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# Dynamic thermal reaction norms and body size oscillations challenge explanations of the temperature–size rule

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## ABSTRACT

**Background:** The temperature–size rule (TSR) describes a decrease in body size with environmental warming. There is little agreement about why the TSR occurs, but potential explanations include that smaller size (1) maintains aerobic scope, (2) is generated by differential responses of development and growth to temperature, and (3) balances the demand for resources with the expected supply.

**Organism:** The ciliate *Tetrahymena thermophila*.

**Methods:** We grew microcosm populations at three temperatures and measured population density and cell volume for 11 days (*c*. 20–53 generations depending on temperature).

**Results:** Populations at all temperatures showed typical sigmoidal population growth, but cell volumes oscillated widely. The oscillations reveal a dynamically shifting relationship between cell volume and temperature, such that the TSR was observed only when population sizes stopped growing, while a reverse TSR was observed during the exponential growth phase.

**Conclusion:** The dependence of the TSR on roughly equilibrium conditions challenges the hypotheses that maintaining aerobic scope and differential responses of growth and development to temperature drive the TSR. Reversals of the TSR and oscillations in cell volume are consistent with the idea that balancing resource demand with environmental supply drives body size changes (the supply–demand model), but the oscillations suggest a role for generational lags in achieving an optimal size.

*Keywords*: body size evolution, climate change, MASROS, protist, supply–demand model, temperature–size rule, thermal asymmetry.

## INTRODUCTION

A common response of ectotherms to environmental warming is a plastic reduction in body size, a pattern known as the temperature–size rule (TSR) (Atkinson, 1994; Atkinson and Sibly, 1997;

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Daufresne *et al.*, 2009; Gardner *et al.*, 2011; Sheridan and Bickford, 2011). This shrinking response (a negative thermal reaction norm) has profound implications for consumer–resource interactions and the overall structure and function of food webs (Yvon-Durocher *et al.*, 2011; Jochum *et al.*, 2012; Cheung *et al.*, 2013; Gibert and DeLong, 2014). Yet there is little agreement on the cause of the TSR and how to integrate the phenomenon with other ecological processes.

There are three non-mutually exclusive explanations for why the TSR occurs. The MASROS (maintain aerobic scope and regulate oxygen supply) hypothesis suggests that aquatic ectotherms become smaller in warmer environments so as to maintain access to oxygen (Atkinson *et al.*, 2006; Forster *et al.*, 2012). The idea is that being smaller is beneficial in warm aquatic environments because oxygen solubility decreases with temperature, reducing oxygen levels in warmer water. Since small size enables higher diffusion of oxygen into the body, organisms might become smaller to allow aerobic metabolism to continue functioning. Another hypothesis is that development rate and growth rate respond differentially to temperature (van der Have and de Jong, 1996; Forster *et al.*, 2011; Zuo *et al.*, 2012). We will refer to this hypothesis as the DDAG (differential development and growth) hypothesis. The idea here is that if development rate increases with temperature faster than growth rate increases with temperature, an organism will mature at a smaller size when it is warmer because the amount of time for growth is reduced. Finally, the supply–demand model suggests that organisms adjust their body size to match their bodily demand for resources to the expected supply of resources from the environment (DeLong, 2012; DeLong and Hanley, 2013; DeLong *et al.*, 2014; DeLong and Walsh, 2016). The supply–demand model suggests that given a fixed resource supply, warming-induced increases in resource demand (through the temperature dependence of metabolism) can be accommodated by smaller size, keeping demand in line with supply. Although all three hypotheses enjoy empirical support, it is not clear whether one of them is a better explanation for the TSR.

The TSR is widespread in protists (James and Read, 1957; Laybourn and Finlay, 1976; Finlay, 1977; Montagnes and Lessard, 1999; Atkinson *et al.*, 2003; Forster *et al.*, 2011). Given the large role protists play in aquatic and soil food webs (Sherr and Sherr, 2002; Caron *et al.*, 2008), their response to temperature is of fundamental importance. Here we report on dynamic shifts in the relationship between cell volume and temperature in the ciliate *Tetrahymena thermophila* (hereafter just *Tetrahymena*) and evaluate these results in light of the three competing hypotheses for the TSR.

