective of AMPK–TOR signaling in the three domains of life. Journal of Experimental Botany 67 (13), 3897–3907.
Savage, V.M., Allen, A.P., Brown, J.H., Gillooly, J.F., Herman, A.B., Woodruff, W.H., West, G.B., 2007. Scaling of number, size, and metabolic rate of cells with body size in mammals. Proc. Natl. Acad. Sci. U.S.A. 104 (11), 4718–4723.
Schmidt-Nielsen, K., 1984. Scaling: why is animal size so important? Cambridge University Press, Cambridge, UK.
Schramm, B.W., Labecka, A.M., Gudowska, A., Antoł, A., Sikorska, A., Szabla, N., Bauchinger, U., Kozlowski, J., Czarnoleski, M., 2021. Concerted evolution of body mass, cell size and metabolic rate among carabid beetles. Journal of Insect Physiology 132, 104272.
Scotese, C.R., Song, H., Mills, B.J.W., van der Meer, D.G., 2021. Phanerozoic paleotemperatures: The earth's changing climate during the last 540 million years. Earth-Science Reviews 215, 103503.
Sears, M.W., Angilletta, M.J., 2004. Body size clines in *Sceloporus lizards*: proximate mechanisms and demographic constraints. Integrative and Comparative Biology 44, 433–442.
Starostova, Z., Kubicka, L., Konarzewski, M., Kozlowski, J., Kratochvil, L., 2009. Cell size but not genome size affects scaling of metabolic rate in eyelid geckos. The American Naturalist 174, E100-105.
Stearns, S.C., 1992. The Evolution of Life Histories. Oxford University Press, Oxford, UK.
Stevenson, R.D., Hill, M.F., Bryant, P.J., 1995. Organ and cell allometry in Hawaiian *Drosophila*: how to make a big fly. Proceeding of the Royal Society B 259, 105–110.
Subczynski, W.K., Hyde, J.S., Kusumi, A., 1989. Oxygen permeability of phosphatidylcholine–cholesterol membranes. Proc. Natl. Acad. Sci. U.S.A. 86 (12), 4474–4478.
Szarski, H., 1983. Cell size and the concept of wasteful and frugal evolutionary strategies. Journal of Theoretical Biology 105 (2), 201–209.
Szlachcic, E., Czarnoleski, M., 2021. Thermal and oxygen flight sensitivity in ageing *Drosophila melanogaster* flies: links to rapamycin-induced cell size changes. Biology 10, 861.
Szlachcic, E., Danko, M.J., Czarnoleski, M., 2023a. Rapamycin supplementation of *Drosophila melanogaster* larvae results in less viable adults with smaller cells. Royal Society Open Science 10, 230080.
Szlachcic, E., Labecka, A.M., Privalova, V., Sikorska, A., Czarnoleski, M., 2023b. Systemic orchestration of cell size throughout the body: influence of sex and rapamycin exposure in *Drosophila melanogaster*. Biology Letters 19, 20220611.
Valentine, J.W., Collins, A.G., Meyer, C.P., 1994. Morphological complexity increase in metazoans. Paleobiology 20 (2), 131–142.
van Dam, T.J.P., Zwartkruis, F.J.T., Bos, J.L., Snel, B., 2011. Evolution of the TOR pathway. Journal of Molecular Evolution 73 (3-4), 209–220.
Verberk, W.C.E.P., Atkinson, D., Hoefnagel, K.N., Hirst, A.G., Horne, C.R., Siepel, H., 2021. Shrinking body sizes in response to warming: explanations for the temperature-size rule with special emphasis on the role of oxygen. Biological Reviews of the Cambridge Philosophical Society 96 (1), 247–268.
Verberk, W.C.E.P., Sandker, J.F., van de Pol, I.L.E., Urbina, M.A., Wilson, R.W., McKenzie, D.J., Leiva, F.P., 2022. Body mass and cell size shape the tolerance of fishes to low oxygen in a temperature-dependent manner. Global Change Biology 28 (19), 5695–5707.
Verspagen, N., Leiva, F.P., Janssen, I.M., Verberk, W.C.E.P., 2020. Effects of developmental plasticity on heat tolerance may be mediated by changes in cell size in *Drosophila melanogaster*. Insect Science 27 (6), 1244–1256.
Walczynska, A., Labecka, A.M., Sobczyk, M., Czarnoleski, M., Kozlowski, J., 2015. The Temperature-Size Rule in Lecane inermis (Rotifera) is adaptive and driven by nuclei size adjustment to temperature and oxygen combinations. Journal of Thermal Biology 54, 78–85.
Wickham, H., 2016. Ggplot2 elegant graphics for data analysis. Springer, New York, NY, USA.
Woods, H.A., Moran, A.L., Atkinson, D., Audzijonyte, A., Berenbrink, M., Borges, F.O., Burnett, K.G., Burnett, L.E., Coates, C.J., Collin, R., Costa-Paiva, E.M., Duncan, M.I., Ern, R., Laetz, E.M.J., Levin, L.A., Lindmark, M., Lucey, N.M., McCormick, L.R., Pierson, J.J., Rosa, R., Roman, M.R., Sampaio, E., Schulte, P.M., Sperling, E.A., Walczyńska, A., Verberk, W.C.E.P., 2022. Integrative Approaches to Understanding Organismal Responses to Aquatic Deoxygenation. The Biological Bulletin 243 (2), 85–103.
Zwaan, B.J., Azevedo, R.B.R., James, A.C., van 't Land, J., Partridge, L., 2000. Cellular basis of wing size variation in *Drosophila melanogaster*: a comparison of latitudinal clines on two continents. Heredity 84 (3), 338–347.