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RESEARCH ARTICLE

Daily temperature cycles prolong lifespan and have sex-specific effects on peripheral clock gene expression in *Drosophila melanogaster*

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ABSTRACT

Circadian rhythms optimize health by coordinating the timing of physiological processes to match predictable daily environmental challenges. The circadian rhythm of body temperature is thought to be an important modulator of molecular clocks in peripheral tissues, but how daily temperature cycles affect physiological function is unclear. Here, we examined the effect of constant temperature (T_{con}, 25°C) and cycling temperature (T_{cyc}, 28°C:22°C during light:dark) paradigms on lifespan of *Drosophila melanogaster*, and the expression of clock genes, *heat shock protein 83* (*Hsp83*), *Frost* (*Fst*) and *senescence marker protein-30* (*smp-30*). Male and female *D. melanogaster* housed at T_{cyc} had longer median lifespans than those housed at T_{con}. T_{cyc} induced robust *Hsp83* rhythms and rescued the age-related decrease in *smp-30* expression that was observed in flies at T_{con}, potentially indicating an increased capacity to cope with age-related cellular stress. Ageing under T_{con} led to a decrease in the amplitude of expression of all clock genes in the bodies of male flies, except for *cyc*, which was non-rhythmic, and for *per* and *cry* in female flies. Strikingly, housing under T_{cyc} conditions rescued the age-related decrease in amplitude of all clock genes, and generated rhythmicity in *cyc* expression, in the male flies, but not the female flies. The results suggest that ambient temperature rhythms modulate *D. melanogaster* lifespan, and that the amplitude of clock gene expression in peripheral body clocks may be a potential link between temperature rhythms and longevity in male *D. melanogaster*. Longevity due to T_{cyc} appeared predominantly independent of clock gene amplitude in female *D. melanogaster*.

KEY WORDS: Amplitude, Circadian rhythms, Clock genes, *Drosophila melanogaster*, Longevity, Temperature

INTRODUCTION

Biological processes that oscillate in phase with environmental variation increase the adaptability of an organism by anticipating predictable fluctuations in the environment (Schibler, 2017). Environmental variation at the daily level is anticipated by circadian rhythms in organisms. Those rhythms are generated by a set of highly conserved clock genes that are almost ubiquitously expressed in cells throughout the body [reviewed in Cox and Takahashi (2019) and Patke et al. (2020) for mammals and *Drosophila melanogaster*, respectively]. The circadian system consists of a central pacemaker in the brain that is primarily entrained to the 24 h day/night cycle by light, and sets the timing of peripheral clocks throughout the body via a combination of internal entraining cues, including body temperature (Schibler, 2017).

Circadian rhythms are intrinsically linked with optimal physiological function because many physiological processes are under circadian control. Several diseases, including cancer (Chan and Lamia, 2020; Fu et al., 2002; Lee et al., 2010), metabolic syndrome (DiAngelo et al., 2011; Kohsaka et al., 2007; Sohail et al., 2015; Turek et al., 2005) and mental illness (Baird et al., 2012; Johansson et al., 2016), have been linked to disruptions of circadian machinery, especially via a dampening of amplitude and shifts in acrophase. Molecular pathways that underlie these conditions, such as Ras in cancer (Relógio et al., 2014; Williams et al., 2001), fat metabolism in obesity (Katewa et al., 2016) and tauopathy in neurological disease (Kim et al., 2018; Means et al., 2015), are also present and entwined with circadian machinery in *D. melanogaster*. In contrast, robust (i.e. high-amplitude) circadian rhythms are associated with resilience to shift work in humans (Reinberg et al., 1980), and better metabolic health (He et al., 2016; Katewa et al., 2016) and longevity (Hurd and Ralph, 1998; Katewa et al., 2016) in rodents and *D. melanogaster*. In addition, ageing is associated with a progressive weakening of circadian rhythmicity, an outcome that is highly conserved in mammals, fish and insects (Hood and Amir, 2017; Kunieda et al., 2006; Stankiewicz et al., 2017; Zhao et al., 2019). Therefore, the manipulation of cues that entrain the amplitude of clock gene expression could have beneficial effects in both mammals and insects.

Temperature is thought to be a universal entraining cue for clock gene expression in heterothermic insects (Glaser and Stanewsky, 2005) and even in homeothermic mammals (Buhr et al., 2010). Indeed, temperature fluctuations within the physiological range have been shown to modulate the amplitude of clock gene rhythms *in vitro* in mammalian tissue (Brown et al., 2002). In flies, cycling ambient temperatures similarly entrained clock gene rhythms (Boothroyd et al., 2007), synchronized activity rhythms (Busza et al., 2007; Sehadova et al., 2009) and led to faster rates of larval development compared with equivalent constant temperatures (Petavy et al., 2001). Thus, the effect of rhythms of temperature on circadian rhythms has important implications for human health, but efforts to investigate the role of temperature as an entraining cue *in vivo* have been impeded by the difficulty of manipulating the circadian rhythm of body temperature in homeotherms. To overcome this challenge, we previously manipulated energy intake in rats to elicit reliable and reproducible changes in the circadian rhythm of body temperature (Goh et al., 2016), aiming to establish

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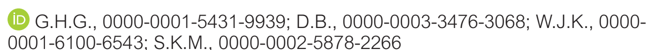
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whether there was an association between the body temperature rhythm and gene expression in peripheral tissues. An association was found, but it was not possible to conclude that the changes in gene expression were because of the altered body temperature rhythm, in part because nutritional signals, such as insulin, can also entrain the circadian clock (Crosby et al., 2019). Therefore, with the rat model, the effects of body temperature rhythms on clock gene expression cannot be disentangled from the effects of diet (Reinke and Asher, 2019).

Drosophila melanogaster is an ideal animal model to test the effects of body temperature on clock gene rhythms and lifespan, because the potential entraining effects of diet can be eliminated. Because *D. melanogaster* is an ectotherm, it is possible to manipulate its body temperature by manipulating its ambient temperature (Goda and Hamada, 2019). In this study, we investigated the effect of daily cycling ambient temperature on the lifespan of male and female *D. melanogaster*. In addition, we aimed to determine whether exposure of the flies to cycling temperatures enhanced the amplitude of clock gene expression (i.e. *Clk*, *cyc*, *per*, *tim*, *Pdp1*, *vri* and *cry*). Given that over-expression of the clock genes *tim* and *per* in abdominal tissues, but not the brain, have been reported to extend lifespan in *Drosophila* (Katewa et al., 2016; Rakshit and Giebultowicz, 2013), we elected to separate the head and body in this study to gain some discrimination between the expression of clocks in different tissues. Because light is regarded as the strongest entrainer of circadian rhythms, we chose to expose the flies to synchronous light and temperature cycles, testing the hypothesis that the circadian rhythms would be enhanced when a temperature cycle is superimposed on the normal light:dark cycle. We also investigated the effect of cycling temperature on the expression of *heat shock protein 83* (*Hsp83*), *frost* (*Fst*) and *senescence marker protein-30* (*smp-30*), which are involved in molecular pathways that may link temperature and lifespan. Heat shock proteins are highly conserved molecular chaperones that play an essential role in cellular responses to stress, making them a universal protective mechanism against cellular damage (Åkerfelt et al., 2010). Furthermore, induced and/or increased rhythmicity in stress response genes has been proposed as an adaptive response to oxidative stress and ageing in *D. melanogaster* (Kuintzle et al., 2017). Heat shock proteins are upregulated during thermal stress and elevated levels of expression have been linked to lifespan extension in *D. melanogaster* under transient heat and cold exposure (Hercus et al., 2003; Klepsatel et al., 2016; Le Bourg, 2007). In particular, *Hsp83* is of interest because it is associated with temperature-mediated circadian entrainment and couples behavioural rhythms to clock gene rhythms in *D. melanogaster* (Goda et al., 2014; Hung et al., 2009). Likewise, *Fst* is a stress-response gene that is upregulated during cold exposure in *D. melanogaster*, and has been implicated in resilience to low temperature (Goto, 2001; Sinclair et al., 2007). Lastly, *smp-30* is a highly conserved marker of senescence that is widely expressed (Ishigami and Maruyama, 2007; Scott and Bahnson, 2011). Mammalian studies have revealed that *smp-30* has various anti-apoptotic, anti-oxidative and anti-inflammatory functions (Ishigami et al., 2002; Jung et al., 2015; Son et al., 2008). In *D. melanogaster*, the gene product of *smp-30* is strongly upregulated during cold acclimation (Goto, 2000), and *smp-30* may thereby play a role in temperature-mediated longevity in flies. We hypothesized that (1) flies housed under cycling temperature conditions (T_{cyc}) will have longer lifespans and increased levels of *smp-30*, and (2) T_{cyc} housing conditions will lead to higher amplitudes of clock gene rhythms and the temperature-responsive *Hsp83* and *Fst*, particularly in aged flies.

MATERIALS AND METHODS

Fly culture and medium

Drosophila melanogaster Meigen 1830 from a stock population that was established in 2012 using wild-caught flies from Innisfail, Queensland, Australia (Dugand et al., 2018), were reared in a controlled temperature (CT) room at 25°C and 60% relative humidity. For experimentation, the flies were placed in temperature-controlled incubators set at 60% relative humidity (A1000 plant growth cabinet; Conviron, Winnipeg, Manitoba, Canada). The incubators were used to expose flies to two regimens of environmental temperature: a constant temperature condition (T_{con}) under which the incubator was kept at 25°C constantly, close to the normal preferred temperature of *D. melanogaster* (Hamada et al., 2008); and a cycling temperature condition (T_{cyc}) under which the incubator altered in a square wave cycle between 28°C during the light phase and 22°C during the dark phase (i.e. a 3°C amplitude oscillating around a daily mean of 25°C).

