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Colder environments did not select for a faster metabolism during experimental evolution of *Drosophila melanogaster*

Lesley A. Alton,[1,2,3] Catriona Condon,[4] Craig R. White,[1,2] and Michael J. Angilletta Jr[4]

[1]*School of Biological Sciences, The University of Queensland, Brisbane, QLD 4072, Australia*

[2]*Current Address: School of Biological Sciences, Monash University, Clayton, VIC 3800, Australia*

[3]*E-mail: lesley.alton@monash.edu*

[4]*School of Life Sciences, Arizona State University, Tempe, Arizona 85287*

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The effect of temperature on the evolution of metabolism has been the subject of debate for a century; however, no consistent patterns have emerged from comparisons of metabolic rate within and among species living at different temperatures. We used experimental evolution to determine how metabolism evolves in populations of *Drosophila melanogaster* exposed to one of three selective treatments: a constant 16°C, a constant 25°C, or temporal fluctuations between 16 and 25°C. We tested August Krogh's controversial hypothesis that colder environments select for a faster metabolism. Given that colder environments also experience greater seasonality, we also tested the hypothesis that temporal variation in temperature may be the factor that selects for a faster metabolism. We measured the metabolic rate of flies from each selective treatment at 16, 20.5, and 25°C. Although metabolism was faster at higher temperatures, flies from the selective treatments had similar metabolic rates at each measurement temperature. Based on variation among genotypes within populations, heritable variation in metabolism was likely sufficient for adaptation to occur. We conclude that colder or seasonal environments do not necessarily select for a faster metabolism. Rather, other factors besides temperature likely contribute to patterns of metabolic rate over thermal clines in nature.

KEY WORDS: ***Drosophila*, experimental evolution, Krogh's rule, metabolic cold adaptation, metabolic rate, temperature.**

A century ago, August Krogh speculated that ectotherms from colder environments should have higher metabolic rates than those from hotter environments, when the two are observed at the same temperature (Krogh 1916). This hypothetical pattern, known as metabolic cold adaptation or Krogh's rule (Gaston et al. 2009), stems from the idea that any effect of temperature on metabolic rate will reduce the performance of an organism and thus will be opposed by natural selection. In contrast to this idea, Clarke (1980, 1991a) argued that natural selection might not favor genotypes with higher metabolic rates at lower temperatures. Rather, Clarke proposed that metabolic rate should decrease at low temperatures if the energetic cost of cellular respiration outweighs the benefit of producing additional ATP (Clarke 1993; Chown and Gaston 1999). These opposing perspectives have sparked much debate among physiologists who study thermal acclimation (Huey and Berrigan 1996).

In seeking evidence of metabolic cold adaptation, biologists have examined inter- and intraspecific variation in the metabolic rates of ectotherms living at different temperatures, such as species distributed along latitudinal clines (e.g., Tsuji 1988; Sokolova and Pörtner 2003; White et al. 2012; Gaitán-Espitia and Nespolo 2014). Evidence has been mixed (Clarke 2003), perhaps because variation in metabolism stems not only from temperature but also the availability of food and other factors (White and Kearney 2013). These other factors, including seasonality, photoperiod, precipitation, and productivity, covary with temperature;

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for example, terrestrial environments at high latitudes not only have low mean temperatures but experience greater seasonality. Addo-Bediako et al. (2002) discovered that metabolic rate was higher for species of insects from environments with colder mean annual temperatures, but the seasonal amplitude of temperature is also greater in colder environments. Consequently, variation in temperature may also contribute to observed geographical patterns in metabolic rate.

Experimental evolution has the potential to test hypotheses about adaptation (Kawecki et al. 2012), such as Krogh's idea that a colder environment selects for a faster metabolism. For certain species, biologists can readily expose populations to various thermal treatments for many generations, while controlling other factors that influence the evolution of metabolism. In an experiment with *D. melanogaster*, Berrigan and Partridge (1997) found that flies from laboratory populations evolving at 18°C had a slightly greater metabolic rate than did flies from populations evolving at 25°C, although this difference might have resulted from inadequate sampling ($P = 0.08$). Furthermore, these researchers observed that the metabolic rate of flies from high latitudes was 9% faster than those from low latitudes when measured at 18°C. These results suggest that either Krogh overestimated the strength of natural selection for a faster metabolism in a colder environment, or that genetic constraints prevented adaptation. Without knowing the genetic variance of metabolic rate, we cannot distinguish between these competing explanations. We also do not know whether the latitudinal clines in metabolic rate resulted from variation in mean temperature or seasonality.

