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Time-Restricted Feeding Extends Longevity in Drosophila Melanogaster

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TIME-RESTRICTED FEEDING EXTENDS LONGEVITY IN *DROSOPHILA MELANOGASTER*

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TIME-RESTRICTED FEEDING EXTENDS LONGEVITY IN *DROSOPHILA MELANOGASTER*

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Time-restricted feeding (TRF) is a dietary intervention in which daily feeding patterns align with behavioral patterns, synchronizing feeding times with periods of higher activity, e.g. humans eating only during the day or rodents (nocturnal animals) at night. TRF has been shown to improve cardiac health in *Drosophila melanogaster,* reduce metabolic markers in rodent models, and reduce glycemic indices in prediabetic men. However, the mechanism and long-term effects of this intervention remain elusive. To understand the effect of TRF on longevity we used the fruit fly *Drosophila melanogaster,* which is a useful model for longevity, sleep and circadian studies because of its well-established sleep behavior, tractable genetics, and short lifespan. We found that TRF extends longevity of fruit flies only in mated females, while showing no effect on mated males or virgin females. We measured the amount of food consumed by flies on TRF and confirmed that TRF does not act through caloric restriction, which has been previously shown to extend longevity. Remarkably, animals undergoing TRF eat more, yet have lower body weight in comparison to animals on constant food. TRF-mediated lifespan extension is dependent on the molecular clock, as arrhythmic clock mutants fail to respond to TRF under light-dark conditions, suggesting that TRF may act as a zeitgeber to improve the animals' health by coordinating activity patterns with food

availability. In addition to its effect on wild-type animals, TRF also improves longevity in animals with reduced lifespan, such as sleep mutants. Further studies show that TRF changes the sleep architecture of wild-type females by increasing the amount of day sleep, while also promoting integrity of the blood-brain barrier. TRF life-extension effects show that this dietary intervention has potential to reveal a deeper understanding of the biology of ageing and how it interacts with feeding and circadian rhythms, placing a larger emphasis on time of intake rather than calories.

To my mother, Astrid Guerrero. “Guerrero” in Spanish means warrior. I don’t think there is a better word to describe my mother. Without her tenacity, determination, and unconditional love, I would not be who I am today or where I am today.

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LIST OF ABBREVIATIONS

PER – period
TIM – timeless
CLK – clock
CYC – cycle
CRY – cryptochrome
TRF – Time-restrited feeding
ALF – Ad libitum feeding
CR – Caloric restriction
IF – Interment fasting
SCN – Suprachiasmatic nucleus
BBB – Blood-brain barrier
ZT – Zeitgeber time
LL – Constant light
DD – Constant darkness
LD – Light-Dark

CHAPTER 1. Introduction

Feeding is a well-studied behavior in biology. From foraging patterns in animals (Breed and Moore, 2016), to the reward that food intake elicits in the brain (Dietrich and Horvath, 2009), scientists have studied this important part of life for decades. However, ubiquitous food availability, in addition to individual susceptibility, manifest in illnesses including metabolic syndrome and obesity (Swinburn et al., 2011). As a result, scientists have studied the impact of different diets on health and longevity, and while there are several types of diets that reduce morbidity, fewer interventions have been studied in the context of longevity and how to extend survival, because longevity is difficult to study in long-lived animals including mice and humans (Fontana and Partridge, 2015). A dietary intervention that has been shown to be effective in treating metabolic illness is time-restricted feeding (TRF) (Sutton et al., 2018).

TRF is a feeding regimen in which food is limited to a time window in which activity is higher, e.g. in mice at night or in humans during the day (Longo and Panda, 2016). Every feeding intervention is compared to animals on a*d libitum* feeding (ALF) – food access at all times – which is the standard condition for lab animals. TRF intervention limits intake of nutrients to a specific time when the animal's locomotor activity is higher, without reducing the number of overall calories. Another dietary intervention is caloric restriction (CR), where animals are fed a diet that contains 10-40% fewer calories. CR extends longevity and improves several health aspects like cardiovascular disease and age related illness through the insulin and mTOR pathways (Speakman and Mitchell,

2011). CR focuses on reducing the number of calories eaten during the day without causing malnutrition. Additionally, there is another intervention called Intermittent fasting (IF), which consists of cycles of long periods of fasting - usually days - followed by days of ALF. These interventions are reviewed in Longo and Panda, 2016; López-Otín et al., 2016. Dietary changes, like intermittent fasting and caloric restriction, have been shown to have positive health outcomes as well as extend longevity in several model organisms (Catterson et al., 2018; Colman et al., 2014). Several molecules that play an important role in nutrient sensing, such as insulin/insulin-like peptides, have been studied in the context of caloric restriction are now important targets for anti-ageing interventions.

The study of different interventions and their effect on ageing has been described for caloric restriction, intermittent fasting, and dietary supplementation. The measurement that the field of ageing uses for measurement of survival, is the median survival. This measurement represents the time at which 50% of the population is alive. Historically, ageing has been defined by, but not limited to, telomere attrition, mitochondrial dysfunction, genomic instability, cellular senescence, loss of proteostasis, deregulated nutrient sensing, among others. To prevent or delay these hallmarks of ageing, the field of gerontology has soldiered many pharmacological, dietary, genetic and metabolic approaches (Partridge, Deelen, and Slagboom, 2018). In addition, much has been said for the critical role of circadian rhythms in ageing (De Nobrega and Lyons, 2018); it has been recently suggested that circadian clocks play a role in the life-extension role of

dietary restriction (Katewa et al., 2016). This, however, has been contended by another group, which claims that they can see extension of longevity in animals with mutations in the circadian clock (Ulgherait et al., 2016). This has yet to be concluded and we should anticipate more interesting data coming from this question.

Circadian rhythms evolved in a world where the earth's rotation takes 24 hours, hence the term "circa-," which means approximate, and "-diem," which means day (Halberg, 1959). These endogenous rhythms evolved as a way for living organisms to anticipate changes in the environment, such as daily light and dark cycles. While observations of circadian behavior had been reported in the literature for centuries (de Mairan, 1729), the first evidence that circadian rhythms has a genetic basis came from mutagenesis experiments in the fruit fly *Drosophila melanogaster,* revealing mutants with short (~19 h) and long (~28 h) rhythms, as well as arrhythmic animals (Konopka and Benzer, 1971). Molecularly, the circadian clock is a negative feedback loop that relies on the PER and TIM proteins self-inhibiting their transcription by dimerizing, entering the nucleus, and inhibiting the action of CLK and CYC, which promote transcription of the *per* and *tim* loci (reviewed in Young, 1998; Jeffrey C. Hall, 1998; Hardin, 1998). In addition to being self-propagating, this pathway is entrainable by the light sensitive CRY protein, which upon exposure to light degrades TIM (Emery et al., 1998). This remarkable ~24 h oscillating pathway is reviewed by Young and Kay in more depth (Young and Kay, 2001).

Zeitgebers are environmental stimuli, such as light and temperature, which reset circadian rhythms (C. S. Pittendrigh, 1960). Furthermore, there has been recent evidence that while food cannot entrain the central clocks in rodent models (Damiola et al., 2000), when food is given at conflicting times with the endogenous rhythms in constant darkness, behavioral rhythms adjust to the feeding schedule by entraining the liver clocks, suggesting the existence of a food-entrainable oscillator (Stokkan et al., 2001). The many inputs and outputs of the clock continue to reveal fascinating mechanisms that regulate physiology and behavior.

Since its discovery, the field of circadian biology or “chronobiology,” has dramatically boomed showing that not only are circadian rhythms in control of many aspects of physiology and behavior, but also how relevant they are in health and several illnesses. Many studies have shown that circadian disruption, as seen in shift workers, can lead to metabolic syndrome, diabetes, cancer and many other morbidities (Murphy et al., 2017; Davis and Mirick, 2006). Consistently, recent studies that illustrate the dramatic impact of circadian rhythms on health, found that the timing of drug administration without change in dosage can improve anti-inflammatory action of drugs and that targeting clocks can be an efficient therapy against cancer (Winter et al., 2018; Sulli, Rommel et al., 2018). As the prevalence for chronic illness increases (van Oostrom et al., 2016), there is a larger need for creative, new, and efficient therapies.

Downstream of the central clock, which in mammalian systems is the suprachiasmatic nucleus (SCN), are the centers for arousal and sleep, and there is large evidence that sleep quality deteriorates and circadian rhythms dampen as animals age. This sleep/circadian disruption is markedly prevalent in neurodegenerative diseases (Mattis and Sehgal, 2016). While the arousal and sleep centers of the brain have been anatomically described, in vertebrate and invertebrate models, and it is known that sleep is a conserved physiological need, the clear reason why living organisms need sleep has yet to be elucidated (Kandel et al., 2000). *Drosophila melanogaster* has been instrumental in understanding the genetics of circadian biology and sleep (Axelrod, Saez, and Young, 2015). Sleep in the fruit fly is defined as consolidated daily periods of inactivity, higher arousal threshold, positional changes, reversibility to wakefulness and homeostatic response (Hendricks et al., 2000; Shaw et al., 2000).

Several genes have been found to be important for sleep and their disruption in *Drosophila* leads to reduced sleep and sometimes reduced longevity. *insomniac (inc^{1}), wide awake (wake),* and *sleepless (sss)* mutants display reduced sleep duration as well as longevity (Stavropoulos and Young, 2011; Liu et al., 2014; Koh et al., 2008). In addition, mutations in the dopamine transporter *fumin (fmn)* also cause a sleep phenotype but no longevity decrease (Kume et al., 2005). These sleep mutations are instrumental in understanding the role of sleep in many physiological pathways and the effects of chronic sleep deprivation in longevity. Recent work from our lab shows that several of these sleep mutants also have a defective blood-brain barrier (Axelrod et al.,

In review). Furthermore, it has also been documented that sleep and blood-brain barrier function deteriorates with age (Koh et al., 2006; Goodall et al., 2018).

The blood-brain barrier is an important organ found in many animals. This organ is what controls the transport of substances/molecules in and out of the brain. While tightly regulating and protecting the brain, the BBB provides the appropriate milieu for optimal function of the brain (Abbott et al., 2010). Furthermore, this remarkable organ has also been studied in *Drosophila melanogaster*, allowing for deeper exploration of the basic biology and physiology (Schirmeier and Klämbt, 2015).

Considering the relationship between diet, ageing, sleep, BBB, and circadian rhythms, TRF has the potential to affect all of the aforementioned aspects of biology. Comparing TRF with all the other feeding interventions shows that TRF is a plausible intervention to fight obesity and metabolic illness because it does not focus on caloric intake, but rather the timing of intake. While TRF shows promise, the mechanism by which this intervention produces its beneficial effects remains elusive. In addition, the long term effects of such intervention have not been studied (Di Francesco et al., 2018).

Feeding cycles as well as circadian rhythms maintain rhythmic gene expression in mouse livers providing a temporal response to food availability (Vollmers et al., 2009). Considering that temporarily restricting access to food changes gene expression in mouse models, initial experiments in the field investigated TRF interventions and their

impact on health. TRF has been studied in the context of regular food and high fat diets. Hatori et al., 2012 showed that mice on high-fat diet (HFD), which normally leads to weight gain and metabolic disease, benefit from TRF. They show that TRF prevents weight gain, improves rhythmicity of metabolic regulators and circadian rhythms, prevents hepatic steatosis, reduces inflammation, and prevents hyperinsulinemia in animals fed high fat diets compared to animals in HFD with unrestricted access to food. This study shows that there are benefits to TRF in the context of high fat diets during short TRF interventions.

To explore whether TRF is beneficial in the context of diverse nutritional challenges, Chaix et al., 2014 investigated TRF in combination with diets that are high in carbohydrates, high in fat, or high in fat and carbohydrates. They found that TRF prevents development of metabolic illness in mice under different nutritional challenges. These findings suggest that TRF has a broader benefit and places emphasis on the timing of food, instead of diet composition or number of calories. In addition, they also tested whether only 5 days of TRF and 2 days of ALF, mimicking human behavior of restricted diet during the week and unrestricted diet during the weekend, also had beneficial effects. They found that this regimen also had beneficial effects in comparison to animals that are never restricted. A very striking finding was also that during the two days of *ad libitum* feeding, the animals continued eating the same amount as the days on TRF.

To study additional effects of TRF on mice on HFD, Sundaram and Yan, 2016 exposed mice to TRF for 9 weeks and then measured body weight, adiposity, respiratory exchange ratio as a proxy for metabolic activity, and plasma levels of ghrelin, leptin and insulin. This study is consistent with the other rodent studies, in which they also show that TRF prevents body weight increase, but it also shows that TRF changes hormonal levels by increasing levels of ghrelin, which usually increase during fasting, and reducing insulin and leptin levels. These experiments play a key role in showing how this temporal feeding intervention can also improve endocrine function, which can be compromised by obesogenic diets.

