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Author for correspondence:

France Dufresne

e-mail: france_dufresne@uqar.ca

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Cell size and ploidy modulate heat tolerance in *Daphnia*

France Dufresne, Sarah-Béatrice Bernier, Alexis Gagnier-Michel and Pierre U. Blier

Dept de Biologie, chimie et géographie, Université du Québec à Rimouski, Rimouski, Quebec G5L 3A1, Canada

FD, 0000-0002-2481-4864; PUB, 0000-0003-1881-2421

The theory of optimal cell size postulates that cell size imposes constraints on oxygen delivery in larger cells owing to their smaller surface-area-to-volume ratios. Smaller cells, with a higher surface-area-to-volume ratio, have an increased capacity to take up oxygen, which is therefore expected to increase their heat tolerance (CT_{max}). However, the precise mechanisms linking cell size, body size and heat tolerance remain unclear. We obtained contrasts in cell size by raising diploid and triploid juvenile *Daphnia* at different temperatures. *Daphnia* raised at lower temperatures had larger cells than those raised at higher temperatures. Triploid clones had larger cells than diploid clones at 16°C, 20°C and 24°C. CT_{max} increased with acclimation temperature and was negatively correlated to both cell and body size. Triploid clones had lower CT_{max} values than both subarctic and temperate diploid clones at 16°C and 20°C. Heat shock significantly increased the expression of Hsp70 and catalase, but these were not correlated with CT_{max} or ploidy, suggesting that heat tolerance was not directly linked to heat-shock proteins or oxidative stress responses. These findings highlight the role of cell size and polyploidy in shaping the heat tolerance and geographic distribution of ectotherms.

1. Introduction

The theory of optimal cell size posits that cell size is a critical factor influencing the metabolic efficiency and physiological performance of ectotherms [1]. Cell size acts as a 'double-edged sword', influencing both resource acquisition and the energetic costs of maintaining life functions [2]. Higher temperatures generally lead to increased metabolic rates, which demand more oxygen. Smaller cells, with their higher surface-area-to-volume ratios, facilitate more efficient oxygen diffusion across the cell membrane [3,4]. This enhanced oxygen uptake is particularly advantageous when oxygen is limited, such as during hypoxia or at elevated temperatures. Additionally, tissues composed of smaller cells possess proportionally more membrane surfaces, serving as preferential pathways for oxygen diffusion and reducing the transport distances [5,6]. However, maintaining smaller cells incurs an energetic cost, requiring more energy to sustain the electrochemical gradients across membranes [7, 8]. This creates a critical trade-off between improved oxygen uptake and increased energy expenditure. Consequently, the optimal cell size probably varies with temperature, striking a balance between the benefits of efficient oxygen diffusion and the metabolic demands of maintaining smaller cells. Variation in cell size patterns could also depend on the different physiological functions of different tissues, requiring specific surface-area-to-volume ratios [9].

One key gap in existing research is our limited understanding of how cell size interacts with body size to influence heat tolerance. While it is known that body size is a function of both cell size and cell number, the relative contributions of these factors to heat tolerance have not been fully

disentangled [10]. For example, smaller-bodied organisms are often more thermally tolerant [11], but it is unclear whether this is owing to their smaller cell size, reduced overall volume or both. Furthermore, the extent to which cell size independently drives heat tolerance, beyond its correlation with body size, remains underexplored. The dissection of the influence of these two factors requires an experimental induction of cell size differences, independent of body size. Both extrinsic (temperature and nutrient levels) and intrinsic (ploidy levels and genome size) factors are known to modulate cell size. Temperature has a pervasive influence on cell size by altering cell growth, division and expression of genes related to cell size [12]. Individuals reared at high temperatures usually attain a smaller body size at maturity, known as the temperature size rule (TSR; [13]). Studies have shown that not only does the body size of organisms decrease at higher rearing temperatures, but so does the size of their cells [14]. As body size is a function of both cell size and cell number, a decrease in cell size may partially explain the temperature–size dependence [10,15–17]. Different cell types within a multicellular body may evolve distinct sizes optimized for their specific functional demands [2]. For example, Czarnoleski *et al.* [9] found that *Drosophila* raised at higher temperatures had smaller epidermal wing cells, flight muscles and Malpighian tubules, but that the epidermal cells of the legs were larger.

Research on the relationship between cell size and heat tolerance has explored how organisms respond to different temperatures by examining strains with natural cell size variations or by rearing individuals in varied thermal regimes. A notable study by Verspagen *et al.* [18] demonstrated that rearing *Drosophila* flies at different temperatures induced changes in cell size, which in turn affected their heat tolerance. Specifically, warm-reared flies had smaller cells and survived longer under intense, acute heat stress. Conversely, cold-reared flies had larger cells and showed better survival under chronic, mild heat stress. These findings can be partially explained by the concept of oxygen delivery efficiency. The enhanced surface-area-to-volume ratio of smaller cells facilitates more efficient oxygen diffusion to mitochondria, which is crucial for meeting the high metabolic demands of acute heat stress. Conversely, larger cells may be more energy-efficient, offering a survival advantage under conditions of chronic, mild stress where resource use is more critical than rapid oxygen delivery.

While the Verspagen *et al.* [18] study provides strong evidence for a link between cell size and heat tolerance, a key limitation is that cell size was altered through developmental acclimation to different temperatures. This makes it difficult to isolate the effect of cell size from other physiological changes induced by temperature acclimation. Therefore, while a relationship between cell size and oxygen diffusion is well-established, the precise mechanisms linking cell size, body size and heat tolerance remain unclear. More research is needed to determine whether the changes in heat tolerance are a direct consequence of cell size variations or a byproduct of the overall physiological acclimation to temperature.