An NS1 LED lighting array (Valoya, Melkonkatu, Helsinki, Finland) was used to maintain a 12 h:12 h light:dark cycle in all experiments, with lights on at 10:00 h being defined as zeitgeber time zero (ZT0), and lights off at 22:00 h defined as ZT12. Environmental data loggers (UA-002-08 and U23-001 Pro V2; Onset, Bourne, MA, USA) were used to record and verify the patterns of light, temperature and humidity in the temperature-controlled room and the incubators. Water vapour pressure (WVP) was calculated using the formula:

$$\mathrm{WVP} = 0.6105 \times e^{\frac{17.27T}{237.3+T}} \times \frac{h}{100}, \quad (1)$$

where T is temperature (°C) and h is relative humidity (%) (Barenbrug, 1974).

All the flies were raised on a standard cornmeal fly medium consisting of 1% (w/v) agar, 2% (w/v) brewer's yeast, 8.5% (w/v) sugar, 6% (w/v) cornmeal and 0.25% (v/v) Nipagin (H5501; Sigma-Aldrich, St Louis, MO, USA). In all experiments, flies were reared at a standard larval density (50 larvae per vial) in 50 ml plastic vials with 10 ml of food medium. Adult flies were maintained at a density of 20 flies per vial.

Lifespan assay

Flies were reared at 25°C in the CT room containing the stock population. Adult flies were then allowed to mate for approximately 24 h after eclosion. The non-virgin adult flies were then anaesthetized using ice, separated by sex and placed in a vial containing 10 ml of food medium at a density of 20 flies per vial. These vials were placed in the incubators, randomly, at either T_{con} or T_{cyc} (N=100 flies per sex per treatment). Every 2–3 days, the number of deaths in each vial was scored when the flies were transferred to new vials containing fresh food medium. Flies that escaped during vial transfers were treated as censures.

Circadian gene expression

For the lifespan assay, mated flies were anaesthetized using ice, separated by sex and placed into vials containing 10 ml of food medium at a density of 20 flies per vial. These vials were randomly assigned to either T_{con} or T_{cyc} in the incubators. Initial experiments began with 96 vials of flies for each sex (N=1920 flies per sex). The flies were transferred to vials containing fresh food medium every 2–3 days. After 14 days (an age that we refer to as 'young'), one-eighth of the flies were transferred to a microfuge tube at each of four ZT points (ZT0=lights on, ZT6, ZT12 and ZT18), flash-frozen in liquid nitrogen, and stored at

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−80°C until RNA isolation. The other half of the flies were processed similarly after 40 days (an age that we refer to as ‘aged’). We processed four to six vials (biological replicates) per time point, per sex, per treatment.

Prior to RNA isolation, the frozen flies were vortexed in a TissueLyser LT (Qiagen, Victoria, Australia) and passed through a pre-chilled no. 25 standard mesh screen to separate the heads from the bodies. Total RNA was isolated separately from the heads and bodies using the TissueLyser LT and QiaZOL (Qiagen) as per the manufacturer’s instructions. Reverse transcription was carried out at 42°C using murine Moloney leukemia virus reverse transcriptase (Promega, Sydney, Australia), 0.25 ng of random hexamers (Promega), and either 500 ng of total RNA from the heads, or 1000 ng of total RNA from the bodies. The resulting cDNA was purified using the QIAquick PCR purification kit (Qiagen).

The expression profile of seven clock genes (*cyc*, *Clk*, *per*, *tim*, *Pdp1*, *vri* and *cry*), as well as *Hsp83*, *smp-30* and *Fst*, was measured. Real-time quantitative PCR (qPCR) using the SYBR Green PCR Kit (Qiagen) was carried out in duplicate in 10 µl reactions, containing 0.5 µmol l^{-1} primers and 1 µl cDNA, on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). A temperature profile of 95°C for 10 min, followed by 40 cycles of 95°C for 1 s, 60°C for 1` 5 s and 72°C for 5 s, was used for amplification of DNA sequences. Reference genes (*SdhA*, *Act5C*, *RpL32* and *eEF1α2*) were selected with the potential effects of ageing in mind (Ling and Salvaterra, 2011; Ponton et al., 2011). Relative quantification of gene expression was performed via interpolation against a seven-point standard curve of 10-fold serial dilutions of purified PCR product. The level of relative gene expression was then normalized against the geometric means of four reference genes (Vandesompele et al., 2002). To account for between-run variations, six samples, obtained from vials (20 flies per vial) of 5- to 10-day-old male (N=3) and female (N=3) flies, were included in each PCR run, and all values were normalized with reference to these six reference samples. The primer sequences, primer concentrations and PCR conditions that were used are provided in Table S1. Primer pairs were designed with Primer-BLAST (Ye et al., 2012) to include introns to avoid amplification from genomic DNA. All gene products were sequenced to confirm specificity of amplification. To compare the level of gene expression between ages and sexes, the mesor (daily mean) and amplitude of gene expression in the young male flies were set to a baseline of 1, and the data from the other groups (the aged males, young females and aged females) were expressed relative to that of the adult male flies. The acrophase of all groups were expressed as the number of hours advanced or delayed compared with that of the adult males.

Statistical analysis

Survival curves were plotted and compared using a log-rank (Mantel–Cox) test in Prism (version 7.03 for Windows; GraphPad Software, La Jolla, CA, USA). A Bonferroni corrected threshold (P=0.008) was used for the six pairwise comparisons between the four groups (male T_{con}, male T_{cyc}, female T_{con}, female T_{cyc}).

To analyse the profiles of gene expression, the expression datasets acquired from qPCR were first tested for rhythmicity using the R package ‘CircaCompare’ (version 3.6.2 for Windows), as described previously (Parsons et al., 2020). If gene expression was rhythmic (at P<0.05), then the estimates and standard errors for the mesor (defined as the mean value around which the daily rhythm oscillates), amplitude (defined as half of the distance between the peak and the nadir of an oscillation) and acrophase (defined as the time when the peak of an oscillation occurs), that describe each profile, were determined. The effect of age, sex and temperature treatment on the mesor, amplitude and acrophase of gene expression was analysed using three-way ANOVAs (with age, sex and culture temperature as sources of variation) in Prism (version 8.4.1 for Windows, GraphPad Software). When significant main effects or interactions were identified, CircaCompare was used to determine whether there was a significant difference (P<0.05) in the rhythmicity of the gene expression profiles of interest by pairwise comparison of the 95% confidence intervals. When one or more of the gene expression profiles was not rhythmic, three-way ANOVA could not be used, and thus two-way ANOVAs were used where possible. When there were multiple non-rhythmic profiles per gene that excluded the use of both three-way and two-way ANOVAs, pairwise comparisons were made using CircaCompare to detect differences in gene expression. All reported P-values are rounded to three significant figures.

RESULTS

Cycling temperature improved the lifespan of *D. melanogaster*

The average ambient temperature, relative humidity and light intensity in the CT room and the incubators during a representative 10-week period of experimentation is given in Table 1. For the T_{cyc} incubators, it took approximately 2 h for the incubators to cool from 28 to 22°C, or to heat from 22 to 28°C, during the transition from the light phase to the dark phase, and vice versa, respectively.

The flies housed at T_{cyc} had a longer median survival (male: T_{con}=38 days, T_{cyc}=47 days; female: T_{con}=34 days, T_{cyc}=47 days) and 90% survival (male: T_{con}=47 days, T_{cyc}=54 days; female: T_{con}=47 days, T_{cyc}=57 days) than flies housed at T_{con} (P<0.0001 for both sexes) (Fig. 1). However, there was no difference in the

Table 1. The average ambient temperature, relative humidity, WVP and light intensity recorded in the fly breeding room and the temperature-controlled incubators

	T_{con} incubator	T_{cyc} incubator	Breeding room
Average temperature (light phase) (°C)	25.6±0.2	28.9±1.0	25.4±0.2
Average temperature (dark phase) (°C)	25.2±0.2	22.6±0.9	25.6±0.2
Daily average temperature (°C)	25.4±0.3	25.7±3.3	25.5±0.3
Average relative humidity (light phase) (%)	61.9±1.4	45.8±4.8	53.4±6.4
Average relative humidity (dark phase) (%)	64.1±1.3	53.3±6.8	53.0±7.1
Daily average relative humidity (%)	63.0±1.7	49.6±7.0	53.3±6.8
Average WVP (light phase) (kPa)	2.03±0.04	1.82±0.16	1.73±0.2
Average WVP (dark phase) (kPa)	2.05±0.03	1.46±0.18	1.74±0.2
Daily WVP (kPa)	2.04±0.04	1.64±0.25	1.73±0.2
Average light intensity (light phase) (lx)	7.024±681	6.504±859	28.9±12.2
Average light intensity (dark phase) (lx)	0±0	0±0	0±0

Values are shown as means±s.d.

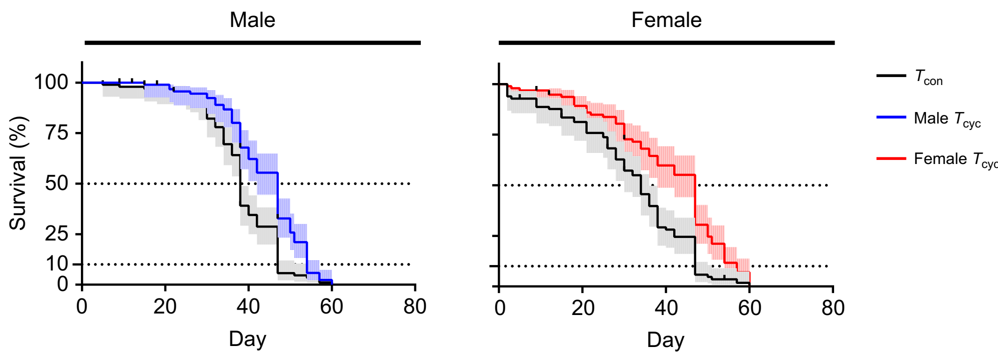

Fig. 1. Housing at cycling temperature (T_{cyc}) extends lifespan in male and female *Drosophila melanogaster*. Survival curves of male and female *D. melanogaster* raised at constant temperature (T_{con}; black lines) and T_{cyc} (coloured lines). Shaded area shows standard error of the mean. Dotted horizontal lines in each panel indicate 50% and 90% mortality. Censured data are shown as ticks in the graphs (male T_{con}, N=7; male T_{cyc}, N=10; female T_{con}, N=10; female T_{cyc}, N=9).

maximum lifespan between T_{con} and T_{cyc} (60 days for all groups). The male and female flies had similar survival curves within each temperature profile (Bonferroni corrected threshold=0.008; T_{con}, P=0.02; T_{cyc}, P=0.92).