## METHODS

We acquired *Tetrahymena* strain SB210 from the *Tetrahymena* Stock Center and grew clonal axenic populations in SPP media [1% proteose peptone, 0.2% glucose, 0.1% yeast extract, 0.003% sequestrene (Cassidy-Hanley, 2012)]. We grew 50-mL cultures at three temperatures (20°C, 26°C, and 32°C) in 80-mL jars with loose-fitting lids. This temperature range includes only the rising part of the species' growth rate thermal performance curve, with 32°C being the temperature at which population growth rate is maximized (Laakso *et al.*, 2003). Each temperature treatment was replicated 10 times (30 jars in total). Jars were inoculated at low densities from a common stock and allowed to grow for 11 days in Percival incubators with no light. Eleven days is roughly 20–53 generations given a maximum growth rate of ~1.8 $day^{-1}$ at 20°C and ~4.8 $day^{-1}$ at 32°C (Laakso *et al.*, 2003). We sampled each jar every 1–2 days by removing 1 mL of well-mixed culture under a sterilized laminar flow hood using sterile techniques. These 1-mL samples were analysed for population size and cell volume.

After sampling, 1 mL of sterile SPP media was replaced into each culture, generating a semi-continuous batch culture.

Population size and cell volume were measured for each sample with a FlowCam (Fluid Imaging Technologies, Scarborough, ME). FlowCams collect data by photographing a sample stream and using image analysis to identify particles in that stream. Particles were identified as *Tetrahymena* using automated tools in the Visual Spreadsheet software associated with the FlowCam; particles were also sorted manually such that the identity of every cell was visually confirmed. The Visual Spreadsheet software automates measurements of cell traits from the photographs including cell volume via measurements of cell width, length, and estimated spherical diameter (ESD). For cell volume, only cells identified as non-dividing, single cells (as opposed to two cells being in an image) that were not cut off by the edge of the photograph were used. For population size, all cells were used. In total, ~345,000 individual cell volume measurements were included in our analysis.

We also assessed how quickly oxygen concentration equilibrated at each temperature. We measured oxygen concentration through time with additional replicate microcosms (without *Tetrahymena* cells) using an optical oxygen sensor (Oxy 10 micro, PreSens Instruments). We used identical test jars with 50 mL of SPP medium, an alternative protist growth medium (protozoan medium, Carolina Biological Supply, Burlington, NC), and locally collected, autoclaved, and filtered pond water. All test jars were equilibrated overnight to room temperature (22°C) and oxygen levels at the start of the test. We then placed jars into incubators at 20°C, 26°C, and 31°C and tracked the temperature and oxygen levels in the water through time. These tests showed that the gradient in oxygen levels generated by temperature was in place within 4–5 hours, or about the length of a typical *Tetrahymena* generation (Fig. 1), regardless of the types or concentrations of different solutes in the medium.

## RESULTS

All *Tetrahymena* populations grew sigmoidally with slight oscillations and entered an approximate ecological equilibrium, where births were roughly equal to deaths on average through time, within the last few days of the experiment (Fig. 2A). Populations at 26°C reached the highest average abundance, and populations at 32°C reached the lowest average abundance. After an initial decrease from starting values, cell volumes oscillated through time at all three temperatures (Fig. 2B). Cell volumes increased for the first few days, then decreased, and finally increased again, stabilizing during the last few days of the experiment. Peak mean cell volumes were 1.6–1.8 times the lowest mean cell volumes.

The changes in cell volume through time generated a shifting relationship between cell volume and temperature (Fig. 2B). During the early increase in cell volumes, the TSR was completely reversed, with cells at 32°C being the largest, cells at 26°C being intermediate, and cells at 20°C being smallest (a positive reaction norm between size and temperature). From day 3 to day 6, there was a peaked reaction norm, with cells at 26°C having the largest volume. Finally, from day 8 onward, the typical TSR (a negative reaction norm) was observed.