To address these issues, we tested Krogh's hypothesis with experimental populations of *D. melanogaster* that evolved at either constant (16 or 25°C) or fluctuating temperatures (temporal fluctuations between 16 and 25°C). These populations diverged genetically in several traits related to thermal physiology (Cooper et al. 2012; Condon et al. 2014; Condon et al. 2015). If Krogh was correct, we expected flies from populations at 16°C to have higher metabolic rates than flies from populations at 25°C when measured at the same temperature, as long as genetic variation in metabolic rate existed within populations (previous estimates of narrow-sense heritability for metabolic rate in *D. melanogaster* range from 0.06 to 0.11, and broad-sense heritability estimates range from 0.07 to 0.62: Khazaeli et al. 2005; Castañeda and Nespolo 2013). If seasonality drives variation in metabolic rate, we expected flies from populations at fluctuating temperatures to have higher metabolic rates than flies from populations at constant temperatures. By quantifying the among-line genetic variance of metabolic rate in our experimental populations, we could infer whether observed patterns of evolution were influenced by genetic constraints.

Materials and methods

EXPERIMENTAL POPULATIONS

The flies in this study came from a selection experiment conducted by Yeaman et al. (2010) at the University of British Columbia. These researchers allowed five populations of *D. melanogaster* to evolve under each of the following selective treatments: a constant 16°C (cold homogenous or C populations), a constant 25°C (hot homogenous or H populations), and temporal fluctuations between 16 and 25°C (temporally heterogeneous or T populations). Within a population, flies were maintained across two cages inside a temperature-controlled room. Within each cage, flies were given eight bottles in which to lay eggs to begin a new generation. Populations at 16 and 25°C began new generations every four and two weeks, respectively. Flies at 16 and 25°C were given three and 1.5 days to lay eggs, respectively, after which, bottles were transferred to new cages. Every four weeks, four bottles were exchanged reciprocally between paired cages such that bottles were exchanged every generation at 16°C and every second generation at 25°C. Within the T selective treatment, paired cages with freshly laid eggs were moved between the 16°C room and 25°C room every four weeks. Flies evolved in the selective treatments for over three years with C, T, and H populations completing 32, 48, and 64 generations, respectively. The photoperiod for each treatment was a 12L:12D. We studied isofemales lines from these populations, created by Cooper et al. (2012). Lines were maintained at 20.5°C and a photoperiod of 12L:12D, in vials containing a cornmeal-yeast medium. Lines were transferred to fresh vials every three weeks between April 2009 and the start of our experiment.

MEASURING METABOLIC RATE

We measured the metabolic rates of male flies from each population. These measurements were completed in two temporal blocks. In the first block, we measured metabolic rates of flies from four isofemale lines per population. These flies had emerged as adults eight to nine days prior. In the second block, we measured metabolic rates of flies from two more isofemale lines per population. These flies had emerged as adults 14–15 days prior. For each isofemale line, we attempted to measure metabolic rate at 16, 20.5, and 25°C. However, measurements were made at one or two measurement temperatures for four or 10 lines, respectively. For each temporal block, measurements were conducted over three days, at one randomly selected temperature per day. Each day, one randomly selected fly from each isofemale line was measured in a random order. At the conclusion of our study, we had measured the metabolic rate of 246 male flies with 166 flies measured in the first temporal block and 80 flies in the second temporal block.