Cycling temperatures had sex-specific effects on clock gene expression

Gene expression in *D. melanogaster* heads

The circadian profiles of gene expression in *D. melanogaster* heads are shown in Fig. 2, while Fig. 3 shows the amplitude, mesor and acrophase that summarize the circadian expression of those genes. The associated P-values for all three-way and two-way ANOVAs are given in Table S2, and P-values for all pairwise comparisons are provided in Table S3.

Clock genes

In male flies, *cyc* expression developed rhythmicity when under T_{cyc} conditions. T_{cyc} also elicited an increase in amplitude in *per* expression in aged male flies (P<0.01). In aged female flies, the amplitudes of *per*, *Pdp1*, *vri* and *cry* were all higher under T_{cyc} compared with T_{con} (P<0.05; P<0.001 for *cry*). Interestingly, in young male flies, T_{cyc} conditions led to lower amplitudes in *Clk*, *tim* and *Pdp1* (P<0.001), with a trend for a lower amplitude in *vri* (P=0.051), and a loss of rhythmicity in *cry*, when compared with T_{con}. Only *Clk* and *tim* in young female flies showed a similar decrease in amplitude when housed under T_{cyc} (P<0.001). None of these lowered amplitudes persisted in the aged flies, regardless of sex (P>0.05).

Compared with T_{con}, young male and young female flies under T_{cyc} conditions had a lower mesor of *Clk*, *tim*, *Pdp1* and *vri* (P<0.01), which was not observed in the aged flies. Conversely, the aged male and female flies showed higher mesor for *per* and *cry* when under T_{cyc} compared with T_{con} (P<0.05).

Housing under T_{cyc} led to varying effects on acrophase. When compared with T_{con}, T_{cyc} led to a delay in the acrophase of *per* in all flies (P<0.05), and an advance in the acrophase of *cry* in young flies (P<0.01). In aged flies, however, T_{cyc} conditions led to a delay in the acrophase of *Clk* and *vri* in both sexes (P<0.01), as well as advances in the acrophase of *tim* and *Pdp1* in females (P<0.05), but a delay in the acrophase of *cry* in males (P<0.05).

Non-clock genes

The amplitude of *Hsp83* expression was higher in all flies under T_{cyc}, with a higher mesor being observed in only the aged males. The expression of *Fst* was rhythmic only in aged males under T_{cyc}, but the mesor of *Fst* was lower under T_{cyc} than T_{con} in females (P<0.05). In aged flies, the mesor of *smp-30* was higher under T_{cyc} than T_{con} in both sexes (P<0.001), and the aged female flies under T_{cyc} rhythmicity of *smp-30* expression. Notably, exposure to T_{cyc} rescued the age-related decrease of *smp-30* in female flies, and reduced the decrease in male flies by 28%. No significant differences in acrophase were observed for *Hsp83*, *Fst* or *smp-30*.

Gene expression in *D. melanogaster* bodies

The circadian profiles of gene expression in *D. melanogaster* bodies are shown in Fig. 4, while Fig. 5 shows the amplitude, mesor and acrophase that summarize the circadian expression of these genes. The associated P-values for all three-way and two-way ANOVAs are given in Table S4, and P-values for all pairwise comparisons are provided in Table S5.

Clock genes

T_{cyc} conditions led to sex-specific changes in clock gene amplitude. In young male flies, *cyc* was the only clock gene that showed an increase in amplitude or rhythmicity under T_{cyc} compared with T_{con} (P<0.01), whereas in young female flies, the amplitude of *per* and *vri* were higher under T_{cyc} compared with T_{con} (P<0.05). The amplitude of all clock genes was higher in aged male flies under T_{cyc} than under T_{con} (P<0.05), except for *vri*, where there was a trend for a higher amplitude (P=0.058). Furthermore, T_{cyc} completely rescued the age-related decrease in the amplitude of *Clk*, *per*, *Pdp1* and *vri* expression, and lessened the degree of the age-related decrease of the remaining two clock genes, *tim* and *cry*, by 33 and 16%, respectively. In aged female flies, higher amplitudes under T_{cyc} were only observed in *per* and *cry* (P<0.05). Unlike the male flies, only the amplitude of *cry* in the female flies decreased with age when under T_{con} (P<0.001).

T_{cyc} conditions had no effect on the mesor of any gene in young male flies. In aged male flies, however, T_{cyc} conditions led to higher mesor of *Clk*, *tim*, *Pdp1* and *cry* (P<0.01), thereby rescuing or reducing the age-related decrease in these four clock genes, but the mesor of *cyc* was lower under T_{cyc} than under T_{con} (P<0.05). The opposite effect was observed in the clock genes of aged females, where T_{cyc} exacerbated the decrease in the mesor of *tim*, *Pdp1* and *vri* in females, and elicited an age-related decrease and increase in the mesor of *per* and *cyc*, respectively.

T_{cyc} conditions elicited few changes in acrophase in *D. melanogaster* bodies. Compared with T_{con}, T_{cyc} led to a delay in the acrophase of *Clk* in young and aged flies of both sexes, as well as a delay in the acrophase of *vri* in aged flies in both sexes (P<0.05). A small advance in the acrophase of *tim* was also observed in young female flies (P<0.05).

Non-clock genes

T_{cyc} conditions consistently elicited higher amplitudes of *Hsp83* in young flies and in aged flies of both sexes. The profile of *Fst* gained

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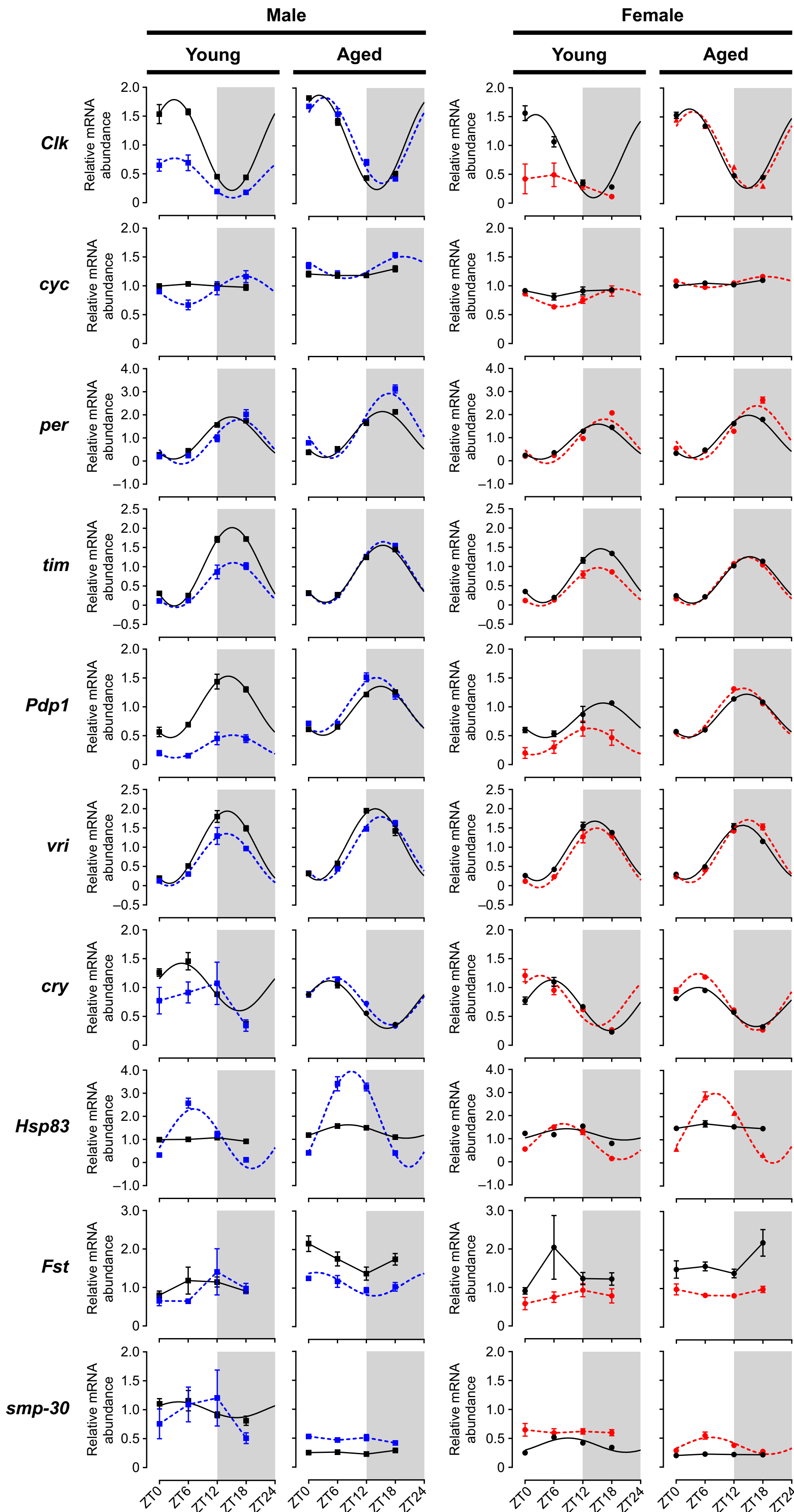

Fig. 2. The circadian profiles of gene expression in *D. melanogaster* heads are altered by age and housing under T_{cyc}. The expression profile of clock genes (*Clk*–*cry*), *Hsp83*, *Fst* and *smp-30* in the heads of male and female *D. melanogaster* at 14 days (young; left panel) and 40 days of age (aged; right panel). The relative mRNA abundance is expressed relative to the young male flies at T_{con}. Graphs for male and female flies are drawn at the same scale. The grey shaded area in each panel indicates the dark phase. Black profiles show *D. melanogaster* housed at T_{con}, and coloured profiles show *D. melanogaster* housed at T_{cyc}. Cosinor curves were not plotted for genes with non-rhythmic expression. Graphs show means±s.e.m. ZT, zeitgeber time.