The aspect ratio (cell width/cell length) of the cells also varied through time at all three temperatures (Fig. 2C). Cell shape oscillated through time between a more oblong form with a low aspect ratio and a rounder form with a high aspect ratio. These shifts were loosely linked to changes in cell volume (Fig. 3). In the populations at 20°C and 26°C, mean

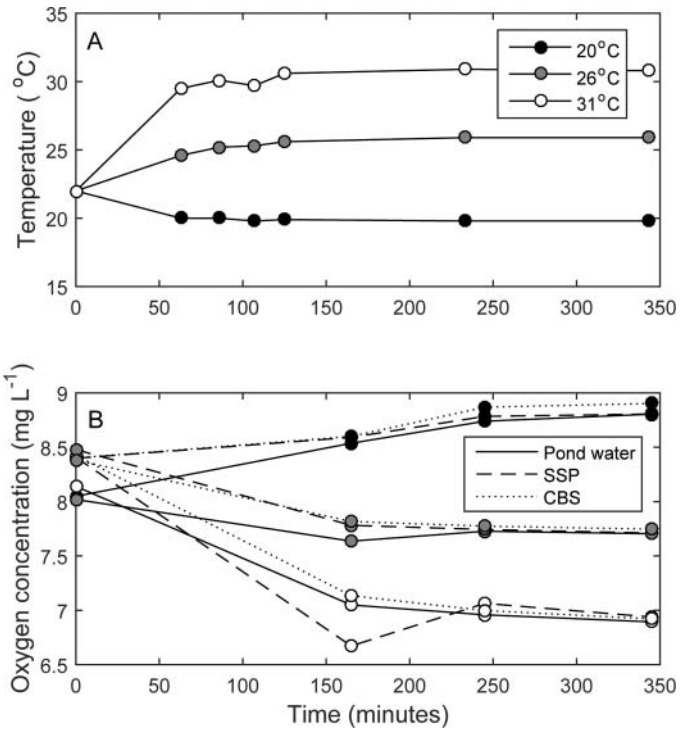


**Fig. 1.** Temperature and oxygen levels of replica microcosms equilibrated very quickly. Within 4–5 hours, the oxygen gradient generated by variation in temperature had equilibrated. SSP is the sequestrene-proteose peptone medium used to grow *Tetrahymena thermophila* in this experiment; CBS is Carolina Biological Supply protozoan medium; pond water is locally collected, autoclaved, and filtered pond water. The oxygen concentration was measured with an optical fluorescent oxygen sensor.

cell volume showed a nearly significant negative correlation with mean aspect ratio across sampling days (20°C: $r = -0.59$, $P = 0.1$; 26°C: $r = -0.64$, $P = 0.06$). For the populations at 32°C, in contrast, cell volume was significantly positively correlated with aspect ratio through time ($r = 0.71$, $P = 0.03$). Variation in aspect ratio was greatest for cells at 20°C and least for cells at 32°C.

At all three temperatures, population growth was density dependent, with the maximum rate of growth seen early on at low population densities (Fig. 4; dashed lines). The maximum rate of increase in cell size, however, lagged behind the population growth rate peak at all three temperatures (Fig. 4; bold lines), indicating that the peak increases in population and cell volume occurred at different times. There was little evidence of cell cycle synchrony in the cultures (Fig. 5).