The metabolic rate of each fly was estimated from the rate of CO_2 production ($\dot{V}_{CO_2}$, μL CO_2 h^{-1}) during open-flow respirometry. Dry CO_2-free air, supplied by an FT-IR purge gas generator (Parker Balston, Model 75-45-12VDC, Haverhill, MA), was pushed through a column of Drierite and Ascarite before passing through a factory-calibrated mass flow controller (Aalborg, Model GFC17, Orangeburg, NY). Flow rate was regulated to 50 mL min^{-1} STPD. After the mass flow controller, the air passed through a humidifying chamber (a syringe of wet cotton), then a respirometry chamber (a 2.2 mL glass vial), and finally a CO_2 analyzer (LI-COR, Model LI-7000, Lincoln, NE). The CO_2 analyzer measured CO_2 concentration at a resolution of 0.1 ppm and a frequency of 1 Hz. Each day, we calibrated the analyzer with air containing 0 ppm CO_2 and air containing 12.3 ppm CO_2 (Praxair, Danbury, CT). The respirometry system was housed in an incubator that maintained the desired temperature within 1°C (Percival Scientific, Model DR36VLC8, Perry, IA). To ensure that the air going into the respirometry chamber reached the desired temperature, air entering the system first passed through a coiled copper tube immersed in water.

Prior to being measured, a fly was transferred from its vial of medium to an unsealed respirometry chamber and maintained in an incubator at 20.5°C for 30 min. The background concentration of CO_2 was measured with an empty respirometry chamber until the CO_2 trace was observed to be stable (≈5 min). Then, the empty respirometry chamber was replaced with a respirometry chamber containing a fly. The CO_2 production of an individual fly was recorded for 20 min in darkness. The first 10 min of each recording was discarded to prevent artifacts in the data caused by handling stress. Following the measurement period, the respirometry chamber containing the fly was exchanged for an empty chamber and the background CO_2 concentration was measured again. This process was repeated until metabolic rates were recorded for all flies. Finally, flies were killed by freezing, dried for 12 h at 50°C, and weighed using a microbalance with a precision of ±0.001 mg (Mettler-Toledo, Model MX5, Columbus, OH).

To calculate $\dot{V}_{CO_2}$ (μL CO_2 h^{-1}), a linear model was fitted to the background CO_2 concentrations measured before and after a fly was inside the respirometry chamber. This model enabled us to interpolate the background CO_2 concentration at the time when the fly was inside the respirometry chamber. The interpolated background CO_2 concentration was then subtracted from the CO_2 concentration of the excurrent air during the 10-min recording. The average factional change in CO_2 concentration of the excurrent air was then multiplied by the flow rate of the air (50 mL min^{-1} STPD) to calculate $\dot{V}_{CO_2}$ (μL CO_2 h^{-1}).

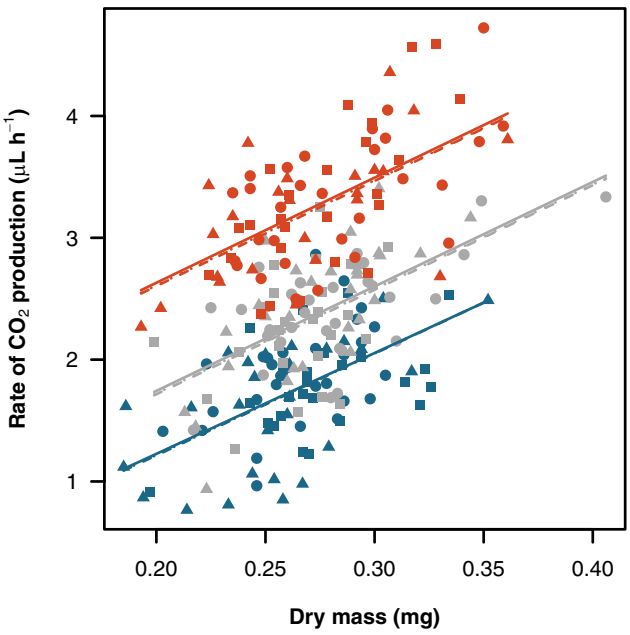

Figure 1. The rate of CO_2 production (μL h^{-1}) of *Drosophila melanogaster* increased with increasing dry body mass. Data are adjusted for the random effects of isofemale line and temporal block. Blue, gray, and red symbols indicate measurement temperatures of 16, 20.5, and 25°C, respectively. The shape of each symbol indicates the selective treatment (● = C, constant 16°C; ▲ = T, temporal fluctuations between 16 and 25°C; ■ = H, constant 25°C). Lines represent the most likely effects of mass for C (solid line), T (dashed line), and H (dotted line) estimated from multimodel averaging. At 16°C, n = 29, 27, and 25 for the C, T and H selective treatments, respectively. At 20.5°C, n = 31, 29, and 27 for the C, T, and H selective treatments, respectively. At 25°C, n = 29, 24, and 25 for the C, T, and H selective treatments, respectively.