Another underexplored area is the role of polyploidy in shaping cell size and heat tolerance. Polyploid organisms, which possess larger genomes owing to chromosome duplication, typically have larger cells than their diploid counterparts [19,20]. For example, experimentally produced triploid zebrafish (*Danio rerio*) had a 50% increase in cell size, resembling the 50% increase in genome size [21]. Under conditions of oxygen limitation, such as high temperature or hypoxia, organisms with larger genomes and, consequently, larger cells may face disadvantages owing to diffusion limitations. This is supported by the finding that large-bodied fish and those with larger genomes exhibit lower hypoxia tolerance in warmer waters [22]. A previous study on zebrafish larvae with different cell sizes and exposed to different rearing and testing temperatures in a full factorial design found that being composed of larger cells could provide metabolic advantages in the cold, whereas smaller cells are metabolically more beneficial in warm water [23].

While some studies suggest benefits for polyploids at low temperatures [23], others indicate that under demanding thermal conditions, the larger cells of polyploids can impose constraints on resource uptake [21]. This is consistent with the idea that larger cells may limit overall physiological efficiency, particularly when environmental demands are high. Polyploid species frequently exhibit distinct life-history traits compared with their diploid relatives (plants [24]; *Daphnia* [25]; freshwater snail [26]; salamanders [27]). These differences are often attributed to nucleotypic effects (conditions of the nucleus impacting the phenotype [28]), including larger cell sizes, larger overall body sizes and delayed maturity in polyploids ([25,27]; reviewed in [29]). Their larger cells and extended development may place them at a competitive disadvantage in warmer and temperate regions [30]. Furthermore, these larger cells could directly influence heat tolerance through increased oxygen limitations at higher temperatures. However, the specific mechanisms by which polyploidy influences heat tolerance are not well understood.

In this study, we aim to address these gaps by investigating the relationships between cell size, body size, ploidy and heat tolerance in the *Daphnia pulex* complex. This species complex includes naturally occurring sympatric diploid and triploid clones in subarctic areas and only diploid clones in temperate zones [31]. This makes it an excellent system for examining how cell size variation, influenced by both temperature and ploidy, affects heat tolerance [32]. We measured the critical thermal maximum (CT_{max}), which is a widely used measure of heat tolerance [33,34]. To test how rearing temperature affects heat tolerance, body size and cell size, we reared *Daphnia* clones from both subarctic and temperate areas under three temperatures: 16°C, 20°C and 24°C. Juveniles with a matched body size across temperatures and ploidy levels were selected to disentangle the effects of cell size from those of body size. Previous cell size studies have focused on *Drosophila* wings [10,15,17,18]. Here, we examined how carapace cells respond to temperature acclimatization and covary with body size. While the TSR posits that high temperatures lead to smaller body sizes, it is likely that the higher temperature tolerance of smaller organisms is dictated by their smaller cell sizes. Therefore, it is crucial to disentangle the impact of body size from cell size to identify the physiological or developmental traits that modulate upper heat tolerance. The association between body size and heat tolerance could stem from a dimensional tension between total organismal volume and the surface area of respiratory surfaces or be because body size acts as a proxy for cell size. Given the established link between oxygen transport capacity, cell size and heat tolerance, we hypothesized that heat tolerance would be dictated by cell size more than by body size in size-matched clones. Specifically, we predicted that (i) individuals reared at lower temperatures will have larger cells and lower heat tolerance; (ii) polyploid individuals will have larger cells than diploid individuals from subarctic and temperate areas and consequently lower tolerance to high temperatures; and (iii) heat tolerance will be negatively correlated with cell size and body size. We further measured the expression of Hsp70

and catalase to explore the underlying physiological mechanisms associated with heat tolerance. In ectotherms, thermal stress typically leads to oxidative stress via the production of reactive oxygen species (ROS) [35,36]. Since physiological acclimation to high temperatures involves the upregulation of stress responses that may be associated with ploidy and cell size, we predicted that CTmax would be positively associated with HSP and catalase expression.

2. Material and methods

(a) Rearing conditions

A total of 17 *D. pulex* clones collected over the years from various sites at temperate (seven diploid clones) and subarctic (five diploid and five triploid clones) locations were chosen for the heat tolerance experiments (table 1). Clones were reared for at least three generations under standardized laboratory conditions prior to the experiments. Each generation started from the second or third brood. *Daphnia* were acclimated at 16°C, 20°C and 24°C. These three temperatures were chosen to allow a large range to detect cell size differences and a maximum temperature well tolerated by both temperate and subarctic clones [32]. Subarctic clones can encounter 24°C during rare heatwave events as water temperatures of small ponds follow closely air temperatures [37]. *Daphnia* were raised at a 12 : 12 photoperiod and at 3000 lx of lighting in 300 ml of FLAMES culture medium with a total of 20 individuals per jar [38]. They were fed 2.0×10^5 cells ml⁻¹ of live *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata* or *Selenastrum capricornotum*) three times a week. One-third of the culture medium was renewed every two weeks. A total of 153 jars were used (3 jars $\times$ 3 temperatures $\times$ 17 clones).

(b) Heat tolerance

Heat tolerance was assessed using the critical thermal maximum (CTmax), which corresponds to the temperature at which an animal loses motor functions and faints [33]. The method for measuring CTmax was modified from that of Geerts *et al.* [39,40]. CTmax was performed using a thermocycler (Biometra®) with a heating block adapted to fit 48 $\times$ 0.5 ml tubes. Individual *Daphnia* were placed in 0.5 ml tubes containing a solution of 0.75 mg ml⁻¹ bovine serum albumin in FLAMES culture medium at the rearing temperature. Bovine serum albumin decreased the surface tension to keep individual adult *Daphnia* out of the surface layer so they could be exposed to the heating temperature. We chose *Daphnia* that were large enough to be well visualized in the tubes and small enough not to bear eggs. Hence, *Daphnia* had similar body sizes among temperatures and between ploidies. The tubes were randomly placed in a thermocycler. The CTmax protocol was fast ramping, with temperature rising one degree per minute from the rearing temperature until 45°C. Each run was performed using *Daphnia* from the same rearing temperature. We obtained 5–25 measures per clone for each temperature. To measure the heating in real time, a temperature probe was placed in a tube containing only the solution. The design was filmed using an HD camera (Fujifilm FinePix HS30EXR) to record each run. The CTmax of individual *Daphnia* was scored by watching these video recordings using video editing software (Movavi Video Editor 14). Even though the thermocycler could fit 48 tubes, a maximum of 25 *Daphnia* could have their CTmax scored in each run because of the camera focus and zoom. CTmax was determined as the temperature (to 1 d.p.) at which the animals stopped swimming and sank to the bottom of the tubes.