As obesity and metabolic illness develops mostly in middle-aged and older adults, Duncan et al., 2016 studied the effect of TRF on aged mice. The 21 to 25 week long intervention on 12-month-old male mice, showed consistent findings with prior reports by also preventing metabolic illness as a consequence of HFD. In addition, they performed memory tests, to see if TRF improved cognitive performance and found no difference between animals on TRF or ALF, showing metabolic improvement and no cognitive benefit to TRF.

TRF synchronizes feeding with periods of higher activity. For this reason, it is likely that circadian clocks may play a role in mediating the response to TRF. To address this question, Chaix et al., 2019 exposed mice on HFD to TRF and ALF, while doing liver-specific or ubiquitous knockout of essential clock genes. After 12 weeks of TRF, they

found that animals lacking a functional circadian clock show improvement and do not develop the metabolic deleterious effects of HFD. In addition, they also found transcriptional and metabolic evidence that TRF prevents accumulation of hepatic lipids and increased resistance to metabolic stress. These experiments show that mice that don't have functional endogenous clocks, also benefit from TRF. However, the question of how arrhythmic animals respond to TRF in constant darkness should also be addressed to continue understanding the relationship between TRF and circadian rhythms.

To study the long term effects of TRF in rodent models, Acosta-Rodríguez et al., 2017 developed an automated feeding system in which they were able to control the timing of feeding, amount fed, duration of feeding, and wheel running activity in mice. With this system, they set up three groups to study how TRF, caloric restricted, and ad libitum fed mice differed from each other. To their surprise, the animals on CR self-imposed a temporal restriction, which made it impossible to discern the effects of TRF from the ones of CR. This shows the difficulty of these types of experiments and the experimental rigor that they require.

TRF has been studied in the context of immune function, exploring how night feeding or day feeding affect response to infection in mice (Cissé et al., 2018). These experiments were performed by exposing male mice to a 4-week feeding regime and then undertaking immunological assays. Their findings indicate that day-feeding in mice

decreases immune response, while night-fed mice (TRF) show improvement in response to bacterial challenges and secretion of proinflammatory cytokines. This study not only shows the promise of TRF beyond metabolic benefits, but also how mistiming of food dampens immune responses. It will be interesting to learn how long-term effects of TRF affect immune function in other animal models as well as in female animals.

While much data come from rodent studies, Gill et al., 2015 studied TRF on *Drosophila melanogaster.* In these experiments they found that TRF reduces body weight, increases sleep duration, and prevents cardiac function decline due to ageing. Furthermore, transcriptional analysis and knockdown experiments show that the cardiac benefit of TRF is mediated via TRiC chaperonin, electron transport chain proteins and the circadian clocks. These were the first experiments that suggest a molecular mechanism as to how TRF mediates health benefits. In addition to improve cardiac function, it was recently reported that TRF also improves muscle function in *Drosophila melanogaster* obesity models (Villanueva et al., 2019). They show that under conditions which lead to obesity, TRF prevents intramuscular fat deposits and reduces metabolic markers of obesity. In addition, they show that TRF is also beneficial under circadian disruption by constant light conditions.

Given the success of TRF in animal models, there has been interest in translating this intervention to humans. Time-restriction interventions in human subjects have been studied in healthy overweight individuals, who after the temporal restriction was

implemented for 16 weeks, were able to lose weight, and reported increased energy and improvement in sleep (Gill and Panda, 2015). In this small study that included both male and female healthy participants, the investigators used a mobile phone app to assess the feeding patterns, quantity and timing.

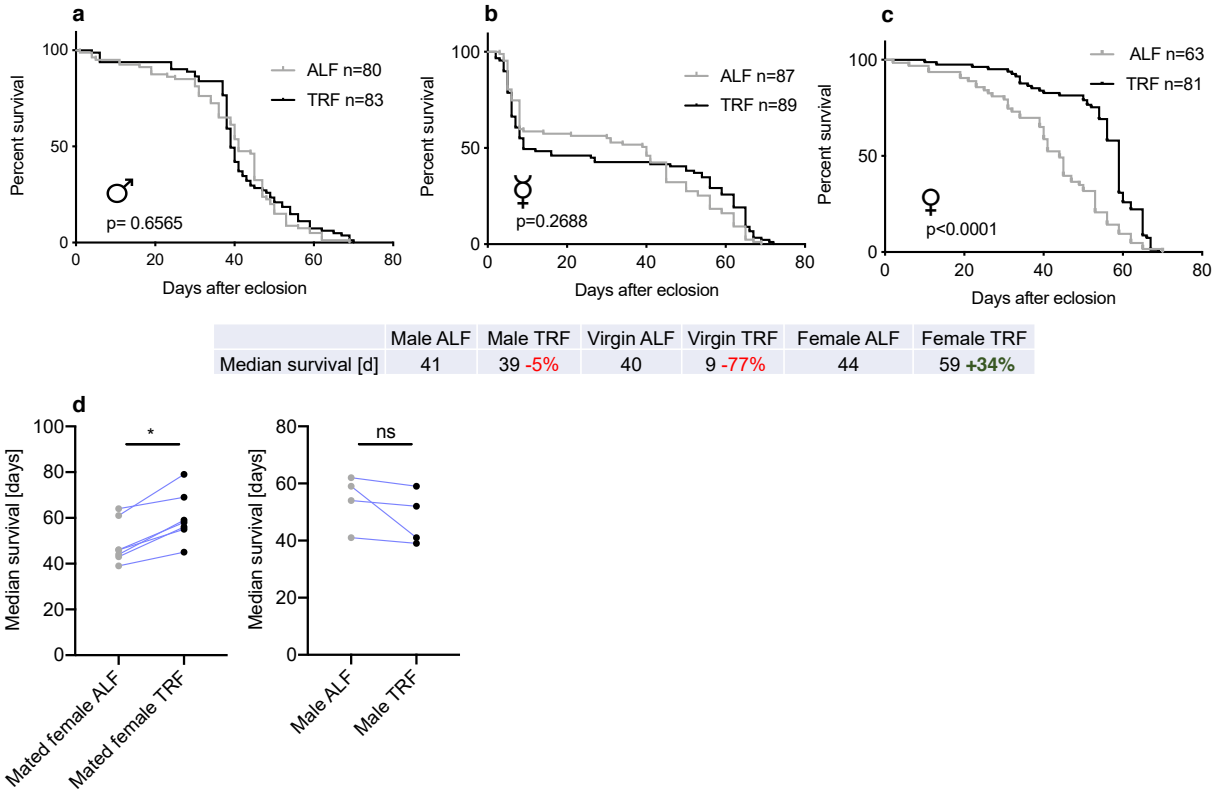
Additional studies have been carried in healthy human subjects, in which physically trained individuals were placed in a TRF regimen of 8 hour feeding time windows and strength and performance were compared between ALF and TRF groups. Performance was compared before and after eight weeks of TRF with training (Moro et al., 2016). While the subjects on TRF maintained strength and performance comparable to ALF after training, subjects in the TRF group showed decreased free testosterone and insulin-like growth factor 1.

While TRF improves metabolic markers, such as hemoglobin A1C, glycemic index, blood pressure and oxidative stress in pre-diabetic men (Sutton et al., 2018), much is left unknown about TRF. Many questions about TRF, such as what are the long-term effects, cellular/molecular changes, and deleterious effects remain to be elucidated. Even more exciting, there is currently a worldwide clinical trial to study the impact of TRF on health (https://mycircadianclock.org/). It will be interesting to learn what this large-scale study will yield.

Considering that sleep, circadian rhythmicity and BBB deteriorate with time, and taking into account that TRF improves cardiac health and sleep in *Drosophila melanogaster*, prevents obesity and metabolic syndrome in mice fed high fat diets, and it can play a preventative role in humans (Gill et al., 2015; Hatori et al., 2012; Sutton et al., 2018), we hypothesized that TRF has the potential to prevent age related changes and extend longevity. In this dissertation, I sought to understand what the role of TRF is on longevity, sleep, circadian clocks and blood-brain barrier function. To do this, I used the fruit fly *Drosophila melanogaster,* which is a genetically tractable organism and a great model for sleep, circadian rhythms, longevity and BBB biology.

CHAPTER 2. TRF Extends Longevity

To study whether TRF extends longevity, we collected wild-type flies after eclosure and separated them into three groups to test mated females, virgin females and mated males. Females and males were allowed to mate for two days, while virgin females were kept separate. Consequently, we separated each group into *ad libitum* feeding (ALF), which are transferred daily into fresh food, and TRF, which only have access to food during the first 12-hour part of the light-dark cycle (ZT0-ZT12) and are transferred to agar-containing vials at night. We followed all the groups for the entire duration of their lives and scored for survival. Males do not show a significant change in median survival when TRF is applied throughout life in comparison to male flies on ALF (Fig.1a). Similarly, virgin females failed to respond to TRF and did not show a significant difference in median survival when compared to the ALF control virgin females (Fig. 1b). Mated females on TRF show a significant ($p<0.0001$) difference in survival and a 34% longer median survival than the mated female controls on ALF (Fig. 1c). While there is a significant effect of TRF on mated females, our experiments do not show a beneficial effect of TRF on wild-type virgin females or males. These experiments were repeated several times and the median survival averaged to confirm that the effect of TRF is reproducible (Fig. 1d). After finding that mated females showed life extension dependent of TRF, we decided to study more closely how this feeding intervention extends longevity in mated females, which from now on will only be called females.



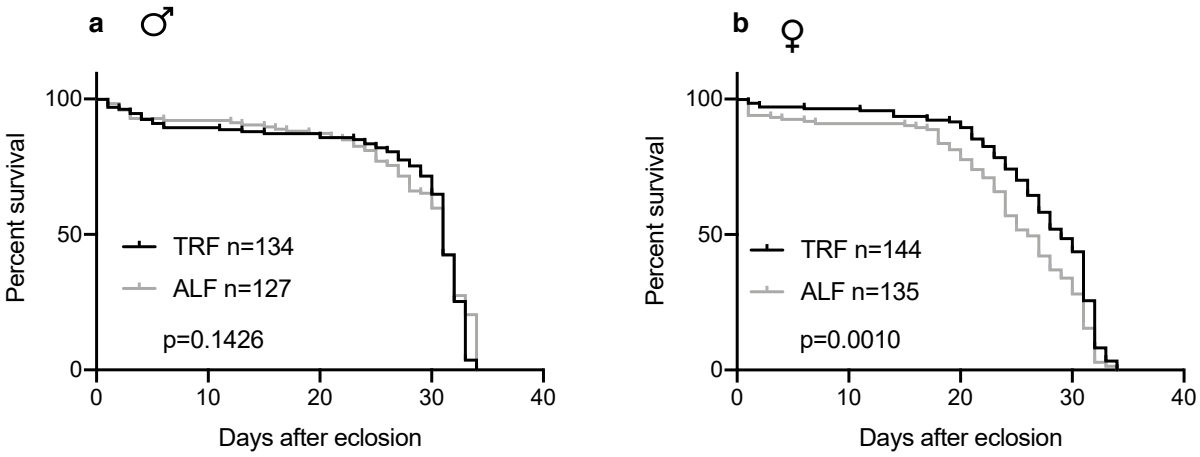
	Male ALF	Male TRF	Virgin ALF	Virgin TRF	Female ALF	Female TRF
Median survival [d]	41	39 -5%	40	9 -77%	44	59 +34%

Figure 1. TRF extends survival of wild-type mated female flies exposed to TRF for life. a, Survival of wild-type mated males subjected to TRF (black) vs ALF (gray) for life and daily scoring for survival. **b**, Survival of wild-type virgin females subjected to TRF vs ALF for life and daily scoring for survival. **c**, Survival of wild-type mated females subjected to TRF v ALF for life and daily scoring for survival. **d**, Average median survival across several independent experiments in mated females and males. Purple connecting lines connect data from same experiments. Survival Log-rank (mantel-cox) tests were performed to compare survival of TRF v ALF groups. Table shows median survival in days of TRF vs ALF and the percent change. Red represents a decrease in TRF compared to ALF and green represents an increase in median survival. Mann-

Whitney tests were performed to compare median survival between TRF and ALF groups.

Different environmental conditions can increase or decrease longevity (Helfand and Rogina, 2003). One that can shorten longevity is higher temperature (Miquel et al., 1976). To test if TRF can also extend longevity under different environmental conditions, such as high temperature, we tested how mated wild-type female and male flies responded to TRF at 29°C (Fig. 2). Male flies did not show any significant changes in median survival when exposed to TRF for life (Fig. 2a). On the other hand, mated females on TRF have a significant ($p=0.001$) 10% increase in median survival when compared to ALF (Fig. 2b). These results are consistent with the life-extension properties of TRF at 25°C on mated females (Fig. 1).