STATISTICAL ANALYSES

To quantify sources of variation in metabolic rate, we fit linear mixed models using the lmer function of the lme4 library (Bates et al. 2015) of the R Statistical Package (R Core Team 2015). We treated selective treatment (C, H, or T) and measurement temperature (16, 20.5, or 25°C) as categorical fixed factors, and dry body mass as a continuous fixed factor (covariate). Temporal block, experimental population, and isofemale line (nested within experimental population) were treated as random factors. We used the MuMIn library (Bartoń 2016) to fit all possible models (including all possible interactions between the fixed factors) to the data, with parameters estimated by maximum likelihood (Zuur et al. 2009). Then, we calculated the second order (small sample) Akaike information criterion (AIC_c) and Akaike weight (w_i) of each model, the latter variable being the probability that the model best describes the data. Following Burnham and Anderson (2002), we used multimodel averaging to calculate the weighted

average of each parameter using estimates from all models. The resulting values of parameters were used to calculate the most likely mean for each group. This approach eliminates the need to interpret *P* values, because all models (including the null model) contributed to the most likely value of each mean.

We next re-fit each of the models using restricted maximum likelihood, which yields estimates of variances that are generally less biased than corresponding maximum-likelihood estimates (Bolker et al. 2009). For each model we calculated the variance associated with the random factors of isofemale line, experimental population and temporal block, conditioned on the fixed effects. The variance associated with isofemale line was used to infer the broad-sense genetic variance within an experimental population (V_G). We used V_G to calculate the broad-sense heritability (H^2) of metabolic rate, which equals V_G divided by the total phenotypic variance (Falconer 1989), conditioned on the fixed effects in the model. We also used V_G to calculate the broad-sense evolvability (e_μ) of metabolic rate, which equals V_G divided by m^2, where m is the grand mean of metabolic rate (i.e., the mean-scaled V_G; Hansen et al. 2011), also conditioned on the fixed effects in the model. Finally, we calculated the weighted average H^2 and e_μ using the estimates from all models.

Results

Body mass and test temperature had the greatest effects on metabolic rate. Although heavier flies generally had higher metabolic rates, the effect of mass was about 6 and 4% greater for flies at 20.5 and 25°C, respectively, than it was at 16°C (Fig. 1). The change in metabolic rate between 16 and 25°C for flies with a mean dry body mass of 0.265 mg was well within the range expected from previous research ($Q_{10} = 1.9$). However, metabolic rates at each measurement temperature were nearly identical for flies from the three selective treatments (Fig. 2). These inferences were based on multimodel averaging. The best two models ($\Sigma w_i = 0.86$) do not include selective treatment; together these models are more than six times as likely as all other models combined ($\Sigma w_i = 0.14$), including all models that include selective treatment or its interactions as predictors (Table 1). Therefore, we confidently conclude that selective treatment had little or no effect on metabolic rate.

The evolution of metabolic rate was not constrained by a lack of genetic variation within populations. Metabolic rates varied substantially among isofemale lines within experimental populations (weighted SD = 0.3). Indeed, removing isofemale line from the full model resulted in a substantial increase in AIC_c (17.2), indicating that there was essentially no support for a model without isofemale line as a random factor. Thus, there should have been sufficient genetic variation for metabolic rate to respond to natural selection. The weighted estimate of the broad-sense heritability of metabolic rate was 0.15, and the weighted estimate of the evolvability of metabolic rate was 0.013.

