An integrative study to understand the temperature-size rule with nonlinear temperature increase: One approach to rule them all?

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Abstract

The temperature-size rule (TSR) describes the inverse relationship between organism size and environmental temperature in uni- and multicellular species. Despite the TSR being widespread, understanding the mechanisms for size responses to environmental warming remain elusive. Here, we experimentally test three hypotheses (differential development and growth [DDG], maintain aerobic scope and regulate oxygen supply [MASROS] and the supply-demand hypothesis [SD]) explaining the TSR using the aquatic protist *Colpidium striatum* in three gradually changing and a constant temperatures crossed with three different nutrient levels. We find the constant and slowly warming environments show similar responses in terms of population dynamics, whereas the linear and fast populations quickly decline and also show a stronger temperature-size reponse. Our analyses suggest that acclimation may have played a role in observing these differences among treatments. Further analyses reveal that the SD hypothesis is most parsimonious with the data, however, neither the DDG nor the MASROS hypothesis can be firmly dismissed. We conclude that the drivers of the TSR are most likely multicausal and recommend the use of structural equation models to inform about the relative contributions of different hypotheses whenever multicausal processes are to be understood in biology.

Keywords: *Colpidium striatum*; development rate; growth rate; supply-demand model, oxygen regulation

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1 Introduction

Temperature and body size jointly influence biological rates from the individual to the ecosystem level (Brown et al. 2004). But temperature and size are not independent. About 80% of ectotherms are smaller when grown in warmer environments (Atkinson 1994). This pattern is so widespread, that is has been named the temperature-size rule (TSR), which describes an inverse, plastic response between body size and environmental temperature observed both in multi- and unicellular organisms (Atkinson 1994, Atkinson et al. 2003, Forster and Hirst 2012, Forster et al. 2013). Despite the TSR being widespread and having important consequences for the structure and functioning of communities, the mechanistic drivers of the TSR remain elusive (Table 1).

Multiple hypotheses have been proposed and tested to explain the mechanisms of the TSR (Figure 1A). The differential development and growth (DDG) hypothesis states that differences in thermal sensitivities of growth and development rates of organismal ontogeny explain the TSR, i.e. development is accelerated more strongly by higher temperatures than the body growth (Forster et al. 2011). If the rates of cell division and differentiation are more sensitive to temperature than cell growth, increased temperature would produce smaller organisms (van der Have and de Jong 1996). While the DDG hypothesis assumes that body size shrinks because the development rate is more temperature-sensitive than the growth rate, in principle, it could go either way. The reversed TSR, where the cell sizes are larger at higher temperatures, has been observed as well (Zuo et al. 2012).

Despite direct temperature effects on the physiology of individuals, it is also possible that temperature affects size indirectly, that is, mediated by another environmental factor that is changed by temperature. The MASROS (maintain aerobic scope and regulate oxygen supply) hypothesis proposes that organisms change their body size to regulate the oxygen demand of their tissues (Atkinson et al. 2006) (Figure 1A). At higher temperatures organisms have greater difficulty meeting tissue oxygen demands due to the increasing metabolic oxygen demand. At the same time, the oxygen solubility decreases in warmer waters, hence limiting the oxygen diffusion to the cell surface, which means that being smaller becomes more beneficial due to a higher surface to volume ratio. The MASROS hypothesis has been suggested to be of great importance in aquatic environments, where the oxygen solubility of water is strongly influenced by temperature (Forster et al. 2012).

Finally, size change with temperature could be driven by the need of organisms to balance their bodily demands for growth with the supply of resources from the environment (the supply–demand model, SD) (DeLong 2012). The supply-demand hypothesis posits that organisms balance their demand and the supply to achieve optimal body size for a given environment. According to this hypothesis, the TSR is consistent with the idea of balancing increasing metabolic demand with a constant or decreasing environmental supply. Unlike the previous two hypotheses, the SD hypothesis provides an explanation for possible reversals or the TSR as well as oscillations in cell size (DeLong et al. 2017).

Although all of these hypotheses have been empirically tested, there is not any consensus

reached about the most likely mechanistic drivers. This is because previous experiments mostly tested a single hypothesis at a time (see Table 1). However, these hypotheses are not all mutually exclusive, but instead might complement each other, i.e. the drivers behind the TSR are most likely a combination of multiple effects, that is, they are multicausal. Therefore, an integrative approach that would simultaneously allow to evaluate all three hypotheses on a single dataset is needed.

Organisms also experience temperature change different to how it is mostly manipulated in experiments (see Table 1). Under natural conditions, temperature change is continuous and gradual. It is therefore possible that the temperature-size response is exaggerated as an experimental artefact due to the abrupt exposure of organisms to large temperature changes and the response may be ameliorated by acclimation of individuals to more gradual temperature change. Therefore, we need studies that evaluate the effect of gradually changing temperatures on the TSR.

Here, we aim to understand the relationship between cell size and gradually increasing temperature using the ciliate *Colpidium striatum* as study organism. We experimentally test all three hypotheses regarding the mechanisms of TSR in aquatic microorganisms using one constant and three gradually increasing temperature treatments, where the rate of temperature change is either slow, linear or fast. We circumvent shortcomings of previous empirical tests by using an integrative approach combining the three hypotheses. We also study the organism's ability to acclimate when the environmental warming affects intra- and intergenerational time scale.

Here we hypothesize that if temperature affects within generation, we expect to see decreasing abundance and/or extinctions, because the lack of time to acclimate. Whereas if temperature affects across generations, then we expect to see TSR, i.e. shrinking cell size. At high speed of increase into sublethal temperatures, we expect to see various responses such as vegetative enlargement to increase viability without inducing sexual reproduction (Atkinson et al. 2003), whereas in the optimal thermal range an inverse relationship between size and temperature is expected. Furthermore, we expect that bacteria, the food source of *Colpidium* can deal with environmental change faster than protists due to quicker, hence maximum density should be comparable across temperature treatments but will differ across nutrient availability.

Authors	Year	Study system	Treatment	Theory tested		
				DDG	MASROS	SD
Pichrtová and Nemcová	2011	*Synura petersenii* and *Mellomonas tonsurata*	five constant temperatures (5, 10 15, 20, 25°C)	X		
Forster et al.	2011	published data were used	constant temperatures	X		
DeLong	2012	*Actinosphaerium*	three constant temperatures (20, 25, 28°C)			X
Forster et al.	2013	*Cyclidium glaucoma*	constant temperature	X		
Huete-Stauffer et al.	2016	bacteria	daily fluctuations with three regimes (ambient, warming (+3°C) and cooling (-3°C))	X		
DeLong et al.	2017	*Tetrahymena thermophila*	three constant temperatures (20, 26, 32°C)	X	X	X
Walczyńska et al.	2016	*Coleps hirtus*	six constant temperatures (10, 15, 20, 25, 30, 35°C)	X		
Skau et al.	2017	*Emiliania huxleyi*, *Chrysochromulina rotalis* and *Prymnesium polylepis*	two constant temperatures (13°and 19°C)			(X)
Tseng et al.	2019	*Scenedesmus obliqus*	three constant temperatures (18, 22, 16°C)			

Table 1: **Literature overview of empirical studies on TSR with an experimental approach on unicellular organisms.**

2 Materials and methods

2.1 Experimental design

Microcosms were loosely capped 250 mL glass jars containing 100 mL of medium (0.28, 0.56 or 1.12 g/L of Carolina Biological Supplies Protist Pellet in Chalkley's medium) (Altermatt et al. 2015) with two autoclaved wheat seeds added for the slow release of carbon and nutrients.

A mix of bacteria (*Bacillus subtilis, Serratia fonticola* and *Brevibacillus brevis*) forms the basal food supply of the bacterivorous protist *Colpidium striatum*. We inoculated bacteria in the medium 2 days prior to the experiment. We then added 1 mL *Colpidium* drawn from stock cultures kept at 15 °C, to obtain an initial concentration of 3 individuals per mL and randomly assigned microcosms to temperature treatments.