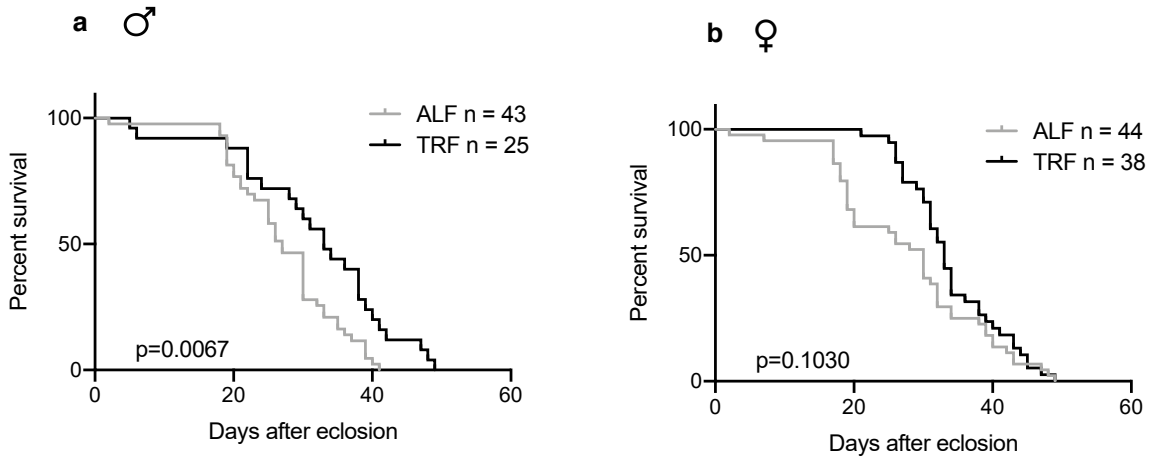
After confirming that TRF can improve longevity under physiological conditions and stress conditions, such as high temperatures, we tested an accelerated model for studying longevity in which the tetanus toxin light chain is expressed using a UAS-TNT-E construct driven by the Gal-4 enhancer trap line DJ651, this UAS-Gal4 system reduces longevity in *Drosophila* and is a useful tool to study ageing in an expedited manner (Bauer et al., 2004). Using these *Drosophila* strains we crossed homozygous UAS-TNT-E flies with DJ651 to create heterozygous progeny. The heterozygous progeny was exposed to TRF or ALF and scored for survival (Fig. 3). Male animals expressing tetanus toxin showed a significant ($p=0.0067$) 22% median survival increment in comparison to ALF flies (Fig. 3a). Similarly, mated females expressing the tetanus toxin light chain show a 10% increase in median survival, although not significant ($p>0.05$) (Fig. 3b). These experiments suggest that TRF can extend median



	Male ALF	Male TRF
Median survival [d] 29 ºC	31	31

Female ALF	Female TRF
26	29 **+11%**

Figure 2. TRF extends survival on wild-type female flies at 29 °C. **a**, Survival of wild-type mated males subjected to TRF (black) vs ALF (gray) for life and daily scoring for survival. **b**, Survival of wild-type mated females subjected to TRF vs ALF for life and daily scoring for survival. Survival Log-rank (mantel-cox) tests were performed to compare survival of TRF v ALF groups. Table shows median survival in days of TRF vs ALF and the percent change. Green represents an increase in median survival.



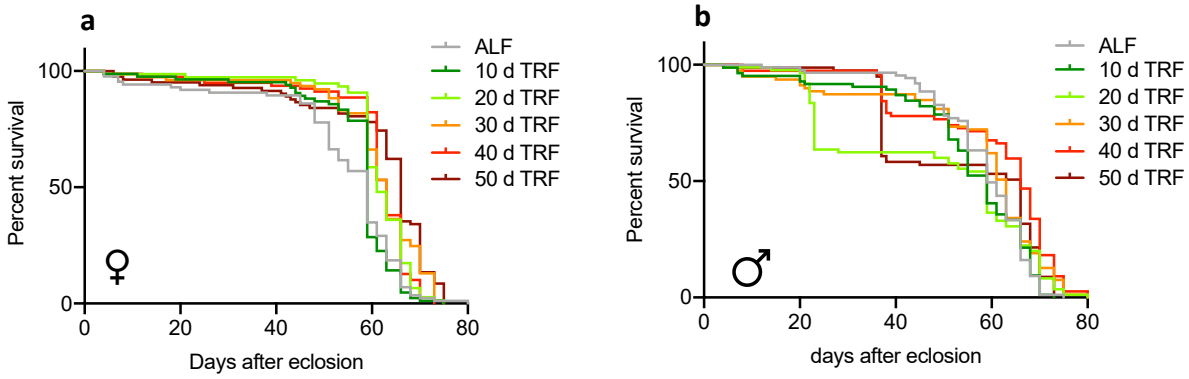
	Male ALF	Male TRF
Median survival [d]	27	33 **+22%**

Female ALF	Female TRF
30	33 **+10%**

Figure 3. TRF improves median survival in combination with accelerated longevity assay. Homozygous UAS-TNT-E flies were crossed to homozygous DJ651-Gal4, progeny was collected, allowed to mate and age for 2 days and TRF regimen undertaken for life. **a**, Survival heterozygous mated males subjected to TRF (black) vs ALF (gray) for life and daily scoring for survival. **b**, Survival of heterozygous mated females subjected to TRF vs ALF for life and daily scoring for survival. Survival Log-rank (mantel-cox) tests were performed to compare survival of TRF v ALF groups. Table shows median survival in days of TRF vs ALF and the percent change. Green represents an increase in median survival.

survival in both males and females under non-physiological conditions. However, these experiments must be carried with higher numbers of flies. This proves difficult as the expression of tetanus toxin compromises the number of progeny in each generation compared to wild-type.

To study how duration of TRF duration affects lifespan, we exposed females and males to 10, 20, 30, 40, and 50 days of TRF, followed by ALF and compared these to flies on ALF for life. We found that as the duration of TRF increased, so did the median survival of mated females. 50 days of TRF increased the median survival to 66 days as compared to 59 days in ALF ($p<0.001$) (Fig 4a). We found that in females there is no significant increase in survival after 10 days of TRF compared to animals on ALF, yet only 20 days of TRF can significantly increase survival ($p=0.001$), compared to animals on ALF. In addition, 30 and 40 days of TRF also significantly improve survival in females ($p<0.001$) in comparison to ALF. Overall, TRF displays a duration-dependent response in females, showing a greater extension of longevity with longer duration of TRF. While females showed a favorable response to increasing duration of TRF, males did not show a similar response (Fig. 4b). Only males that were exposed to TRF for 40 days showed a significant ($p<0.001$) life extension response to TRF, increasing the median survival from 59 days to 66 days. The TRF improvement that we see in the group of male flies exposed to 40 days of TRF, differs from what we see when we



Female response to TRF duration						
	ALF	10 d	20 d	30 d	40 d	50 d
Median survival [days]	59	59	61	63	63	66
p=		0.6528	0.0010	<0.001	<0.001	<0.001

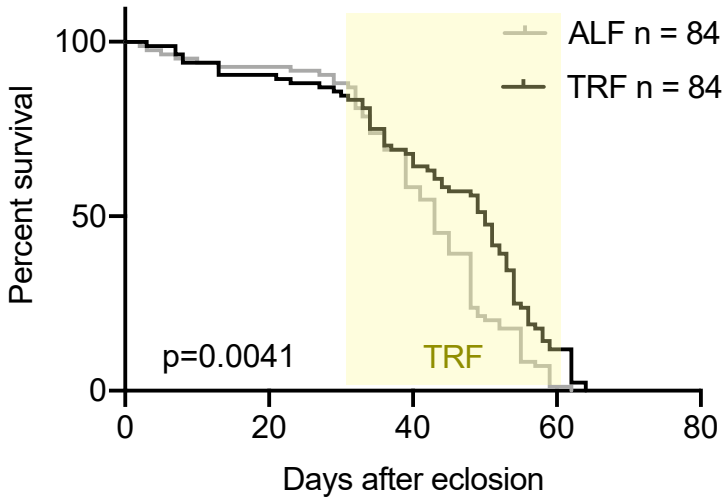
Male response to TRF duration					
ALF	10 d	20 d	30 d	40 d	50 d
59	59	59	63	66	66
	0.6051	0.9874	0.0852	<0.001	0.1197

Figure 4. TRF shows a duration-dependent response in wild-type females. a, b, Survival of wild-type mated females (left panel) and males (right panel) subjected to different durations of TRF compared to ALF. ALF (gray), 10 day TRF (dark green), 20 day TRF (light green), 30 day TRF (orange), 40 day TRF (red) and 50 day TRF (maroon). Table displays median survival and p values for comparison to ALF. **a** ALF n = 86, 10d TRF n = 84, 20d TRF n = 75, 30d TRF n = 77, 40d TRF n = 79, 50 d TRF n =82. **b,** ALF n = 87, 10d TRF n = 84, 20d TRF n = 85, 30d TRF n = 79, 40d TRF n = 77, 50 d TRF n =79. Log-rank (mantel-cox) tests were performed to compare survival of (n) day TRF v ALF groups.

exposed the animals to TRF for life (Fig. 1a), which might be due to a specific TRF window that is beneficial in males but becomes deleterious in longer durations. Males show a dramatic drop of survival at different time points, e.g. day 20 in the 20d TRF group, which could be explained by a higher sensitivity to starvation due to smaller body-size (Chandegra et al., 2017). The sexually dimorphic dose-response to TRF in *Drosophila melanogaster* points to a mechanism which shows higher benefits for females when exposed to TRF and shows variable response in males.

As increasing TRF durations early in life increase median-survival, we explored if TRF late in life can also increase median survival. To study late TRF, we allowed flies to age on ALF for 30 days, followed by 30 days of TRF, and switched again to ALF. 30-day TRF on aged female flies significantly increases survival by 16% ($p=0.0041$) when compared to ALF flies (Fig. 5). Therefore, both late and early TRF interventions extend median survival.

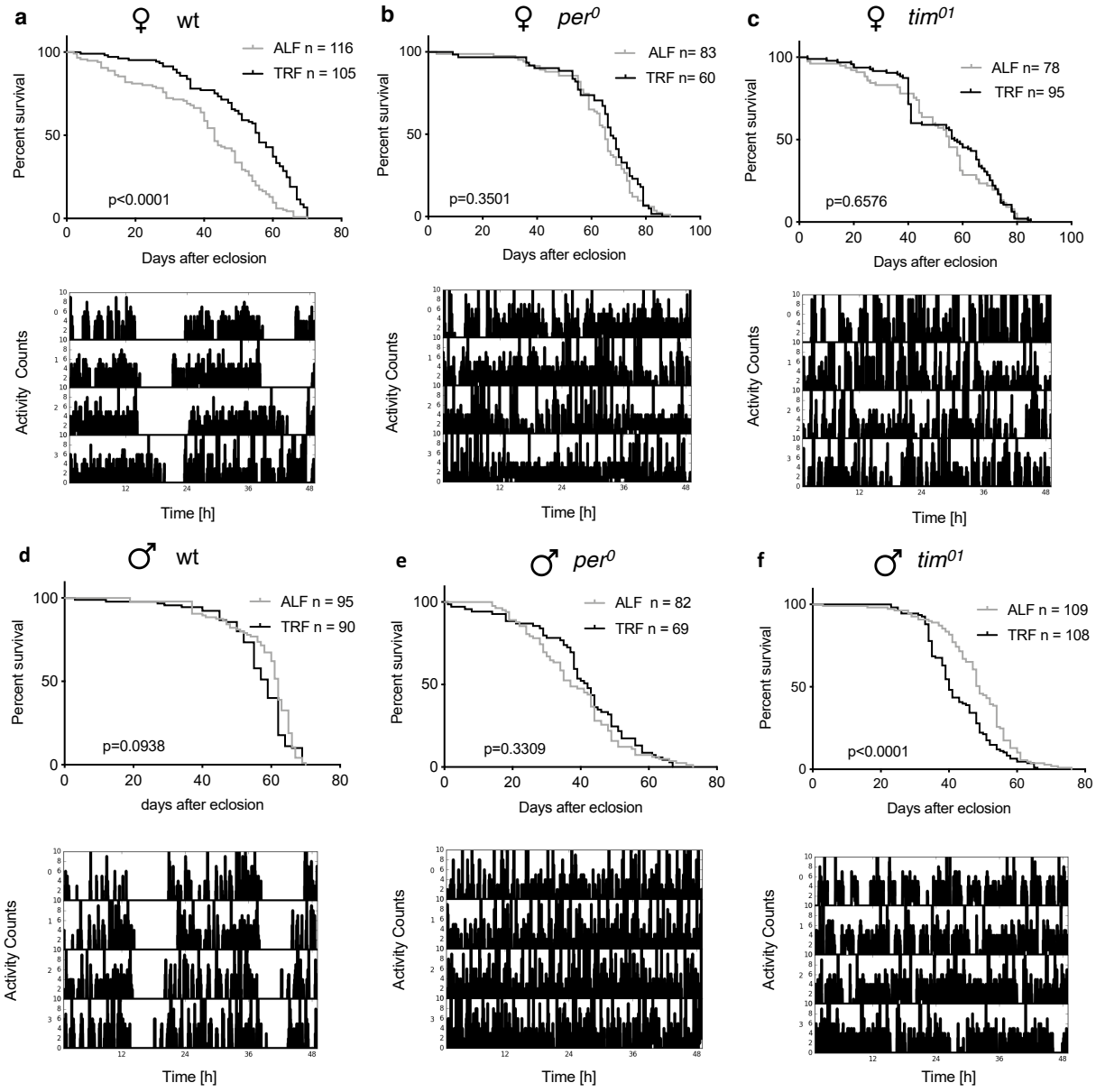
TRF aligns feeding with the time of increased locomotor activity, which is orchestrated by the circadian rhythms and zeitgebers. Thus, we wanted to investigate if endogenous clocks play a role in the longevity extension of TRF. To investigate the role of circadian clocks in TRF, we exposed the arrhythmic clock mutants, per^0 and tim^{01} (Konopka and Benzer, 1971; Sehgal et al., 1994) to TRF and evaluated the survival of TRF vs ALF groups. Female per^0 and tim^{01} mutants, which show arrhythmic behavior, on TRF for life do not show a significant change increase in median survival ($p>0.05$) (Fig. 6b, c).