(c) Size measurements

Body and cell sizes were measured for each *Daphnia* individual using a microscope with a digital microscope camera A35100U3 (OMAX) and ToupView software (OMAX, v. 3.7). The total length of *Daphnia* was measured at 2.5 $\times$ from the top of the compound eye to the base of the caudal spine. Cell sizes were obtained using the modified protocols of Omilian [41]. Briefly, individual *Daphnia* were placed under a microscope at 10 $\times$. Diamond-shaped prints on *Daphnia*'s carapace (figure 1) were used as proxies for epidermal cell size under the carapace [41,42]. Preliminary measures revealed the presence of a single DAPI-stained nucleus in each diamond-shaped print on the carapace, confirming the prints as valid proxies for epidermal cells. In *Daphnia*, cell imprints found at the base of the caudal spine, near the labrum and directly at the margin of the shell, differ in pattern and have high levels of endopolyploidy (somatic polyploid cells) [31]. To avoid endopolyploid cells, the prints measured were those of the anterior region of the ventral margin. Only regular-sized and shaped prints were taken. The mean vertical length of 10 regular-shaped prints was used as a cell size proxy for each *Daphnia*. The cell volume was also estimated by raising the length measures to the power of 3. A mean ratio of 1.51 (95% CI: 1.17–1.94) was obtained when comparing triploid with diploid cells, close to the theoretically expected ratio of 1.5, suggesting that shape prints on the carapace could be reliably used as a proxy for cell size (electronic supplementary material, figure S1).

(d) Heat-shock experiment

To induce the expression of Hsp70 and catalase, *Daphnia* were individually placed in 10 ml tubes filled with FLAMES medium and heat-shocked in a 30°C water bath for an hour. We used three replicates of four individual *Daphnia* for each clone (except for A5–7, which only had two individuals per replicate). An untreated control group comprising *Daphnia* unexposed to heat shock was included to measure the basal expression levels of Hsp70 and catalase. All samples were flash-frozen in liquid nitrogen and stored at –80°C until RNA extraction. For the heat-shock experiment, we used only clones reared at 20°C.

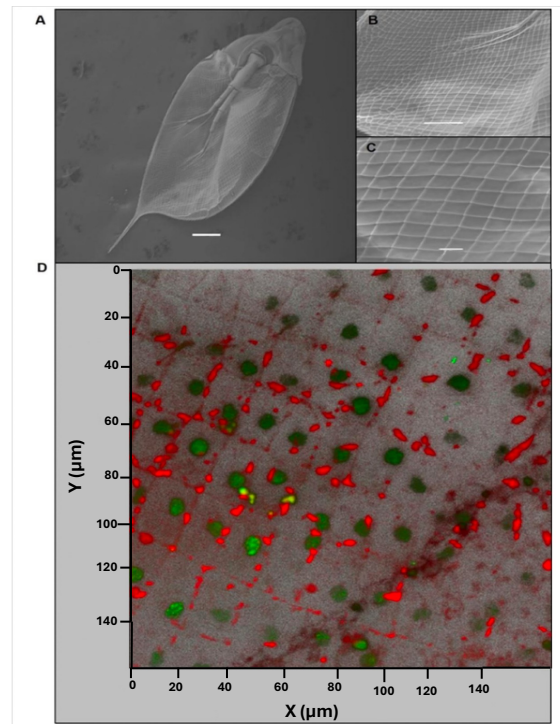


Figure 1. (A–C) Scanning electron microscope images of *Daphnia pulex* with diamond-shaped prints on the carapace. Scale bars: A, 200 μm; B, 100 μm; C, 20 μm. (D) Membranes were stained with Nile Red and nuclei with Sybr Green I following permeabilization with ethanol 70%. Picture was taken on a JEOL 6460 LV scanning microscope and a Carl Zeiss LSM700 confocal laser scanning microscope.

Table 1. List of *Daphnia pulex* clones sampled for the CTmax measurements.

clone	ploidy level	climatic zone	location	latitude	longitude
C145-01	triploid	subarctic	Churchill, Manitoba, Canada	58.76764	−93.9752
A38-10	triploid	subarctic	Churchill, Manitoba, Canada	58.76764	−93.9752
A5-7m	triploid	subarctic	Churchill, Manitoba, Canada	58.76764	−93.9752
IQ12	triploid	subarctic	Kuujuarapik, Nunavik	55.27667	−77.7764
R202	triploid	subarctic	Kuujuarapik, Nunavik	55.27667	−77.7764
R210	diploid	subarctic	Kuujuarapik, Nunavik	55.27667	−77.7764
R206	diploid	subarctic	Kuujuarapik, Nunavik	55.27667	−77.7764
B104-05	diploid	subarctic	Churchill, Manitoba, Canada	58.76764	−93.9752
A06-06	diploid	subarctic	Churchill, Manitoba, Canada	58.76764	−93.9752
WP2	diploid	subarctic	Kuujuarapik, Nunavik	55.27667	−77.7764
Bus15	diploid	temperate	Urbana, Illinois, USA	40.12989	−88.2065
Bus79	diploid	temperate	Urbana, Illinois, USA	40.12989	−88.2065
NFL68	diploid	temperate	Webster, Indiana, USA	39.90351	−84.9415
SPS100	diploid	temperate	Homer, Illinois, USA	40.75394	−73.9821
KAP-53	diploid	temperate	Danville, Illinois, USA	40.18278	−87.6462
Ste-Luce	diploid	temperate	Sainte-Luce, Québec, Canada	48.54757	−68.3942
Stukely	diploid	temperate	Bolton, Québec, Canada	45.28688	−72.4201