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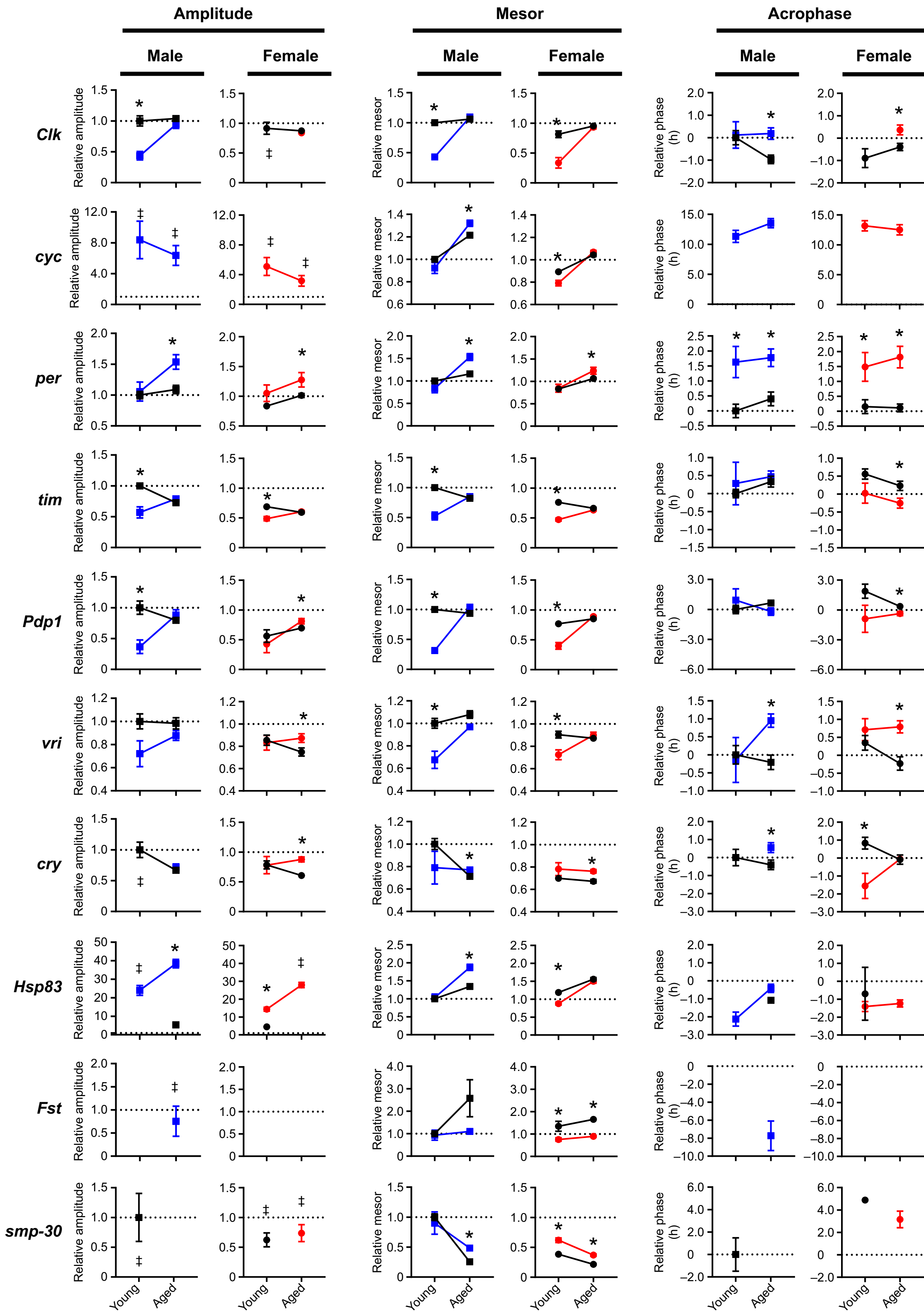

Fig. 3. See next page for legend.

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Fig. 3. The variables describing circadian gene expression in the heads of *D. melanogaster* housed under T_{con} or T_{cyc}. The amplitude, mesor and acrophase of circadian gene expression, derived from CircaCompare, in the heads of male (squares; left panels) and female (circles; right panels) flies housed at T_{con} (black) or at T_{cyc} (coloured). Gene expression was measured in young (14 days) and aged (40 days) flies. All values are expressed relative to the young male flies at T_{con} (horizontal dotted lines). Graphs for male and female flies are drawn at the same scale. When values in amplitude or acrophase were missing, the expression was non-rhythmic (P>0.05). The daily average mesor of non-rhythmic genes is plotted. *P<0.05, young T_{con} versus young T_{cyc}, or aged T_{con} versus aged T_{cyc}. ‡Either T_{cyc} was rhythmic and T_{con} was non-rhythmic, or vice versa when T_{con} was rhythmic and T_{cyc} was non-rhythmic. Graphs show means±s.e.m.

rhythmicity under T_{cyc} in young males and in aged flies of both sexes. For *smp-30*, gain of rhythmicity was observed in the amplitude of young females and in aged males under T_{cyc} compared with T_{con}.

In both sexes, ageing under T_{con} led to a lower mesor for *Hsp83* and *smp-30* (P<0.01), but not *Fst*. The mesor of *smp-30* decreased by 48% between young and aged males under T_{cyc}, compared with 78% under T_{con}. In female flies, ageing under T_{cyc} conditions completely rescued the age-related decrease in *smp-30* mesor. Similarly, the age-related decrease in the mesor of *Hsp83* that was observed under T_{con} (P<0.001) was reduced in male and female flies under T_{cyc} conditions. Conversely, T_{cyc} elicited a decrease in the mesor of *Fst* in both aged male and female flies (P<0.05).

DISCUSSION

We tested the effect of daily cycling temperature on lifespan and the profile of clock gene expression in young and aged *D. melanogaster*. We found that the superimposition of daily temperature cycles, on top of the light:dark cycle, increased longevity. Temperature cycling rescued the age-related decrease in amplitude of almost all clock genes that occurred in the bodies of male flies at T_{con}, an outcome that aligns with the hypothesis that an increase in the amplitude of molecular clock rhythms by manipulation of the strength of a temperature zeitgeber had beneficial effects on physiological function. The robust rhythms of *Hsp83* and the rescue of *smp-30* expression in the aged male and female flies under T_{cyc} potentially indicate that cycling temperatures elicit an increased capacity to cope with age-related oxidative stress.

An inverse relationship between constant ambient temperature and lifespan in ectotherms, including *D. melanogaster*, is well established (Klass, 1977; Liu and Walford, 1966; Miquel et al., 1976). Even in homeotherms such as mammals, a reduction in body temperature of 0.5°C extends lifespan, independently of caloric intake (Conti et al., 2006). One potential mechanism of ageing postulates that ageing is caused by the production of reactive oxidative species from oxidative metabolism (Harman, 1956; Liguori et al., 2018; Sohal and Weindruch, 1996). Therefore, temperature should have a direct effect on the rate of ageing via the Q_{10} effect, which describes how the rate of chemical processes changes with temperature (IUPS Thermal Commission, 2001). Indeed, increased metabolism, and therefore increased production of reactive oxygen species (ROS) at higher temperature, accelerates ageing (Buttemer et al., 2010; Malek et al., 2004; Van Voorhies and Ward, 1999). Because the Q_{10} relationship is exponential, the increase in metabolism from 25 to 28°C is larger than the decrease in metabolism from 25 to 22°C. Accordingly, the fact that we observed longer lifespan in the T_{cyc} flies argues that the beneficial effect of spending half the day at 22°C, or the effect of T_{cyc} as an additional zeitgeber that reinforces circadian physiology, or a combination of both, alleviated the deleterious effect of exposure to higher temperatures during the day. Previous findings that temperature cycles rescue behavioural rhythms in aged male and female *Drosophila* (Luo et al., 2012) and rescue *per* (but not *tim*) expression in aged male flies (Zhao et al., 2018), lend further support to the latter conclusion that the benefits of exposure to T_{cyc} strengthens circadian rhythms and outweighs the harmful effects of exposure to higher daytime temperature. Likewise, our finding that the expression of circadian systems was amplified under T_{cyc} does not appear to be a simple thermodynamic effect.

Our observation that the extension in lifespan under T_{cyc} conditions was associated with the maintenance of the amplitude of all clock genes (except in *vri*, where a similar trend was observed) in the bodies, but not the heads, of aged male flies supports the hypothesis that the maintenance of robust circadian rhythms via temperature cycles had beneficial effects. Our findings are broadly supported by previous studies showing that whole-body over-expression of *cry* increased lifespan, prevented the age-related dampening of activity rhythms in *D. melanogaster*, and was associated with an increase in amplitude of *per*, *tim*, *Pdp1* and *vri* (but not *Clk* or *cyc*) (Rakshit and Giebultowicz, 2013). Similarly, reinforcing the circadian clock via the over-expression of *tim*, but not *per*, also led to longer lifespan (Katewa et al., 2016). Consistent with the general principle that peripheral clocks are more plastic than central clocks in mammals and *D. melanogaster* (Dibner et al., 2010; Xu et al., 2008), neuron-specific over-expression of *cry* and *per* in both of the aforementioned studies did not promote longevity (Katewa et al., 2016; Rakshit and Giebultowicz, 2013). In contrast, the over-expression of *per* in the fat body, gut and Malpighian tubules was sufficient to increase lifespan (Katewa et al., 2016).