	ALF	Late TRF
Median survival [d]	43	50 **+16%**

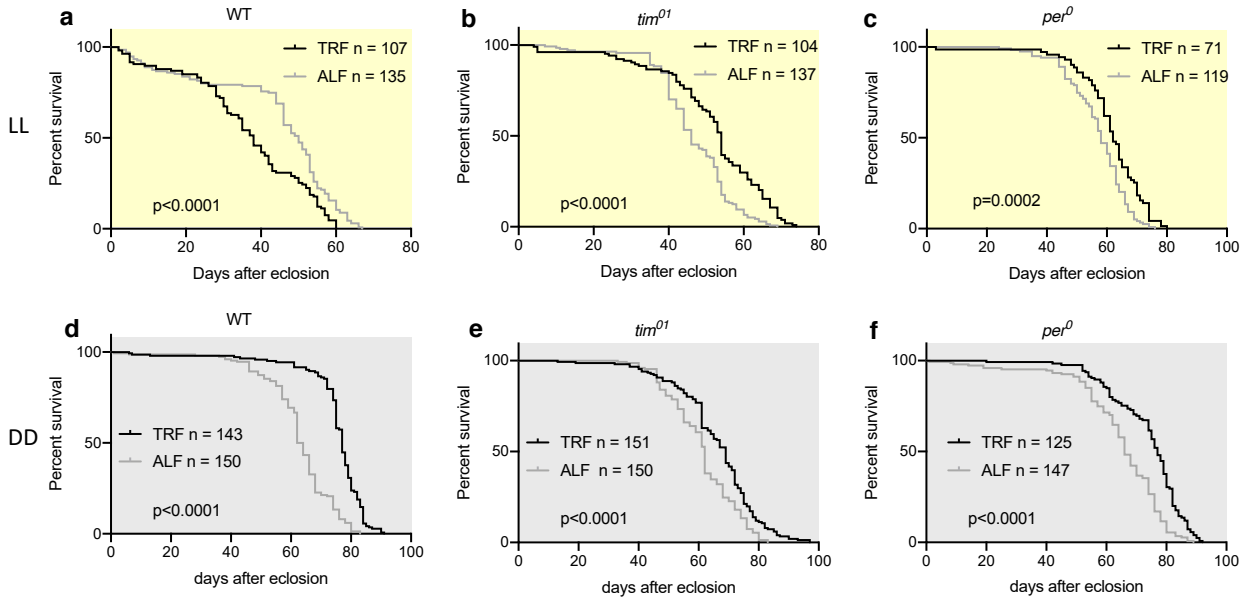
Figure 5. TRF late in life extends median survival. Wild-type mated females exposed to TRF (black) for 30 days after 30 day of constant food and then transferred to constant food. ALF (gray) flies on constant food for the entire duration of the experiment. Tan area represents time TRF flies received intervention. Log-rank (mantel-cox) tests were performed to compare survival of TRF v ALF groups. Table shows median survival in days of late TRF vs ALF and the percent change. Green represents an increase in median survival.

Figure 6. Lifespan extension by TRF is abrogated in clock mutants in light-dark conditions. a, upper panel shows survival of wild-type females subjected to TRF (black) v ALF (gray) for life and daily scoring for survival. Lower panel shows representative actogram showing rhythmic behavior. **b, c,** upper panels show survival of per^{0} and tim^{01} females subjected to TRF (black) v ALF (gray) for life and daily scoring for survival. Lower panels show representative actogram of arrhythmic behavior. **d,** upper panel shows survival of wild-type males subjected to TRF (black) v ALF (gray) for life and daily scoring for survival. Lower panel shows representative actogram showing rhythmic behavior. **e, f,** upper panels show survival of per^{0} and tim^{01} males subjected to TRF (black) v ALF (gray) for life and daily scoring for survival. Lower panels show representative actogram of flies with arrhythmic behavior per^{0} and tim^{01}. Log-rank (mantel-cox) tests were performed to compare survival of TRF v ALF groups.


a
♀ wt
ALF n = 116
TRF n = 105
p<0.0001
Percent survival
Days after eclosion
Activity Counts
Time [h]
b
♀ per0
ALF n= 83
TRF n= 60
p=0.3501
Percent survival
Days after eclosion
Activity Counts
Time [h]
c
♀ tim01
ALF n= 78
TRF n= 95
p=0.6576
Percent survival
Days after eclosion
Activity Counts
Time [h]
d
♂ wt
ALF n = 95
TRF n = 90
p=0.0938
Percent survival
days after eclosion
Activity Counts
Time [h]
e
♂ per0
ALF n = 82
TRF n = 69
p=0.3309
Percent survival
Days after eclosion
Activity Counts
Time [h]
f
♂ tim01
ALF n = 109
TRF n = 108
p<0.0001
Percent survival
Days after eclosion
Activity Counts
Time [h]

On the other hand, wild-type female flies with intact clocks and rhythmic behavior, consistently show a significant response ($p<0.001$) to TRF in comparison to ALF (Fig. 6a). To see if the effect of TRF was also absent in arrhythmic male clock mutants we exposed males to TRF for life and quantified the survival. We evaluated arrhythmic per^0 and tim^{01} male mutants and found no significant difference between ALF and TRF groups in per^0, while we saw a decrease in longevity in tim^{01} male mutants exposed to TRF (Fig. 6e, f). In addition, male wild-type flies, which show rhythmic behavior, consistently fail to respond to TRF in comparison to TRF (Fig. 6 d). These findings suggest an important role of circadian clocks in life-extension function of TRF. Female flies with a functioning circadian clock benefit from the cycling of food that TRF offers, as it aligns with their behavioral rhythms.

To continue learning about what role circadian rhythms play in TRF we looked at the effect of constant light, which makes rhythmic animals arrhythmic, and constant darkness in which animals don't have light stimuli to entrain them (Winfree 1974; Pittendrigh 1981; Saunders 1977; Konopka, Pittendrigh, and Orr 1989; Power, Ringo, and Dowse 1995). After doing TRF in constant light conditions, we found that TRF has deleterious effects in median survival in wild type animals by significantly ($p<0.0001$) reducing the median survival from 50 days in ALF to 38 days in TRF (Fig. 7a). In addition to testing wild-type flies, we also tested how TRF affected the median survival of clock mutants, tim^{01} and per^0, under constant light conditions (Fig. 7b, c). TRF significantly ($p<0.001$) extends median survival from 46 days in ALF to 54 days in the



Female	WT ALF	WT TRF	*tim*^{01} ALF	*tim*^{01} TRF	*per*^{0} ALF	*per*^{0} TRF
Median survival LL [d]	50	38 -24%	46	54 +14%	58	62 +6%
Median survival DD [d]	63	77 +22%	62	69 +11%	66	77 +16%

Figure 7. TRF effects are light and clock-dependent. Shown are survival plots of females kept in constant light conditions represented with the yellow areas, subjected to TRF (black) vs ALF (gray) for life and scored daily for survival **a-c**, Constant light (LL) reverses TRF effects. In LL, TRF shortens lifespan in wild-type flies **(b)**, but lengthens it in clock mutants **(b, c). d-f**, TRF in constant darkness (DD), represented by gray boxes, improves longevity in a clock-independent fashion. In DD, TRF significantly prolongs median lifespan in wild-type flies **(d)** and clock mutants **(e, f)**. Table shows median survival in days of TRF vs ALF and the percent change. Red represents a decrease in TRF compared to ALF and green represents an increase in median survival. Log-rank (mantel-cox) tests were performed to compare survival of TRF v ALF groups.

tim^{01} TRF group in comparison to ALF (Fig. 7b). Similarly, *per*^{0} mutant flies also show a significant (p=0.0002) increase in median survival from 58 days on ALF to 62 day on TRF.

To examine how TRF affects animals in constant darkness, where there are no light or temperature stimuli that could entrain the animals, we also exposed wildtype and clock mutants to TRF for life in constant darkness and examined the effect of this intervention on median survival. TRF shows an increase in median survival in constant darkness by significantly ($p<0.0001$) extending median survival from 63 days in ALF to 77 on TRF (Fig. 7a). Clock mutants *tim*^{01} and *per*^{0} also show a significant ($p<0.0001$) increase in median survival from 62 days on ALF to 69 days on TRF for *tim*^{01}*. per*^{0} also showed a significant ($p<0.0001$) increase in median survival by extending a median survival of 66 days on ALF to 77 on TRF.

There is extensive research that shows the beneficial effects of caloric restriction on survival in different model organisms, including yeast, *Drosophila, C. elegans,* rodents and primates (Balasubramanian, Howell, and Anderson 2017), allowing the possibility that TRF could be another form of caloric restriction. To determine whether the life extension is due to the effect of TRF or because the animals on TRF had decreased food intake, thereby masking a calorically restrictive diet, we quantified feeding in flies after TRF. After 45 days on TRF or ALF, we fed the animals 2.5% yeast/ 2.5% sucrose liquid diet and used the “Activity Recording Capillary Feeder or CAFE (ARC)” (Murphy

et al. 2017) to measure food intake. In this novel set up, we can simultaneously record feeding and activity in real time. To measure food intake over 24 hours for ALF and 12 hours for TRF, we extrapolated the change in food over time while controlling for evaporation. Flies on TRF eat significantly more in the 12 hours that they are exposed to food than their ALF counterparts over 24 hours (Fig. 8). These findings indicate that TRF acts through a pathway independent of caloric restriction. We also found that after 45 days of TRF flies consolidate their feeding to the time before the end of the day between ZT 10 and ZT 12.

In addition to assessing food consumption, we measured body weight after 45 days on TRF. To measure the body weight of the flies, we kept the flies on food for at least 10 hours from ZT 0-10. We then measured weight in groups of 10 flies and found the average weight per fly by dividing the total weight by the number of flies. Wild-type mated females flies on TRF have a significantly lower (10%) body weight ($p=0.0286$) in comparison to control ALF flies (Fig. 9). Body weight after TRF was not significantly changed in tim^{01}, but lower (12%) in per^{0} animals ($p=0.0286$) (Fig. 9). We also measured body weight changes in sleep-deficient inc^{1} animals, which also show a positive longevity response to TRF for life (Fig. 10a), but we did not see a significant change in body weight in inc^{1} mated females after 45 days of TRF (Fig. 9), suggesting that the body weight reduction is a consequence rather than the cause of TRF's beneficial effects on the animals' physiology.

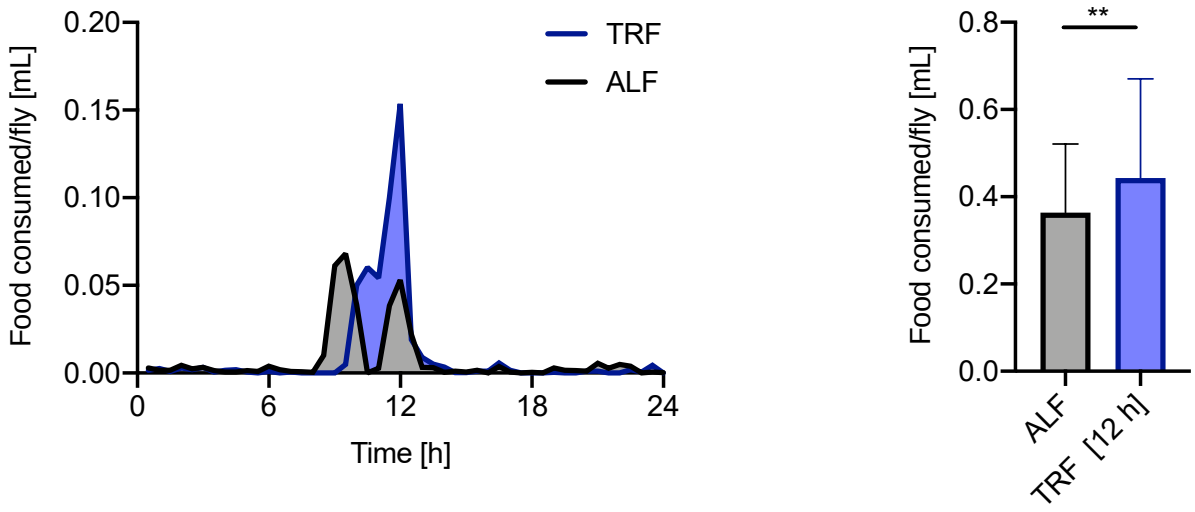


Figure 8. TRF animals eat more than animals on constant food. Total daily food consumption of wild-type females subjected to TRF (purple) n=24 v ALF (gray) n=24. ALF and TRF flies feeding is measured ZT 0-24. ** represent $p \leq 0.01$. Mann-Whitney tests were performed to compare total daily feeding between TRF and ALF groups.