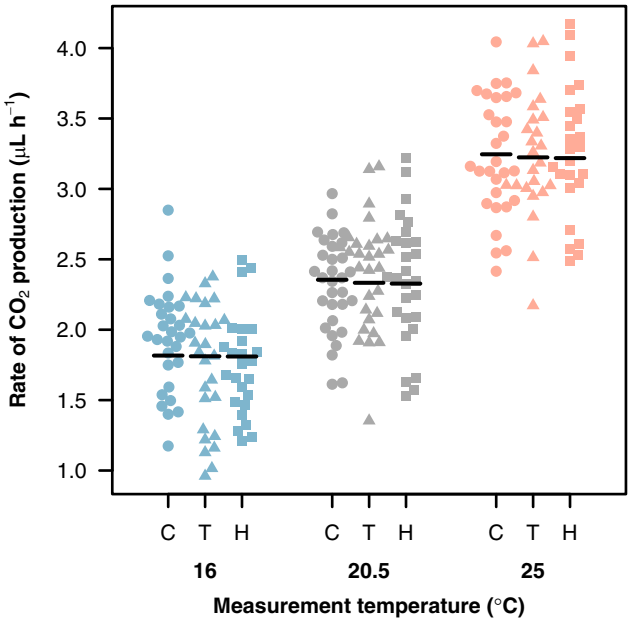

Figure 2. The rate of CO_2 production (μL h^{-1}) of *Drosophila melanogaster* increased with increasing measurement temperature, but was nearly identical for flies from populations that evolved in the three selective treatments. Data are adjusted for the fixed effect of dry body mass (to a mean dry body mass of 0.265 mg) and the random effects of isofemale line and temporal block. Blue, gray, and red symbols indicate measurement temperatures of 16, 20.5, and 25°C, respectively. The shape of each symbol indicates the selective treatment (● = C, constant 16°C; ▲ = T, temporal fluctuations between 16 and 25°C; ■ = H, constant 25°C). Black horizontal lines denote the most likely means estimated from multimodel averaging. At 16°C, n = 29, 27, and 25 for the C, T, and H selective treatments, respectively. At 20.5°C, n = 31, 29, and 27 for the C, T, and H selective treatments, respectively. At 25°C, n = 29, 24, and 25 for the C, T, and H selective treatments, respectively.

Discussion

Our results contradict Krogh's hypothesis that a colder environment selects for a faster metabolism. At each measurement temperature, the metabolic rate of flies from populations at a constant 16°C was similar to that of flies from populations at a constant 25°C. This observation agrees reasonably well with that of Berrigan and Partridge (1997), who reported a small and potentially insignificant effect of environmental temperature on the evolution of metabolic rate in populations of *D. melanogaster*. They observed a 5–7% difference in mass-specific metabolic rate between populations evolving at 18°C and those evolving at 25°C, noting that this small difference could have resulted by chance

Table 1. Likely models of the metabolic rate (CO_2 production) of *Drosophila melanogaster*.

Model	nP	logLik	AIC_c	ΔAIC_c	w_i
(1) Mass + temp	8	–189.7	396.0	0	0.72
(2) Mass + temp + (mass · temp)	10	–189.2	399.3	3.3	0.14
(3) Mass + temp + selection	10	–189.6	400.2	4.2	0.09
(4) Mass + temp + selection + (mass · selection)	12	–188.9	403.1	7.0	0.02
(5) Mass + temp + selection + (mass · temp)	12	–189.1	403.6	7.6	0.02

All models contain an intercept term and the random factors of temporal block, experimental population, and isofemale line. Models are ranked according to AIC_c, and only models with ΔAIC_c values less than 8 are presented. All of these models include an effect of dry body mass and measurement temperature on metabolic rate. The two best models (summed Akaike weight, $\Sigma w_i = 0.86$) do not include selective treatment.