The experiment has a full-factorial design of four levels of temperature treatment (constant 15°C, three rising temperature between 15-30°C, see Figure 1C) and three levels of nutrient concentration (low = 0.28, medium = 0.56, and high = 1.12 g Protist Pellet medium per liter). We choose constant 15°C as the reference temperature as this is representative for the temperature

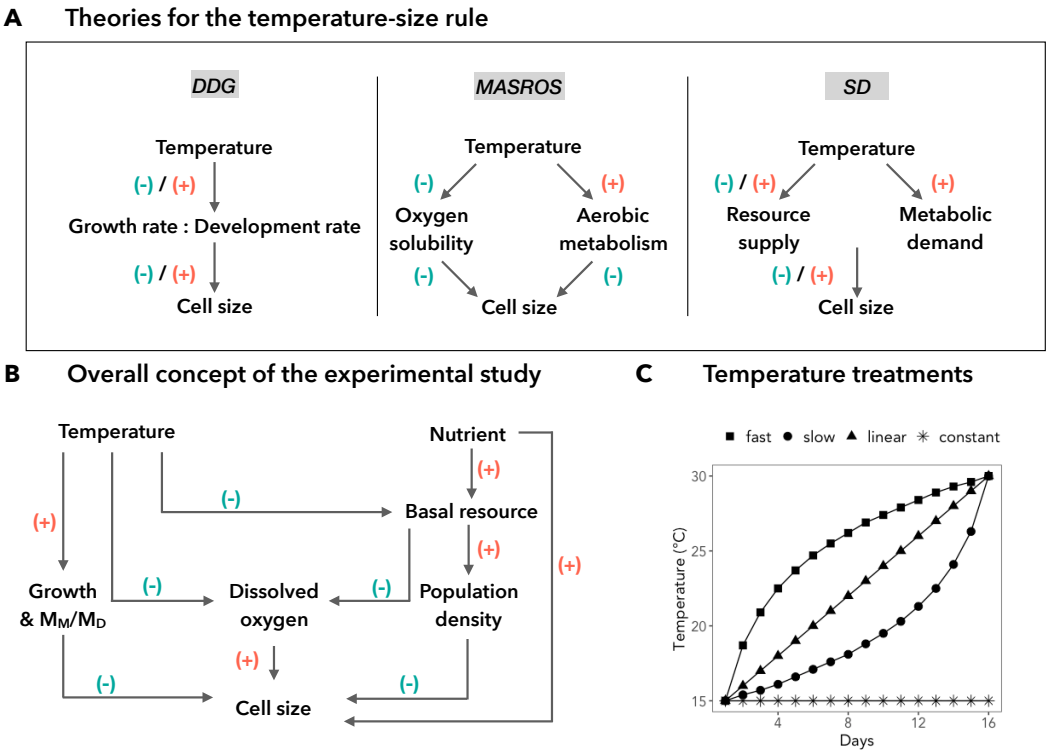

Figure 1: **Theoretical and conceptual background of this study. (A)** Several hypotheses have been proposed to explain the mechanistic drivers of the TSR. (1) The differential development and growth (DDG) hypothesis states that in warm environments an organism has less time to grow, since warming accelerates the rate of development faster than that of growth, which results in a smaller cell size. However, a reversed TSR has been observed in some cases, which might indicate that growth has higher sensitivity relative to development. (2) Elevated temperature affects oxygen availability through decreasing oxygen solubility in the water and also through increasing aerobic metabolism, which both bring about smaller cell size. (3) Increased temperature also has a positive effect on the metabolic rate, which increases resource demand of the organism relative to the available supply. This mismatch of demand and supply then leads to a smaller cell size (supply-demand model). **(B)** In this study we jointly test empirically these three theories, in order to identify the major drivers of TSR and their interconnections. Furthermore, we also account for the effect of nutrient enrichment and the density-dependence of the cell size. **(C)** Four different temperature treatments were applied in this study: constant 15°C, linear, slow and fast rising treatment between 15-30°C. The fast and slow rising treatments have opposite shapes.

Colpidium striatum experiences in natural freshwater environments. All three rising temperature treatments start at 15°C and end at 30°C. The linear speed is +1°C per day, while the fast rising temperature increases at the beginning of the experiment faster then it slows down and the slowly warming treatment goes in reversed fashion. The selected temperature treatments reflect the predicted per generation rates of increase over the next decades (IPCC 2007). Furthermore, this temperature range is suitable for studying dynamical changes based on the thermal response of the selected species. Based on previous experiments, the abundance and growth rate of *Colpidium striatum* increases up to 17°C and then decreases in the 17-30°C temperature range (Jiang and Morin 2004, Plebani 2015). Temperature was manipulated by use of four incubators. This design results in 12 treatment combinations, with five replicates each, and 60 experimental units in total.

During the experiment, 5 mL of medium (of a total of 100 mL medium) was taken every day and replaced with 5 mL of sterile media. 1 mL of the sample was transferred to a counting chamber and filmed under a stereo microscope with a 25× magnification mounted digital CMOS camera (Hamamatsu Orca C11440; Hamamatsu Photonics, Japan) to take a 5s video (25 frames per second). The following properties were measured with the R package BEMOVI (Pennekamp et al. 2015): individual morphology (cell size and shape), and population size (individuals per mL). Furthermore, samples were processed to measure bacterial abundance using flow cytometry with an Accuri C6 flow cytometer and dissolved oxygen (DO) concentration with oxygen sensors (from PreSens - Precision Sensing GmbH); for details refer to Tabi et al. (2019) .

2.2 Response and predictor variables to understand the TSR

Cell size was estimated for each individual using length and width information assuming an ellipsoid form (Laakso et al. 2003): $V = \frac{4}{3}\pi a^2 b$, where a is the radius of width and b is the radius of length. Then, we calculated the cell size as the median cell volume (MCV) for each day in each population. The mother and daughter cells were estimated as the upper and lower 20% of cell size distribution for each day. Cell shape was measured by the aspect ratio (cell length / cell width) of each cell. The aspect ratio closer to 1 represents a rounder form, whereas >1 means a more oblong cell shape.

The growth rates for each population were estimated by fitting logistic growth model with changing intrinsic growth rates to the time series of *Colpidium*'s density (Werker and Jaggard 1997):

$$\frac{dy}{dt} = \mu y(1 - \frac{y}{K}) \tag{1}$$

$$\frac{d\mu}{dt} = -k(\mu - \mu_{min}) \tag{2}$$

where μ is the growth rate, K is the carrying capacity and k is a constant which determines the speed with which the initial intrinsic growth rate, approaches the final minimum intrinsic growth rate, μ_{min}. The carrying capacities were calculated as the maximum density of the time series of each population (see more details in Supplementary Material).

The growth rate was used as the demand proxy to test the SD hypothesis. Bacterial abundance was used as the supply in SD model. To test the DDG, we rely on the equation by (van der Have and de Jong 1996), i.e. $\frac{g}{D} = ln(\frac{M_M}{M_D})$, where g is the mass-specific growth rate of the individual (day^{-1}), D is the development rate (day^{-1}, that is, 1/doubling time), M_M is the mass of the mother cells and M_D is the mass of the daughter cell. The dissolved oxygen concentration of the medium was used for testing the MASROS hypothesis.

2.3 Data analyses

2.3.1 Linear model analysis

In order to understand the relationship between cell size and population abundance, temperature, time and nutrients, we used a general linear model:

$$ln(MCV) = t + log(P) * T * N \tag{3}$$

where MCV is the median cell volume, t is time (days), P is the cell density (cells mL^{-1}), T is temperature (°C) and N is the nutrient concentration (three levels: low, medium, high). MCV and density were log-transformed to meet assumptions of homoscedastic residuals. For the constant temperature treatment, we added a small amount of noise to the constant 15°C time series to be able to estimate the temperature term. Noise was modelled as a Gaussian distribution (mean = 0, SD = 0.5), representative for the small temperature fluctuations which naturally occur in the incubators. For the linear treatment, we dropped the day term because the perfect correlation with the temperature term did not allow to estimate both independently.