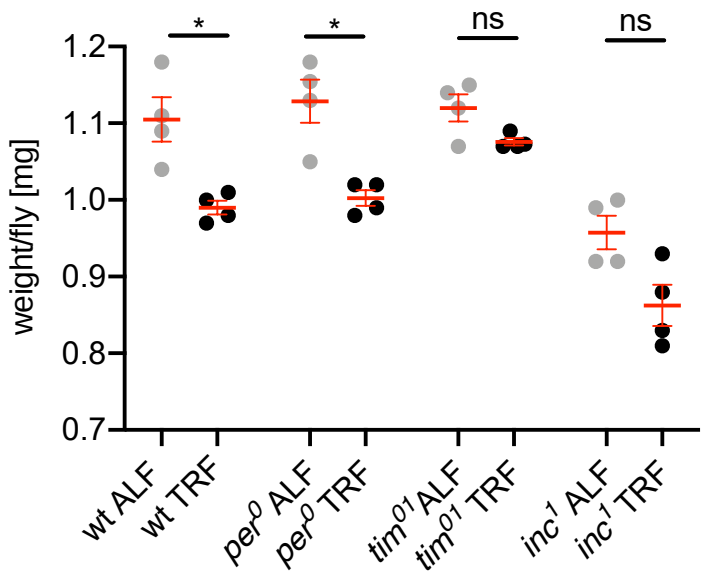
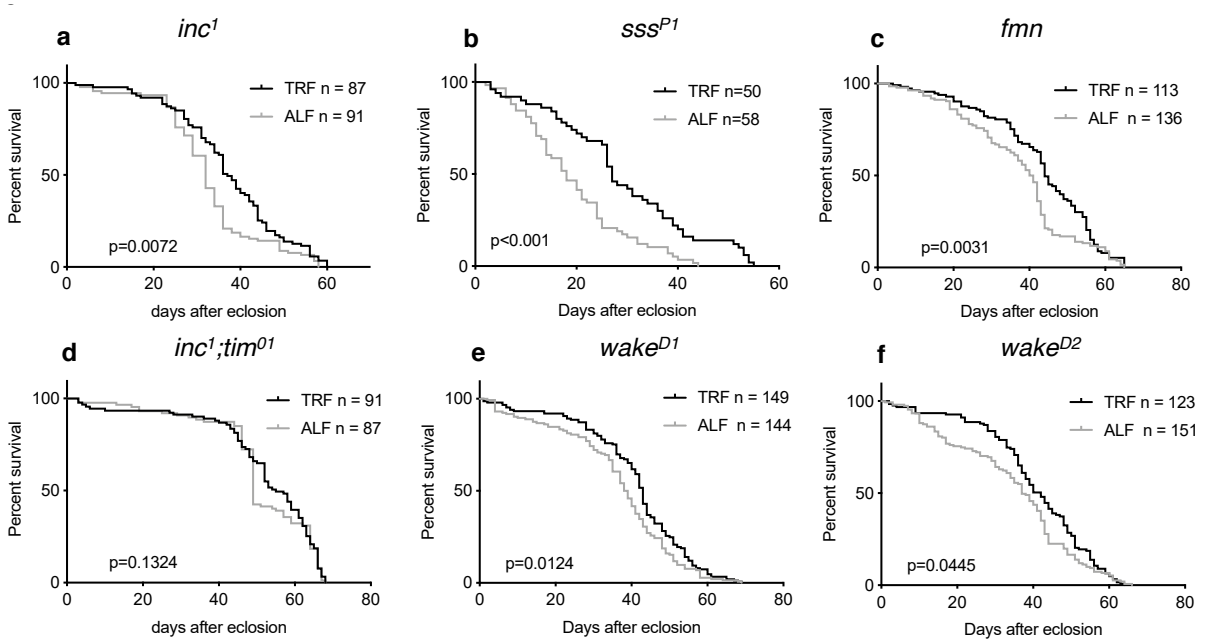


Figure 9. TRF decreases body weight. Weight of wild-type, *per*^{0}, *tim*^{01} and *inc*^{1} females subjected to TRF (black) v ALF (gray) for 45 days. Red error bars show mean and standard error of the mean. * represents $p \leq 0.05$. Mann-Whitney tests were performed to compare weight between TRF and ALF groups.

CHAPTER 3. TRF and Sleep

Sleep has been shown to deteriorate as organisms age, suggesting an important role in ageing (reviewed in Kondratova and Kondratov, 2012). Furthermore, chronic sleep deprivation, as seen in many *Drosophila* sleep mutants, reduces the lifespan of these animals. As TRF extends longevity in wild-type flies, we wanted to test if TRF can improve longevity in animals with reduced survival. *Drosophila* sleep mutants provide a great tool to study longevity as they show reduced lifespan. To study if TRF can increase lifespan in sleep mutants, we used inc^{1}, sss^{P1}, *fmn*, $wake^{D1}$, $wake^{D2}$, and $inc^{1};tim^{01}$ double mutants. To understand if the effect is sexually dimorphic as in wild-type, we studied both mated females and males. Female sleep mutants show TRF-mediated life extension compared to ALF (Fig. 10). inc^{1} mated females show a 13% ($p=0.0072$) median survival increase (Fig. 10a). sss^{P1} show a 50% increase ($p<0.001$) in median survival (Fig. 10b). *fmn* flies show a 9% ($p=0.0031$) increase in median survival (Fig. 10c). $wake^{D1}$ flies show a 12% ($p=0.0124$) increase in median survival (Fig. 10e). $wake^{D2}$ flies show a 14% ($p=0.0445$) increase in median survival (Fig. 10f). To learn if an intact molecular clock is important for TRF-mediated life extension in sleep mutants, we examined $inc^{1};tim^{01}$ double mutants, which show both the arrhythmic phenotype in constant darkness and reduced sleep (Fig 11a, b). These double mutants do not show a significant increase in survival (Fig. 10d), which is consistent with our prior experiments that show that endogenous clocks are necessary for the response to TRF in light-dark conditions. It is rather interesting that sleep mutants with no reduced longevity



Female	*inc*¹ ALF	*inc*¹ TRF	*inc*¹;*tim*⁰¹ ALF	*inc*¹;*tim*⁰¹ TRF	*sss*P1 ALF	*sss*P1 TRF	*wake*D1 ALF	*wake*D1 TRF	*wake*D2 ALF	*wake*D2 TRF	*fmn* ALF	*fmn* TRF
Median survival [d]	32	37 +13%	49	55 +11%	18	27 +50%	38.5	43 +12%	37	42 +14%	40.5	44 +9%

Figure 10. TRF extends longevity in female sleep mutants in a clock dependent manner. a-f, Survival of *inc*¹, *sss*P1, *fmn*, *inc*¹;*tim*⁰¹, *wake*D1, and *wake*D2 mated females subjected to TRF (black) v ALF (gray) for life and daily scoring for survival. Table shows median survival as well as percent change between ALF and TRF groups. Red represents a not significant change in TRF compared to ALF and green represents a significant increase in median survival. Log-rank (mantel-cox) tests were performed to compare survival of TRF v ALF groups.

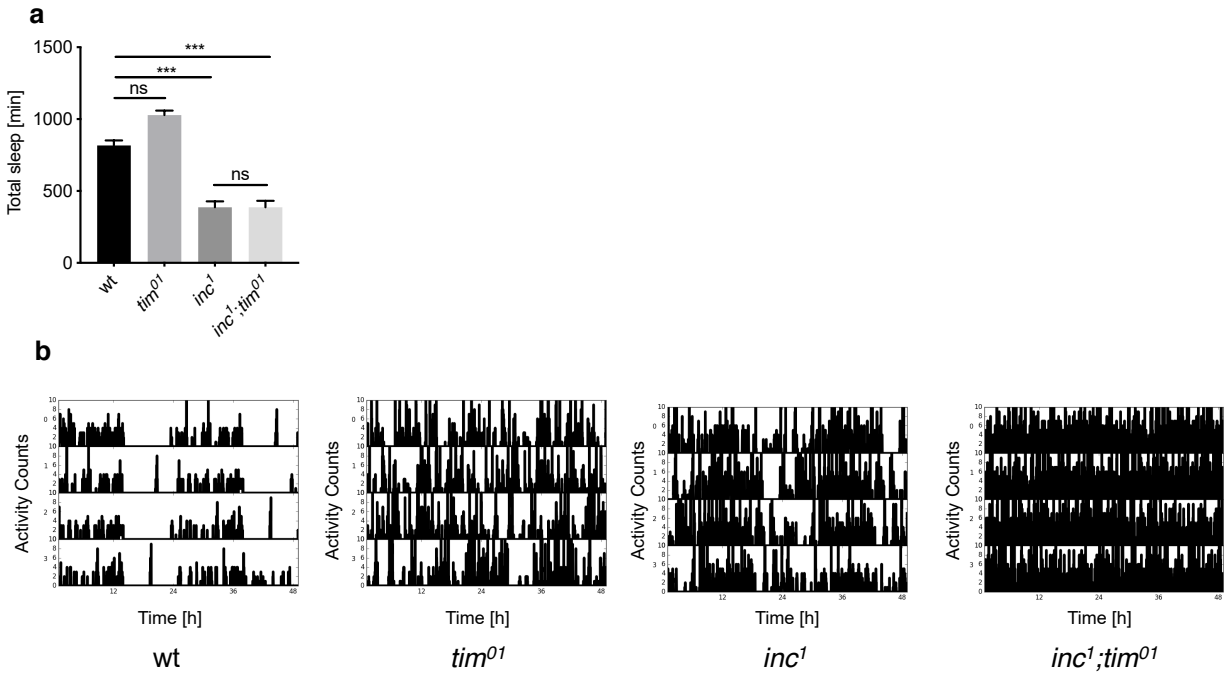
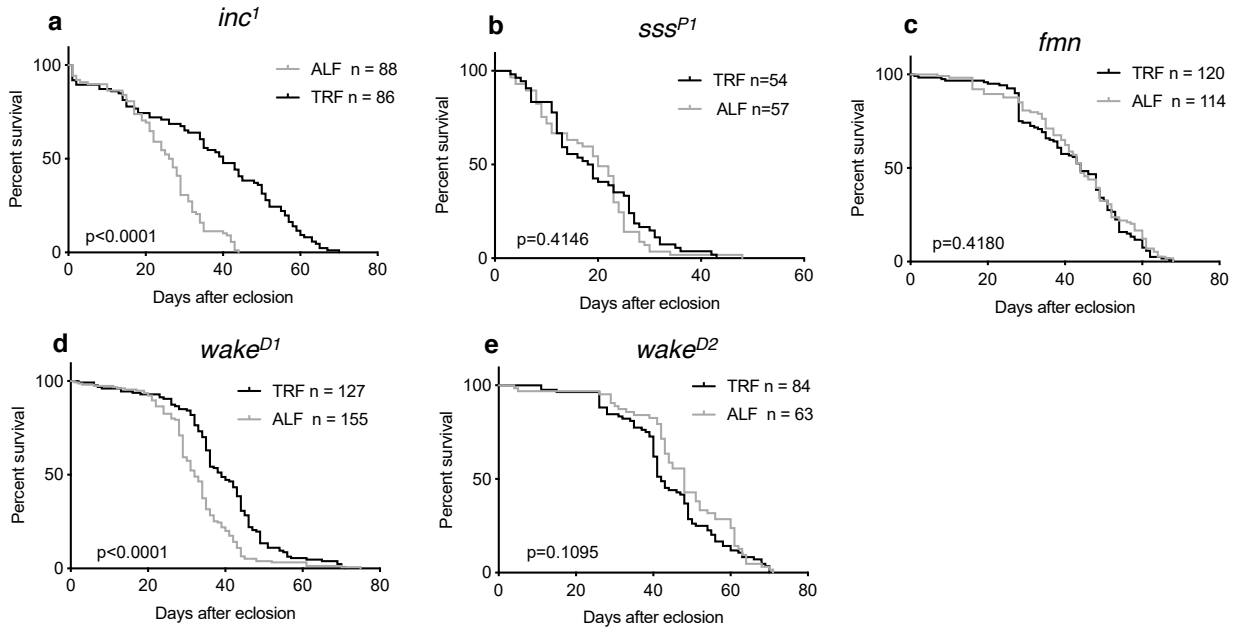


Figure 11. Phenotypic confirmation of *inc¹;tim⁰¹* double-mutant. a, total sleep measurement in wild-type, *tim⁰¹*, *inc¹,* and *inc¹;tim⁰¹*, n=16 for each group. Males were assayed to measure sleep. Sleep phenotype was evident in *inc¹* with a mean total sleep per day of 387.35 minutes per day vs 816.9 min in wild-type (p= 0.008). *inc¹;tim⁰¹* animals sleep 386.8 minutes per day showing a significant difference with wild-type (p=0.0008) and no difference (p=0.8916) with *inc¹* animals. **b,** actograms showing rhythmic behavior of wild-type and *inc¹,* while *tim⁰¹* and *inc¹;tim⁰¹* show arrhythmic behavior as expected. Krustal-Wallis multiple comparison tests were performed to compare sleep between wild-type and *tim⁰¹*, *inc¹,* and *inc¹;tim⁰¹*. Mann-Whitney tests were performed to compare sleep between *inc¹,* and *inc¹;tim⁰¹*. *** represent p ≤ 0.001. Actograms and rhythmicity were determined using a custom-written Python script (S.A., unpublished).

phenotype, such as *fmn,* we see a median survival extension, which suggests that it also has an effect on sleep mutants that don't have a reduced longevity phenotype. TRF also has an effect on some male sleep mutants; however, to a lesser degree (Fig. 12). As seen in prior experiments, we did not see a significant change in median survival in sss^{P1}, or *fmn* males in comparison to ALF (Fig. 12b, c). However, inc^{1} and $wake^{D1}$ males showed a median survival increase of 51% ($p<0.001$) and 22% ($p<0.001$), respectively (Fig. 12a, d). On the other hand, $wake^{D2}$ males showed a significant decrease in median survival of 12% after TRF (Fig. 12e). These findings support the role of TRF in extending longevity not only in healthy animals, but also in those with reduced longevity, including males with reduced longevity. Importantly, positive TRF effects in some sleep mutant males suggest that TRF has the potential to help males with compromised longevity.