(e) RNA extraction and RT-quantitative polymerase chain reaction

RNA was extracted from four replicates of *Daphnia* following modified protocols from Lopez & Bohuski [43] and Escobar & Hunt [44]. Following extraction, RNA was treated with DNase I (Thermo Fisher, #EN0525) by adding 1 μl of RNA to 1 μl of 10× reaction buffer with 1 μl DNase. The reaction volume was adjusted to 10 μl with DEPC-treated water before incubation at 37°C for 30 min. After incubation, 1 μl of EDTA 50 mM was added to the mix and incubated once more at 65°C for 10 min. To obtain cDNA, DNase-treated RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, cat: 436614) with MultiScribe™ Reverse Transcriptase and 10× RT random primers without an RNase inhibitor, following the manufacturer's instructions. Quantitative real-time PCR was performed on a LightCycler 480 (Roche) using the

SensiFAST SYBR® No-ROX mix (98020, Bioline) under the following conditions: 95°C for 10 min and 45 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. The total reaction volume was 15 µl. Target genes used were *Hsp70* (forward: 5'-TGATGCCAAGCGTCTTATTG-3'; reverse: 5'-CGGCAACCTCCTTCATTTA-3') and *catalase* (forward: 5'-TCTCTTCGACCGGAAACAAC-3'; reverse: 5'-GCCCCGATCTAAATTACGAA-3'). Target gene expressions were normalized to two reference genes: *Stx16* (forward: 5'-CCTGCCACAATTGCATAAAA-3' reverse: 5'-AACGTTCTCGCGTGATTTA-3') and *CAPON* (forward: 5'-GCACAACCATC AACATCTGG-3'; reverse: 5'-CCATTTCATCGCGAATTCT-3'). All primers were designed using Primer3 (v. 4.0.0) [45]. The mean normalized fold change of target gene expression between heat-shocked and untreated individuals was calculated using the $2^{-\Delta\Delta CT}$ method described by Livak & Schmittgen [46], where $\Delta\Delta CT = (CT_{\text{target}} - CT_{\text{reference genes}})_{\text{Treatment}} - (CT_{\text{target}} - CT_{\text{reference genes}})_{\text{Control}}$. Technical duplicates were included to test the quality of the manipulations and pipetting errors.

(f) Statistical analyses

A total of 702 individual *Daphnia* (1–12 individuals × jar × clone × temperature) were used for heat tolerance measurements, body size and average cell size. The average cell size per *Daphnia* was calculated using ten measurements (SE of each individual varied from 0.11 to 2.21 µm). We used mixed linear models with clones and jars as random effects. The effect of acclimation temperature was also considered as random among clones. Climate and ploidy factors were treated as fixed effects in all models. Climate and ploidy were combined into a single factor with three levels (subarctic 2n, subarctic 3n and temperate 2n). Considering the relationships between CT_{max}, body size and cell size, cell size was used as a covariable to adjust the mean body size, and both body and cell sizes to adjust the mean CT_{max}. The partial effects of body size and cell size on CT_{max} were calculated using the 'visreg' package [47]. The BIC and AIC criteria were used to compare several mixed models to determine the importance of the variables on CT_{max}. The effects of heat-shock treatment and ploidy level on the normalized mean fold change expression of catalase and Hsp70 were also tested in a linear mixed model with clone as a random effect. All statistical analyses were performed using R 4.5.1 [48] with the 'lme4' [49] and 'lmerTest' [50] packages. The degree of freedom of the dominator was approximated using the Satterthwaite method, and type III ANOVA was used. We visually checked normality, homoscedasticity and independence of residuals with the package 'performance' [51]. Tukey *post-hoc* tests and linear contrasts were performed using the 'emmeans' package [52].

3. Results

(a) Effects of cell size, climate, ploidy and temperature on body size

We tested the effects of cell size, acclimation temperature (random effect among clones), climate–ploidy group with clone and jar as random effects on body size (first model, electronic supplementary material, table S2). There was a significant relationship between body size and cell size ($F_{1,582} = 108.91$, $p < 0.001$); however, this relationship did not vary significantly with acclimation temperature ($F_{2,442} = 2.68$, $p = 0.070$) and climate–ploidy grouping ($F_{2,576} = 0.77$, $p = 0.464$; figure 2A). The slope of this relationship was positive and increased slightly (from 0.031 to 0.047) as a function of temperature. There were generally no significant differences between the estimated marginal means of body size among the climate–ploidy groups for the same temperatures ($p > 0.064$) and among the temperatures for the same climate–ploidy group ($p > 0.262$; figure 2B). The only significant differences observed were between subarctic-2 n and temperate-2 n at 24°C ($p = 0.023$) and between 16°C and 24°C for subarctic-2 n ($p = 0.035$). The mean cell size varied significantly with temperature ($F_{2,18} = 18.88$, $p < 0.001$) and climate–ploidy ($F_{2,14} = 16.01$, $p < 0.001$; figure 3; second model in electronic supplementary material, table S1). The interaction of temperature and climate–ploidy group was not significant ($F_{4,19} = 2.39$, $p = 0.087$). Clones had significantly larger cells at 16°C than at 20°C ($p = 0.0001$) and at 16°C than at 24°C ($p = 0.011$). The estimated marginal mean of cell size was larger for subarctic clones 3 n than for subarctic and temperate clones 2 n ($p < 0.006$). There was no significant difference in cell size between the temperate and subarctic 2 n clones ($p = 0.307$).