2.3.2 Structural equation modeling

We fitted structural equation models (SEM) to the data to understand the drivers of changes in median cell volume (Grace et al. 2010). Relevant predictor were the time series of temperature, the nutrient availability (three levels: low, medium, high), the mean cell density (individuals per mL), the bacterial abundance (cells per mL as a supply proxy), the growth rates (as a demand proxy), as well as the dissolved oxygen content measured in the medium. We started with an initial SEM including all causal drivers and relevant links and fitted the model simultaneously to the four temperature change groups. We then evaluated model fit with goodness-of-fit metrics till a model was found that included all relevant links. We then simplified the model by removing links which were included in the initial model but had relatively low explanatory power, using AIC-based model comparisons. Finally, we tested whether coefficients were different across groups, by comparing models where certain path coefficients were forced to be the same, against freely estimated coefficients. Model comparison was based on the AIC. All structural equation models were fitted with the lavaan package (Rosseel 2012) in R.

3 Results

3.1 Cell size, shape, and population abundance through time

In the constant treatment population dynamics closely followed logistic growth, where after an initial growth phase, carrying capacity was reached and maintained till the end of the experiment. The slow warming treatment showed remarkably similar dynamics, despite the fast increase in temperature at the end of the experiment. The fast warming treatment showed initial growth and a short stable phase, whose length depended on the nutrient treatment. The linear treatment showed dynamics intermediate to the slow and the fast increase (Figure 2A).

Cell size decreased with time in the constant, slow and fast warming temperature treatments (Figure 2D, Figure 3, Tables S1-S2). The temperature effect was not significant for all but the fast treatment, where it was positive, but note that temperature interacted with other factors rendering the interpretation of the main effect rather difficult. The effect of enrichment was only visible in the linear and fast temperature treatments, where it negatively affected cell size in the medium enrichment case, and positively in the high enrichment case, respectively (Figure 3). Density and temperature interacted in the fast change treatment only, whereas both nutrient enrichment interacted with temperature in a positive way, hence alleviating the negative effect of temperature, for all but the fast treatment. Density interacted with enrichment in a negative fashion in the fast treatment only, while the three-way interaction was positive only for the fast treatment (Table S2).

To better understand the consequences of interactions among factors on cell size, we show plots of the estimated effects for the four treatments in the supplement (Figure S2-S5). The interaction plots suggest that slowly increasing temperature had a positive effect on cell size in the medium and high nutrient enrichment, but the temperature effect reverses to be negative in the low enrichment treatments, when warming is linear or fast. Increasing density reinforced the temperature effect across all increasing temperature treatments.

Cell shape also varied with time (Figure 2E). The changes in cell shape were generally negatively correlated with the cell size over time (see Table S4 in Supplementary Material). In constant 15°C, both the cell size and shape stabilized showing a strong negative correlation across all nutrient enrichment. In fast and linear warming combined with low nutrient enrichment, no strong pattern was observed. In both cases, the aspect ratio changed from rounder to more oblong and then back to rounder, while cell size decreased. Variation in aspect ratio was highest in the fast treatment with medium and high nutrient enrichment, which also mirrored the changes in cell sizes. In linear temperature treatment, after reaching a more oblong shape, cell shapes returned to rounder form at the end of the experiment across all nutrient enrichment.

3.2 Understanding the drivers of the temperature-size rule

The log ratio of mother and daughter cells showed no decoupling of mother and daughter sizes (Figure S1), even after removing the effect of population abundance on cell sizes. Instead, the log ratio of mother and daughter cells did not change over time but fluctuated around the

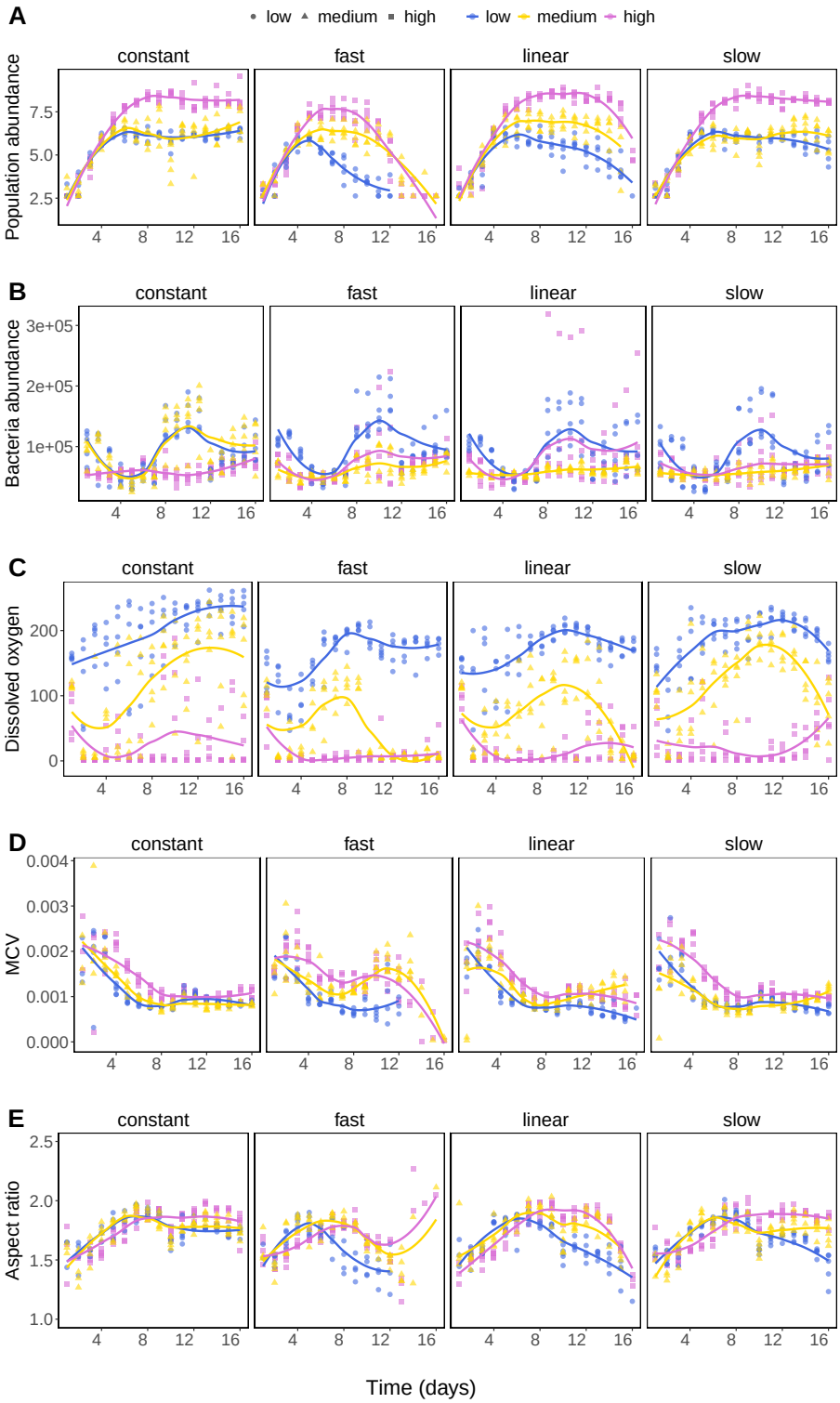

Figure 2: ***Colpidium striatum* abundance, cell size (MCV) and aspect ratio (AR) over time**. A) Population abundance is plotted on log scales (ind. mL^{-1}). B) Bacteria abundance per mL. C) Dissolved oxygen content in the medium. D) Changes in cell size. E) Changes in aspect ratio. Different nutrient treatments are colour coded. The fitted lines are splines.