## DISCUSSION

Our results indicate that the response of body size (through phenotypic plasticity) to increasing temperature is not fixed but rather can vary with ecological conditions. The standard TSR pattern was observed by the end of the experiment after population growth

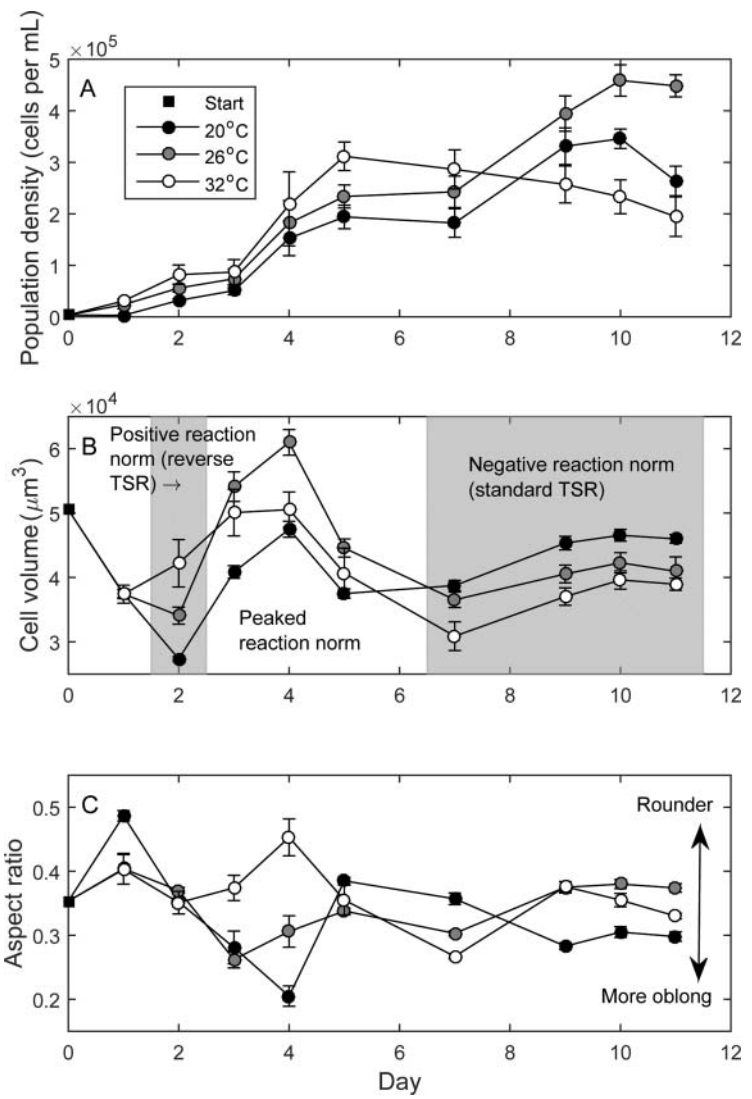


**Fig. 2.** Time-series of (A) population density, (B) cell volume, and (C) aspect ratio. All points are replicate level means with standard error of the mean.

had stopped, and at that time the effect of temperature on size was similar in magnitude to that seen with *T. pyriformis* [~1% decrease in volume per degree (James and Read, 1957)]. Prior to this stabilization, however, the reaction norm shifted qualitatively through time (Fig. 2). Reversals of the TSR have been documented for many species (Atkinson, 1995; DeLong and Hanson, 2011). For example, a reversed TSR was seen during the early growth phases of *Schizosaccharomyces pombe*, but it was not shown whether the reaction norm then changed as time progressed (Baumgärtner and Tolić-Nørrelykke, 2009). Our study documents not just a reversed TSR, but also a variable thermal reaction norm arising through time within a