(b) Effects of cell size, body size, climate, ploidy and temperature on CT_{max}

To determine which factor best explained the variation in CT_{max}, we tested the effects of cell size, body size, acclimation temperature (random effect among clones), ploidy and climate with clone and jar as random effects. The Bayesian information criterion (BIC) supported a more parsimonious model $CT_{\text{max}} \sim \text{CellSize} + \text{Acc. Temperature}$ (model A in table 2; third model, electronic supplementary material, table S1). The Akaike Information Criterion (AIC) for the best model supported a linear mixed model $CT_{\text{max}} \sim \text{CellSize} + \text{Acc. Temperature ClimatePloidy}$ (model D in table 2). Likelihood ratio tests revealed that climate–ploidy provided statistically significant improvements ($\chi^2_6 = 21.00$, $p = 0.002$). Models with body size were less well supported than those with cell size. The model $CT_{\text{max}} \sim \text{CellSize} \times \text{Acc. Temperature}$ (fourth model, electronic supplementary material, table S1), despite no significant interaction between cell size and acclimatization temperature ($F_{2,332} = 0.78$, $p = 0.458$), revealed that the effect of cell size on CT_{max} was slightly modulated with temperature. The slope of CT_{max} as a function of cell size varied from −0.084 to −0.105 between 16°C and 24°C.

The mean CT_{max} varied significantly with temperature ($F_{2,14} = 44.04$, $p < 0.001$) and climate ploidy ($F_{2,14} = 3.92$, $p < 0.001$, figure 4, fifth model electronic supplementary material, table S1). The interaction temperature–climate–ploidy was not significant ($F_{4,14} = 1.90$, $p = 0.167$). The partial effects of body size and cell size on CT_{max} were similar (electronic supplementary material, figure S2). For each climate–ploidy combination, the estimate marginal means of CT_{max} increased with temperature

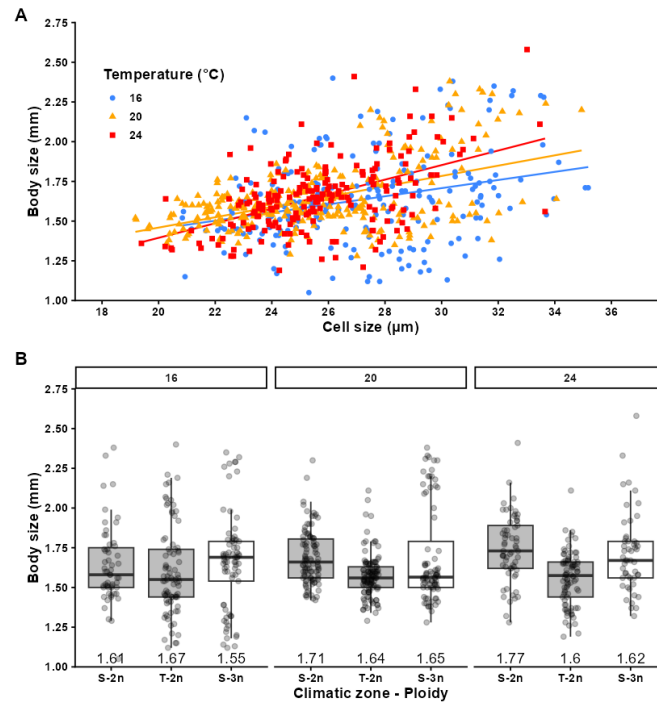


Figure 2. (A) Relationships between body size and cell size at three temperatures and (B) distribution of body size according to temperature and climate–ploidy combination. The estimated marginal means of body size from Model A (table 1) are also presented.

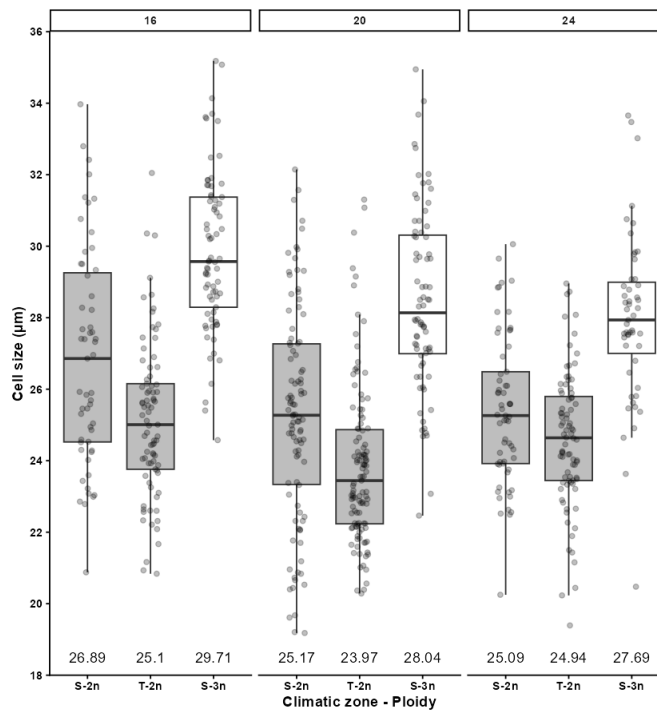


Figure 3. Cell size as a function of ploidy levels (2 n, 3 n) and acclimation temperature (16°C, 20°C and 24°C) with error bars. The estimated marginal means of cell size from the second model (electronic supplementary material, table S1) are also shown.

(linear contrasts, $p < 0.0010$). All contrasts, except CTmax between 16°C and 20°C for temperate clones 2 n and subarctic 3 n, were significant. Significant differences in adjusted CTmax among climate–ploidy contrasts were found between subarctic 3 n clones and temperate 2 n at 16°C and 20°C ($p < 0.023$) and between subarctic 2 n clones and subarctic 3 n at 20°C ($p = 0.003$). All other contrasts were not significant ($p > 0.05$).