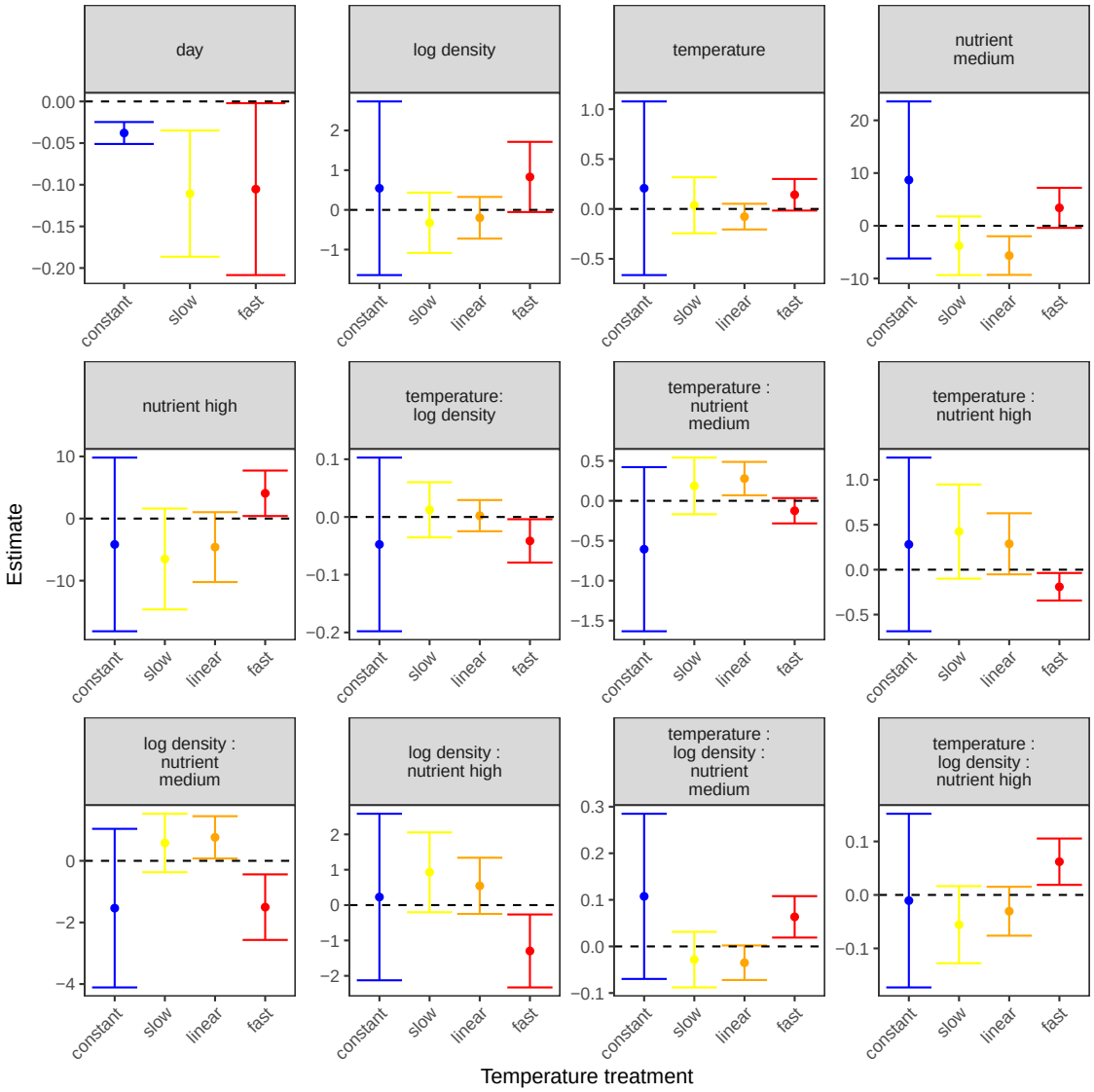

Figure 3: **Linear model parameters estimating effects of time (in days), density, temperature and nutrient enrichment**. The dashed lines indicate zero. Error bars show 95% confidence intervals for the estimates.

expected value of ln(2) in constant and all rising temperatures, regardless of nutrient treatment. Therefore, we excluded the mother and daughter cell ratio from the structural equation model.

The final structural equation model was a good fit to the data (comparative fit index: 0.999, test statistic 15.947, df = 14, p = 0.317) and explained between 30% and 45% of the variation observed in cell size (see Table 2). Variation in cell size was best explained in the constant and slow warming treatments. Across treatments, variation in oxygen content of the medium was best explained (R^2: 0.56 - 0.63), whereas abundance of bacteria had the lowest explanatory power (R^2: 0.08 - 0.11).

Table 2: Coefficients of determination (R^2) for the five endogenous variables in the SEM.

variable	fast	linear	slow	constant
Cell size	0.30	0.31	0.43	0.45
Dissolved Oxygen content of medium	0.63	0.61	0.60	0.56
Consumer abundance	0.22	0.44	0.50	0.43
Bacteria abundance	0.11	0.11	0.08	0.08
Growth rate	0.77	0.66	0.37	0.11

The SEM indicated that cell size was impacted directly and indirectly by temperature (see Figure 4 and Table S3). The direct effect of temperature on cell size (MCV) was negative in the fast and linear warming treatments. However, temperature also affected cell size indirectly, mediated by the dissolved oxygen content of the medium. Temperature increased the DO content in the linear and slow treatments, whereas it had no effect in the other treatments. Dissolved oxygen content itself affected cell size in a consistently negative fashion across treatments, meaning higher oxygen content led to lower cell size. Temperature had a negative effect on growth rate for all but the constant treatment, resulting in more strongly decreased growth rates with increasing speed of warming. All increasing temperature treatments had a positive effect on bacteria abundance, whereas the consumer abundance remained relatively constant across warming treatments. Higher bacteria abundance did not directly translate into larger cell size. Surprisingly, the direct effect of growth rate on cell size was positive in the constant and slow treatment, opposite to the expectation for a demand proxy. Consumer abundance had the expected negative effect on cell size, for all but the fast treatment where it was positive.

Nutrients had a positive effect on consumer abundance, but a negative effect on bacteria abundance, potentially indicating a trophic cascade. This was also reflected in higher growth rates in all but the fast warming treatment. Higher nutrient concentrations nevertheless only translated into larger cells in the slow and linear treatments.

