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Natural populations of *Drosophila melanogaster* reveal features of an uncharacterized circadian property: the lower temperature limit of rhythmicity

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Abstract

Most cyclic biological processes are under control of a circadian molecular timing system that synchronizes these phenomena to the 24hr day. One generic property of circadian-controlled processes is that they operate within a specific temperature range, below which the manifestation of rhythm ceases. Little is known about the functional significance of the lower temperature limit of rhythmicity or about the mechanism underlying the loss of overt circadian behavior below this lower-limit, especially in one model organism of chronobiology, *Drosophila melanogaster*. Natural populations of *Drosophila* are evolving under divergent selection pressures and so provide a source of diversity necessary to address these issues. Using lines derived from the wild, we find that there is natural variation in the expression of rhythmic behavior under low temperature conditions. Interestingly, this variability appears ecologically relevant at *extremely* low temperature (12°C) such that lines originating from high altitude sources are twice as likely to maintain rhythm than those from low altitude populations. Lines resistant to 15°C show an additional layer of diversity in their response to temperature extremes because some lines are resistant to low temperature only whereas others are cross-resistant to high and low temperature. Genetic analysis of one cold-resistant circadian line at 15°C reveals that the phenotype maps to the X-chromosome but not to the core clock genes, *per* and *sgg*. Analysis of the central clock cells of this line reveals that maintenance of rhythm is associated with robust clock function, which is compromised in a standard laboratory strain. These data indicate that the cold-resistant circadian phenotype is clock-based. This study highlights the importance of using natural populations to inform us of the basic features of circadian traits, especially those that might be under temperature-based selection.

Keywords

circadian clock; lower temperature limit of rhythm; temperature resistance; altitude; s-LNVs

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Introduction

Most biochemical, physiological, and behavioral phenomena exhibit daily cycles that are under control of an internal circadian timing system that serves to synchronize these processes to peak at critical times of the 24hr day. The ability to anticipate and coordinate biological processes according to light:dark transitions of the environment confers a fitness advantage in several organisms (Ouyang et al., 1998; Woelfe et al., 2004; Dodd et al., 2005), which is presumably why the internal timing mechanism is evolutionarily conserved from bacteria to mammals. In flies (*Drosophila melanogaster*), the molecular clock is characterized by a well-established negative feedback loop in which core clock proteins – PERIOD (PER) and TIMELESS (TIM) – inhibit the transcription of their own mRNAs. The ~24hr cycle begins when two transcription factors (CLK and CYC) bind to E-boxes upstream of the *period* (*per*) and *timeless* (*tim*) genes and transcribe *per* and *tim* mRNA. These mRNAs are translated into proteins which accumulate in the cytosol, form a heterodimer complex, and translocate into the nucleus. Phosphorylation plays a central role in the accumulation of the proteins as well as their nuclear transfer. Once the complex translocates into the nucleus, PER-TIM bind to CLK-CYC, inhibiting their binding affinity for the *per* and *tim* E-boxes, thereby negatively regulating the transcription of their own mRNAs. The negative inhibition is removed once other proteins degrade the PER-TIM complex, allowing the cycle to begin again. For a detailed review of the molecular clock, see (Zheng and Sehgal, 2012).

In the *Drosophila* brain, several neuronal clusters exhibit oscillations of the molecular clock and these ‘clock cells’ coordinate the timing of circadian-controlled processes. Within the environment, there are a number of stimuli such as light, chemicals, and temperature that affect the timing and efficacy of circadian outputs. Of these, temperature is especially interesting because it influences overt rhythms in several ways, one of which is that low temperature blocks rhythm, which resumes once returned to permissive temperatures. Thus, the overt manifestation of circadian rhythms occurs within specific temperature limits (Njus et al., 1977; Francis and Sargent, 1979; Martino-Catt and Ort, 1992). With the exception of one study in the fungus *Neurospora crassa* (Liu et al., 1997), little is known about the functional significance of this circadian property nor why species lose their ability to maintain rhythm below the lower limit of rhythmicity. Although *Drosophila* has been the focus of intense research on the evolutionary and molecular biology of circadian rhythms and some studies have addressed mechanisms and selection pressure for temperature-dependent changes in activity patterns (Majercak et al., 1999; Low et al., 2008) – the lower temperature limit property of circadian rhythms has not been investigated.

Natural populations of *Drosophila melanogaster* are evolving in diverse environments across the world (Kyriacou et al., 2008) and their molecular clocks have been shown to be under selection pressure based on how well they resonate with the ~24hr cycle of the environment (Sawyer et al., 1997; Sawyer et al., 2006; Low et al., 2008). In addition, cold tolerance and correlated phenotypes (reproductive diapause expression, longevity, starvation tolerance, lipid content, fecundity profiles (Schmidt et al., 2005) are directly relevant to *Drosophila* populations in the wild and are affected by selection pressures that vary spatially and temporally (Mitrovski and Hoffman, 2001; Schmidt and Conde, 2006; Schmidt and Paaby,

2008). This suggests that the maintenance of fundamental biological processes (including circadian rhythmicity) during exposure to cold temperatures is directly relevant to performance and fitness of *Drosophila* evolving in temperate environments. Here we tested the response of wild-derived populations of *Drosophila* to low-temperature stress and used lines rhythmic at low temperatures to address the ecological relevance as well as the mechanism underlying maintenance of rhythm under these conditions. We found tremendous variability in the response of natural populations to temperature, and determined that the variability at *extremely* low temperature is ecologically relevant. The differences in rhythmicity are likely due to differences in clock function: a standard laboratory strain that is arrhythmic at low temperatures has reduced clock function while a wild-derived line that displays rhythmicity has robust cycling of clock proteins.