(c) Expression of Hsp70 and catalase

Heat-shock treatment significantly increased the expression level of Hsp70 ($F_{1,75} = 72.74$, $p < 0.001$) and catalase ($F_{1,74} = 14.83$, $p < 0.001$; 6th and 7th Model, electronic supplementary material, table S1). The constitutive expression level (untreated) or heat-shocked expression level of Hsp70 and catalase did not differ between climate–ploidy levels ($F_{2,14} = 3.63$, $p = 0.054$, $F_{2,14} =$

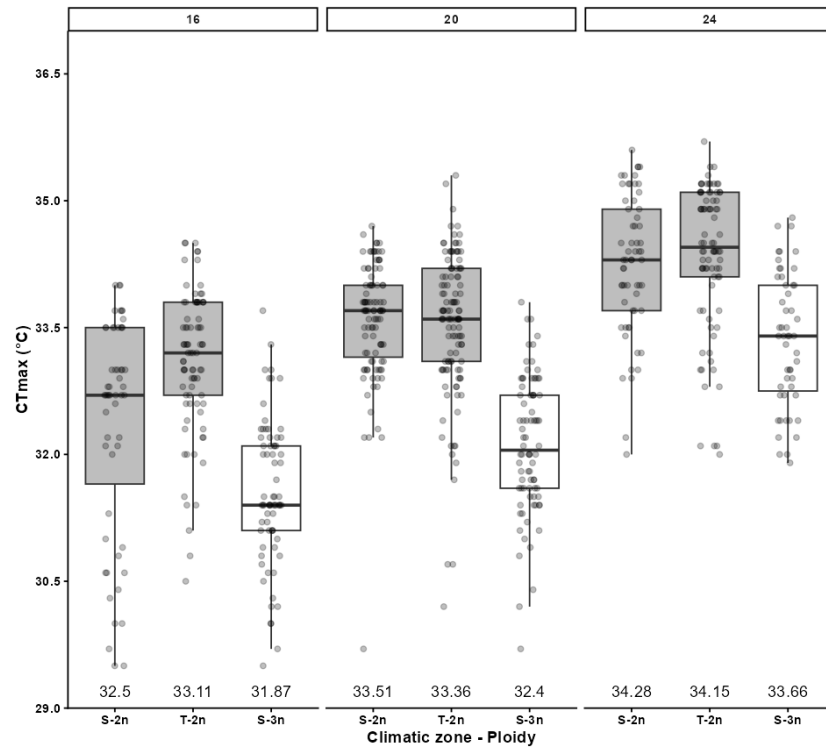


Figure 4. CTmax measurements by ploidy levels (2n, 3n), climatic zone (S: subarctic, T: temperate) and acclimation temperature (16°C, 20°C and 24°C). Estimated marginal means of CTmax from the fifth model (electronic supplementary material, table S1) are shown.

Table 2. Model predictors of thermal tolerance (CTmax; CS = cell size; AT = acclimation temperature; CP = climate–ploidy; BS = body size). Bold values indicate best models.

model	k	R^2_{marginal}	BIC	ΔBIC	weight	LL	AIC
(A) CS + AT	12	0.565	1444.20	0	0.996	−682.8	1389.69
(B) CS × AT	14	0.566	1455.88	11.67	0.003	−682.1	1392.29
(C) BS + AT	12	0.553	1458.77	14.57	0.001	−690.1	1404.13
(D) CS + AT × CP	18	0.689	1462.46	18.25	0.000	−672.3	1380.69
(E) BS × AT	14	0.396	1463.05	18.84	0.000	−685.6	1399.29
(F) BS + AT × CP	18	0.695	1473.37	29.16	0.000	−677.7	1391.40
(G) BS × AT × CP	26	0.610	1502.68	58.48	0.000	−666.1	1384.28
(H) CS × AT × CP	26	0.693	1504.43	60.23	0.000	−667.2	1386.33
(I) AT + CP	13	0.641	1542.75	98.54	0.000	−728.8	1483.54
(J) AT	11	0.460	1544.57	100.36	0.000	−736.2	1494.48
(K) CS × BS	7	0.150	1555.78	111.57	0.000	−755.0	1523.98
(L) AT × CP	17	0.640	1561.40	117.20	0.000	−725.0	1483.99
(M) CS + BS	6	0.118	1579.55	135.35	0.000	−770.1	1552.30
(N) CS	5	0.105	1615.08	170.88	0.000	−791.2	1592.37
(O) CS + CP	7	0.279	1621.36	177.15	0.0006	−787.8	1589.56
(P) CS × CP	9	0.282	1633.04	188.83	0.000	−787.1	1592.16
(Q) BS	5	0.058	1643.23	199.03	0.000	−805.2	1620.46
(R) BS × CP	9	0.242	1653.45	209.25	0.000	−797.2	1612.47
(S) null	4	0	1718.26	274.05	0.000	−846.0	1700.04
(T) CP	6	0.204	1720.03	275.83	0.000	−840.4	1692.71

0.29, $p = 0.750$, figure 5). Clones differed significantly in their expression of Hsp70 and catalase ($p < 0.001$). The clone ‘Ste-Luce’ had the highest induction of Hsp70 and catalase following heat shock (electronic supplementary material, figure S3). There was no correlation between CTmax at 20°C and mean heat-shocked expression levels of Hsp70 or catalase.