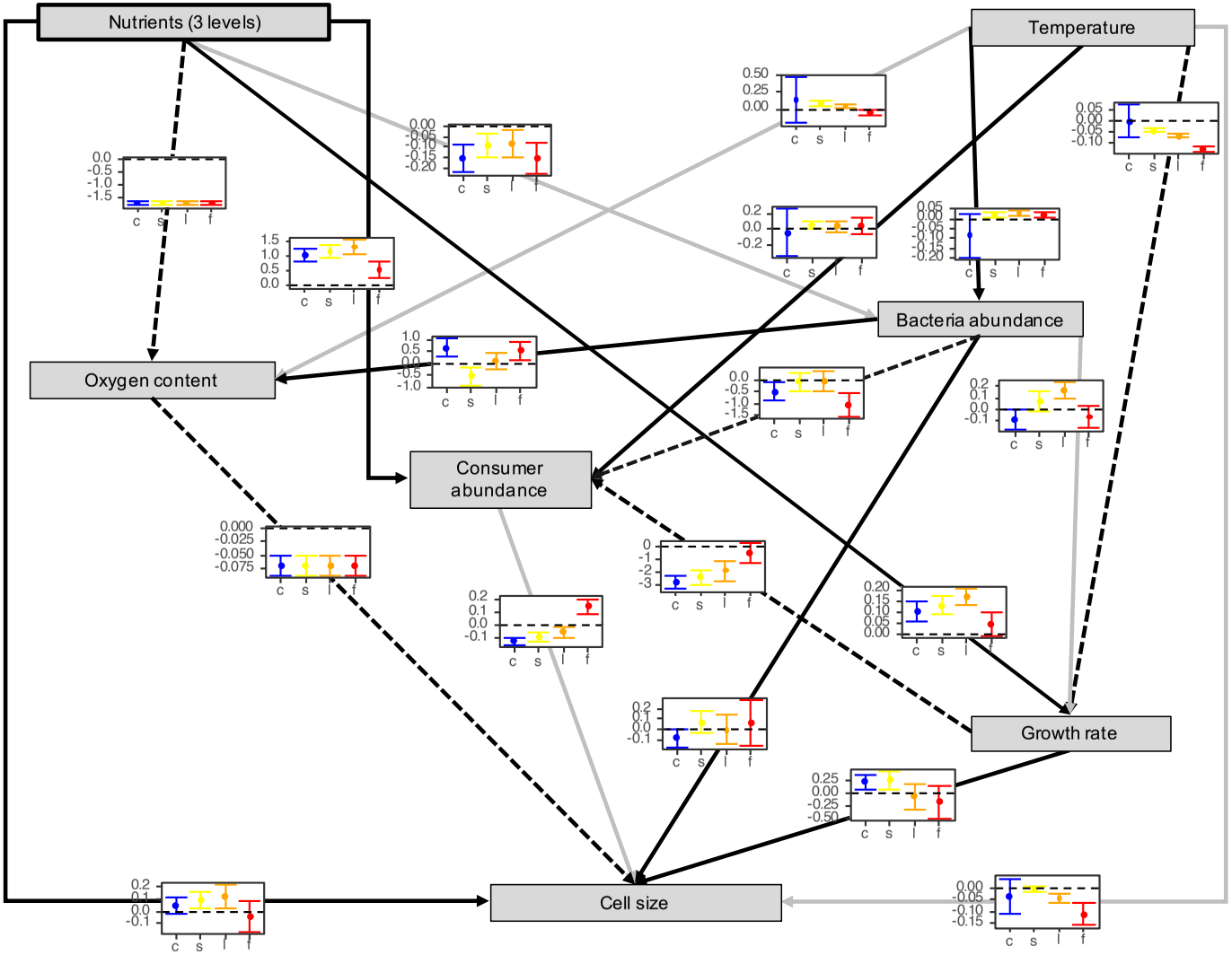

Figure 4: The causal network based on structural equations. The path coefficients are shown in the small inset plots, with the different temperature regimes (groups) shown with different colours (constant = blue, slow = yellow, linear = orange, fast = red). Dashed arrows indicate a predominantly negative effects, while solid arrows indicate a positive effects across temperature change. Grey arrows indicate a switch of sign in the coefficients across treatments.

4 Discussion

The temperature-size rule is a widely observed phenomenon, however, the mechanisms behind the TSR remain elusive. Here, we provided the first comprehensive analysis integrating components of different theoretical frameworks on the same experimental dataset to pinpoint the mechanisms of size changes in an unicellular model organism.

We observed substantially different dynamics among the rising temperature treatments, which might give an indication of the time lag acclimation needs. As for our hypothesis regarding the temperature effects in intra- vs intergenerational time scale, we showed that if the warming occurred at higher speed affecting the generation time during the growth phase, it led to extinctions. Moreover, cell sizes and shapes showed the strongest response in the fast change treatment, supporting our hypothesis. Interestingly, the slow temperature increased and the constant 15°C temperature affected *Colpidium* in a very similar fashion, which suggests that cells were able to acclimate to the slowly warming environment.

4.1 Evidence for different hypotheses

Our analysis of the log ratios of mother and daughter cells did not show any sign of decoupling across the four treatments (see Figure S1). Rather mother-to-daughter cell size ratios fluctuated around the expected ratio of $ln(2)$, regardless of the nutrient enrichment. In a previous study with a unicellular protist, support was found for the DDG hypothesis (Forster et al. 2013). However, organisms were exposed to different constant temperatures, unlike the gradually changing environmental temperatures as in our study. It is possible that acclimation to gradually changing temperature masked the pronounced decoupling visible when cells were exposed immediately to higher temperatures. Nevertheless, we would have expected to see a decoupling at least in the linear and fast changing temperature treatments. Therefore, we cannot fully exclude or support the DDG hypothesis.

While the pathway between temperature and dissolved oxygen content in the medium was supported by the SEM, the dissolved oxygen negatively affected cell sizes, i.e. higher dissolved oxygen content led to smaller cell size. This response was consistent across the four temperature treatments. *Colpidium* has tricarboxylic acid cycle allowing the synthesis of proteins in anaerobic conditions (Seaman 1949), however, in the presence of bacteria its oxygen uptake can be limited. Therefore the MASROS hypothesis might have a low importance in explaining shrinking cell sizes. The negative effect of DO on cell sizes can be an indirect effect of increasing metabolism of the protists which resulted in higher consumption of bacteria and therefore lower level of DO in the medium. Furthermore, the DO level showed no consistent pattern with either the bacteria or the the protist abundance and also cannot explain the decreasing cell sizes with temperature in our study. A potential explanation for not seeing any effect may be that *Colpidium* is only limited at very low dissolved oxygen concentrations, which we did not reach in this experiment. Therefore, we cannot fully exclude or support the MASROS hypothesis.

The supply-demand model predicts changes in cell size as a function of temperature-dependent demand and supply (DeLong 2012). Higher temperature will generally lead to higher metabolic rates and hence demand. Temperature may also affect the supply of resources. We found that temperature had a negative effect on growth rate, i.e. quickly increasing temperatures decreased growth rates more than linearly or slowly increasing temperatures. Regarding supply, temperature had a positive effect on bacteria abundance, with increasing abundance in the slow compared with the fast treatments. As expected, higher bacteria abundance (supply) had a positive effect on cell size, however, the effect was only significant in the fast treatment. The effect of temperature on the balance of supply and demand determines whether cell size should increase or decrease. Given that demand was higher in slow and linear treatments but bacteria abundance was also higher in these treatments one could expect these drivers to cancel out. Hence, although our results are consistent with the mechanisms of the SD model, we would expect to see little effect in our study. Previous experiments using the SD model found evidence for the balance between supply and demand explaining size variation in protists (DeLong 2012), including possible reversals of the temperature-size rule (DeLong et al. 2017)

In summary, our results are consistent with the mechanisms of the SD model, while the MASROS and differential growth and development hypotheses cannot be ruled out but neither be supported. Instead, other factors like the density of the consumer mediate the effect of temperature in our study. The direct effect of temperature on cell size also hints at other, still unidentified factors being important.

4.2 Acclimation to gradual temperature change

Previous attempts to study how cell size is affected by temperature have relied on designs where cells were immediately exposed to higher temperatures and the response compared to cell size in control temperatures to which cells were adapted (see examples in Table 1). Our design allows us to investigate the dynamic acclimation process as we exposed cells to gradually changing temperature regimes with a faster temperature increase occurring during either the population growth phase or the stationary phase (i.e. "fast" and "slow" treatment respectively). Our results indicate that linear and fast temperature increase leads to the occurrence of frequent interactions among temperature, density and nutrients potentially suggesting that cells were unable to adapt to the fast temperature increase, while simultaneous acclimation processes in the slow and linear treatment lead to additive processes unfold. Investigating the three-way interactions revealed that nutrient enrichment buffers some of the adverse temperature effects, but as soon as temperature change accelerates (linear and fast treatment), a negative temperature-size effect was observed. We interpret this as a signal of the acclimation process unfolding too slowly in the linear and fast change treatments, resulting in the population collapse observed as well as the negative temperature-size rule. Indeed, in our study, *Colpidium* experienced daily a temperature change (Figure 1C), whereas (Forster et al. 2013) observed an acclimation period between 70.5 and 120h for *Cyclidium glaucoma*.