TRF improves sleep in male wild-type *Drosophila* (Gill et al., 2015). In addition, as animals age, sleep deteriorates - fragmentation is increased and duration is reduced -in several model organisms including *Drosophila melanogaster* (Koh et al., 2006). To learn if TRF extends lifespan by increasing sleep duration, we exposed females to 14, 30, and 45 days of TRF and found that the sleep architecture changes as a consequence of TRF (Fig. 13a). Wild-type female flies do not show a significant increase in total or nighttime sleep at any of the times measured (Fig. 13b). However, we observed a significant change in total sleep during the day from ZT 0-12. As the flies age, daytime sleep is reduced in ALF flies, with an average day sleep of 140.8 minutes on day 14, to



Male	sss^{P1} ALF	sss^{P1} TRF	inc^{1} ALF	inc^{1} TRF	$wake^{D1}$ ALF	$wake^{D1}$ TRF	$wake^{D2}$ ALF	$wake^{D2}$ TRF	*fmn* ALF	*fmn* TRF
Median survival [d]	20	18.5 -7%	26.5	40 +51%	32	39 +22%	48	42 -12%	44	44 +/-0%

Figure 12. TRF improves longevity in some male sleep mutants. **a-e,** Survival of wild-type, sss^{P1}, inc^{1}, *fmn*, $wake^{D1}$, and $wake^{D2}$ males subjected to TRF (black) v ALF (gray) for life and daily scoring for survival. Table shows median survival as well as percent change. Red represents a significant decrease in TRF compared to ALF, blue represents a not significant change, and green represents an increase in median survival. Log-rank (mantel-cox) tests were performed to compare survival of TRF v ALF groups.

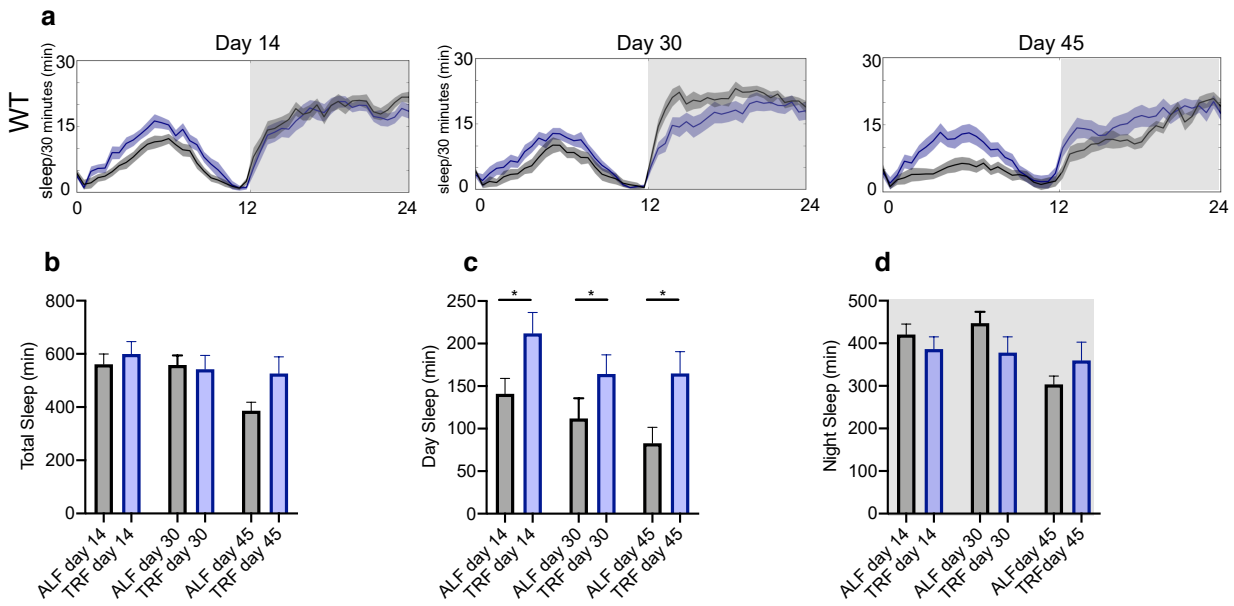


Figure 13. TRF increases day sleep in wild-type females. **a**, show sleep architecture of wild-type flies with ALF (gray) n=16 and TRF (purple) n=16. **b,** illustrates total sleep. **c, d,** Day sleep and night sleep in ALF v TRF in wild-type flies. Graphs with no label for significance did not show a significant change ($p>0.05$) after statistical analysis. * represents $p \leq 0.05$. Mann-Whitney tests were performed to compare sleep between TRF and ALF groups.

an average 82.7 minutes on day 45. In contrast, day sleep remains elevated in TRF relative to ALF groups during aging: with an average day sleep of 212 minutes on day 14, to an average of 164.9 minutes on day 45 (Fig. 13c). We also assayed the sleep duration of wild-type female flies during the night and did not find a significant change in the amount that these flies sleep during the night (Fig. 13d).

To test if the observed TRF-associated sleep change is causal for TRF's effect of longevity, we tested sleep in the clock mutant per^0 (Fig. 14a), which doesn't respond to TRF in light-dark conditions (Fig, 6b). We found that only 30 days of TRF significantly increases the total sleep in per^0 (Fig. 14b). In addition, we did not see a change in day sleep (Fig. 14c). However, contrary to what we saw in wild-type, we saw a significant increase in night sleep in per^0 mutants after 14, 30, and 45 day TRF (Fig. 14d). Thus, TRF in per^0 mutants shows a change in sleep that is different to that in wild-type, increasing night sleep as opposed to day sleep, suggesting that age-related day sleep is important for longevity and aided by TRF, which is abrogated in clock mutants.

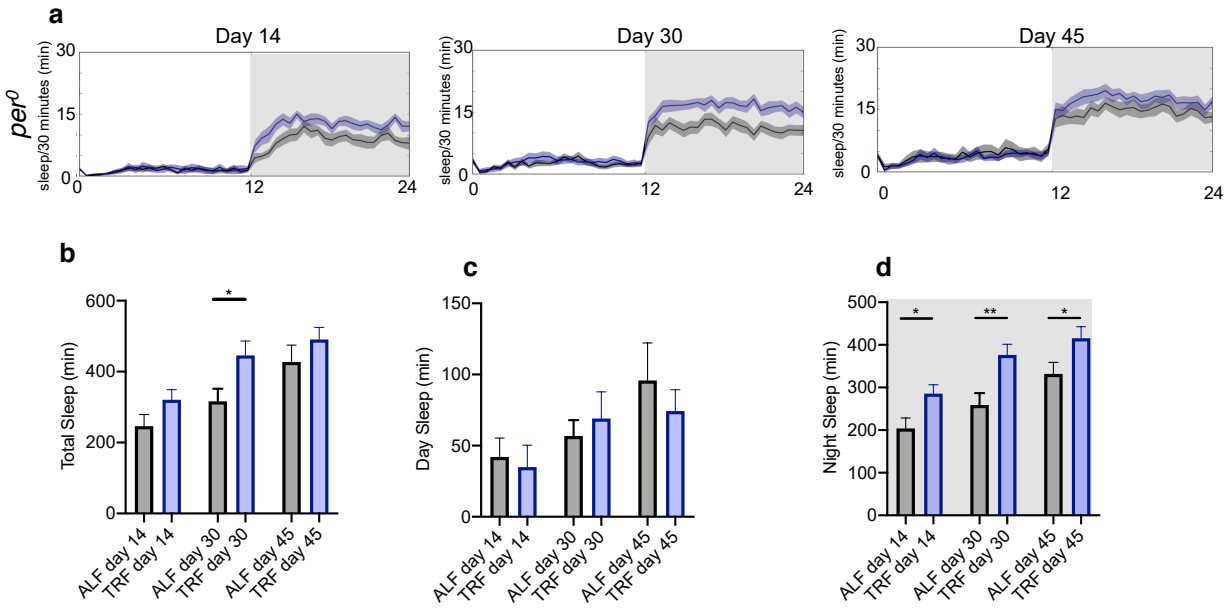


Figure 14. **TRF increases night sleep in *per^0* females**. Effects of TRF on mated *per^0* mated female flies over time. **a,** show sleep architecture of per0 flies with ALF (gray) n=16 and TRF (purple) n=16. **b,** illustrates total sleep. **c, d,** Day sleep and night sleep in ALF v TRF in wild-type flies. Graphs with no label for significance did not show a significant change ($p>0.05$) after statistical analysis. * represents $p \leq 0.05$. ** represent $p \leq 0.01$. Mann-Whitney tests were performed to compare sleep between TRF and ALF groups.

CHAPTER 4. TRF and Blood-Brain Barrier

Ageing has been shown to correlate with blood-brain barrier (BBB) dysfunction in mice and humans (Goodall et al., 2018). Furthermore, work from our lab demonstrated that BBB permeability shows circadian rhythmicity (Axelrod et al., *in review*). With this in mind, we wanted to know if the life-extension effect of TRF could also improve BBB function. To learn what the effect of TRF is in wild-type flies and clock mutants, we exposed female flies to 14, 30, and 45 days of TRF, and assessed BBB permeability. After 14 days of TRF, wild-type flies show significantly (p=0.0229) decreased BBB permeability, while per^0 and tim^{01} showed significant increases in BBB permeability (p=0.0337 and p=0.0012, respectively) (Fig. 15a). After 30 days of TRF we did not see a change in wild-type BBB permeability, while per^0 and tim^{01} on TRF show significantly increased permeability relative to ALF (p=0.0412 and p<0.0001, respectively) (Fig. 15b). After 45 days of TRF, wild-type flies did not show a significant change, while paradoxically we saw a decrease in BBB permeability in per^0 and tim^{01} (p=0.0017 and p=0.0149, respectively) (Fig. 15c). 14 days of TRF significantly reduce BBB permeability but increase it in per^0 and tim^{01} after 14 and 30 days TRF relative to ALF. Interestingly, as flies age (45 days TRF), wild-type flies do not show a difference in permeability after TRF, while per^0 and tim^{01} exhibit an opening of the BBB. This difference in BBB permeability suggests that arrhythmic flies adapt differently to cycling food availability, potentially associated to a barrier breakdown. In contrast, the BBB integrity is aided by TRF in young flies, which could be important for longevity later in life.

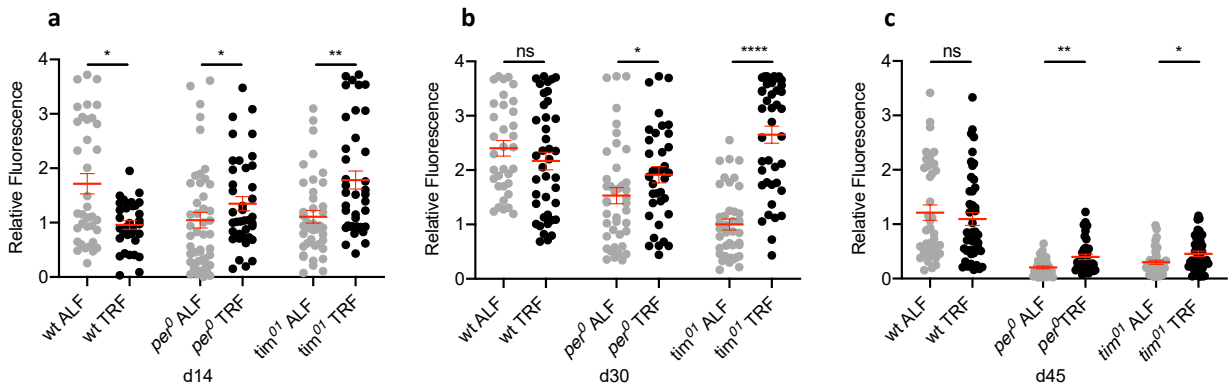


Figure 15. TRF reduces BBB permeability in wild-type *Drosophila* but increases it in clock mutants. a-c, Relative fluorescence as a proxy for BBB permeability wild-type, *per*^{0} and *tim*^{01} mated females after 14, 30, and 45 days of TRF. ALF (gray) n=20 and TRF (black) n=20. * represents $p \leq 0.05$. ** represent $p \leq 0.01$. **** represent $p \leq 0.0001$. Mann-Whitney tests were performed to compare relative fluorescence between TRF and ALF groups.