We observed several sex differences in the mesor and amplitude of gene expression in *D. melanogaster*. First, age did not dampen the amplitude of clock gene expression in the bodies of female flies, which precluded a rescue of age-dampened rhythms by T_{cyc} similar to that observed in the male flies. Second, T_{cyc} elicited small but significant increases in the amplitude of *Pdp1*, *vri* and *cry* in the heads of the aged female flies, which were not observed in the aged male flies. Lastly, *per* expression, which is temperature driven in *D. melanogaster*, partially via temperature-dependent splicing of the 3′-UTR region (Goda et al., 2014; Majercak et al., 2004; Yoshii et al., 2007), was increased in the heads and bodies of both sexes under T_{cyc}, albeit to a greater extent in the males. The sex differences in the bodies may be explained by the ovaries in the females, which contribute approximately 70% of the RNA in the female abdomen, and do not appear to have oscillating levels of clock genes (Beaver et al., 2003; Hardin, 1994). Therefore, any temperature-driven changes in the rhythmicity of clock genes in the female body may have been masked by the non-cycling ovarian RNA.

The beneficial effect of daily temperature cycles on lifespan may be partly attributable to an increase in the levels of *smp-30* expression. The *smp-30* gene is highly conserved between *D. melanogaster* and vertebrates and is widely expressed (Scott and Bahnson, 2011). Under T_{con}, our flies experienced an age-related decrease in *smp-30* expression, as has been reported for mammalian SMP30 in aged rats, and its homologs in aged zebrafish (Fujisawa et al., 2011; Fujita et al., 1992). In contrast, the level of the *smp-30* protein has been reported to increase with age in *D. melanogaster*, but the comparability of these results is confounded by differences in the light regimens used (Goto, 2000). In mammals, SMP30 deficiency leads to shortened lifespan (Ishigami et al., 2004) and higher levels of oxidative damage in the

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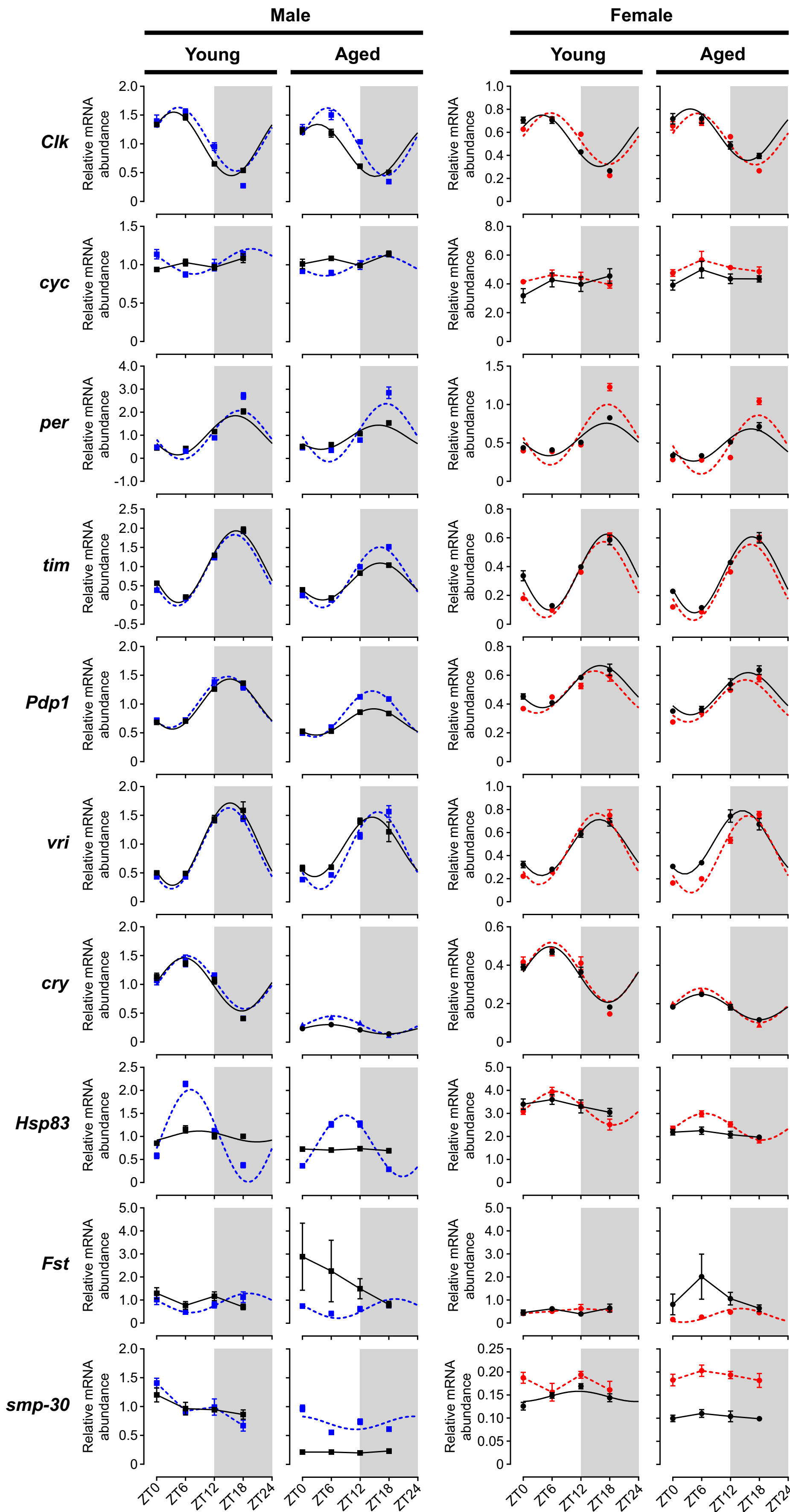

Fig. 4. The circadian profiles of gene expression in *D. melanogaster* bodies are altered by age and housing under T_{cyc}. The expression profile of clock genes (*Clk*–*cry*), *Hsp83*, *Fst* and *smp-30* in the bodies of male and female *D. melanogaster* at 14 days (young; left panel) and 40 days of age (aged; right panel). The relative mRNA abundance is expressed relative to the young male flies at T_{con}. The bodies of female flies tended to show lower rhythmicity and lower overall levels of gene expression compared with the male flies, therefore graphs for male and female flies are drawn at different scales. The grey shaded area in each panel indicates the dark phase. Black profiles show *D. melanogaster* housed at T_{con}, and coloured profiles show *D. melanogaster* housed at T_{cyc}. Cosinor curves were not plotted for genes with non-rhythmic expression. Graphs show means±s.e.m. ZT, zeitgeber time.

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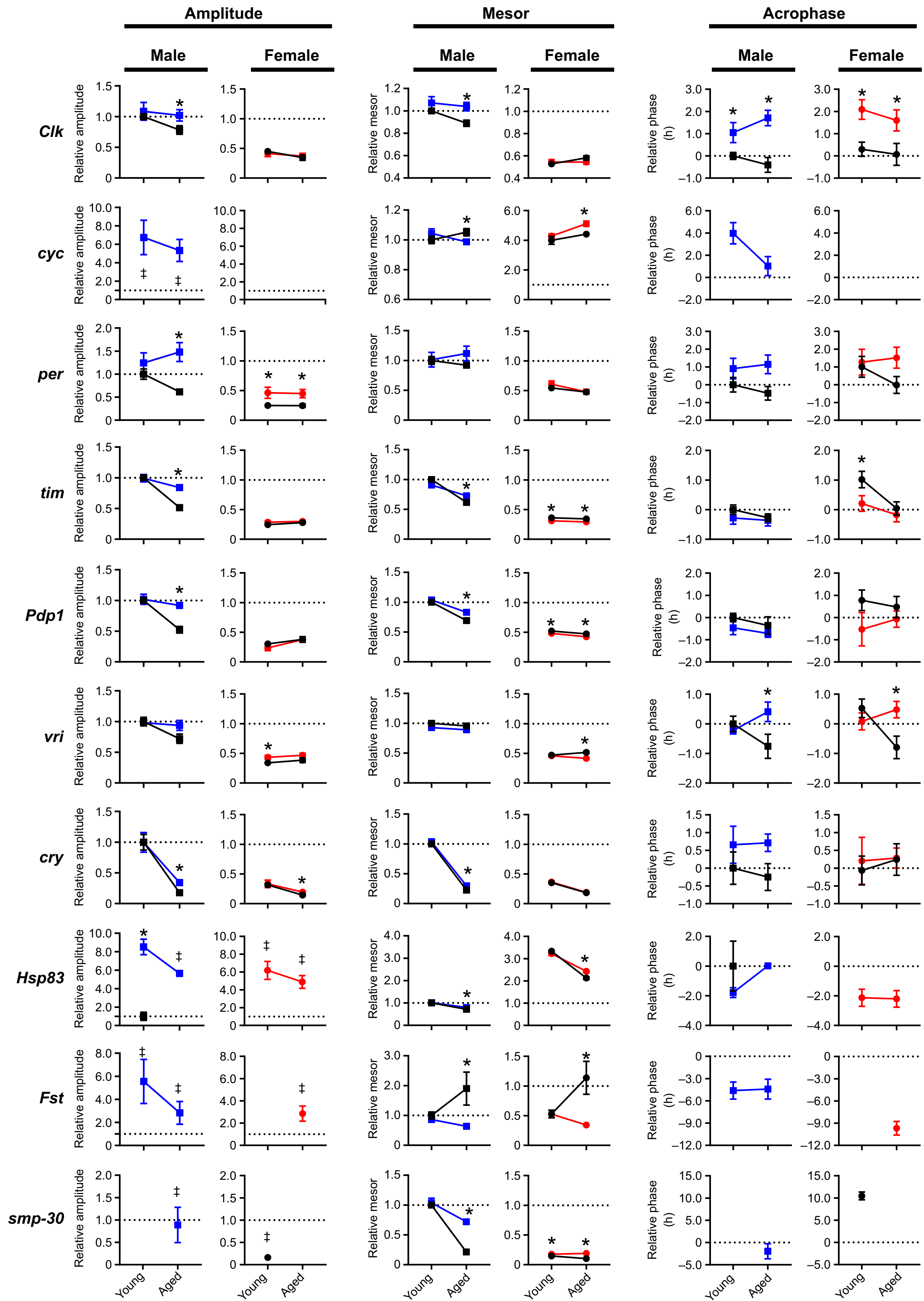

Fig. 5. See next page for legend.