4.3 Limitations

Our study is the first to take an integrative approach which compares the ability of different frameworks to understand the effect of temperature on cell size, combining distinct physiological pathways like oxygen consumption and the balance of supply and demand. The structural equation models allow us to compare the relative size of effects and a causal interpretation given the experimental nature. However, not all of the key variables could be manipulated, hence some of the estimated effects have to be evaluated with caution. Foremost, we only measured oxygen availability in the experimental cultures, which can co-vary with other variables such as bacterial density. While we controlled for some of these effects, our approach remains partly observational and hence future experiments should evaluate the oxygen effect by manipulating oxygen availability. The previous comment also applies to other variables that co-vary with the temperature change, such as time and density, which are difficult to disentangle due to the inherent covariation due to the growth process. While we integrate three main hypothesis regarding causal drivers of the temperature-size rule, our experiment cannot account for all possible hypotheses regarding temperature-size responses. For example, lacking measurements of DNA content, we were not able to test whether temperature induces changes at the the genomic level, which trigger subsequent changes in cell size to maintain the genome size to cell volume ratio (Hessen et al. 2013).

4.4 Conclusions

Our study revealed the multicausal nature of the temperature-size rule in gradually warming environments. The SEM suggests that the SD hypothesis is most consistent with the observed changes in cell size for our experimental system, but both MASROS and DDG may have played a role too. We recommend the use of structural equation models to inform about the relative contributions of different hypotheses to observed changes in cell size, especially the role of acclimation and possibly adaptation whenever complex, possible multicausal processes are to be understood in biology.

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Supplementary Material

1 Supplementary tables

Table S1

	Dependent variable:	
	log(MCV)	
	Constant	Slow
	(1)	(2)
temperature	0.207 (0.441)	0.036 (0.142)
log_density	0.544 (1.108)	−0.325 (0.385)
nutrientmedium	8.690 (7.549)	−3.782 (2.823)
nutrienthigh	−4.166 (7.088)	−6.512 (4.120)
days	−0.038*** (0.007)	−0.111*** (0.038)
temperature:log_density	−0.048 (0.076)	0.012 (0.024)
temperature:nutrientmedium	−0.606 (0.521)	0.186 (0.180)
temperature:nutrienthigh	0.281 (0.490)	0.424 (0.266)
log_density:nutrientmedium	−1.535 (1.305)	0.580 (0.481)
log_density:nutrienthigh	0.228 (1.194)	0.927 (0.572)
temperature:log_density:nutrientmedium	0.108 (0.090)	−0.028 (0.030)
temperature:log_density:nutrienthigh	−0.011 (0.082)	−0.056 (0.037)
Constant	−8.791 (6.404)	−6.246*** (2.238)
Observations	202	205
R^2	0.529	0.546
Adjusted R^2	0.499	0.518
Residual Std. Error	0.277 (df = 189)	0.280 (df = 192)
F Statistic	17.669*** (df = 12; 189)	19.273*** (df = 12; 192)
Note:		*p<0.1; **p<0.05; ***p<0.01

Table S2

	Dependent variable:	
	log(MCV)	
	Linear	Fast
	(1)	(2)
temperature	−0.077 (0.065)	0.142* (0.080)
log_density	−0.199 (0.266)	0.830* (0.448)
nutrientmedium	−5.649*** (1.857)	3.401* (1.926)
nutrienthigh	−4.600 (2.853)	4.068** (1.854)
days		−0.105** (0.052)
temperature:log_density	0.002 (0.014)	−0.041** (0.019)
temperature:nutrientmedium	0.278*** (0.106)	−0.124 (0.080)
temperature:nutrienthigh	0.288* (0.172)	−0.191** (0.078)
log_density:nutrientmedium	0.763** (0.348)	−1.504*** (0.539)
log_density:nutrienthigh	0.545 (0.403)	−1.297** (0.522)
temperature:log_density:nutrientmedium	−0.035* (0.019)	0.063*** (0.023)
temperature:log_density:nutrienthigh	−0.030 (0.023)	0.062*** (0.022)
Constant	−4.541*** (1.235)	−8.993*** (1.665)
Observations	205	183
R^2	0.393	0.399
Adjusted R^2	0.358	0.357
Residual Std. Error	0.383 (df = 193)	0.394 (df = 170)
F Statistic	11.336*** (df = 11; 193)	9.414*** (df = 12; 170)
Note:		*p<0.1; **p<0.05; ***p<0.01

Table S3: Table with SEM coefficients and their standard errors for the different temperature treatments

	Constant		Slow		Linear		Fast	
path	estimate	SE	estimate	SE	estimate	SE	estimate	SE
Cell size ~bacteria abundance	-0.079	0.044	0.071	0.054	0.003	0.068	0.061	0.109
Consumer abundance ~bacteria abundance	-0.492	0.178	-0.091	0.2	-0.063	0.221	-1.044	0.261
Oxygen content ~bacteria abundance	0.629	0.194	-0.527	0.2	0.099	0.18	0.534	0.192
Growth rate ~bacteria abundance	-0.087	0.043	0.07	0.046	0.167	0.036	-0.066	0.048
Consumer abundance ~Growth rate	-2.811	0.27	-2.402	0.283	-1.869	0.396	-0.482	0.397
Cell size ~Growth rate	0.211	0.078	0.246	0.086	-0.07	0.128	-0.184	0.16
Oxygen content ~temperature	0.129	0.173	0.082	0.018	0.044	0.018	-0.04	0.021
Growth rate ~temperature	-0.001	0.037	-0.041	0.004	-0.066	0.003	-0.126	0.005
Consumer abundance ~temperature	-0.048	0.153	0.049	0.021	0.031	0.033	0.036	0.058
Cell size ~temperature	-0.033	0.036	-0.008	0.006	-0.041	0.01	-0.111	0.023
Bacteria abundance ~temperature	-0.085	0.056	0.018	0.006	0.029	0.006	0.021	0.008
Cell size ~Consumer abundance	-0.122	0.016	-0.085	0.017	-0.049	0.021	0.143	0.029
bacteria abundance ~nutrients	-0.152	0.035	-0.093	0.029	-0.086	0.033	-0.151	0.037
Cell size ~nutrients	0.041	0.033	0.088	0.036	0.119	0.05	-0.043	0.06
Oxygen content ~nutrients	-1.682	0.049	-1.682	0.049	-1.682	0.049	-1.682	0.049
Consumer abundance ~nutrients	1.013	0.101	1.142	0.097	1.297	0.126	0.549	0.14
Growth rate ~nutrients	0.101	0.024	0.13	0.021	0.169	0.018	0.047	0.025
Cell size ~Oxygen content	-0.068	0.01	-0.068	0.01	-0.068	0.01	-0.068	0.01

Table S4: Spearman's rank correlation coefficients (*rho*) between aspect ratio (AR) and cell size (MCV) and corresponding p-values by treatment.