Mass, dry body mass; temp, measurement temperature; selection, selective treatment; nP, number of parameters; logLik, log-likelihood; AIC_c, Akaike information criterion; ΔAIC_c, the difference in AIC value relative to the best model; w_i, Akaike weight, the probability that a model describes the data better than other models.

given their sample of three populations per selective treatment ($P = 0.08$). Our comparison of metabolic rates between populations at a constant 25°C and populations at fluctuating temperatures, which experienced a lower mean and higher variance of temperature, also failed to support the hypothesis that a colder environment selects for a faster metabolism. The similarity of metabolic rates between populations in stable and fluctuating temperatures also fails to support the hypothesis that seasonality selects for a higher metabolic rate. Taken together, these results suggest one of two possibilities: either insufficient genetic variation prevented the expected response to selection, or colder or seasonal environments do not select for higher metabolic rates in *D. melanogaster*.

The experimental populations likely had sufficient genetic variation in metabolic rate to respond to natural selection. We estimated the broad-sense heritability of metabolic rate as 15%, which lies well within the range of 4–88% for broad-sense heritabilities of metabolic rates among ectothermic animals (median = 29%; White and Kearney 2013) and *D. melanogaster* (0.07–0.62; Khazaeli et al. 2005). We estimated the broad-sense evolvability of metabolic rate as 1.3%, which is above the median estimate of narrow-sense evolvability of physiological traits (0.49 ± 0.14%; Hansen et al. 2011). Of course, the broad-sense genetic variance might exceed the additive genetic variance, which reflects the true potential for adaptation. The narrow-sense heritabilities of metabolic rate in cockroaches ($h^2 = 0.13$–0.24: Schimpf et al. 2013), crickets *Gryllodes sigillatus* ($h^2 = 0.14$: Ketola and Kotiaho 2009), and *D. melanogaster* ($h^2 = 0.06$–0.11: Castañeda and Nespolo 2013) were comparable to the broad-sense heritability for *D. melanogaster* in the present study (0.15). Our populations, therefore, likely had some additive genetic variation in metabolic rate, which forces us to question the assumptions of Krogh's idea of thermal adaptation. Given that the metabolic rates of flies from the constant 16°C or fluctuating selective treatments were nearly identical to those of flies from the constant 25°C selective treatment, our findings suggest that colder environments per se do not necessarily select for higher metabolic rates.

The lack of divergence observed in the metabolic rate of our fly populations is surprising given the documented association between temperature and metabolic rate among insect species (Addo-Bediako et al. 2002), and the observed latitudinal clines in allele frequency of metabolic genes in *D. melanogaster* (Kolaczkowski et al. 2011; Fabian et al. 2012; Cogni et al. 2015). We cannot dismiss the possibility that a selective treatment with a constant temperature below 16°C might select for a higher metabolic rate, especially if there are metabolic costs associated with cold hardiness (Williams et al. 2016a, b); however, 16°C is close to the minimum temperature for reproduction in *D. melanogaster* (~13°C: Cooper et al. 2010; Condon et al. 2014). Furthermore, we have reasons to believe that the 9°C difference between our constant selective treatments should have been sufficient to observe a change in metabolic rate. First, a 9°C decrease in temperature causes a substantial (almost twofold) reduction in metabolic rate (Figs. 1, 2). Assuming the effect size was similar to that estimated through the interspecific analysis by Addo-Bediako et al. (2002), a decrease in temperature of 9°C should have caused an evolutionary increase in metabolic rate of 22% at each measurement temperature. Given the variance in our phenotypic data, such a change would have been detected if present. Second, several other traits have diverged among our experimental populations in response to the selective treatments, including cell size (Adrian et al. 2016), membrane fluidity (Cooper et al. 2012), flight performance (Le Vinh Thuy et al. 2016), reproductive rate (Condon et al. 2014), and thermal tolerance (Condon et al. 2015). Therefore, the treatments would likely have caused metabolic rates to diverge if colder temperatures select for a faster metabolism.