In addition to showing a circadian oscillation, the BBB is also defective in sleep mutants such as *inc¹,* and improving sleep in these mutants by different genetic and pharmacological manipulations improve BBB function (Axelrod et al., *in review*). With this in mind, we wanted to learn whether the beneficial effects of TRF also affected the BBB and potentially reduce its deterioration. To explore how TRF affected *inc¹* females, which show extended median survival with TRF (Fig. 10a), we performed 10, 20, and 30 days of TRF and assessed sleep and BBB permeability (Fig. 16 a-d). Interestingly, we found that only after 10 days of TRF in *inc¹* females there is a significant decrease in BBB permeability ($p=0.0180$) (Fig. 16a). In addition to the difference between ALF and TRF groups, we also saw a significant decrease in BBB permeability as a function of time. We did not find any significant changes in sleep at any of the durations of TRF (Fig. 16b). To further study how sleep changed, we looked at day sleep and night sleep and found that there are no differences between ALF and TRF groups in day or night sleep (Fig. 16c, d). However, it is interesting that day sleep significantly reduces as the animals age.

To further investigate the effects of TRF on BBB, we exposed *inc¹* male flies, which also show an increase in longevity (Fig. 12a) to TRF for life and assessed weekly BBB permeability and total sleep in male *inc¹* male flies (Fig 17a, b). While we did not see a significant change in permeability or sleep between ALF and TRF groups during the first five weeks, we saw a significant decrease in permeability as well as a significant change in total amount sleep on week 6 between ALF and TRF groups (Fig. 17a, b).

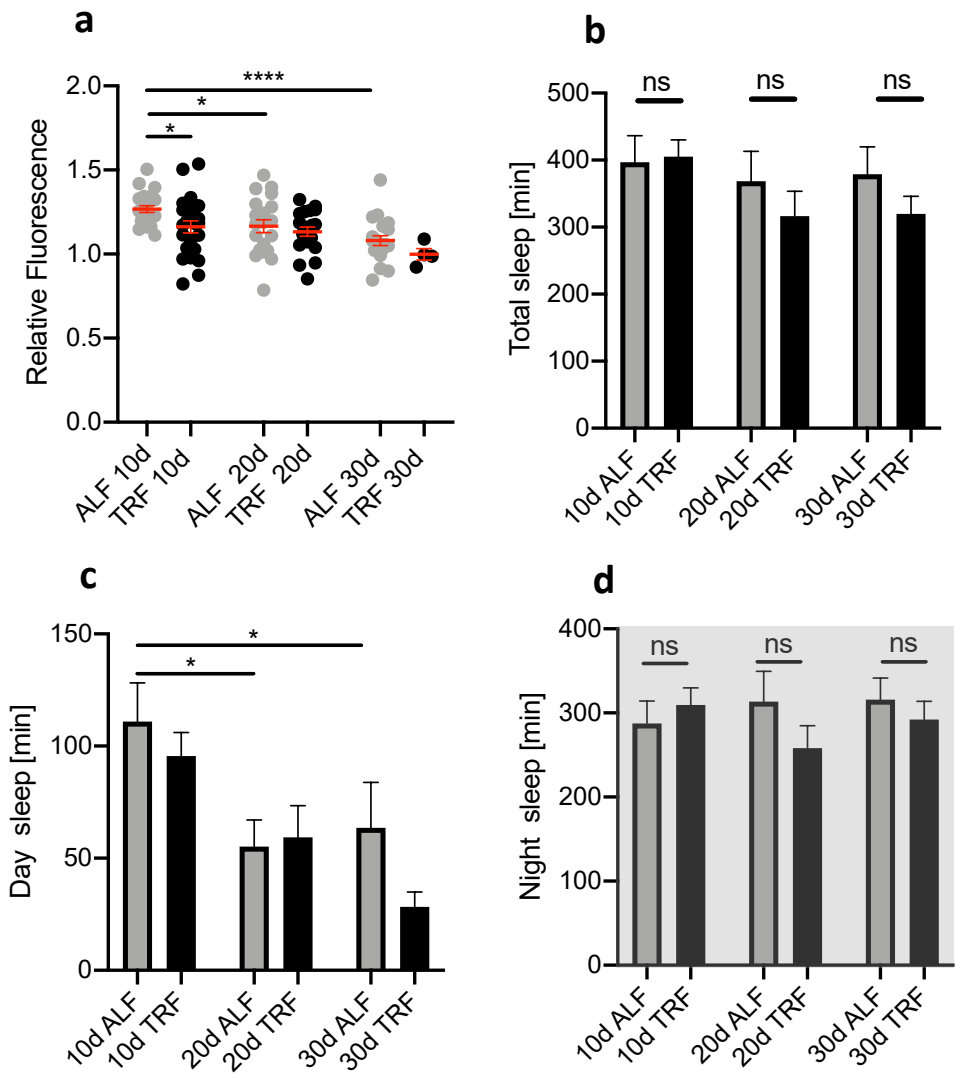


Figure 16. BBB permeability and sleep in *inc*^{1} females after TRF. a, shows relative fluorescence measured at day 10, 20 and 30 as a proxy for BBB permeability, ALF (gray) n=20 and TRF (purple) n=20. **b,** shows total sleep measured at day 10, 20 and 30 in ALF (gray) n=16 and TRF (purple) n=16. **c, d,** Show day and night sleep measured at day 10, 20 and 30 in ALF (gray) n=16 and TRF (purple) n=16. * represents $p \leq 0.05$. **** represent $p \leq 0.0001$.Graphs with no label for significance did not show a significant change ($p>0.05$) after statistical analysis. Mann-Whitney tests were performed to compare relative fluorescence and sleep between TRF and ALF groups.

Furthermore, we see that there is a reduction in BBB permeability in *inc*^{1} as a function of time and after six weeks there is a significant reduction in permeability (Fig.17a). Similarly, we see an increase in sleep as the flies age and a significant increase in TRF after 5 weeks of age relative to ALF (Fig. 17b). To further study how sleep changes *inc*^{1} males when exposed to TRF, we looked at day sleep and night sleep (Fig 17c,d). We found that day sleep shows variable changes throughout life and increased sleep after week three, followed by a reduction in week six (Fig. 17c). On the other hand, we did not see any significant changes between ALF and TRF groups in night sleep (Fig. 17d). However, it is interesting that the night sleep of this mutants correlates with the increase in total sleep.

In summary, TRF in wild-type and *inc1* leads to decreased permeability of the BBB, while we see increased permeability in clock mutants, suggesting that TRF changes the BBB permeability and this may play a role in lifespan extension. It is challenging to understand how TRF is changing sleep or BBB permeability, as the connection between TRF and BBB function has not been explored. Higher resolution experiments, in which we assay the BBB dynamics more often and at different times, and with different durations of TRF may help elucidate this phenomenon. This encouraging response to TRF in BBB indicates that TRF also changes this important biological barrier and potentially more physiological mechanisms. While it would be premature to make any direct conclusion of this correlation, our data open the window for further questions in regard to BBB function and TRF in ageing.

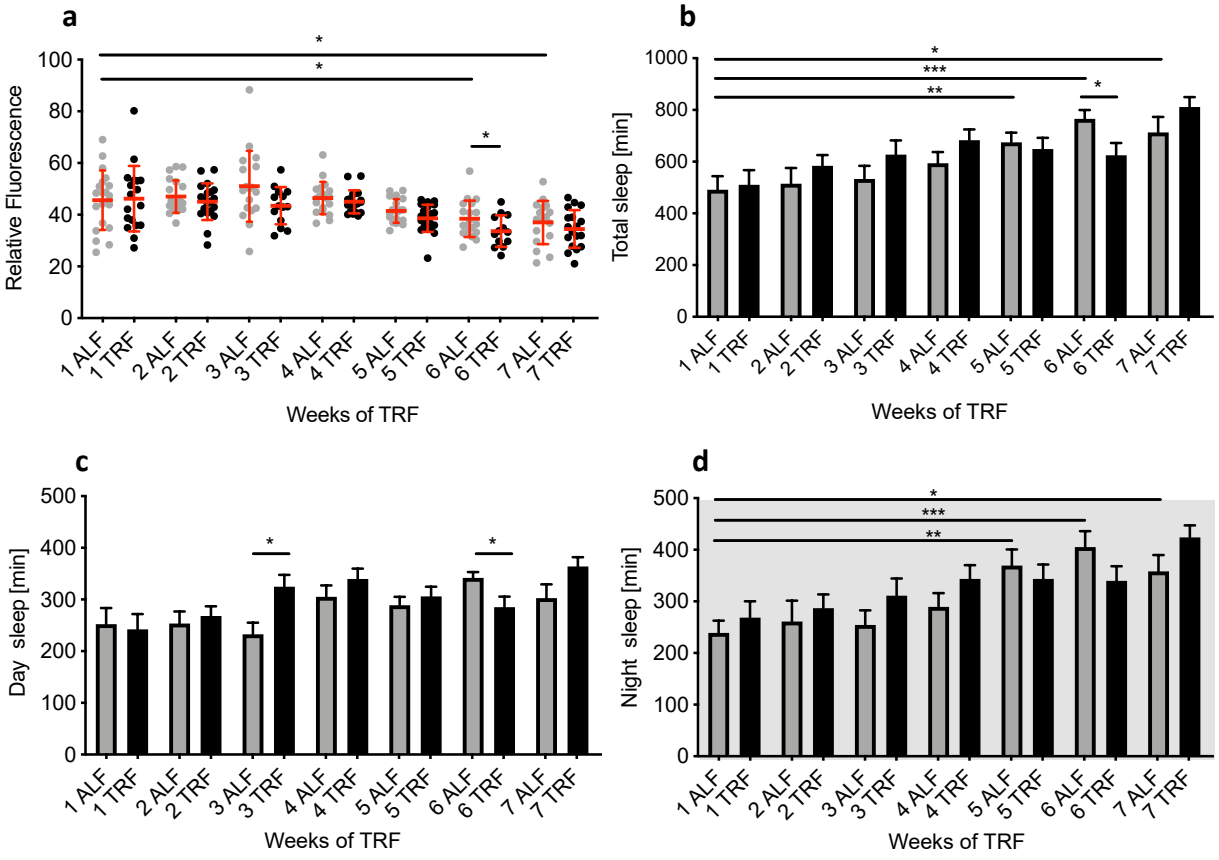


Figure 17. BBB permeability and sleep in *inc*^{1} males after TRF. a, shows relative fluorescence measured weekly as a proxy for BBB permeability, ALF (gray) n=20 and TRF (purple) n=20. **b,** shows total sleep measured weekly in ALF (gray) n=16 and TRF (purple) n=16. **c,d,** Show day and night sleep measured weekly in ALF (gray) n=16 and TRF (purple) n=16. Graphs with no label for significance did not show a significant change ($p>0.05$) after statistical analysis. Mann-Whitney tests were performed to compare relative fluorescence and sleep between TRF and ALF groups.

Discussion

The field of chronobiology continues to look for ways to target clocks as a means to improve health and treat illness. More and more diseases, from metabolic to psychiatric, show a circadian component, which speaks to the future of applied circadian biology (Sulli, Manoogian et al., 2018). Time-restricted feeding has been shown to improve metabolic markers in humans, rodent models, and Drosophila (Longo and Panda, 2016).

In this thesis, I show that TRF also extends longevity in the fruit fly *Drosophila melanogaster* in a dose dependent manner. In addition, TRF intervention late in life can significantly extend longevity, suggesting that the effect of TRF is not dependent on age. Not only is the effect of TRF present in wild-type females, but also it increases both male and female mutant flies with reduced longevity. Flies on TRF consume more food relative to ALF, yet have reduced body weight. This suggests a mechanism independent of caloric restriction - in TRF the most influential factor is not calorie count, but rather food timing.