Methods

Isofemale lines

Isofemale lines are strains derived from a single, multiply mated, wild-caught female and are often used to investigate the genetic architecture of natural populations (David et al., 2005). In this study, 46 Isofemale lines derived from six natural populations in Africa [9 from CO: Oku, Cameroon (6.25, 10.43; altitude: 2169m); 3 from ED: Dodola, Ethiopia (6.98, 39.18; altitude: 2492m); 11 from GA: Franceville, Gabon (−1.65, 13.6; altitude: 350); 9 from NG: Maiduguri, Nigeria (11.85, 13.16; 295m); 9 from RG: Gikongoro, Rwanda (−2.49, 28.92; 1927); SP: 5 from Phalaborwa, South Africa (−23.94, 31.14; 350m)] were used to investigate the functional significance of this circadian property. All 46 lines were used in the 12°C dataset and a random subset of these lines was used for analyses at 15°C. J. Pool collected lines from CO and ED in 2004 as well as RG in 2008; lines from GA were collected by B. Ballard and S. Charlat in 2002; lines from NG were collected by D. Gwary and B. Sastawa in 2004; and SP lines were collected by R. Corbett-Detig in 2010 (Pool et al., 2012). All *Drosophila* lines used in this study were maintained according to the principles and practices of the US National Institute of Health's (NIH) *Guide for the Care and Use of Laboratory Animals*.

Behavioral analysis

The following behavioral assays were conducted under *extremely* low (12°C), low (15°C), standard (25°C), or high (30°C) temperature. Adult males between 1 to 5 days old were entrained in TriKinetics Activity Monitors (TriKinetics, Waltham, MA) under a 12hr:12hr light:dark schedule for three days in a temperature-regulated Precision incubator model 818 (Thermo Electron Corporation; Marietta, Ohio). Flies were then placed in constant darkness (DD) and allowed to free-run for 4 days, after which locomotor activity – a commonly used metric of circadian behavior in *Drosophila* (Frenkel and Ceriani, 2011) – was scanned into 5 minute bins using the DAM File Scan (v.1.1.06, TriKinetics). We used ClockLab software (v.2.61; Actimetrics, IL) to score an individual fly as either rhythmic or arrhythmic, which we characterized based on the strength of the rhythm (FFT). Flies were scored as rhythmic only if they achieved an FFT value of ≥ 0.01 . For each fly, we reported its behavioral state (rhythmic/arrhythmic); and, if it achieved rhythmicity, the strength of its rhythm.

Mapping the NG7 cold-resistant circadian behavior

Establishing chromosomally isolated genetic lines—We mapped the chromosome responsible for the NG7 cold-resistant circadian behavior by establishing chromosomally isolated genetic lines of the NG7 isofemale line. To do this, we first crossed a group of NG7 males that exhibited rhythm (FFT = 0.01) at 15°C to virgins balanced for each of the three fly chromosomes. Balancer lines were obtained from Bloomington Drosophila Stock Center (<http://flystocks.bio.indiana.edu/>) and each was initially outcrossed to our isogenized *w¹¹⁸* background (Iso31) for 7 generations.

To isolate the NG7 X-chromosome, we used the attached X line XX/Y (Bloomington stock #988); to isolate the 2nd chromosome, we used the balancer line, *Sco/Cyo* (Bloomington stock #5907); and to isolate the 3rd chromosome, we used the balancer line TM2/TM6b (Bloomington stock #5906). F1 males were sorted by the presence of the chromosomal balancer and then crossed to virgin females with the appropriate chromosomal balancer line used in the P1 cross. This process was repeated for a total of three generations. To achieve homozygosity of each chromosome after the third generation (F3), we sorted and then crossed males and virgin females based on the absence of the chromosomal balancer, thereby generating three outcrossed genetic lines of X, 2nd, and 3rd chromosomal isolates for NG7. We then phenotyped each chromosomally isolated stock at 15°C to see which could maintain rhythmicity (see ‘behavioral analysis’ section above).

Generating recombinants—To test whether the NG7 *per* or *sgg* locus accounts for its cold-resistant circadian behavior, we created recombinant lines of the NG7 and Iso31 X-chromosome. To do this, we first crossed a male from the chromosomally isolated X NG7 line positive for the behavior to a female Iso31 virgin negative for the behavior. We then selected female virgins from the F1 generation (which are all heterozygous for the NG7 and Iso31 X-chromosome) and crossed them to a male from the Iso31 strain. Because recombination occurs in the female sex of this species, the resulting males of this F2 progeny carry an X-chromosome that underwent one round of meiosis and was therefore recombinant for the Iso31 and NG7 X-chromosome. We used eye color as a marker for *per* and *sgg* allelic identity because the genes are linked. We then assayed behavior of 64 white and 62 red-eyed males from the cross at 15°C.