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Fig. 5. The variables describing circadian gene expression in the bodies of *D. melanogaster* housed under T_{con} or T_{cyc}. The amplitude, mesor and acrophase of circadian gene expression, derived from CircaCompare, in the bodies of male (squares; left panels) and female flies (circles; right panels) housed at T_{con} (black) or T_{cyc} (coloured). Gene expression was measured in young (14 days) and aged flies (40 days). All values are expressed relative to the young male flies at T_{con} (horizontal dotted lines). Graphs for male and female flies are drawn at the same scale, except for *cyc* mesor. When values in amplitude or acrophase were missing, the expression was non-rhythmic (P>0.05). The daily average mesor of non-rhythmic genes is plotted. *P<0.05, young T_{con} versus young T_{cyc}, or aged T_{con} versus aged T_{cyc}. ‡Either T_{cyc} was rhythmic and T_{con} was non-rhythmic, or vice versa when T_{con} was rhythmic and T_{cyc} was non-rhythmic. Graphs show means±s.e.m.

brain (Son et al., 2006), whereas the over-expression of SMP30 leads to lower levels of ROS (Handa et al., 2009; Son et al., 2008). Based on the oxidative stress theory of ageing (Harman, 1956), higher levels of *smp-30* expression or activity may confer protection against oxidative insults in aged organisms.

In the present study, exposure to T_{cyc} led to robust expression and rhythmicity of *Hsp83* in flies of both sexes, which contrasts with the low rhythmic or arrhythmic expression in fly heads and bodies under T_{con}. Taken together with the well-known relationship between the heat shock response and longevity in invertebrates (Baldi et al., 2017; Steinkraus et al., 2008; Tower, 2011; Zhao et al., 2005), the increased expression and rhythmicity of *Hsp83* in aged flies in our study could have provided an increased capacity to respond to ageing-related cellular stresses (Kuintzle et al., 2017). In contrast, we found no effect of T_{cyc} on *Fst* expression. Thus, it appears that *Hsp83*, but not *Fst*, is implicated in mediating the physiological effects of temperature rhythms in our study.

As with many other species (Piper et al., 2011), dietary restriction protocols extend lifespan in *D. melanogaster* (Bass et al., 2007). Interestingly, energy intake and metabolism scale linearly in *D. melanogaster* over the ambient temperatures range from 13 to 33°C (Klepsatel et al., 2019). Furthermore, feeding behaviour in *D. melanogaster* is strongly rhythmic, and the majority of food intake occurs between ZT0 and ZT2 (Xu et al., 2008). Therefore, despite their longevity, the flies housed at T_{cyc} probably consumed more than flies at T_{con}, because they were at 28°C during this critical feeding window, while the flies at T_{con} were at 25°C during that window. The interaction between age, ambient temperature and food intake remains to be empirically verified, especially as the effects of ambient temperature and calorie restriction on lifespan in *D. melanogaster* appear to utilize overlapping pathways (e.g. 4E-BP and IGF-1) (Carvalho et al., 2017; Cintron-Colon et al., 2017).

Conclusions

The current study shows that temperature rhythms modulate lifespan in *D. melanogaster*, and data from the male flies support the notion that circadian amplitude may link temperature rhythms with longevity. The female flies also lived longer under T_{cyc}, but did not show as many increases in the amplitude of clock gene expression as the males, and further study is required to determine if any changes in the amplitudes were masked by the contribution of non-cycling RNA from the ovaries. It may also be that multiple pathways are present. The expression of *smp-30* and *Hsp83* are implicated as potential effectors of ambient temperature rhythms, and may confer increased resistance to age-related oxidative and inflammatory damage. Further study that discriminates the effects of T_{cyc} on sleep, activity, food intake and interaction with the light:dark cycle is required to disentangle the specific mechanisms that are responsible for modulating the various known pathways that modulate lifespan.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: G.H.G., D.B., P.J.M., S.K.M.; Methodology: G.H.G., D.B.; Validation: G.H.G., D.B., S.K.M.; Formal analysis: G.H.G., D.B., P.J.M.; Investigation: G.H.G.; Resources: W.J.K.; Writing - original draft: G.H.G.; Writing - review & editing: G.H.G., D.B., P.J.M., W.J.K., S.K.M.; Supervision: D.B., P.J.M., W.J.K., S.K.M.

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References

Åkerfelt, M., Morimoto, R. I. and Sistonen, L. (2010). Heat shock factors: integrators of cell stress, development and lifespan. *Nat. Rev. Mol. Cell Biol.* **11**, 545-555. doi:10.1038/nrm2938

Baird, A. L., Coogan, A. N., Siddiqui, A., Donev, R. M. and Thome, J. (2012). Adult attention-deficit hyperactivity disorder is associated with alterations in circadian rhythms at the behavioural, endocrine and molecular levels. *Mol. Psychiatry* **17**, 988. doi:10.1038/mp.2011.149

Baldi, S., Bolognesi, A., Meinema, A. C. and Barral, Y. (2017). Heat stress promotes longevity in budding yeast by relaxing the confinement of age-promoting factors in the mother cell. *eLife* **6**, e28329. doi:10.7554/eLife.28329

Barenbrug, A. W. T. (1974). *Psychrometry and Psychrometric Charts*. Cape Town: Chamber of Mines of South Africa.

Bass, T. M., Grandison, R. C., Wong, R., Martinez, P., Partridge, L. and Piper, M. D. W. (2007). Optimization of dietary restriction protocols in *Drosophila*. *J. Gerontol. A Biol. Sci. Med. Sci.* **62**, 1071-1081. doi:10.1093/gerona/62.10.1071

Beaver, L. M., Rush, B. L., Gvakharia, B. O. and Giebultowicz, J. M. (2003). Noncircadian regulation and function of clock genes period and timeless in oogenesis of *Drosophila melanogaster*. *J. Biol. Rhythms* **18**, 463-472. doi:10.1177/0748730403259108

Boothroyd, C. E., Wijnen, H., Naef, F., Saez, L. and Young, M. W. (2007). Integration of light and temperature in the regulation of circadian gene expression in *Drosophila*. *PLoS Genet.* **3**, e54. doi:10.1371/journal.pgen.0030054

Brown, S. A., Zumbrunn, G., Fleury-Olela, F., Preitner, N. and Schibler, U. (2002). Rhythms of mammalian body temperature can sustain peripheral circadian clocks. *Curr. Biol.* **12**, 1574-1583. doi:10.1016/S0960-9822(02)01145-4

Buhr, E. D., Yoo, S.-H. and Takahashi, J. S. (2010). Temperature as a universal resetting cue for mammalian circadian oscillators. *Science* **330**, 379-385. doi:10.1126/science.1195262

Busza, A., Murad, A. and Emery, P. (2007). Interactions between circadian neurons control temperature synchronization of *Drosophila* behavior. *J. Neurosci.* **27**, 10722-10733. doi:10.1523/JNEUROSCI.2479-07.2007

Buttemer, W. A., Abele, D. and Costantini, D. (2010). From bivalves to birds: oxidative stress and longevity. *Funct. Ecol.* **24**, 971-983. doi:10.1111/j.1365-2435.2010.01740.x

Carvalho, G. B., Drago, I., Hoxha, S., Yamada, R., Mahneva, O., Bruce, K. D., Soto Obando, A., Conti, B. and Ja, W. W. (2017). The 4E-BP growth pathway regulates the effect of ambient temperature on *Drosophila* metabolism and lifespan. *Proc. Natl. Acad. Sci. USA* **114**, 9737-9742. doi:10.1073/pnas.1618994114

Chan, A. B. and Lamia, K. A. (2020). Cancer, hear my battle CRY. *J. Pineal Res.* **69**, e12658. doi:10.1111/jpi.12658

Cintron-Colon, R., Sanchez-Alavez, M., Nguyen, W., Mori, S., Gonzalez-Rivera, R., Lien, T., Bartfai, T., Aïd, S., François, J.-C., Holzenberger, M. et al. (2017). Insulin-like growth factor 1 receptor regulates hypothermia during calorie restriction. *Proc. Natl. Acad. Sci. USA* **114**, 9731-9736. doi:10.1073/pnas.1617876114

Conti, B., Sanchez-Alavez, M., Winsky-Sommerer, R., Morale, M. C., Lucero, J., Brownell, S., Fabre, V., Huitron-Resendiz, S., Henriksen, S., Zorrilla, E. P. et al. (2006). Transgenic mice with a reduced core body temperature have an increased life span. *Science* **314**, 825. doi:10.1126/science.1132191

Cox, K. H. and Takahashi, J. S. (2019). Circadian clock genes and the transcriptional architecture of the clock mechanism. *J. Mol. Endocrinol.* **63**, R93-R102. doi:10.1530/JME-19-0153

Crosby, P., Hamnett, R., Putker, M., Hoyle, N. P., Reed, M., Karam, C. J., Maywood, E. S., Stangherlin, A., Chesham, J. E., Hayter, E. A. et al. (2019). Insulin/IGF-1 drives PERIOD synthesis to entrain circadian rhythms with feeding time. *Cell* **177**, 896-909.e20. doi:10.1016/j.cell.2019.02.017

DiAngelo, J. R., Erion, R., Crocker, A. and Sehgal, A. (2011). The central clock neurons regulate lipid storage in *Drosophila*. *PLoS ONE* **6**, e19921. doi:10.1371/journal.pone.0019921

Dibner, C., Schibler, U. and Albrecht, U. (2010). The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu. Rev. Physiol.* **72**, 517-549. doi:10.1146/annurev-physiol-021909-135821