The life extension effect of TRF is not observed in clock-deficient flies in normal LD cycles, but increases in constant darkness or constant light conditions. Data from TRF on wild-type flies in constant light point to an important role of circadian clocks because we see that the effect of TRF is lost as the animals become arrhythmic. However, we also see that TRF increases median survival in clock mutants under constant light

conditions. These experiments in constant light and constant darkness must be complemented with measurements of feeding at different times throughout the experiments, to see how TRF affects feeding under constant light conditions to ensure that we are not calorically restricting them as well. Another potential explanation as to why TRF is extending median survival in clock mutants in constant light is that they are under potential high stress conditions and TRF is beneficial in this context. On the other hand, we also see that TRF extends longevity on wild-type and clock mutants in constant darkness. While this is consistent with our hypothesis that TRF synchronizes behavior with feeding schedules and thereby extending median survival, it is interesting that clock mutants also show a positive effect. To learn how the median survival extension is mediated by TRF, we must also examine the feeding quantity, duration and timing at different times in constant darkness during the duration of the experiment. After elucidating how feeding changes under constant darkness and constant light, experiments looking at transcriptional levels of clock genes in these conditions should be performed to see how these change after TRF. Furthermore, protein level analysis of clock proteins at different times on these same animals could explain the different responses to TRF to different environmental conditions. These results suggest an important role of endogenous clocks in the life extension mechanism of TRF.

Barber et al., 2016 showed an interesting connection between the central clocks in the Drosophila brain, where insulin producing cells (IPC) in the pars intercerebralis are under circadian regulation and are also deeply affected by feeding. IPCs produce insulin

which also communicates to the fat body producing a rhythmic connection between the brain and peripheral tissues. IPCs being under circadian modulation, but also highly responsive to feeding, provides a plausible mechanism by which TRF works. By providing food in synchrony with the circadian rhythms, this might provide a physiological oscillation, which in turn extends longevity via insulin. To test this hypothesis, it will be interesting to test how TRF affects the rhythmicity of IPCs or if this communication with the peripheral organs is enhanced and maintained as the animals age, preventing the dampening of circadian rhythmicity in peripheral organs. To test this one could use luciferase as a reporter for circadian cycling in different neuronal and peripheral clusters.

The Takahashi group attempted to tease apart the difference between CR and TRF in mice and found an interesting behavior in which the animals in CR self-imposed a temporal restriction by eating all the pellets at the beginning of the night followed by a long fasting period (Acosta-Rodríguez et al., 2017). This points to the difficulty to tease apart these different diets and their effects, and in fact caloric restriction effects could be attributed to time-restricted feeding. A remaining argument for CR and TRF not being identical is the fact that CR but not TRF still prolongs longevity in clock mutants (Katewa et al., 2016) (Fig. 6).

The effect of TRF is more salient in mated females than in males. This could be explained by the fact that males are more sensitive to periods of starvation (Chandegra

et al. 2017), suggesting that shortening the daily period of starvation could lead to TRF life extension in males. Metabolic differences could also underlie TRF's sexual dimorphism, which is consistent with other life-extending interventions, such as CR, where both males and female *Drosophila* show a response to CR, but there is a larger effect in females with 60% longer lives and males only showing 30% longer than controls (Magwere, Chapman, and Partridge, 2004). Furthermore, there has been extensive research into the difference in lifespan between females and males (Regan and Partridge, 2013), showing a role for steroid hormones and nutrient sensing pathways. Future work will elucidate this puzzling interaction between sex and longevity.

We also found that a beneficial longevity effect of TRF is only seen in mated but not virgin females, which could be explained by the finding that mated females have higher resistance to starvation than their virgin female counterparts (Rush et al., 2007). Another explanation for the different response between mated and virgin females, could also be that mated females use their ovaries as an energy source in times of starvation and egg production decreases during nutritional challenges (Drummond-Barbosa and Spradling, 2001). To approach the question of why mated status affects the results of TRF, experiments should be undertaken looking at the effect of TRF on egg-laying behavior, quantity of eggs laid, and quality of eggs laid in TRF vs ALF animals at different points in life. In addition, dissecting and analyzing morphology and anatomy of the ovaries and embryos could also shed light on this matter.

A recent report also explored how TRF extended longevity. In agreement with our results, they found that TRF does not extend longevity in males of virgin females (Villanueva et al. 2019). They did, however, not use mated females in their experiments, which are the group responsive to TRF.

As we evaluated the role of TRF on sleep, we found that TRF increases day sleep in wild-type females, while we see an increase in night sleep in per^{0}. It is interesting to see that changing the timing at which food is available has such an impact on sleep. In inc^{1} mutants, sleep is unchanged by TRF, while longevity is markedly extended. This suggests that TRF's mechanism of lifespan extension in inc^{1} is independent of sleep, and the observed sleep effect in wild-type flies is likely correlative rather than causal. Alternatively, TRF could affect longevity through different mechanisms in wild-type *Drosophila* and *insomniac*.

The BBB is degraded in a number of neurodegenerative diseases as well as aging in humans, pointing to a role in longevity. We found that TRF transiently decreases BBB permeability after 14 days of TRF, which is dependent on an intact circadian clock. In contrast, TRF in clock mutants leads to BBB permeability increases, which is in line with their unchanged lifespan. While there is no effect on BBB permeability on wild-type flies after prolonged intervention, we see that there is a decrease in clock mutants after TRF. This phenomenon could be due to the late in life cycling of *moody*, an important BBB

GPCR in *Drosophila,* which shows increased cycling in old flies (Kuintzle et al. 2017). *Moody* or other important regulators of the BBB might be aided by TRF, but this is abrogated in clock mutants. Further evidence from mouse models show that the circadian clock is necessary for BBB integrity and deletion of BMAL, leads to BBB breakdown (Nakazato et al. 2017). Moreover, the invertebrate blood-brain barrier in *Drosophila melanogaster* also shows cycles with higher permeability at night under circadian regulation in addition to cycling expression of gap junctions (Zhang et al. 2018). These findings are consistent with our data; however, it will be important to distinguish whether BBB improvement is merely correlated or causal for TRF-mediated longevity improvement.

A three month TRF intervention in Huntington's disease (HD) mouse model improves locomotor activity, sleep, heart rate variability and motor performance, all hallmarks of HD in patients and mouse models (Wang et al. 2018). This is consistent with prior findings that TRF improves cardiac function in *Drosophila;* however, in this study they perform a short intervention in mice. It would be interesting to look at neurodegeneration markers in TRF v ALF as these animals continue to deteriorate. There is evidence that the brain vascularity increases and cleanses toxic metabolites and even beta amyloid in mice as they sleep (Xie et al. 2013). With these in mind and given that we have shown that TRF affects BBB permeability, we should further explore the role of TRF on brain physiology and in neurodegeneration.

While we showed that TRF extends longevity by setting a temporal restriction to food access, which coincides with the same period of time when the flies are more active, additional experiments must be done to continue building on this knowledge. One experiment, which will continue informing this question is to do TRF on per^S and per^L alleles of the *per* gene (Konopka and Benzer, 1971). To do these experiments one would need two incubators, one with a shorter day for per^S and one with and longer day for per^L. In the case of per^S, the daily light-dark cycle would be 19 hours-long with cycling food accordingly every 9.5 hours, while for per^L the incubator would be set up to a daily light-dark cycle every 28 hours, changing food every 14 hours. This experiment wasn't performed because the dealignment of the short, long, and the regular cycle of the experimenter would place extreme physical constraint or potential health hazard on the person performing this experiment.

Because of the high time demand that TRF experiments have, to increase the number of experiments that are simultaneously carried, and to be able to test different variations of the food cycling, we are currently developing an automated feeder system (S. Pletcher et al, *unpublished*) in collaboration with Scott Pletcher from University of Michigan. By automating the cycling of food, we will be able to perform high-throughput experiments, continue to test different variations of diets, cycle large number of flies for molecular biology studies, test longer or shorter daily rhythms with TRF, and elucidate the molecular mechanism of TRF.

To continue learning about how TRF extends longevity, we are currently doing tissue knockdown of the *tim* gene using different neuronal, ubiquitous and peripheral Gal-4 drivers (Brand and Perrimon, 1993), exposing flies to TRF or ALF, and scoring for longevity. The results of this experiment will inform as to whether TRF requires circadian clocks in neurons, in all tissues or only in peripheral tissues. If there is a specific tissue that is responsible for the effect of TRF, then the follow up to this study will be a tissue specific transcriptional analysis. In this experiment, we will perform TRF and ALF on mated females until the ALF population has decreased by 25%. We hypothesize that at this point, there will be transcriptional changes that are caused by TRF and could point to the specific molecular pathway that drives TRF life extension.

Time-restricted feeding offers benefits that have been well documented in basic organisms like *Drosophila,* mammalian models like rodents, and also as a part of clinical trials in humans. However, the biology of TRF and the long-term effects have not been studied. Here we show the beneficial effects of TRF in extending longevity. However, we must now focus on understanding the underlying biological mechanism by which this simple intervention can have such impactful effects.

Methods and Materials

Drosophila strains

Isogenic *(Iso1CJ) (Yin et al. 1994)* was used as wild-type. Circadian mutants per^{0} and tim^{01} (Konopka and Benzer, 1971; A. Sehgal et al., 1994) were used to study the effects of TRF on clock mutants. Sleep mutants inc^{1} *(Stavropoulos and Young 2011)*, *fmn (Kume et al. 2005)*, sss^{P1} (Koh et al., 2008), and $wake^{D1}$ and $wake^{D2}$ *(Liu et al. 2014)* were used to assay TRF on sleep mutants. All mutants were backcrossed to wild-type flies for at least 5 generations. UAS-TNT-E (RRID:BDSC_28838) and DJ651 (RRID:BDSC_8613) strains for accelerated longevity assay were obtained from Bloomington Drosophila Stock Center.

Longevity Assay

Animals were collected after eclosing and allowed to mate and age for 2 days in standard cornmeal vials. Subsequently, animals were lightly anesthetized with CO_2 and separated into vials with 30 animals of the same sex, which were kept in LD cycles at 25°C. Flies on TRF were transferred to vials with 1.1% agar at the end of the light part of the LD cycle, and transferred back to standard cornmeal at the begging of the light part of the LD cycle. Animals on ALF were transferred daily to fresh food. The number of dead animals was recorded every day. Experiments in constant light conditions were carried in an incubator at 25°C and lights on for the entirety of the experiment. Experiments in constant darkness were performed in dark incubator and animals manipulated with a safe light in dark room for scoring of survival and transfer to new

vials. Survival Log-rank (mantel-cox) tests were performed on Graphpad Prism to compare survival of TRF v ALF groups.

Sleep and Circadian Analysis

Single age matched animals from TRF experiments in LD were loaded into glass tubes containing standard cornmeal and assayed for four days in LD for sleep measurement and for five days in DD for rhythmicity using DAM5 monitors (Trikinetics). Locomotor data were collected in 1 min bins, and 5 min period of inactivity (Shaw et al. 2000; Huber et al. 2004) was defined as sleep. Sleep data were analyzed with custom software in Python (Axelrod S. *unpublished).* Dead animals were excluded from analysis either by the software or by visual inspection. Mann-Whitney tests were performed to compare sleep between TRF and ALF groups.

For period analysis in constant darkness, LD entrained animals were placed in glass tubes as stated above and placed in constant darkness. To assess rhythmicity and period length, data were binned at 30 min and analyzed by computing the autocorrelation function of the data, fitting the result with a four-term Fourier series. The period is given by the fundamental frequency of the Fourier series fit.

Feeding Assay

Feeding measurements were performed as described in (Murphy et al. 2017). 45 day old animals were loaded onto the ARC chambers, with capillary tubes containing 2.5%

yeast extract and 2.5% sucrose. Measurements of feeding and activity were recorded and analyzed as described by Murphy and colleagues. Mann-Whitney tests were performed to compare food intake between TRF and ALF groups.

Fly weight

To measure fly weight, we allowed all fly groups (ALF and TRF) to remain on fresh food for approximately 10 hours. Flies were separated into groups of 10 flies and weighted on a balance (Mettler AT20). Data reported are for quadruplicates of the average weight of 10 flies. Mann-Whitney tests were performed to compare weight between TRF and ALF groups.

Fly BBB injection

Modified from (Bainton et al. 2005) CO_2 anesthetized adult flies were injected with MPPI-3 pressure injector (Applied Scientific Instrumentation) with 1 mm borosilicate needles (FHC-Co) containing fluorescent dyes under a dissecting microscope. An average volume of 100 ± 25 nL dye per injection (range 70-130 nL) of dye was injected into the lateral thorax between wing socket and haltere. For all BBB permeability assessment we used 2.5 mM Dextran TexasRed (MW 10,000, Thermo Fisher D1863) Flies were allowed to recover for precisely 30 min before decapitation and imaging on a Zeiss LSM710 confocal microscope. Laser and acquisition settings remained unchanged for all samples in the same experiment. Stacks of 6-20 confocal slices of 16 µm were taken and maximum projection images generated using custom Metamorph

(Molecular Devices) script (gift from T. Tong, Bioimaging Resource Center, Rockefeller University). Average pixel intensity in the eye was measured using ImageJ software. For each experiment, data were normalized to experimental group with lowest average fluorescence.

Statistics

Statistical analysis was performed using GraphPad Prism. For comparisons between two genotypes, Student's T-tests were used. For comparisons between three or more genotypes, One-way ANOVAs with Dunn's post hoc tests were used. Log-rank (mantel-cox) tests were performed to compare survival between two or more groups.

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