Immunohistochemistry and confocal microscopy

For the 15°C and 25°C immunohistochemistry experiments, we entrained Iso31 and X-chromosome isolated NG7 males according to the behavioral analysis section above. Once we characterized the behavior of the two genotypes, we then re-entrained these animals for one day on a 12hr:12hr light:dark schedule, followed by a return to constant darkness (DD1). All samples were collected on DD1. IHC preparation and quantification protocols for PER in the Pigment Dispersing Factor (PDF)-positive small ventral-lateral neurons (s-LNVs) have been described previously (Benito et al., 2007; Blanchard et al., 2010; Zhang et al., 2013). We quantified PER in this cell cluster because it is essential for robust circadian behavior in DD (Zheng and Sehgal, 2012). Briefly, to compare PER intensity in the s-LNVs, NG7 and Iso31 were sacrificed at 4-hour intervals starting at CT0 (CT0, 4, 8, 12, 16, and 20). For the 15°C experiment, all Iso31 males (which were arrhythmic) were sampled for

immunohistochemistry, whereas both arrhythmic and rhythmic NG7 males were sacrificed (NG7A and NG7R respectively). For the 25°C experiment, males from both genotypes were rhythmic so figure 6a,b reflects the behavior of rhythmic Iso31 and NG7 males.

After dissection in chilled PBS-T (PBS with 0.1% Triton), brains were fixed in 4% paraformaldehyde diluted in PBS-T for 25 minutes at room temperature. Subsequently, the brains were rinsed and washed with PBS-T three times (5 minutes each wash) and then incubated with 5% normal donkey serum in PBS-T for 30 minutes and then incubated with primary antibodies at 4°C overnight. To stain for PER, we used rat anti-PER (1:1000). Because the PDF-containing s-LNVs control circadian behavior in DD (Renn et al., 1999; Stoleru et al., 2005), we co-stained the brains for PDF expression using rabbit anti-PDF (1:250). The following day, brains were washed 3 times with PBS-T (5 minutes each wash) then incubated in the following secondaries at room temperature for 1.5hrs: Alexa 488-conjugated donkey anti-rat (2:1000) and Alexa 555-conjugated donkey anti-rabbit (2:1000) (LifeTechnologies. 1:500 dilution). Brains were mounted on a glass slide using Vectashield (Vector Laboratories, Burlingame, CA).

Images of the s-LNVs were acquired on a Leica TCS SP5 confocal microscope with a 40X oil immersion lens by sequentially collecting z-sections at 1.5µm for both Alexa fluorophores. To avoid oversaturating the image, we calibrated the gain and offset for the PER detector to the s-LNV with the maximal amount of PER expression for each experiment. Therefore the scales in figures 5b, d and 6b are not comparable with each other. The calibration of the detector remained constant throughout the given experiment for all groups. Once collected, the images were digitally projected using ImageJ v1.42q (<http://rsb.info.nih.gov/ij>). Mean PER fluorescence was taken for each s-LNV and normalized to the field adjacent to the imaged neuron. PDF (magenta channel) was used as a marker for the neuron. For each time point, signal intensity was measured bilaterally in up to 4 neurons from at least 5 brains.

Statistical analyses

All statistical analyses were performed using JMP PRO v.10 (SAS Institute, Cary, NC). To compare the differences among the proportion of rhythmic individuals between two or more independent groups, we used a Chi-square test. When the proportions between the independent groups consisted of sparse data (cells<5), we used a Fisher's exact test. To model the relationship between altitude and a fly's likelihood of maintaining rhythm under *extremely* cold (12°C) or cold temperature stress (15°C), we used a nominal logistic regression model. To take into account the nested structure of the immunohistochemistry data we used a linear mixed effects model to analyze differences in PER intensity at each timepoint for all groups.

Results

Rhythmicity at low temperatures varies in naturally occurring *Drosophila* populations

To determine if natural populations of *Drosophila* display variation in the manifestation of circadian behavior under *extremely* low temperature stress, we evaluated the locomotor

rhythm of isofemale strains derived from ancestral African populations at 12°C. We phenotyped between 10–128 individuals for each of the 46 isofemale lines, recording the individual's behavioral state (rhythmic/arrhythmic). We found that the proportion of rhythmicity in these lines varies under *extremely* low temperature stress (fig. 1a).

The effect of altitude on selection for fitness related traits (e.g. desiccation, heat knock-down and starvation resistance; developmental time) in *Drosophila* has been reported previously (Sørensen et al., 2004; Sambucetti et al., 2006); however, it is unknown whether altitude also influences an individual's likelihood of maintaining rhythmic behavior under *extremely* low temperature stress. Because the circadian clock is known to be under selection based on its ability to maintain a ~24hr rhythm regardless of the temperature variance (Sawyer et al., 1997; Sawyer et al., 2006), we predicted that extreme environmental flux associated with high altitude would select for cold-resistant circadian traits at 12°C. To test this hypothesis, we performed a logistic regression to determine the effect of a fly's altitude of origin [high [>1900m: Cameroon (CO), Ethiopia (ED), Rwanda (RG)] and low [<350m: Gabon (GA), Nigeria (NG), South Africa (SP)] on its likelihood of maintaining rhythm. In support of our hypothesis, we found that flies from high altitude sources are twice as likely to maintain behavioral rhythm under extreme cold stress than those from low altitude sources (fig. 1b, table 1).

Next we sought to determine the extent to which natural populations display variation in their ability to maintain rhythm at a slightly higher temperature (15°C) and so we phenotyped a random subset of our original pool under this less-stressful cold condition. Similarly to the 12°C dataset, we found that natural populations of *Drosophila* display variation in the manifestation of circadian behavior (fig. 1c). However, unlike the 12°C dataset, altitude is not a significant predictor of a fly's likelihood of maintaining behavior rhythm under 15°C cold-temperature stress (table 2). For the remainder of the study in which we investigate the mechanistic aspects of the lower-limit trait, we chose to phenotype the isofemale lines under 15°C, as this temperature yielded more penetrant and robust rhythms.