If temperature alone does not select for a faster metabolism, other factors likely contribute to latitudinal clines in metabolic rate. In natural environments, low temperature combines with

other abiotic and biotic conditions that affect the evolution of metabolism. Clarke (1991a, 1993) repeatedly cautioned that an animal's metabolic rate depends on its need for energy; no organism produces ATP for the sake of doing so. Thus, the evolution of metabolic rate depends on how a combination of environmental conditions affects the benefit of using energy relative to the cost of obtaining that energy. During experimental evolution, flies were exposed to an abundance of food and an absence of predators. Both factors seem more likely than not to favor high metabolic capacity. With abundant food, an animal could easily fuel the production of ATP for activity and the gametes for reproduction (Burton et al. 2011). Consistent with this idea, a comparative study of mice revealed that species from productive environments had evolved faster metabolisms during rest (Mueller and Diamond 2001). In the absence of predators, flies could forage, mate, and reproduce without a greater risk of mortality. For these reasons, we conclude that genotypes able to consume more food and produce more ATP would have enjoyed greater fitness at 16°C, which suppressed metabolism relative to 25°C (see Fig. 2).

Conceivably, the colder treatments in this experiment created no selective pressure for flies to biochemically compensate for a slowed metabolism. In other words, a fly with a slower metabolism at 16°C could have been as likely to survive, grow, and reproduce as one with a faster metabolism at 16°C. Such an outcome might have resulted from the design of the selection experiment. Yeaman et al. (2010), who generated the selection lines, allowed flies to reproduce at set intervals by introducing bottles of medium on which flies could lay eggs. Because flies moved slower at 16 than at 25°C, they gave flies at 16°C twice as much time to lay eggs in these bottles before transferring them to a new cage to establish the next generation. This difference in time would have offset any locomotor disadvantage resulting from a slower metabolism at 16°C. Still, each fly competed only against other flies in its population, meaning that a faster metabolism could have given a fly an advantage over other flies laying eggs for the same period. Moreover, we measured the metabolic rates of male flies, the sex which would have faced direct selection for metabolic capacity to survive and mate before bottles were introduced to the cages. Thus, the nearly identical metabolic rates among flies from the three selective treatments seem more consistent with the idea that a faster metabolism conferred no significant gain in fitness. This experimental result implies that geographic patterns of metabolism result from factors other than environmental temperature. Similarly, Kellermann et al. (2015) were also unable to replicate observed latitudinal patterns of cold, heat, and starvation resistance, development time, and body size in laboratory populations of *D. melanogaster* that had evolved under thermal regimes that mimicked environmental temperatures along the east coast of Australia. In the present study, there was also no effect of selective treatment on dry body mass (Supporting Information), even though there is clear evidence of latitudinal body size clines in *D. melanogaster* (e.g. James et al. 1995, 1997; van't Land et al. 1999).

To understand interspecific variation in metabolism, such as the higher metabolic rates of insect species from colder environments (Addo-Bediako et al. 2002), researchers will have to deconstruct the suite of environmental factors that covary along a latitudinal cline. Addo-Bediako et al. (2002) drew attention to the weakness of the interspecific relationship that they found, suggesting that some species have evolved elevated metabolisms in cold environments whereas others have not. They noted that differences among species could reflect the selection of microhabitats or the availability of resources. Although others envisioned that abundant food would favor a faster metabolism, a latitudinal cline in metabolic rate could also result from a suppression of metabolism in warm environments. If food becomes scarce or risky to acquire, natural selection might favor genotypes that resist starvation by requiring little energy for maintenance. In this scenario, animals in a colder environment with more food would evolve a faster metabolism (at a given body temperature), than that of animals in a hotter environment with less food. To test this hypothesis, one could design a selection experiment in which temperature and other factors are manipulated simultaneously. Under such conditions, we predict that the evolution of metabolic rate will depend on the interaction between environmental temperature and food availability. In the hotter treatment, a low metabolic rate should evolve under restricted food. In the colder treatment, where thermodynamics suppress metabolism, natural selection should not lead to an evolutionary reduction of metabolic rate, especially within populations with abundant food. This interaction between environmental temperature and food availability would generate a pattern that resembles the metabolic cold adaptation predicted by Krogh (1916). In other words, when compared at the same temperature, genotypes from populations at a colder temperature and abundant food would have a faster metabolism than would genotypes from populations at a hotter temperature and restricted food. Such experiments would reveal what role, if any, temperature plays in the evolution of metabolism.

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. The linear mixed models that explain variation in dry body mass (mg) of *Drosophila melanogaster*.