Dugand, R. J., Kennington, W. J. and Tomkins, J. L. (2018). Evolutionary divergence in competitive mating success through female mating bias for good genes. *Sci. Adv.* **4**, eaaq0369. doi:10.1126/sciadv.aaq0369

Fu, L., Pelicano, H., Liu, J., Huang, P. and Lee, C. C. (2002). The circadian gene Period2 plays an important role in tumor suppression and DNA damage response *in vivo*. *Cell* **111**, 41-50. doi:10.1016/S0092-8674(02)00961-3

Fujisawa, K., Terai, S., Hirose, Y., Takami, T., Yamamoto, N. and Sakaida, I. (2011). Senescence marker protein 30 (SMP30)/regucalcin (RGN) expression decreases with aging, acute liver injuries and tumors in zebrafish. *Biochem. Biophys. Res. Commun.* **414**, 331-336. doi:10.1016/j.bbrc.2011.09.067

Fujita, T., Uchida, K. and Maruyama, N. (1992). Purification of senescence marker protein-30 (SMP30) and tis androgen-independent decrease with age in the rat liver. *Biochim. Biophys. Acta (BBA) Gen. Subj.* **1116**, 122-128. doi:10.1016/0304-4165(92)90108-7

Glaser, F. T. and Stanewsky, R. (2005). Temperature synchronization of the *Drosophila* circadian clock. *Curr. Biol.* **15**, 1352-1363. doi:10.1016/j.cub.2005.06.056

Goda, T. and Hamada, F. N. (2019). *Drosophila* temperature preference rhythms: an innovative model to understand body temperature rhythms. *Int. J. Mol. Sci.* **20**, 1988. doi:10.3390/ijms20081988

Goda, T., Sharp, B. and Wijnen, H. (2014). Temperature-dependent resetting of the molecular circadian oscillator in *Drosophila*. *Proc. R. Soc. B Biol. Sci.* **281**, 20141714. doi:10.1098/rspb.2014.1714

Goh, G. H., Mark, P. J. and Maloney, S. K. (2016). Altered energy intake and the amplitude of the body temperature rhythm are associated with changes in phase, but not amplitude, of clock gene expression in the rat suprachiasmatic nucleus *in vivo*. *Chronobiol. Int.* **33**, 85-97. doi:10.3109/07420528.2015.1112395

Goto, S. G. (2000). Expression of *Drosophila* homologue of senescence marker protein-30 during cold acclimation. *J. Insect Physiol.* **46**, 1111-1120. doi:10.1016/S0022-1910(99)00221-8

Goto, S. G. (2001). A novel gene that is up-regulated during recovery from cold shock in *Drosophila melanogaster*. *Gene* **270**, 259-264. doi:10.1016/S0378-1119(01)00465-6

Hamada, F. N., Rosenzweig, M., Kang, K., Pulver, S. R., Ghezzi, A., Jegla, T. J. and Garrity, P. A. (2008). An internal thermal sensor controlling temperature preference in *Drosophila*. *Nature* **454**, 217-220. doi:10.1038/nature07001

Handa, S., Maruyama, N. and Ishigami, A. (2009). Over-expression of Senescence Marker Protein-30 decreases reactive oxygen species in human hepatic carcinoma Hep G2 cells. *Biol. Pharm. Bull.* **32**, 1645-1648. doi:10.1248/bpb.32.1645

Hardin, P. E. (1994). Analysis of period mRNA cycling in *Drosophila* head and body tissues indicates that body oscillators behave differently from head oscillators. *Mol. Cell. Biol.* **14**, 7211-7218. doi:10.1128/MCB.14.11.7211

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* **11**, 298-300. doi:10.1093/geronj/11.3.298

He, B., Nohara, K., Park, N., Park, Y.-S., Guillory, B., Zhao, Z., Garcia, J. M., Koike, N., Lee, C. C., Takahashi, J. S. et al. (2016). The small molecule nobiletin targets the molecular oscillator to enhance circadian rhythms and protect against metabolic syndrome. *Cell Metab.* **23**, 610-621. doi:10.1016/j.cmet.2016.03.007

Hercus, M. J., Loeschcke, V. and Rattan, S. I. S. (2003). Lifespan extension of *Drosophila melanogaster* through hormesis by repeated mild heat stress. *Biogerontology* **4**, 149-156. doi:10.1023/A:1024197806855

Hood, S. and Amir, S. (2017). The aging clock: circadian rhythms and later life. *J. Clin. Investig.* **127**, 437-446. doi:10.1172/JCI90328

Hung, H.-C., Kay, S. A. and Weber, F. (2009). HSP90, a capacitor of behavioral variation. *J. Biol. Rhythms* **24**, 183-192. doi:10.1177/0748730409333171

Hurd, M. W. and Ralph, M. R. (1998). The significance of circadian organization for longevity in the golden hamster. *J. Biol. Rhythms* **13**, 430-436. doi:10.1177/074873098129000255

Ishigami, A. and Maruyama, N. (2007). Significance of SMP30 in gerontology. *Geriatr. Gerontol. Int.* **7**, 316-325. doi:10.1111/j.1447-0594.2007.00420.x

Ishigami, A., Fujita, T., Handa, S., Shirasawa, T., Koseki, H., Kitamura, T., Enomoto, N., Sato, N., Shimosawa, T. and Maruyama, N. (2002). Senescence marker protein-30 knockout mouse liver is highly susceptible to tumor necrosis factor-α-and Fas-mediated apoptosis. *Am. J. Pathol.* **161**, 1273-1281. doi:10.1016/S0002-9440(10)64404-5

Ishigami, A., Kondo, Y., Nanba, R., Ohsawa, T., Handa, S., Kubo, S., Akita, M. and Maruyama, N. (2004). SMP30 deficiency in mice causes an accumulation of neutral lipids and phospholipids in the liver and shortens the life span. *Biochem. Biophys. Res. Commun.* **315**, 575-580. doi:10.1016/j.bbrc.2004.01.091

IUPS Thermal Commission (2001). Glossary of terms for thermal physiology (3rd edition). *Jpn. J. Physiol.* **51**, 245-280.

Johansson, A.-S., Owe-Larsson, B., Hetta, J. and Lundkvist, G. B. (2016). Altered circadian clock gene expression in patients with schizophrenia. *Schizophr. Res.* **174**, 17-23. doi:10.1016/j.schres.2016.04.029

Jung, K. J., Lee, E. K., Kim, S. J., Song, C. W., Maruyama, N., Ishigami, A., Kim, N. D., Im, D.-S., Yu, B. P. and Chung, H. Y. (2015). Anti-inflammatory activity of SMP30 modulates NF-κB through protein tyrosine kinase/phosphatase balance. *J. Mol. Med.* **93**, 343-356. doi:10.1007/s00109-014-1219-1

Katewa, S. D., Akagi, K., Bose, N., Rakshit, K., Camarella, T., Zheng, X., Hall, D., Davis, S., Nelson, C. S., Brem, R. B. et al. (2016). Peripheral circadian clocks mediate dietary restriction-dependent changes in lifespan and fat metabolism in *Drosophila*. *Cell Metab.* **23**, 143-154. doi:10.1016/j.cmet.2015.10.014

Kim, M., Subramanian, M., Cho, Y.-H., Kim, G.-H., Lee, E. and Park, J.-J. (2018). Short-term exposure to dim light at night disrupts rhythmic behaviors and causes neurodegeneration in fly models of tauopathy and Alzheimer's disease. *Biochem. Biophys. Res. Commun.* **495**, 1722-1729. doi:10.1016/j.bbrc.2017.12.021

Klass, M. R. (1977). Aging in the nematode *Caenorhabditis elegans*: major biological and environmental factors influencing life span. *Mech. Ageing Dev.* **6**, 413-429. doi:10.1016/0047-6374(77)90043-4

Klepsatel, P., Gáliková, M., Xu, Y. and Kühnlein, R. P. (2016). Thermal stress depletes energy reserves in *Drosophila*. *Sci. Rep.* **6**, 33667. doi:10.1038/srep33667

Klepsatel, P., Wildridge, D. and Gáliková, M. (2019). Temperature induces changes in *Drosophila* energy stores. *Sci. Rep.* **9**, 5239. doi:10.1038/s41598-019-41754-5

Kohsaka, A., Laposky, A. D., Ramsey, K. M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F. W. and Bass, J. (2007). High-fat diet disrupts behavioral and molecular circadian rhythms in mice. *Cell Metab.* **6**, 414-421. doi:10.1016/j.cmet.2007.09.006

Kuintzle, R. C., Chow, E. S., Westby, T. N., Gvakharia, B. O., Giebultowicz, J. M. and Hendrix, D. A. (2017). Circadian deep sequencing reveals stress-response genes that adopt robust rhythmic expression during aging. *Nat. Commun.* **8**, 14529. doi:10.1038/ncomms14529

Kunieda, T., Minamino, T., Katsuno, T., Tateno, K., Nishi, J.-i., Miyauchi, H., Orimo, M., Okada, S. and Komuro, I. (2006). Cellular senescence impairs circadian expression of clock genes *in vitro* and *in vivo*. *Circ. Res.* **98**, 532-539. doi:10.1161/01.RES.0000204504.25798.a8

Le Bourg, É. (2007). Hormetic effects of repeated exposures to cold at young age on longevity, aging and resistance to heat or cold shocks in *Drosophila melanogaster*. *Biogerontology* **8**, 431-444. doi:10.1007/s10522-007-9086-6

Lee, S., Donehower, L. A., Herron, A. J., Moore, D. D. and Fu, L. (2010). Disrupting circadian homeostasis of sympathetic signaling promotes tumor development in mice. *PLoS ONE* **5**, e10995. doi:10.1371/journal.pone.0010995

Liguori, I., Russo, G., Curcio, F., Bulli, G., Aran, L., Della-Morte, D., Gargiulo, G., Testa, G., Cacciatore, F. and Bonaduce, D. et al. (2018). Oxidative stress, aging, and diseases. *Clin. Interv. Aging* **13**, 757-772. doi:10.2147/CIA.S158513