In natural populations, cold-buffered circadian behavior at 15°C is resistant only to low temperature stress or cross-resistant to high and low temperature extremes

Drosophila respond adaptively to selection for heat (Hoffman et al., 1997) or cold stress (Watson and Hoffman, 1996), resulting in a change of the resistance to high or low temperature respectively. Less understood in insect thermal physiology is the concept of 'cross-resistance,' in which adaptation to one stressor (e.g. low temperature) confers an advantage in counteracting the deleterious effects of another (e.g. high temperature). This property may be due to selection of genes that curb the effects of stressors that induce similar physiological responses, predicting positive genetic correlations between the traits of interest. In this experiment, we tested whether cold-buffered circadian behavior at 15°C (fig. 1c) is resistant to low temperature stress only or is 'cross-resistant' to high and low temperature extremes. If the cold-buffered rhythm is resistant only to low temperature, we predicted that either of the two behaviors related to rhythm – the line's proportion of rhythmicity or average rhythm strength (FFT) – would degrade from low (15°C) to high

(30°C) temperature stress. In contrast, if the cold-buffered rhythm is cross-resistant to high and low temperature stress, we predicted that there would be no change in the two behaviors between temperature extremes. From the isofemale lines we phenotyped in fig. 1c, we identified 4 —CO1, NG8, NG10, and NG7— that were highly rhythmic at 15°C, meaning that the proportion of rhythmic individuals in the line exceeded 70%. We consider these lines as resistant to low temperature stress because they have cold-buffered rhythm.

For the first assay, we compared the difference in each line's proportion of rhythmic individuals between low and high temperature stress. Of the 4 lines assayed, NG8 shows a significant decrease in rhythmicity from low to high temperature and so we categorize this line as cold-resistant only (fig. 2a). Next, we used rhythm strength as an output measure of how the circadian behavior of each isofemale line responds to temperature stress. We found that the rhythms of NG8 and NG7 show a decrease in strength when placed under high temperature stress, indicating that their rhythms are cold-resistant only (fig. 2b). The results of figs. 2a-b suggest that rhythms of the NG7 and NG8 lines are resistant to cold temperature stress only because rhythmic behavior degrades in at least one of the assays we used to detect clock output: (NG8: proportion rhythmicity, FFT; NG7: FFT). And, while slightly non-significant ($P=0.09$), NG10 shows the same trend in having a higher proportion of rhythmic individuals (fig. 2a) under cold temperature stress and so we consider the rhythm in this line as 'slightly cross-resistant' to cold and hot temperature stress. In contrast, our data suggest that the rhythm of CO1 is cross-resistant to high and low temperature extremes because there was no difference in the proportion of rhythmic individuals or rhythm strength between 15 and 30°C. Taken together, these results suggest that in nature, cold-buffered circadian lines at 15°C can be either resistant to cold temperature stress or cross-resistant to both high and low temperature extremes.

Cold-buffered rhythmic lines withstand low temperature stress better than a generic wild-type laboratory line

Next, we determined whether the cold-buffered rhythmic lines withstand cold temperature stress better than our wild-type laboratory line, Iso31. We predicted that if these lines are better able to withstand cold temperature stress, then either the proportion of rhythmic individuals or the rhythm strength would be higher than Iso31 at 15°C. Our data are consistent with this prediction: cold-buffered rhythmic lines have a higher proportion of rhythmic animals under low temperature stress (fig. 3a) and higher rhythm strength (fig. 3b). Next we determined if the rhythm of Iso31 is better buffered against high temperature stress than our cold-buffered lines. If so, then either the proportion of rhythmic individuals or the rhythm strength would be higher in Iso31 than any of the cold-buffered rhythmic lines at high temperature. Figure 3c is consistent with this prediction because at 30°C, Iso31 is more rhythmic than two cold-buffered rhythmic lines (NG10 and NG8) (fig. 2). The rhythm strength was not different among the lines at this temperature (fig. 3d). Our 30°C data support the idea that our laboratory line can buffer high temperature extremes better than some of our cold-buffered isofemale lines.

Mapping the genetic locus of a 15°C cold-resistant rhythmic line (NG7)

To determine whether the cold-resistant circadian behavior of one line (NG7) could be mapped to a single chromosome or if it represented a quantitative output of any combination of the three major fly chromosomes (X, 2nd, or 3rd), we isolated each NG7 chromosome into three independent lines in an Iso31 background (see Methods). As we determined that Iso31 is negative for the cold-resistant rhythmic phenotype (fig. 3a, b), isolating each NG7 chromosome in an Iso31 background would reveal the source of the behavior. We found that the X-chromosome of NG7 contributes to the phenotype while the other isolated chromosomes do not (fig. 4a).

Next we tested whether the *period* and *shaggy* loci are functionally important for the phenotype because these are two important clock genes on the X-chromosome and they are implicated in interactions of the clock with temperature (Cheng et al., 1998; Majercak et al., 1999; Low et al., 2008). Because *per* and *sgg* are linked to the eye color gene, *white* (*w*) – meaning that they are located so positionally close together that they do not recombine – *w* can be used as a marker of *per* and *sgg* allelic identity: NG7 contains a wild-type copy of the allele (*w*⁺), which produces red-eyes and Iso31 contains the mutant allele *w*¹¹⁸, which results in a white-eyed phenotype. Therefore red-eyed recombinants of the NG7 and Iso31 X-chromosome are expected to have NG7 *per* and *sgg* alleles. If the cold-resistant rhythmic phenotype maps to *per* or *sgg*, then recombination of the NG7 X-chromosome with the Iso31 X-chromosome (see Methods) should yield only red (and no white) eyed recombinants that are positive for the behavior. However, we were able to recombine the *w*¹¹⁸ gene of Iso31 with the NG7, cold-resistant circadian behavior (fig. 4b), suggesting that the phenotype is not linked to NG7 *per* or *sgg*.