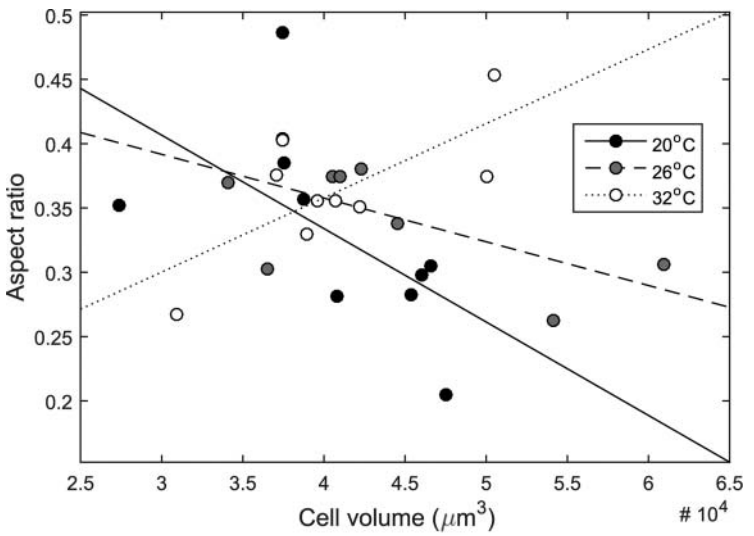


**Fig. 3.** Across sampling days, mean aspect ratio was not consistently related to mean cell volume. Populations at 32°C showed a significant positive relationship between aspect ratio and cell volume (dotted line), while populations at 20°C and 26°C showed nearly significant negative relationships (solid and dashed line respectively).

clonal organism, which indicates that this form of phenotypic plasticity is more complex than previously understood. The shifts in reaction norm appeared to be linked to an overall pattern of oscillation in cell volume. In particular, the early reversal of the TSR was linked to the fact that cell volume increased most quickly in the warmest cultures (Fig. 2B). Thus, to understand our results, it is essential to consider the reaction norms and the oscillations in cell volume together in the context of the models competing to explain the TSR.

The MASROS hypothesis appears incompatible with the shifting reaction norms and the oscillations in cell volume. This hypothesis is built upon the idea that oxygen concentrations decrease in warmer water, and an oxygen gradient generated by temperature was in place in our experiment within about a generation for *Tetrahymena* (Fig. 1). This means that the reversed TSR was not generated by a reverse oxygen gradient. Furthermore, for any given temperature, there would have to be oscillations in oxygen concentration through time to produce oscillations in cell volume, which is unlikely given that cultures were maintained with the potential for gas exchange and population abundance did not cycle. Although our results clearly do not rule out a role for oxygen in setting size, the varying thermal reaction norms and size oscillations are not consistent with this model. Measurements of oxygen levels through time would have provided additional insights into the role of oxygen in setting size, but we were unable to measure oxygen levels repeatedly in the cultures and maintain axenic conditions.

The DDAG hypothesis can predict reversals of the TSR, if growth rate increases with temperature faster than development (Zuo *et al.*, 2012). However, because both a standard and a reverse TSR were observed, growth rate would have to increase with temperature faster than development at one time, while at other times the reverse would have to be true. The DDAG hypothesis provides no indication of how a flipping of the asymmetric responses of growth

