Flies developed smaller cells when temperature fluctuated more frequently

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Abstract

Changes in cell size might be an important component of adaptation to thermal heterogeneity. Although *Drosophila melanogaster* develops smaller cells at fluctuating temperatures, we do not know whether this response depends on the frequency or amplitude of thermal change. In a laboratory experiment, we exposed flies to either frequent or infrequent fluctuations between 17° and 27°C, while controlling the total exposure to each temperature. Flies emerged from these treatments with similar body sizes, but flies at more frequent fluctuations emerged earlier and had smaller epidermal cells for a given body size. Tissue built from small cells has more nuclei for transcription, shorter distances between cell compartments, and a larger surface area for transport across membranes. Therefore, we hypothesize that physiological effects of small cells reduce lags in metabolic activity and enhance performance of flies during warming. For plasticity of cell size to confer a fitness advantage, this hypothetical benefit must outweigh the cost of maintaining a greater area of plasma membrane.

Keywords: body size; cell size; *Drosophila melanogaster*; thermal fluctuations; metabolism; oxygen diffusion; plasticity; membranes, thermal adaptation

1. Introduction

To understand the potential impacts of climate change, biologists have started to consider how organisms respond to the variance of temperature as well as how they respond to the mean. Today's climates include more extreme temperatures than previous climates did (Hansen *et al.* 2012), and an increasing range of temperatures can be more detrimental than an increasing mean. After all, animals rarely experience the mean air temperature. Instead, environmental temperatures combine with other physical and phenotypic factors to change an organism's distribution of body temperatures (Bakken 1976; Gates 1980; Angilletta 2009). For many organisms, fluctuations in temperature constitute a daily environmental stress; for example, the larvae of *Drosophila melanogaster* (Meigen) warm by as much as 20°C in fewer than four hours while developing in rotting fruits (Feder 1997). Such fluctuations drive ecological and evolutionary phenomena. On an ecological scale, thermal fluctuations influence the phenotypes expressed throughout the life cycle (Niehaus *et al.* 2012). These ecological responses to thermal variance are complicated by the nonlinear relationship between an animal's temperature and its performance (Martin & Huey 2008; Kingsolver *et al.* 2009): an increase in the variance of body temperature enhances fitness at low mean temperatures but depresses fitness at high ones (Siddiqui & Barlow 1972, 1973; Siddiqui *et al.* 1973; Bozinovic *et al.* 2011). On an evolutionary scale, thermal fluctuations select for certain physiological and life historical phenotypes (Levins 1968; Lynch & Gabriel 1987; Stearns 1992; Angilletta 2009), some of which have been documented through experimental evolution (Bennett & Lenski 1993; Duncan *et al.* 2011; Cooper *et al.* 2012). Although we have much to learn about these ecological and evolutionary responses, one thing is certain: thermal fluctuations do matter.

Although many theories address how organisms respond to fluctuating temperatures (reviewed by Angilletta 2009), much less attention has been focused toward cellular

responses. To understand how thermal fluctuations affect cells, we focus on *Drosophila melanogaster*, whose cellular responses to temperature have been better described than those of any other species. An increase in either the mean or the variance of temperature during development causes flies to emerge smaller (Economos & Lints 1986; Petavy *et al.* 2001; Petavy *et al.* 2004; Czarnoleski *et al.* 2013); this reduction in body size stems primarily from a reduction in size of cells rather than the number of cells (French *et al.* 1998; Azevedo *et al.* 2002; Czarnoleski *et al.* 2013). A theory has emerged to address the development of smaller cells in warmer environments. This theory assumes that smaller cells, with their greater surface area relative to volume and shorter paths for diffusion, better meet increased demands for resources (Szarski 1983; Woods 1999; Kozłowski *et al.* 2003; Atkinson *et al.* 2006; Hessen *et al.* 2013). Still, this theory cannot explain why organisms should develop smaller cells at fluctuating temperatures than they do at constant temperatures. To explain this pattern, Czarnoleski and colleagues (2013) proposed that flies with smaller cells benefit by completing more metabolism during brief periods at high temperature. When organisms must quickly process large quantities of resources, the relatively large plasma membrane of a small cell increases the exchange of molecules, and especially oxygen, between the cell and its environment. Although plausible, this hypothesis assumes that when temperatures fluctuate, the magnitude of thermal maxima and the time spent at these maxima drives changes in cell size.