Ling, D. and Salvaterra, P. M. (2011). Robust RT-qPCR data normalization: validation and selection of internal reference genes during post-experimental data analysis. *PLoS ONE* **6**, e17762. doi:10.1371/journal.pone.0017762

Liu, R. K. and Walford, R. L. (1966). Increased growth and life-span with lowered ambient temperature in the annual fish, *Cynolebias adloffi*. *Nature* **212**, 1277-1278. doi:10.1038/2121277a0

Luo, W., Chen, W. F., Yue, Z., Chen, D., Sowcik, M., Sehgal, A. and Zheng, X. (2012). Old flies have a robust central oscillator but weaker behavioral rhythms that can be improved by genetic and environmental manipulations. *Aging Cell* **11**, 428-438. doi:10.1111/j.1474-9726.2012.00800.x

Majercak, J., Chen, W.-F. and Edery, I. (2004). Splicing of the period gene 3′-terminal intron is regulated by light, circadian clock factors, and phospholipase C. *Mol. Cell. Biol.* **24**, 3359-3372. doi:10.1128/MCB.24.8.3359-3372.2004

Malek, R. L., Sajadi, H., Abraham, J., Grundy, M. A. and Gerhard, G. S. (2004). The effects of temperature reduction on gene expression and oxidative stress in skeletal muscle from adult zebrafish. *Comp. Biochem. Physiol., C: Toxicol. Pharmacol.* **138**, 363-373. doi:10.1016/j.cca.2004.08.014

Means, J. C., Venkatesan, A., Gerdes, B., Fan, J.-Y., Bjes, E. S. and Price, J. L. (2015). *Drosophila* spaghetti and doubletime link the circadian clock and light to caspases, apoptosis and tauopathy. *PLoS Genet.* **11**, e1005171. doi:10.1371/journal.pgen.1005171

Miquel, J., Lundgren, P. R., Bensch, K. G. and Atlan, H. (1976). Effects of temperature on the life span, vitality and fine structure of *Drosophila melanogaster*. *Mech. Ageing Dev.* **5**, 347-370. doi:10.1016/0047-6374(76)90034-8

Parsons, R., Parsons, R., Garner, N., Oster, H. and Rawashdeh, O. (2020). CircaCompare: a method to estimate and statistically support differences in mesor, amplitude and phase, between circadian rhythms. *Bioinformatics* **36**, 1208-1212. doi:10.1093/bioinformatics/btz730

Patke, A., Young, M. W. and Axelrod, S. (2020). Molecular mechanisms and physiological importance of circadian rhythms. *Nat. Rev. Mol. Cell Biol.* **21**, 67-84. doi:10.1038/s41580-019-0179-2

Petavy, G., David, J. R., Gibert, P. and Moreteau, B. (2001). Viability and rate of development at different temperatures in *Drosophila*: a comparison of constant and alternating thermal regimes. *J. Therm. Biol.* **26**, 29-39. doi:10.1016/S0306-4565(00)00022-X

Piper, M. D. W., Partridge, L., Raubenheimer, D. and Simpson, S. J. (2011). Dietary restriction and aging: a unifying perspective. *Cell Metab.* **14**, 154-160. doi:10.1016/j.cmet.2011.06.013

Ponton, F., Chapuis, M.-P., Pernice, M., Sword, G. A. and Simpson, S. J. (2011). Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in *Drosophila melanogaster*. *J. Insect Physiol.* **57**, 840-850. doi:10.1016/j.jinsphys.2011.03.014

Rakshit, K. and Giebultowicz, J. M. (2013). Cryptochrome restores dampened circadian rhythms and promotes healthspan in aging *Drosophila*. *Aging Cell* **12**, 752-762. doi:10.1111/acel.12100

Reinberg, A., Andlauer, P., Guillet, P., Nicolai, A., Vieux, N. and Laporte, A. (1980). Oral temperature, circadian rhythm amplitude, ageing and tolerance to shift-work. *Ergonomics* **23**, 55-64. doi:10.1080/00140138008924718

Reinke, H. and Asher, G. (2019). Crosstalk between metabolism and circadian clocks. *Nat. Rev. Mol. Cell Biol.* **20**, 227-241. doi:10.1038/s41580-018-0096-9

Relógio, A., Thomas, P., Medina-Pérez, P., Reischl, S., Bervoets, S., Gloc, E., Riemer, P., Mang-Fatehi, S., Maier, B., Schäfer, R. et al. (2014). Ras-mediated deregulation of the circadian clock in cancer. *PLoS Genet.* **10**, e1004338. doi:10.1371/journal.pgen.1004338

Schibler, U. (2017). Interaction between central and peripheral clocks in mammals. In *Biological Timekeeping: Clocks, Rhythms and Behaviour* (ed. V. Kumar), pp. 337-363. New Delhi: Springer.

Scott, S. H. and Bahnson, B. J. (2011). Senescence marker protein 30: functional and structural insights to its unknown physiological function. *Biomol. Concepts* **2**, 469-480. doi:10.1515/BMC.2011.041

Sehadova, H., Glaser, F. T., Gentile, C., Simoni, A., Giesecke, A., Albert, J. T. and Stanewsky, R. (2009). Temperature entrainment of *Drosophila*'s circadian clock involves the gene nocte and signaling from peripheral sensory tissues to the brain. *Neuron* **64**, 251-266. doi:10.1016/j.neuron.2009.08.026

Sinclair, B. J., Gibbs, A. G. and Roberts, S. P. (2007). Gene transcription during exposure to, and recovery from, cold and desiccation stress in *Drosophila melanogaster*. *Insect Mol. Biol.* **16**, 435-443. doi:10.1111/j.1365-2583.2007.00739.x

Sohail, S., Yu, L., Bennett, D. A., Buchman, A. S. and Lim, A. S. P. (2015). Irregular 24-h activity rhythms and the metabolic syndrome in older adults. *Chronobiol. Int.* **32**, 802-813. doi:10.3109/07420528.2015.1041597

Sohal, R. S. and Weindruch, R. (1996). Oxidative stress, caloric restriction, and aging. *Science* **273**, 59-63. doi:10.1126/science.273.5271.59

Son, T. G., Kim, S. J., Kim, K., Kim, M.-S., Chung, H. Y. and Lee, J. (2008). Cytoprotective roles of senescence marker protein 30 against intracellular calcium elevation and oxidative stress. *Arch. Pharm. Res.* **31**, 872-877. doi:10.1007/s12272-001-1240-3

Son, T. G., Zou, Y., Jung, K. J., Yu, B. P., Ishigami, A., Maruyama, N. and Lee, J. (2006). SMP30 deficiency causes increased oxidative stress in brain. *Mech. Ageing Dev.* **127**, 451-457. doi:10.1016/j.mad.2006.01.005

Stankiewicz, A. J., McGowan, E. M., Yu, L. and Zhdanova, I. V. (2017). Impaired sleep, circadian rhythms and neurogenesis in diet-induced premature aging. *Int. J. Mol. Sci.* **18**, 2243. doi:10.3390/ijms18112243

Steinkraus, K. A., Smith, E. D., Davis, C., Carr, D., Pendergrass, W. R., Sutphin, G. L., Kennedy, B. K. and Kaeberlein, M. (2008). Dietary restriction suppresses proteotoxicity and enhances longevity by an hsf-1-dependent mechanism in *Caenorhabditis elegans*. *Aging Cell* **7**, 394-404. doi:10.1111/j.1474-9726.2008.00385.x

Tower, J. (2011). Heat shock proteins and *Drosophila* aging. *Exp. Gerontol.* **46**, 355-362. doi:10.1016/j.exger.2010.09.002

Turek, F. W., Joshu, C., Kohsaka, A., Lin, E., Ivanova, G., McDearmon, E., Laposky, A., Losee-Olson, S., Easton, A., Jensen, D. R. et al. (2005). Obesity and metabolic syndrome in circadian clock mutant mice. *Science* **308**, 1043-1045. doi:10.1126/science.1108750

Van Voorhies, W. A. and Ward, S. (1999). Genetic and environmental conditions that increase longevity in *Caenorhabditis elegans* decrease metabolic rate. *Proc. Natl. Acad. Sci. USA* **96**, 11399-11403. doi:10.1073/pnas.96.20.11399

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, research0034. doi:10.1186/gb-2002-3-7-research0034

Williams, J. A., Su, H. S., Bernards, A., Field, J. and Sehgal, A. (2001). A circadian output in *Drosophila* mediated by neurofibromatosis-1 and Ras/MAPK. *Science* **293**, 2251-2256. doi:10.1126/science.1063097

Xu, K., Zheng, X. and Sehgal, A. (2008). Regulation of feeding and metabolism by neuronal and peripheral clocks in *Drosophila*. *Cell Metab.* **8**, 289-300. doi:10.1016/j.cmet.2008.09.006

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. and Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**, 134. doi:10.1186/1471-2105-13-134

Yoshii, T., Fujii, K. and Tomioka, K. (2007). Induction of *Drosophila* behavioral and molecular circadian rhythms by temperature steps in constant light. *J. Biol. Rhythms* **22**, 103-114. doi:10.1177/0748730406298176

Zhao, Y., Sun, H., Lu, J., Li, X., Chen, X., Tao, D., Huang, W. and Huang, B. (2005). Lifespan extension and elevated hsp gene expression in *Drosophila* caused by histone deacetylase inhibitors. *J. Exp. Biol.* **208**, 697-705. doi:10.1242/jeb.01439

Zhao, J., Warman, G. and Cheeseman, J. (2019). The development and decay of the circadian clock in *Drosophila melanogaster*. *Clocks Sleep* **1**, 489-500. doi:10.3390/clockssleep1040037

Zhao, J., Warman, G. R. and Cheeseman, J. F. (2018). Clock gene expression and locomotor activity predict death in the last days of life in *Drosophila melanogaster*. *Sci. Rep.* **8**, 11923. doi:10.1038/s41598-018-30323-x