A more robust circadian clock underlies the 15°C cold-resistant behavioral rhythm of NG7

Stronger behavioral rhythm at lower temperature could result from enhanced clock function or from better transmission of output signals from the clock. To distinguish between these two possibilities we assayed molecular cycling of the clock protein, PER, in the small ventral-lateral neurons (s-LNVs), which are central clock cells of the *Drosophila* brain. As not all NG7 flies are rhythmic at lower temperature, we first assayed all flies for behavior to separate them into rhythmic and arrhythmic groups. Briefly, we entrained flies from the NG7 X-chromosome cold-resistant circadian strain (generated in fig. 4a) as well as Iso31 to 3 days of 12hr:12hr light:dark cycles. On the fourth day, we moved the flies into constant darkness and let them free-run for four days, after which we scored the behavior of the flies as either rhythmic or arrhythmic based on the strength of the rhythm. All Iso31 flies were arrhythmic in this experiment. Once we characterized the behavior of individual flies, we re-entrained them for one day to resynchronize molecular oscillations across flies and then returned them to constant darkness. On the first day of constant darkness (DD1), we dissected the brains of rhythmic and arrhythmic NG7 as well as Iso31 flies at four-hour intervals. We then examined PER expression by immunohistochemistry in the s-LNVs, which are central clock cells of the *Drosophila* brain that maintain free-running rhythm (Grima et al., 2004) and can be identified through their expression of a neuropeptide, Pigment Dispersing Factor (PDF). Representative images of the immunohistochemistry are shown in fig. 5a. We found that rhythmic and arrhythmic NG7 flies have stronger PER

oscillations in central clock cells at 15°C than Iso31 (fig. 5b), indicating that the strong behavioral rhythm of NG7 is due to stabilization of the central clock as well as the clock output under cold-temperature stress. Table 3 illustrates the linear mixed-effects model regression weights and significance levels for each treatment group in predicting mean PER expression for each timepoint.

In comparison with NG7, PER expression appeared low and noncyclic in Iso31 at 15°C (fig. 5a,b). To determine whether PER oscillations in Iso31 are indeed disrupted or merely reduced in amplitude, we enhanced the detection of PER expression in Iso31 using a calibration specific to an Iso31 s-LNV cell with maximal PER expression. A disrupted molecular profile would produce a flat protein expression profile throughout the circadian time course. Representative images of this re-quantification are shown in fig. 5c. The group means of these samples show that PER cycling is not disrupted but considerably dampened, due to an overall reduction in levels relative to expression in NG7.

Enhanced PER expression of the NG7 cold-resistant clock is specific to cold temperature stress

To determine whether enhanced PER expression of the NG7 cold-resistant clock relative to Iso31 (fig. 5) is specific to low temperature stress, we performed IHCs on NG7 and Iso31 s-LNVs at room temperature (25°C) using the same methods as above. Both genotypes were rhythmic in this experiment. Representative images of NG7 and Iso31 flies are shown in fig. 6a. Group means of the two genotypes show that there is no difference in PER expression at room temperature (fig. 6b). Table 4 illustrates the linear mixed-effects model weights and significance levels for each treatment group in predicting mean PER expression for each timepoint.

Discussion

Little is known about the functional and mechanistic features of the lower temperature limit property of circadian rhythm, especially in *Drosophila*, in which there has been no research effort. In this study, we used 46 isofemale lines isolated from 6 different natural populations of *Drosophila melanogaster* to address these issues. Previous work has shown that physiological traits influenced by temperature (such as the temperature compensation property of the clock, cold-tolerance, and reproductive diapause) are under predictable selection pressures of the environment so natural populations offer a unique medium to understand the functional (variability and ecological distribution) and mechanistic (why *Drosophila* experience overt loss of behavior rhythm) features of this trait.

Our study is the first to capture the diversity in how the *Drosophila* circadian system responds to low temperature stress: under both *extremely* low (12°C) and low (15°C) temperatures, some isofemale lines are better at maintaining behavioral rhythm than others (fig. 1a, c). Interestingly at 12°C, we found that altitude is one parameter associated with this variability because populations derived from high altitude sources are twice as likely to maintain rhythmic behavior than those from low altitude sources (figure 1b, table 1). However, the effect of altitude on behavioral rhythmicity is only applicable under *extreme* temperature stress because there is no difference in the proportion of rhythmic high and low

altitude flies at 15°C (table 2). One possible explanation for this difference is that populations at high and low altitudes may experience temperatures of 15°C or higher. In this scenario, both high and low populations would express the same variation of gene(s) that drive circadian rhythm. However, high altitude populations might experience *extreme* cold temperature extremes (<15°C) while low populations do not or do so less frequently. This condition might then cause a differential selection pattern for gene(s) specialized to buffer extreme cold in the high population group.

For the lines that withstand cold temperature stress under 15°C, we found additional diversity in how they respond to temperature stress in general. Some of these cold-buffered lines display rhythms that are resistant only to low temperature stress (NG7, NG8; fig. 2) whereas others are cross-resistant to both high and low temperature extremes (CO1, fig. 2). Extension of this work to natural populations on other continents and from other habitats would be required to further evaluate the significance of resistance to a specific temperature extreme and cross-resistance to temperature extremes.