Here, we address a simple yet important distinction about biological responses to thermal fluctuations. When animals develop smaller bodies and smaller cells in fluctuating environments, are they responding to the frequency or magnitude of thermal change? Although previous experiments showed that thermal fluctuations triggered the development of smaller cells in *D. melanogaster*, they conflated the frequency and magnitude of change (Czarnoleski *et al.* 2013). An environment that fluctuates between two temperatures has a

greater variance than one that remains constant. Nevertheless, an environment that fluctuates frequently between two extremes can have the same mean and variance of temperature as one that fluctuates less frequently. Since natural environments differ not only in their mean and variance of temperature but also in the frequency of warming and cooling, we must decouple these effects to develop a more accurate picture of thermal plasticity. Here, we report an experiment of *D. melanogaster* in which the frequency of thermal fluctuations was manipulated independently of the magnitude to isolate its effects on development time, body size, and cell size.

2. Materials and methods

Female flies were collected in bait traps in Marlton, New Jersey, and were shipped to Indiana State University. To form isofemale lines, each female was transferred to a vial containing standard medium (Indiana University, Bloomington). Vials were kept at 21°C on a 12:12 light cycle. New generations were created every three weeks by transferring emerging adults to fresh vials. Females from the third generation supplied eggs for our experiment. To obtain these eggs, we allowed 12-day-old females from 31 isofemale lines to oviposit for 24 h at 22°C. Replicate vials were used for each line, with only one female in each vial.

We raised two vials from each isofemale line in each of two environments, which were maintained by programmable incubators (Precision Scientific, Chicago, USA). Although both treatments fluctuated between 17° and 27°C, one treatment fluctuated frequently while the other did so infrequently (six versus two shifts per 24 h; Fig. 1). Other factors were the same for both treatments: the light cycle (15L:9D), the duration per day at each temperature (13.5 h and 10.5 h at 17° and 27°C, respectively), and the durations at each temperature during scotophase (6.5 h and 2.5 h at 17° and 27°C, respectively) and photophase (7 h and 8 h at 17°

and 27°C, respectively). To confirm the mean temperatures of the frequently and infrequently fluctuating treatments, we immersed iButton thermochrons (Dallas Semiconductors, Dallas, USA) in the medium within vials.

We measured development and morphological traits of flies in each treatment. The minimal developmental time was estimated as the number of days between oviposition and the first emergence. For morphological measurements, we sampled up to four females from each vial at four to six days after eclosion. An ocular micrometer of a dissecting microscope (Leica Microsystems, Buffalo Grove, USA) was used to measure thorax length to the nearest 0.025 mm. The left wing of each fly was fixed to a slide with Permount (FisherScientific, Fair Lawn, USA), and the dorsal surface was digitized by a microscopic camera (Olympus Corporation, Tokyo, Japan). We used ImageJ software (National Institutes of Health, USA) to measure the area of the wing in mm² (Fig. 2B). Following (Dobzhansky 1929), we estimated the mean area of epidermal cells from the reciprocal of trichome density in the 0.01-mm² area between the distal segments of the fourth and fifth wing veins. Epidermal cells in wings are commonly used to infer relative cell size in drosophilids (Arendt 2007), because their size correlates with the sizes of other cells (Stevenson *et al.* 1995). Moreover, the sizes of cells in different organs of flies respond in concert to developmental conditions (Azevedo *et al.* 2002; Heinrich *et al.* 2011). The discovery of strong correlations between the sizes of cells from different tissues in animals (Kozłowski *et al.* 2010) and plants (Brodribb *et al.* 2013; John *et al.* 2013) suggests that the mechanisms coordinating cell size in organs might be evolutionarily conserved among metazoans. Nevertheless, some cell types can change independently (Kozłowski *et al.* 2010; Maciak *et al.* 2014).

We used nlme library of the R Statistical Package (R Development Core Team 2011) to examine the effects of thermal fluctuations on developmental time, thorax length, wing size, and cell size. Two random factors were included in our models: 1) isofemale line and 2) female nested within line and treatment. Thorax length was a covariate in the analysis of wing size, and wing size was a covariate in the analysis of cell size. AIC was used to identify the most likely model for each variable.

3. Results and discussion

More frequent changes in temperature caused flies to develop more quickly ($F_{1,86} = 19.0$, $P < 0.0001$) and produce smaller epidermal cells in wings ($F_{1,78} = 4.7$, $P = 0.03$; Fig. 2A).