Temperature	Nutrient	Rho	p-value
constant	low	-0.82	5.894670e-20
constant	medium	-0.60	5.025890e-09
constant	high	-0.70	2.873411e-12
fast	low	0.14	3.056213e-01
fast	medium	-0.72	1.886859e-12
fast	high	-0.48	4.441602e-05
linear	low	0.09	4.628842e-01
linear	medium	-0.77	1.575486e-15
linear	high	-0.58	3.752201e-08
slow	low	-0.38	5.730952e-04
slow	medium	-0.61	3.105849e-09
slow	high	-0.85	5.979670e-23

2 Supplementary figures

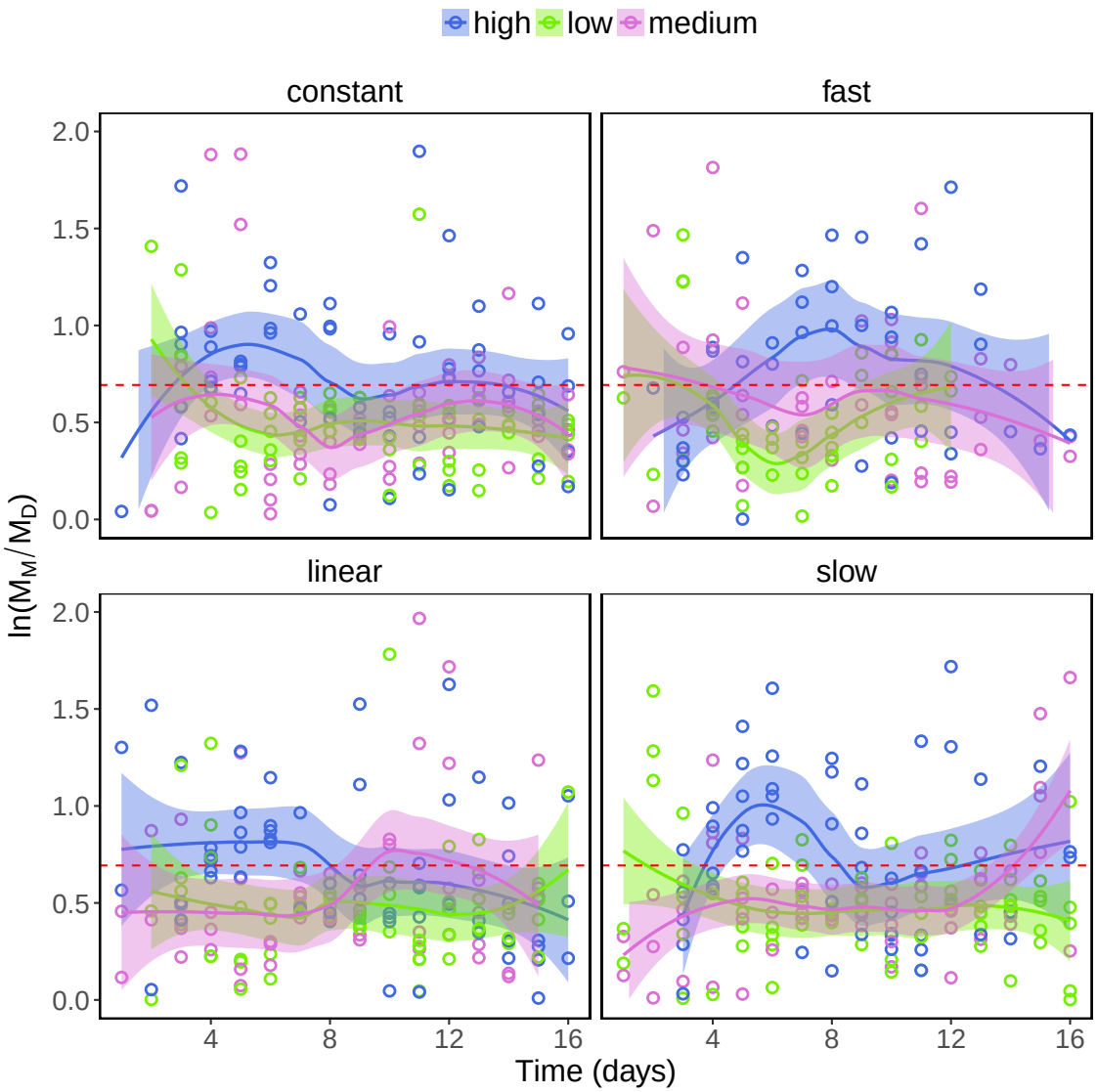

Figure S1: **The ratio of mother and daughter cell sizes of Colpidium striatum**. Cell sizes were corrected for the effect of population abundance. The horizontal red dashed line depicts the expected ratio of mother and daughter cell sizes ($ln(2)$).