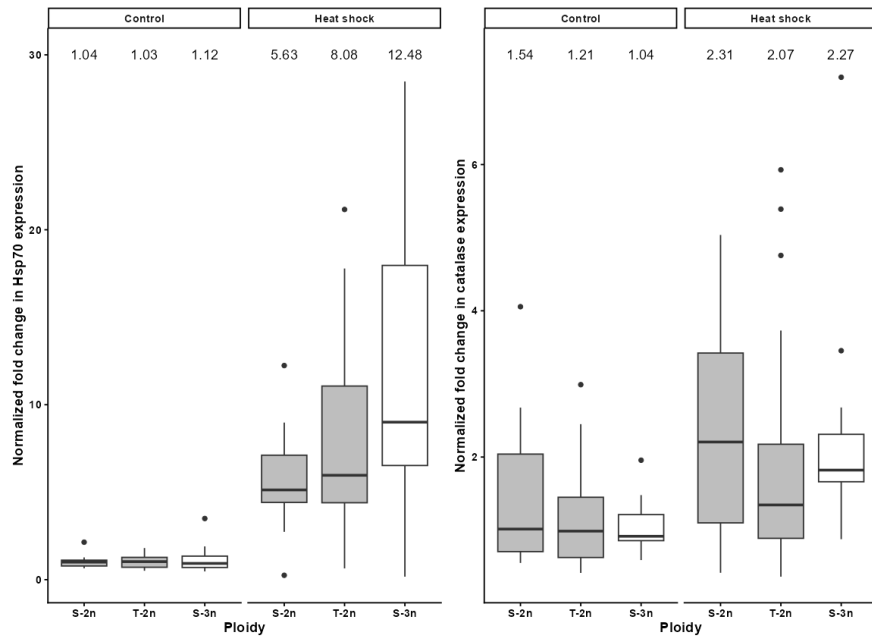


Figure 5. Mean normalized expression fold change of Hsp70 and catalase by ploidy levels (2n, 3n) and treatment (heat shock, control). Estimated marginal means are shown (sixth and seventh model, electronic supplementary material, table S1).

4. Discussion

It has been proposed that tissues composed of smaller cells have better oxygen diffusion [30,53] and this should be advantageous under higher temperatures despite the higher costs. We show here that reduction in cell size through temperature changes and through ploidy differences is associated with higher heat tolerance.

(a) Temperature and cell size

This is one of the first studies to use the imprints on the carapace as a measure of cell size in *Daphnia* (but see [42]). We showed that the size of cells composing the carapace was positively correlated with body size, and this relationship did not change with acclimation temperature. Our results also showed that carapace cell size varied with acclimation temperature, with *Daphnia* raised under lower temperatures (16°C) having larger cells than those raised under moderate to high temperatures (20°C and 24°C). As the inner wall of the carapace is a major site of respiratory gas exchange [54], this organ is highly relevant to examine the effects of temperature on cell size. Temperature-induced effects on cell size have been reported in different organisms such as protists [55], dungflies [56], Madagascar ground geckos [57], *Drosophila* [10,15], nematodes [58] and frogs [59]. Different tissues, cells and ontogenetic stages can have different thermal sensitivities and responses to temperature [60–63]. In eutelic organisms (with fixed cell number), such as copepods, temperature-mediated changes in body size occur exclusively through changes in cell size. In non-eutelic organisms, changes in cell size also correspond to temperature-mediated changes in body size depending on the tissues or organs considered. In *Drosophila*, the cells of the wing epidermis have been used as a proxy for body size change measures as wings grow by increasing cell size rather than cell number (e.g. [10,15,64]). It is not completely clear how cells become smaller under higher temperature. In *Drosophila*, the mTOR pathway (mammalian target of rapamycin) is thought to be an important player with the activation of the hypoxia-inducible factor (HIF) [65]. When *Drosophila* flies are treated with rapamycin (a component of the mTOR pathway), they have smaller bodies and smaller cells in all tissues [17].

(b) Polyploidy and cell size

Ploidy had an important effect on cell size, with triploid clones having cells that were 1.5× the size of diploid ones as expected with a genome size that is 1.5× larger. Cell size differences between triploid and diploid clones were observed under the three acclimation temperatures. This is consistent with other findings showing correlations between genome size and cell sizes [19,66]. Triploid hybridogenetic frogs, *Pelophylax esculentus*, had larger erythrocytes and epidermal cells than diploids at 19°C and 24°C [59]. In our study, diploid and triploid clones differed in their cell size but not in body size because we specifically chose individuals in the same size range. As triploid clones have larger body size at maturity than diploids [25], our diploid individuals had to be one instar older than the triploid ones. It is not clear whether *Daphnia* with different instars show differences in CT_{max} after accounting for mass differences. Additional studies should help to reveal age-related changes in CT_{max}.

(c) CTmax association with cell and body size

Acclimation temperature had a significant effect on CTmax. As expected, *Daphnia* raised at 16°C had lower CTmax than those raised at 20°C or 24°C (except for temperature 2 n). Cell size (but not body size) was also larger at 16°C than at 20°C or 24°C. Even if it is difficult to disentangle the effects of cell size and body size, our experimental design using size-matched *Daphnia* allowed us to determine that linear models with cell size had greater support than those with body size. Body size also contributed to CTmax differences, as found in several *Daphnia* studies that have reported negative correlation between heat tolerance and body size [11,39,40,67]. Burton & Einum [11] confirmed that younger/smaller individuals were more tolerant of both brief (~5 min) and also more moderate and prolonged (>15 h) exposure to higher temperatures. Negative associations between body size and survival under heat stress have been reported in other species such as *Drosophila* [68]. In stoneflies (*Plecoptera*), larger nymphs displayed lower temperature tolerance than smaller ones [69]. A recent meta-analysis of 500 species of ectotherms exhibiting an extreme range in body size revealed that heat tolerance was negatively correlated with body mass in water breathers [70]. A study on eight species of crustaceans found that larger species had lower CTmax, but only under hypoxia [71]. Lower heat tolerance in large-bodied organisms could be owing to the lower surface-area-to-volume ratios of their cells, which constrain their capacity to deliver oxygen to their mitochondria [22,60,70]. Fewer studies have examined the impact of cell size on heat tolerance. In some studies, genome size has been used as a proxy of cell size. For example, reduction in hypoxia tolerance in warmer water has been found to be particularly pronounced for fishes with large genomes [22]. In this study, we sought to isolate the individual role of cell size, body size and ploidy in determining CTmax. However, this separation is somewhat artificial, since cell size and body size are intimately tied through developmental processes and both depend on the organism's ploidy level. The most logical interpretation is that these traits evolved together under shared developmental constraints and selective pressures. Progress in understanding adaptive cell size evolution is challenging owing to the tight covariation between cell size, genome size and body mass [2].