Nevertheless, thermal fluctuations did not affect thorax or wing sizes. Indeed, the best model of thorax or wing size omitted an effect of thermal fluctuations, while the second best models indicated a statistically insignificant effect ($P > 0.40$ for both traits; ΔAIC was 11.1 and 1.0 for thorax size and wing size, respectively). Importantly, we can attribute the observed differences in cell size to the frequencies of thermal fluctuations rather than to the temperatures themselves, since both treatments had the same mean and variance of temperature. Not only were the mean temperatures almost identical (21.6° and 21.9°C for frequently vs. infrequently fluctuating temperatures, respectively), the difference in mean temperatures should have caused flies to develop larger cells in the more fluctuating treatment instead of smaller cells (see, Partridge *et al.* 1994; Czarnoleski *et al.* 2013). Therefore, we doubt that the observed difference in cell size was caused by mean temperatures as much as by fluctuations in temperature.

Although we do not know whether changes in epidermal cells reflect changes in other cells, we hypothesize that flies emerging from frequently fluctuating temperatures comprised

more, smaller cells. If cells grow and divide according to thermal fluctuations, how might these fluctuations regulate growth? In *D. melanogaster* and many other organisms, pathways involving insulin and TOR (target of rapamycin) regulate the size and number of cells (De Virgilio & Loewith 2006; Grewal 2009). In Australia and North America, genes that control these pathways in flies differentiated along latitudinal clines in conjunction with cell size and body size (De Jong & Bochdanovits 2003; Paaby *et al.* 2010; Fabian *et al.* 2012). Activities of these pathways depend on many factors, including the energetic state of cells, the history of heat shock, and extracellular concentrations of nutrients and oxygen (De Virgilio & Loewith 2006). These factors likely vary with the frequency and magnitude of thermal fluctuations, providing a molecular basis for the plasticity of cell size.

If the plasticity of cell size is adaptive, what are the benefits to an organism? According to theory, small cells increase metabolic performance at high temperatures by providing sufficient surface area to transport substrates (Atkinson *et al.* 2006; Czarnoleski *et al.* 2013). Many ectotherms - including bacteria (Sjostedt *et al.* 2012), protists (Butler & Rogerson 1996; Atkinson *et al.* 2003), rotifers (Stelzer 2002), planarians (Romero & Baguna 1991), nematodes (vanVoorhies 1996), fish (vanVoorhies 1996), lizards (Goodman & Heath 2010), and flies (Partridge *et al.* 1994; Blanckenhorn & Llaurens 2005) - develop smaller cells in warm constant environments. In such environments, demands for resources are high but supplies of oxygen are low (Woods 1999; Atkinson *et al.* 2006). This explanation, however, cannot account for the patterns observed in our experiment, because flies in both treatments experienced the same temperatures for the same duration yet still developed smaller cells. Therefore, we must consider mechanisms related to the frequency of thermal change rather than the time spent at extreme temperatures.

We propose that smaller cells minimize lags in metabolic activity as animals warm. The peak of metabolism would lag behind the onset of warming if cells require more

transcripts, ribosomes, ATP, and substrates. This lag would last as long as it takes cells to acquire and distribute these resources, which depends on the capacity of cells to produce transcripts and move molecules between compartments (Ben-Ari *et al.* 2010; Dill *et al.* 2011). Tissues comprising numerous small cells would have many nuclei to transcribe genes and a small volume of cytoplasm per nucleus, shortening distances for transcripts and other molecules to move among nuclei, ribosomes, and mitochondria. Consistent with this idea, rates of transcription and translation at higher temperatures were less limited by access to genes in small cells than in large cells (Zhurinsky *et al.* 2010). Furthermore, developmental responses of cellular architecture to metabolic demands reduce the distances required for diffusion of oxygen, ATP and transcripts; for example, the hypertrophy of muscles involves a redistribution of mitochondria and nuclei in the fibres (Kinsey *et al.* 2007; Hardy *et al.* 2009; Kinsey *et al.* 2011). These potential benefits of small cells during periods of intense metabolic demand could explain why flies exposed to more frequent fluctuations in temperature produced more, smaller cells.

Tissues made of small cells would also have a greater density of cell membranes, relaxing constraints on transport imposed by the area of plasma membrane. Moreover, membranes provide a better medium for oxygen diffusion than does the aqueous environments of cells, especially at higher temperatures (Subczynski *et al.* 1989); therefore, small cells can build a dense network of pathways for perfusion of tissue with oxygen, speeding the delivery of oxygen from capillaries (vertebrates) or tracheoles (insects) to mitochondria. Consistent with this view, flies that developed in a hypoxic environment produced small cells in two types of tissues (Heinrich *et al.* 2011). Moreover, flies with small cells consumed oxygen more rapidly during hypoxia than did flies with large cells (Zhou *et al.* 2007). The role of cell size in maintaining a sufficient partial pressure of oxygen within

tissues is an intriguing avenue of research that complements current research on the developmental plasticity of the tracheal system (reviewed by Harrison *et al.* 2012).