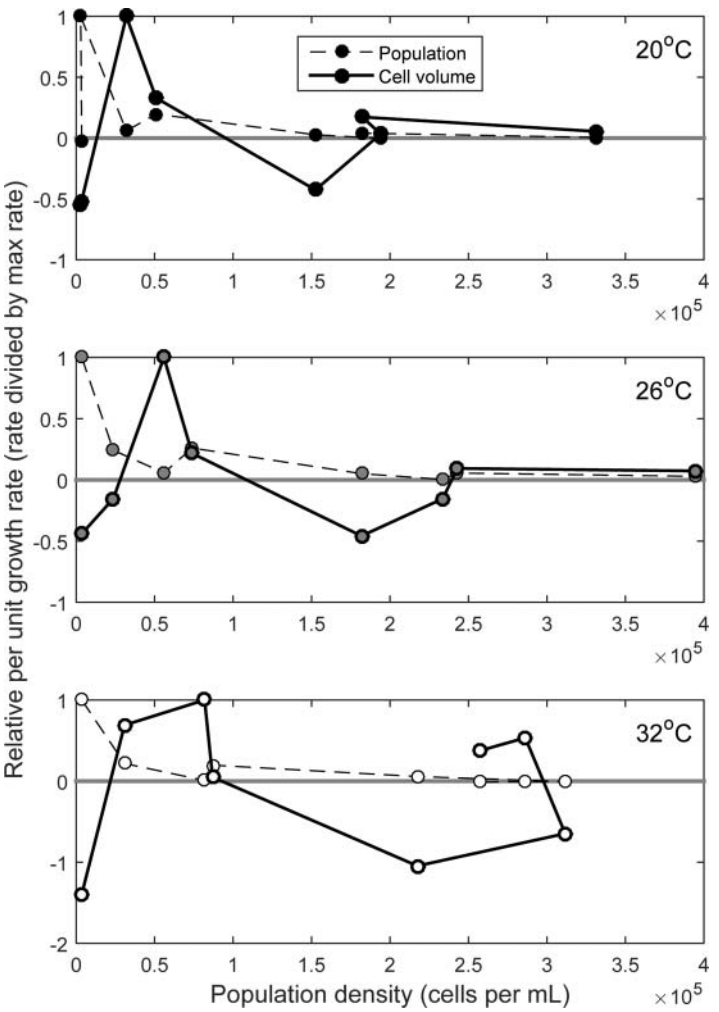


**Fig. 4.** Density dependence of population growth rate and cell size change at (A) 20°C, (B) 26°C, and (C) 32°C. The lines connect observations through time. All rates are rescaled to a maximum of one. There is a clear lag between the peak rates of population growth and the peak rates of increase in cell volume at all temperatures.

and development to temperature would occur, making it incompatible with these data. Also, it is not clear how the DDAG hypothesis would predict oscillations in cell volume through time, although ontogenetic growth models linked to the DDAG hypothesis have been linked to food levels (Hou *et al.*, 2011).

Unlike the MASROS and DDAG hypotheses, the supply–demand model appears compatible with both aspects of our results. The supply–demand model invokes an energetic optimum where the right size is that which matches bodily demand for resources $D$ with the expected environmental supply of resources $S$. If we assume that demand is an allometric function of body size $M$, a demand curve can be written as $D = b_0 M^b$, where $b_0$ is the

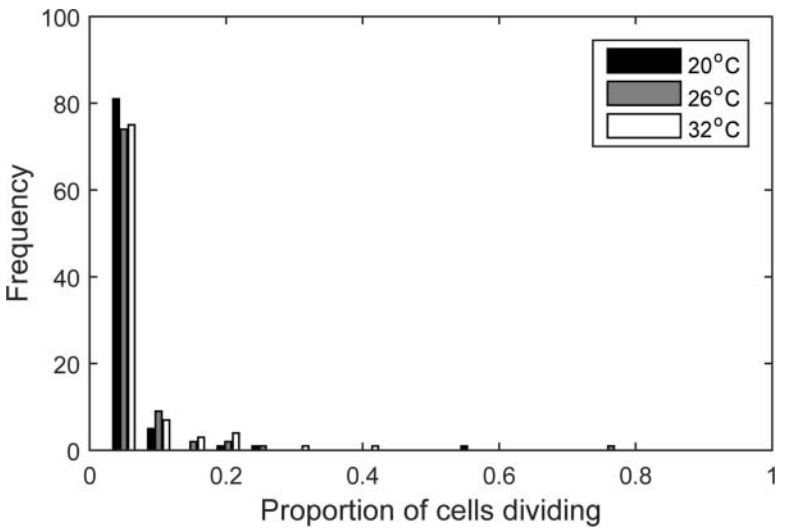


**Fig. 5.** The frequency distribution of the proportion of cells dividing across all samples shows that cell division was not synchronous. At most sampling times for most replicates, the proportion of cells dividing was less than 10%. In very few instances, many cells were dividing at a time. This distribution indicates that patterns of synchrony are not the source of variation in cell size through time.