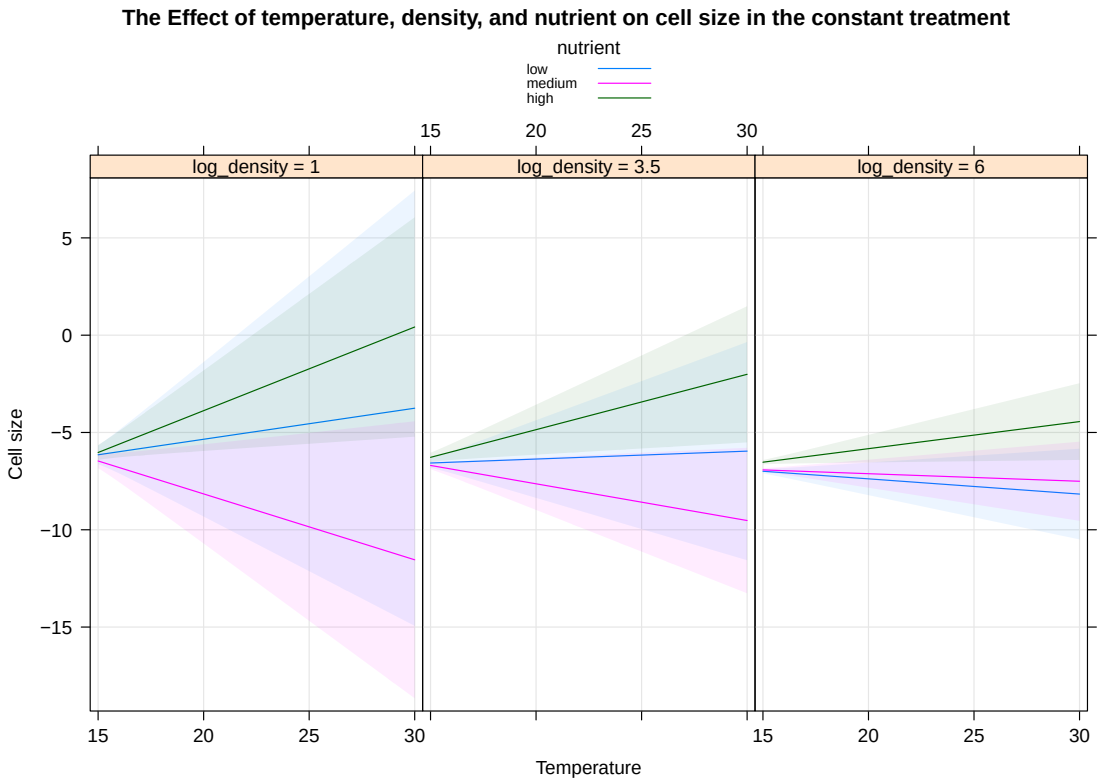

Figure S2: **Three-way interactions in the constant temperature treatment.**

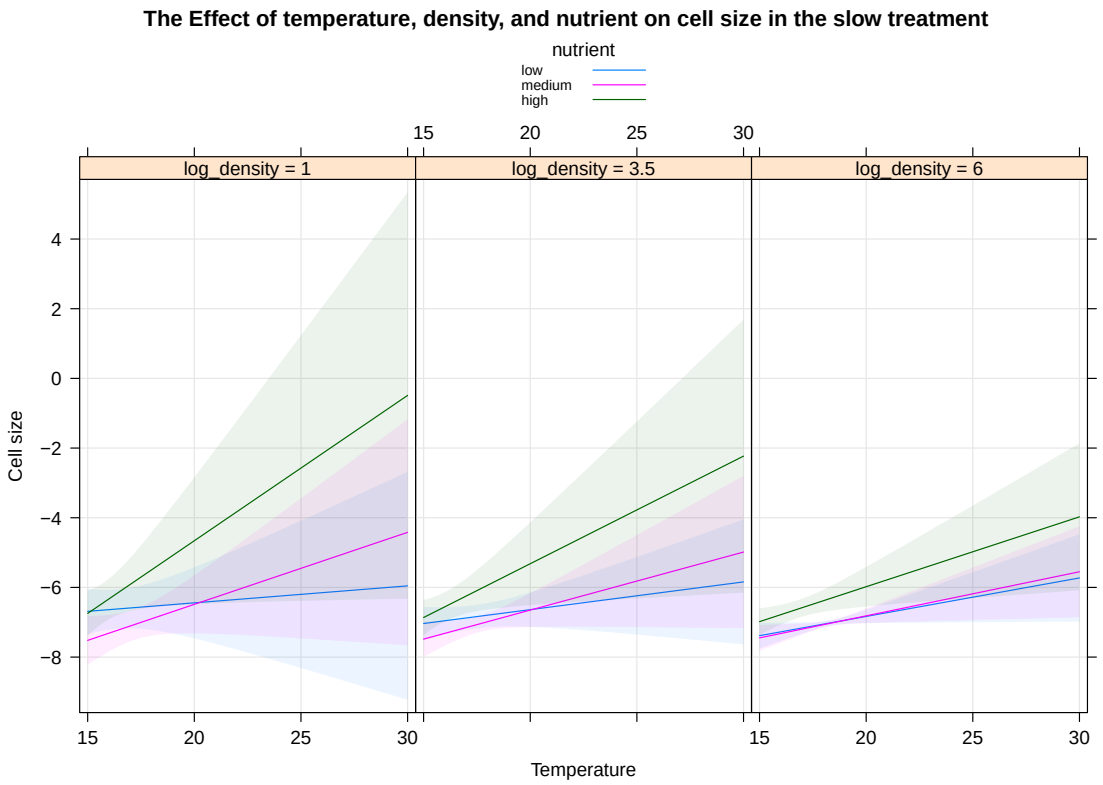

Figure S3: **Three-way interactions in the slow temperature treatment.**

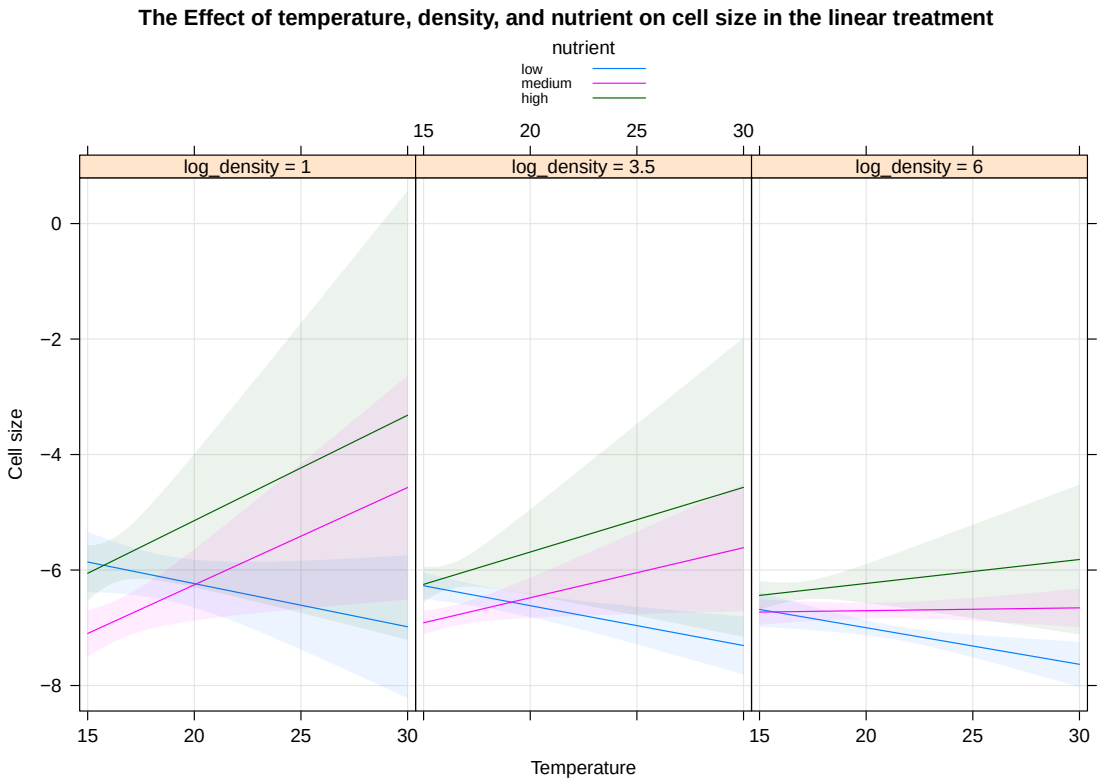

Figure S4: **Three-way interactions in the linear temperature treatment.**

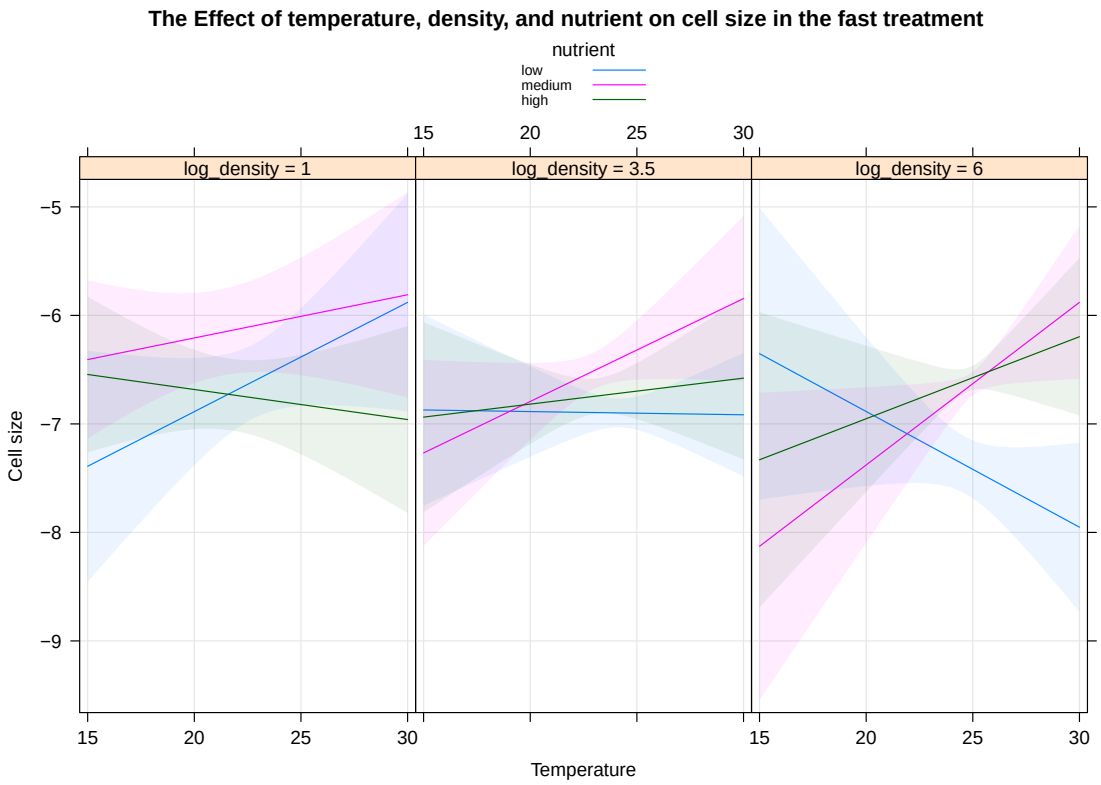

Figure S5: **Three-way interactions in the fast temperature treatment.**