Our research also highlights the advantage of using lines derived from natural populations to identify novel loci relevant to temperature-influenced circadian traits. Although the locus responsible for the phenotype of one cold-resistant strain (NG7) maps to the X chromosome, it does not appear to be the canonical clock gene, *period* (*per*). This is distinct from findings in *Neurospora crassa*, where the determinants of the lower limit are linked to the central clock gene, *frequency* (*frq*) (Liu et al., 1997). In this study, we have shown that the rhythm of at least one isofemale line is resistant to low temperature stress due to a locus unrelated to *per* (fig. 4c), which is surprising given that this gene is important for other ways the clock interacts with temperature. For example, the structure of *per* is fundamentally important for temperature-dependent changes in the distribution of daily activity patterns (Majercak et al., 1999; Low et al., 2008) whereby temperature-sensitive splicing of *per* (Cheng et al., 1998) facilitates the temperature-mediated advance and delay in behavior.

Although *per* is not the allelic source of NG7, we believe that the enhanced PER expression we observed in NG7 at low temperature (fig. 5a,b, table 3) is functionally important for maintaining rhythmicity. The cold-resistant circadian behavior of NG7 is most likely due to more robust oscillations of core clock genes. As shown from our immunohistochemistry data, PER expression is significantly higher in the s-LNVs of NG7 than Iso31 during times at which it is typically elevated, from CT20-4 (fig. 5a,b; table 3). As shown previously (Liu et al., 1997; Lim and Allada, 2013), a threshold amount of PER is required for the circadian rhythm to persist, and this level changes as a function of temperature. Our wild-type line is likely arrhythmic under cold temperature stress because their s-LNVs do not reach sufficient PER expression to achieve rhythmicity. We note that we observed oscillations (fig. 5c,d) when imaging the circadian cells under a lower calibration for PER.

Interestingly, our data also suggest that while enhanced clock function is necessary for overt rhythm to persist under low temperature stress, it is not sufficient because there is no difference in PER levels between rhythmic and arrhythmic NG7 flies throughout the circadian cycle (fig. 5a,b; table 2). Thus, mechanisms downstream of the clock also need to be resistant to low temperature to produce rhythmic behavior. Given that the overall behavior

can be recombined with *w* (and, therefore, is not linked to *per/sgg*), we suggest that the NG7 line contains component(s) distinct from *per* and *sgg* on the X-chromosome (fig. 4c) that enhance PER oscillations in the s-LNVs at low temperature and stabilize output circuits necessary for rhythmic behavior. Furthermore, our data also suggest that these component(s) are active in response to cold-temperature stress only, since we did not observe a difference in PER cycling between NG7 and Iso31 under standard temperature conditions (fig 6a,b; Table 4).

What underlies the NG7 clock's ability to buffer cold temperature? It is possible that transcriptional and/or translational modifications within its molecular clock circuit enhance clock oscillations. Under low temperature stress, numerous insults could interfere with the clock circuit's transcriptional machinery, which must be maintained for precise spatiotemporal control of clock genes. For example, under low temperature, single enhancers sometimes fail to drive the complete expression of genes, and studies have shown that there are several regulatory mechanisms that counteract these insults (Lagha et al., 2012), two of which include intra-enhancer redundancy (Ludwig et al., 2011) and shadow enhancers (Frankel et al., 2010; Perry et al., 2010). In *Drosophila*, shadow enhancers are good candidates for the NG7 cold-resistant clock phenotype because some have been shown to stabilize gene expression of certain loci specifically at 15°C (Frankel et al., 2010).

Once clock genes are transcribed, clock RNA structures might also encounter problems unique to cold stress. Under low temperature conditions, misfolded single stranded RNA structures become overstabilized and certain transcript variants do not always produce optimally behaving proteins for the given abiotic condition. Therefore post-transcriptional modification mechanisms might play a key role in modulating clock gene expression by generating different mRNA variants with altered stability, translation efficiency, and/or distribution. Some candidates include alternative splicing (which is already known to be relevant to the *Drosophila* circadian clock (Majercak et al., 1999)) and RNA editing (Savva et al., 2012). Similarly, tight translational control mechanisms might play a key role in optimizing clock gene expression. Cold-temperature stress in general reduces protein synthesis and so tight translational control mechanisms of NG7 clock genes might guarantee that a sufficient amount of clock expression is produced to maintain circadian oscillations.

Low temperatures also result in abnormally high levels of misfolded or damaged proteins which could affect the efficacy of the molecular clock. Molecular chaperones can assist with protein folding and protein degradation by capturing damaged proteins and refolding them back into their native confirmation. If this re-folding fails, molecular chaperones, with the help of other co-factors can target such proteins for degradation. In the plant *Arabidopsis thaliana*, targeted degradation of certain proteins installs new regulatory mechanisms that upregulates the expression of cold-responsive genes, mediating tolerance to cold conditions (Lyzenga and Stone, 2012). To our knowledge, it is unknown whether post-translational modifications due to molecular chaperones or other cofactors are involved with the *Drosophila* response to low temperature stress.