Fluctuating environments have consequences for fitness, because even a modest change in temperature exerts immediate effects on biochemical machinery. Still, ectotherms can remodel their cells to adjust biochemical reactions as temperatures change. If this process of acclimation occurs too slowly, a mismatch between body temperatures and optimal temperatures arises. Rates of acclimation differ between organisms and types of performance (reviewed by Angilletta 2009). For example, heat tolerance of flies was found to acclimate rapidly to continuous warming (Overgaard *et al.* 2011), but thermal optima for locomotion or fecundity often fail to acclimate even during long periods (Cooper *et al.* 2010). We propose that small cells would speed physiological acclimation. Studies based on breeding designs or artificial selection could be used to test whether a genetic correlation exists between cell size and the capacity for physiological acclimation. Alternatively, selective pressure on cell size can be quantified by comparing the fitnesses of genotypes with different reaction norms in constant and fluctuating environments. In a recent experiment, the evolution of *D. melanogaster* at fluctuating temperatures resulted in genotypes with a greater capacity to shift the composition of cell membranes in response to developmental temperatures (Cooper *et al.* 2012). Whether these changes were accompanied by changes in cell size remains unknown. However, future studies that examine plasticities of organismal, cellular, and molecular traits should help to define the role of cell size in thermal adaptation.

These hypothetical benefits of small cells in fluctuating environments complement the benefits that small cells confer in warm environments (Szarski 1983; Woods 1999; Kozłowski *et al.* 2003; Atkinson *et al.* 2006; Hessen *et al.* 2013). As such, we should expect the frequency and magnitude of thermal fluctuations to influence cell size independently. When increasing solely the frequency of thermal change, we observed a reduction of cell size by

1.4%. By contrast, Czarnoleski and colleagues observed a reduction in cell size of 3.2–3.5% when increasing the frequency and magnitude of thermal fluctuations at the same time (Czarnoleski *et al.* 2013). These results suggest that multiple aspects of thermal change exert a cumulative effect on cell size. Still, we do not know how much these changes in cell size affect fitness, although we can generally expect that the fitness landscapes are highly nonlinear. Moreover, the potential benefits of small cells could be offset by an energetic cost (Davison 1955; Starostova *et al.* 2009), because cells require ATP and other substrates to maintain the fluidity of their membranes and generate gradients of ions (Rolfe & Brown 1997).

Finally, the thermal plasticity of cell size has broad implications for studies of biogeographic patterns. Although flies in the lab evolved larger cells and larger bodies at lower temperatures (Partridge *et al.* 1994), a more complex picture has emerged from comparative studies of flies throughout their geographic ranges (reviewed by Arendt 2007). In North America, populations of *D. simulans* at higher latitudes evolved larger cells, but no such cline evolved among European and South American populations (Calboli *et al.* 2003). In *D. melanogaster*, cell size increases with latitude more strongly among South American populations than it does among Australian populations (Zwaan *et al.* 2000). At high latitudes, selection for smaller cells, driven by thermal fluctuations, would counter selection for larger cells, driven by low temperatures. Thus, disparate clines in cell size might result from geographic patterns of climate, since sharper latitudinal clines in temperature and its frequency of change occur on certain continents (Ghalambor *et al.* 2006). Future studies that compare cell sizes among populations should address the consequences of not only means and variances of temperature, but also of the temporal patterns of thermal change.

Acknowledgements

Brandon Cooper helped maintaining flies and Diana Hews provided facilities. The Fulbright and Kosciuszko Foundations supported MC; the National Science Foundation (IOS-0616344) supported MJA; the National Science Centre in Poland (NN304 373238) and Jagiellonian University (DS/WBINS/INOS/757/2013) supported the research.

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Figure legends

Figure 1. Flies in our experiment experienced different frequencies of thermal fluctuations while spending the same durations at two temperatures. The central row depicts the durations of photophase and scotophase (denoted by the sun and moon, respectively). The stippled blocks depict the durations at 17° and 27°C (denoted by white and black backgrounds, respectively).

Figure 2. A) Flies that experienced frequent fluctuations in temperature developed smaller epidermal cells for a given wing area than did flies that experienced infrequent fluctuations. Lines depict the effects estimated by the most likely model. B) The area of a wing was delimited by the outer edge of a wing blade (dashed line) and two straight lines linking the points indicated by arrows. The black circle marks the area used to estimate the area of epidermal cells.

Figure 1

Frequent fluctuations

Infrequent fluctuations

Figure 2