metabolic demand at unit size and $b$ is a scaling exponent. An optimal size $M_\infty$ occurs when $S = D$, so we can write $S = b_0 M_\infty^b$ and solve for $M_\infty$, giving an explicit expression for an energetically optimal size: $M_\infty = (S/b_0)^{1/b}$ (DeLong, 2012). This model shows that for a given supply of resources, an increasing mass-specific metabolic demand induced by higher temperature will lower the energetic optimal size. However, if supply changes at the same time or is linked to temperature, then the response to temperature may be stronger, counteracted, or reversed. Furthermore, for any given metabolic demand, oscillations of resource supply through time should generate oscillations in body size, which have been shown to occur in protists (DeLong *et al.*, 2014). Although it would be expected that total resource levels should decline through time in these experiments, it is unclear whether resource levels would oscillate or what would cause this. Resources in the SPP medium are a mix of amino acids that are difficult to quantify; thus we cannot rule out a role for variation in resource levels in driving oscillations in cell volume.

Alternatively, the lag between peak growth rate and peak increase in cell volume (Fig. 4) may implicate a generational lag in cell volume response to changes in growth conditions, causing the oscillations in cell volume. Such a lag might cause cell volumes to continue to increase for a period of time even if resource levels have begun to decline. This would create an overshoot in size, generating a stronger mismatch between cell volume and the resource supply, causing the cells to then decrease in size for a while. Once again, a lag in tailoring cell volume to resource conditions might cause the cells to continue to decline beyond what can be supported by the resources. Finally, as resource levels stabilize at the steady state, cell volumes may arrive at a stable cell volume distribution. This mechanism is consistent with the lag between peak population growth and cell size increases seen at all temperatures (Fig. 4).

An alternative hypothesis for the reversed TSR seen in the early growth phase is an interaction of the response to abundant resources during early growth and a shortening generation time with increasing temperature. Since it is clear that protists become larger when resources are plentiful (Jiang and Morin, 2005; Kimmance *et al.*, 2006), it makes sense that cell volume would increase at all temperatures during the early exponential growth phase. Given that populations cannot adjust immediately but rather require time to change across generations, the shorter generation times at the warmer temperatures would enable warmer populations at these temperatures to get large more quickly in absolute time, generating a temporary reversal of the thermal reaction norm.

Cell aspect ratios changed through time along with cell volume (Fig. 2C). *Tetrahymena thermophila* is not known to show the discrete morphological forms seen in some parasitic *Tetrahymena* (e.g. trophont forms), so such life-history shifts are unlikely to explain these changes (Lynn, 1975). It is possible that the variation in aspect ratio may have arisen because cells tend to be rounder immediately after mitosis and become somewhat elongated as they begin to divide, which would generate a negative relationship between cell volume and aspect ratio. This explanation is unlikely, however, because the observed relationship between cell volume and aspect ratio was variable and only significant when positive (Fig. 3). In addition, the lack of synchrony in the cell cycle indicates that an abundance of large elongate cells or small rounder cells would be detected only rarely (Fig. 5). Notably, none of the hypotheses for the TSR also explain variation in organism shape.

Our results challenge current perspectives about the temperature–size rule. First, the reaction norm between size and temperature is not a fixed relationship, even for a single genotype. This finding suggests that any mechanistic explanation for the pattern must have the ability to account for variable reaction norms within genotypes as well as size changes through time. Leading hypotheses for the TSR, including the MASROS and DDAG hypotheses, cannot account for the variable reaction norms or oscillations in cell volume that we observed. These results suggest that although the role of oxygen and developmental responses to temperature are likely still to be important for understanding many features of phenotypic response to temperature, they may not be the mechanisms generating temperature–size reaction norms. In contrast, the supply–demand model does predict variable reaction norms given oscillating resources and generational lags in the response of size to temperature. Second, warming should be expected to influence the body size of ectotherms in a variable way. Although many species in the laboratory and in the field show declines in size (Atkinson, 1994; Daufresne *et al.*, 2009), many others become larger or remain the same size, and this variability should be expected. These results, along with others, should caution against a general expectation of size reductions everywhere that persist through time (Gardner *et al.*, 2011; Sheridan and Bickford, 2011). We suspect that in nature, body size may still mostly decline with warming, but as environmental changes alter resource levels, species interactions, and population dynamics, shifts in body size may be highly variable.

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