Our data also suggest that stability of the output pathway is functionally important for cold-resistant clock behavior. Downstream of the molecular clock, circadian pacemaker neurons

form a functional circuit necessary for the ~24hr output of circadian behavior and our data suggest that the NG7 line also regulates its output circuit during temperature stress. Temperature stress is generally associated with massive changes in the *Drosophila* transcriptome, which include upregulation of several physiological relevant genes (Kelty et al., 2002; Colinet and Hoffman, 2010). For example, the senescence marker protein *smp-30* – which is important for Ca^{2+} ion homeostasis and neuron signaling – is thought to be relevant to cold tolerance in *Drosophila* because *smp-30* mRNA accumulates after cold acclimation to 15°C (Goto, 2000). In addition, there is an association between genetic variation at *smp-30* and with variation in chill-coma recovery (Clowers et al., 2010). These or other relevant mechanisms might be important for how the NG7 clock circuit and/or circadian output maintains physiological homeostasis during low temperature stress.

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Figure 1. Rhythmicity at lower temperatures varies in naturally occurring *Drosophila* populations

(a) 12°C Using a Chi-square test, we found that the proportion of rhythmic individuals differs among 46 isofemale lines at 12°C $\chi^2(df=45, N=1937)=145, P<0.001$. **(b) Isofemale lines derived from high altitude populations are more likely to maintain rhythm under extreme temperature stress.** Results of the logistic regression can be found in table 1. **(c) At 15°C, rhythmicity varies in naturally occurring *Drosophila* populations** Using a Chi-square test, we found that the proportion of rhythmic individuals differs under low temperature stress among a random 27 line subset of our isofemale strains $\chi^2(df=28, N=862)=145, P<0.001$.

Figure 2. In natural populations, cold-buffered circadian behavior at 15°C is resistant only to low temperature stress or cross-resistant to high and low temperature extremes

(a) Proportion of rhythmic individuals. We used a Fisher's exact test to compare differences in the proportion of rhythmic individuals between low and high temperature stress for CO1, NG8, NG10, NG7. * $P < 0.05$. There is a slightly non-significant trend for the proportion of rhythmic individuals in NG10 to decrease from 15 to 30°C ($P = 0.09$). **(b) Rhythm strength.** Independent sample t-tests were conducted to compare the rhythm strength (FFT) between isofemale lines at 15°C and 30°C. *** $P < 0.05$. CO1 does not change its circadian behavior in either assay.

Figure 3. Cold-buffered lines are more resistant to cold-temperature stress than a generic wild-type laboratory line

(a) Cold-buffered lines are more rhythmic at 15°C. We used Fisher's exact tests to compare differences in the proportion of rhythmic individuals between Iso31 and CO1, NG8, NG10, and NG7 respectively. *** $P < 0.0005$. **(b) Cold-resistant lines have stronger rhythm strength at 15°C.** We used independent sample t-tests to compare the rhythm strength of Iso31 to CO1, NG8, NG10, and NG7, respectively. *** $P < 0.0005$, * $P < 0.05$. **(c) Iso31 can buffer high temperature extremes better than some of our cold-buffered**

isofemale lines. We used Fisher's exact tests measuring the proportion of rhythmic individuals between Iso31 and CO1, NG10, NG8, NG7 under high temperature stress. ** $P < 0.005$ There is a non-significant trend for the Iso31 line to be more rhythmic at 30°C than the cold-resistant NG7 line ($P = 0.13$). **(d) Rhythm strength is not different among lines at 30°C.** Independent sample t-tests were conducted to compare the rhythm strength (FFT) between each isofemale line and Iso31.

Figure 4. Mapping the genetic locus of a 15°C cold-resistant circadian line (NG7)

(a) The X-chromosome of NG7 underlies cold-resistant circadian behavior. A one-way between subjects ANOVA was conducted to compare the effect of low temperature (15°C) on rhythmicity for the 4 groups (X-isolated NG7, Iso31, 2nd-isolated NG7, and 3rd-isolated NG7). There was a significant effect of low temperature on rhythmicity at the $p < 0.05$ level for the 4 groups [$F(3)=71.1$, $P < 0.0001$]. Post hoc comparisons using the Tukey HSD test indicate that the mean proportion of rhythmic individuals for the X-Isolated NG7 line ($M=0.60$, $SD=0.49$) was significantly higher than that of Iso31 ($M=0.07$, $SD=0.24$), the 2nd-

isolated NG7 line ($M=0.63$, $SD=0.25$) and the 3rd-isolated NG7 line ($M=0.31$, $SD=0.18$); ***, $P<0.0001$. There is no difference in the proportion of rhythmic individuals at cold temperature among Iso31, the 2nd-isolated NG7 line, and the 3rd-isolated NG7 line. **(b) The NG7 clock genes *per* and *sgg* do not underlie the cold-resistant rhythmic behavior.** Fisher's exact test, $p=0.07$.

Figure 5. A more robust circadian clock underlies the 15°C cold-resistant behavioral rhythm of NG7

(a) Representative images of PER (green) and PDF (magenta) staining in the s-LNVs of rhythmic and arrhythmic NG7 flies (NG7R and NG7A respectively) as well as Iso31.

Rhythmic and arrhythmic NG7 flies express classic cycling of PER protein in the s-LNVs throughout the circadian cycle: PER is primarily nuclear between CT0 and CT4 (panels A, B, and D, E), absent in the middle of the subjective day at CT8-16 (panels G, J, M and H, K, N) and then cytoplasmic towards the end of the cycle at CT 20 (panels P and Q). In contrast, PER is absent in the s-LNVs in Iso31 throughout the circadian cycle (panels C, F, I, L, O, R) under the same detection level for PER.

(b) NG7 has enhanced PER oscillations relative to Iso31 at cold temperature. Group means of PER expression in the s-LNVs of rhythmic and arrhythmic NG7 flies as well as Iso31.