In addition, few studies have successfully unravelled the effects of cell size and body mass on heat tolerance. A recent study by Leiva *et al.* [10] on the effects of hypoxia in *Drosophila* lines that naturally vary in cell size offers revealing insights into this relationship. They found that flies with larger cells showed stronger reductions in body mass under warm and hypoxic conditions, indicating that larger cells are more prone to oxygen limitations. Hypoxia tolerance is dictated by the accessibility of mitochondria to available oxygen, which in turn depends on the cell surface area for gas exchange. The smaller surface-area-to-volume ratio in larger cells may limit oxygen diffusion to mitochondria. The same structural constraints probably apply to thermal stress, as higher temperatures increase the mitochondrial respiration rate and, consequently, oxygen demand [72,73]. Thus, the lower diffusion rate in larger cells may be detrimental at higher temperatures, lowering thermal limits. Additional studies are needed to clarify the relative importance of cell size and body size to heat tolerance in *Daphnia*. These studies should assess the total number of somatic cells, cell size and body mass in *Daphnia* clones raised under different temperatures and at various ages. In addition, selection experiments designed to decouple body size from cell size would allow more direct tests of the independent effects of cell size and body size on thermal tolerance [2].

(d) CTmax and polyploidy

Triploid clones had lower CTmax than diploid clones at 16°C and 20°C, probably resulting from their larger cells limiting oxygen diffusion under warmer temperatures. The lower temperature tolerance of polyploids could not be solely ascribed to their larger cells, as when CTmax was adjusted for cell and body size, triploid clones still had lower CTmax than diploid clones. Studies investigating heat tolerance and ploidy in ectotherms have shown mixed results. Bowden *et al.* [74] found no differences in the CTmax of triploid and diploid salmon. Ellis *et al.* [75] found that triploid brook charr (*Salvelinus fontinalis*) had lower CTmax than diploid brook charr, but only under hypoxic conditions. Other studies on different fish species showed that triploids have lower tolerance at high temperatures than diploids [76,77]. As suggested by Leiva *et al.* [70], the duration of the thermal assays needs to be considered because short chronic tests under high temperature may favour small-celled individuals, whereas longer chronic assays under the same temperatures may favour larger-celled individuals. In the spined loach (*Cobitis taenia*), triploid individuals had larger erythrocytes and lower standard metabolic rate than diploids [78]. Diploid tadpoles of the edible frog *P. esculentus* had a higher standard metabolic rate (SMR) than triploids, whereas in froglets, ploidy did not affect the SMR, suggesting that triploids are more vulnerable to oxygen limitations in aquatic than in terrestrial areas [79]. Triploid *Xenopus laevis* containing fewer, larger cells than diploids had a lower metabolic rate [80]. This difference was abolished when inhibiting the Na⁺/K⁺ ATPase pump, suggesting that less energy is required to maintain plasma membrane potential in triploids due to the lower cell surface-area-to-volume ratio [80].

(e) CTmax and oxidative stress

Besides cell size, we hypothesized that polyploids might have increased expression of important genes such as *Hsp* and *catalase* that may affect heat tolerance. Heat-shock treatment significantly increased the expression of *Hsp70* and *catalase* compared with the control group, meaning that exposure to 30°C for an hour successfully induced a heat-shock response in *Daphnia*. These genes are usually upregulated during thermal stress in numerous ectotherm species, including *Daphnia* [81]. Higher *catalase* activity in response to temperature changes or to long-term acclimation to higher temperatures has been reported in daphnids [36,82,83], consistent with our findings. Ploidy did not affect *Hsp70* or *catalase* gene expression in both heat-shocked

and untreated individuals. This indicates there is no difference in constitutive expression levels or in the heat-shock response in triploids. Few studies have investigated differences in Hsp70 and catalase activity between diploid and polyploid species. Malerba & Marshall [84] found that larger cells had more accumulation of ROS and could therefore be more susceptible to oxidative stress. Triploid fish had lower Hsp70 levels than diploids [85]. Nunes *et al.* [86] found consistently higher catalase activity in the liver of diploid rainbow trout (*Oncorhynchus mykiss*) compared with triploids when investigating the effects of ploidy and diet on different enzymes. The lack of relationship between CTmax and Hsp and catalase expression suggests that these enzymes do not set the heat tolerance limit but might play a role in its modulation.

5. Conclusions

Our results imply that cell size-mediated heat tolerance could be an important contributing factor explaining the distribution of polyploid ectotherms. Polyploid *Daphnia* with larger cells have lower heat tolerance and could thus be disadvantaged under warmer temperatures compared with diploids. This could potentially explain their absence from temperate regions [31]. Maintaining the polarization of membranes with trans-membrane gradients takes up a substantial amount of energy [87]. This process could be more costly for smaller cells because of their larger surface-area-to-volume ratio, so larger genomes and cells could be more cost-efficient in colder temperatures and display better cold tolerance. In accordance with this, a companion study found that triploid *Daphnia* clones had increased cold tolerance compared with diploid clones [32].

Ethics. Ethical approval is not required for experimental work with *Daphnia*. The animals used have been in culture in the lab for several years.

Data accessibility. Measures of CTmax and gene expression in *Daphnia* acclimated to three temperatures are available in Dryad [88].

Supplementary material is available online [89].

Declaration of AI use. We used AI to improve the grammar.

Authors' contributions. F.D.: conceptualization, funding acquisition, project administration, resources, supervision, writing—original draft, writing—review and editing; S.-B.B.: formal analysis, investigation, methodology, software, visualization, writing—original draft, writing—review and editing; A.G.-M.: software, visualization; P.U.B.: conceptualization, funding acquisition, project administration, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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