(c) Representative images of the Iso31 s-LNVs captured under enhanced detection. When we captured images of the Iso31 samples using a stronger PER detector than in fig.5a, b we found that PER expression is not disrupted at low temperature.

(d) Iso31 has a dampened (not disrupted) circadian clock at cold temperature. Group means of the re-quantified Iso31 genotype show that PER oscillations persist, suggesting that Iso31 produces lower levels of PER protein in the s-LNVs than NG7 throughout the circadian cycle. The pattern of PER expression is the same between Iso31 and the NG7 individual: PER is present at CT0, 4, and 20 but absent at CT 8, 12, and 16.

Note: figures 5a,b and 5c,d were analyzed using a different calibration of mean PER intensity. Therefore scales of 5b and d are not the same and are not comparable.

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Figure 6. Enhanced PER expression of the NG7 cold-resistant clock is specific to low temperature stress

(a) Representative images of NG7 and Iso31 PER expression in the s-LNVs under room temperature. (b) The higher level of PER expression in the s-LNVs of NG7 observed in figure 5a, b is a specific response to cold temperature stress. Group means reveal that there is no difference in PER expression between the genotypes throughout the circadian cycle. Both groups exhibit classic patterns of PER nuclear and cytoplasmic localization. *Note: figures 5a,b and 5c,d and 6a,b were analyzed using a different calibration of mean PER intensity. Therefore scales of 5b,d, and 6b are not the same and are not comparable.*

Table 1
Results for the nominal logistic regression model for fig. 1b

Altitude is a significant predictor of a fly's likelihood of rhythmicity under extreme cold temperature stress (12°C).

Figure 1b				
Coefficient	Estimate	Std. Error	ChiSquare	p-value
Intercept	3.82	0.23	271	<0.0001*
Altitude[low]	0.75	0.22	12.2	0.0005*
Altitude[low]:population [GA]	0.41	0.56	0.53	0.47
Altitude[low]:population [NG]	0.15	0.56	0.08	0.78
Altitude[high]:population[CO]	0.24	0.25	0.86	0.35
Altitude[high]:population[ED]	-0.48	0.25	3.47	0.06
Assay[GV11]	-0.37	0.23	2.67	0.10
Assay[GV12]	0.67	0.30	5.00	0.03*

Table 2
Results for the nominal logistic regression model

Altitude is not a significant predictor of a fly's likelihood of rhythmicity under cold temperature stress (15°C).

Coefficient	Estimate	Std. Error	ChiSquare	p-value
Intercept	0.72	0.09	59.1	<0.0001*
Altitude [low]	-0.14	0.09	2.25	0.13
Altitude[low]:population[GA]	-0.02	0.14	0.02	0.89
Altitude[low]:population[NG]	-0.33	0.13	5.97	0.01*
Altitude[high]:population[CO]	-0.29	0.19	2.47	0.12
Altitude[high]:population [ED]	0.08	0.27	0.08	0.77

Table 3

Results of the linear mixed-effects model for fig 5b

PER mean signal intensity is significantly higher in rhythmic and arrhythmic NG7 individuals than in Iso31 animals at CT0, CT4, and CT20: times when PER classically reaches maximal expression in the circadian cycle. Mean PER intensity between arrhythmic and rhythmic NG7 individuals is not significantly different at any point throughout the circadian cycle. There is no difference among the three groups between CT8-16.

Figure 5b	Value	Std. Error	t-value	p-value
CT0	Intercept	1.75	4.10	0.43
	NG7 Arrhythmic	42.7	5.54	7.71
	NG7 Rhythmic	31.9	6.00	5.31
CT4	Intercept	1.84	7.17	0.26
	NG7 Arrhythmic	21.4	9.05	2.37
	NG7 Rhythmic	24.3	8.77	2.77
CT8	Intercept	1.04	1.81	0.57
	NG7 Arrhythmic	2.71	2.50	1.08
	NG7 Rhythmic	0.49	2.80	0.17
CT12	Intercept	0.53	0.40	1.30
	NG7 Arrhythmic	0.04	0.51	0.08
	NG7 Rhythmic	-0.53	0.59	-0.90
CT16	Intercept	0.00	3.23	0.00
	NG7 Arrhythmic	3.40	3.72	0.92
	NG7 Rhythmic	0.57	3.60	0.16
CT20	Intercept	1.34	3.82	0.35
	NG7 Arrhythmic	29.9	5.23	5.73
	NG7 Rhythmic	15.9	4.88	3.25

Table 4

Results of the linear mixed-effects model for Fig. 6b

There is no difference in PER mean intensity between NG7 and Iso31 individuals throughout the circadian timecourse.

Figure 6b		Value	Std. Error	t-value	p-value
CT0	Intercept	29.2	6.25	4.67	0.00
	NG7	-2.18	10.4	-0.21	0.83
CT4	Intercept	19.3	5.34	3.62	0.00
	NG7	-5.24	8.25	-0.64	0.54
CT8	Intercept	6.83	4.86	1.40	0.19
	NG7	-0.17	5.33	-0.03	0.98
CT12	Intercept	4.35	1.01	4.32	0.00
	NG7	-0.54	1.58	-0.34	0.74
CT16	Intercept	17.0	6.68	2.54	0.01
	NG7	-1.99	8.84	-0.22	0.83
CT20	Intercept	35.8	5.53	6.46	0.00
	NG7	-7.11	5.47	-1.30	0.20