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Research Report

The effects of reduced *rpd3* levels on fly physiology

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Abstract.
BACKGROUND: Rpd3 is a conserved histone deacetylase that removes acetyl groups from lysine residues within histones and other proteins. Reduction or inhibition of Rpd3 extends longevity in yeast, worms, and flies. Previous studies in flies suggest an overlap with the mechanism of lifespan extension by dietary restriction. However, the mechanism of *rpd3*'s effects on longevity remains unclear.
OBJECTIVES: In this study we investigated how *rpd3* reduction affects fly spontaneous physical activity, fecundity, and stress resistance.
METHODS: We examined the effects of *rpd3* reduction on fly spontaneous physical activity by using population monitors, we determined female fecundity by counting daily egg laying, and we determined fly survivorship in response to starvation and paraquat.
RESULTS: In flies, *rpd3* reduction increases peak spontaneous physical activity of $rpd3^{def}$ male flies at a young age but does not affect total 24 hour activity. Male and female $rpd3^{def}$ mutants are more resistant to starvation on low and high calorie diets. In addition, increased resistance to paraquat was observed in females of one allele. A decrease in *rpd3* levels does not affect female fecundity.
CONCLUSIONS: A decrease in *rpd3* levels mirrors some but not all changes associated with calorie restriction, illustrated by an increased peak of spontaneous activity in $rpd3^{def}$/+ heterozygous male flies but no effect on total spontaneous activity and fecundity.

Keywords: *rpd3*, dietary restriction, aging, *Drosophila melanogaster*

1. Introduction

Aging is characterized with progressive decline of physiological responses. Dietary restriction (DR) without malnutrition delays age-related pathophysiology and increases mean and maximal lifespan in a number of species [1, 2]. DR affects many physiological processes, including mitochondrial function, preserved protein homeostasis, enhanced genomic stability, increased insulin sensitivity, and nutrient metabolism. DR improves organismal health, and it delays the development of Type 2 diabetes, cardiovascular disease, neurodegeneration, and cancer. Similar health benefits have been observed in humans on a strict DR regimen without malnutrition, as well as in short-term, randomized clinical trials [3]. The effects of DR depend on the timing and particular nutrients in diet [2]. Some of the possible mediators of beneficial effects of DR include the nutrient-sensing insulin/insulin-like signaling (IIS), the Tor signaling pathway, AMPK, HSF, and sirtuins [2].

Members of class I of the zinc-dependent histone deacetylase (HDAC) family are vital regulators

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of chromatin structure and gene expression [4–6]. Class I HDACs have a role in development, as well as in pathophysiology of human disorders. Mutations that affect the normal function of HDAC1 have been implicated in development of human cancers, Alzheimer's, Huntington's, and Parkinson's diseases. Thus HDAC inhibitors have the potential to be used in therapy for cancer, neurodegenerative disorders, muscular atrophy, and heart disease [6–9]. Underexpression of Rpd3, the *Drosophila* HDAC1 homologue, is associated with a longer lifespan in yeast and fruit flies [10–15]. Males heterozygous for either the hypomorphic or null *rpd3* mutations live 33% and 41% longer compared to controls, respectively [11]. Female flies heterozygous for the hypomorphic allele have a 52% longer lifespan, while females carrying the null mutation have a longer maximum but not median lifespan [11]. Longer lifespan was also observed in flies fed 4-phenylbutyrate (PBA), a histone deacetylase inhibitor [16]. Others and our lab have shown that longevity extension associated with reduced *rpd3* overlaps with longevity effects of a dietary restricted diet [11, 13–15, 17, 18]. This hypothesis was supported by findings that DR and *rpd3* under-expression are not additive in their lifespan effects [11]. They also cause a similar change in transcription levels of *dSir2*, *takeout*, 4E-BP, and members of the insulin/insulin-like signaling (IIS) pathway such as *InR*, *chico,* and *dFoxo* [11, 13, 15, 17]. It was also reported that levels of *rpd3* mRNA are decreased and *dSir2* mRNA levels are increased in starved flies, while the opposite was found in flies when re-fed; they have increased levels of *rpd3* and reduced *dSir2* mRNA levels [19]. Flies mutant for both *rpd3* and *dSir2* live shorter compared to *rpd3* mutant flies, suggesting that dSir2 mediates some effects of *rpd3*'s effects on fly physiology and longevity [13]. However, when longevity studies were done on diets with varying caloric content, *rpd3* mutant flies live longer on some DR diet levels suggesting that *rpd3* and DR have distinct but interacting effects on fly longevity [14]. This suggests that the relationship between the mechanism of longevity observed in *rpd3* flies and DR is more complex than previously thought.

2. Materials and methods

2.1. Fly strains and maintenance

rpd3-deficient ($rpd3^{def24}$*/TM6,Sb)* and *rpd3–* hypomorphic ($rpd3^{P\text{-}UTR}$*/TM3,Sb,Ser*) flies and their genetic controls, were used in the experiments. Heterozygous $rpd3^{P\text{-}UTR}$/+ and $rpd3^{P\text{-}1.8}$/+ flies were F1 generation from the crosses between male $rpd3^{P\text{-}UTR}$*/TM3,Sb,Ser* or $rpd3^{P\text{-}1.8}$*/TM6,Tb* and *CS* virgin females. Male $rpd3^{def24}$*/TM6,Sb* flies were crossed to *yw* females. Genetic controls for $rpd3^{def}$ were progeny generated by crossing F1 $rpd3^{def}$*/yw* littermates. *CS* and *yw* flies are wild type for the *rpd3* gene, thus flies heterozygous for $rpd3^{def24}$/yw, $rpd3^{P\text{-}UTR}$/CS and $rpd3^{P\text{-}1.8}$/CS are referred as $rpd3^{def}$/+, $rpd3^{P\text{-}UTR}$/+, and $rpd3^{P\text{-}1.8}$/+ in the text. The hypomorphic $rpd3^{P\text{-}UTR}$ allele has a P-element inserted in the 5'UTR region of the *rpd3* gene, which affects expression throughout the fly's body. The control $rpd3^{P\text{-}1.8}$ ($rpd3^{P\text{-}1.8}$*/TM6,Tb*) allele has a P-element inserted 1.8 Kb upstream from the transcriptional start site, which only decreases expression in the eye [20]. *Canton S* and *yw* were kindly provided by the Bloomington Stock center. Flies were collected within 24 hours of eclosion and maintained using standard corn culture media, low calorie (0.5 N, 0.7 N) or high calorie (1.5 N) diet in plastic vials. The caloric content of 0.5 N food is 50% that of the 1.0 N food. Flies were kept at 25°C in a humidified incubator with a 12 hour light-dark cycle. 25 males and 25 females are kept together in each vial, and they are passed to a fresh vial every Monday, Wednesday, and Friday. Fly diets were prepared as previously reported [21].

2.2. Spontaneous physical activity

Flies were aged until 10 or 40 days of age on a corn diet. 10 males or 10 females were placed in separate glass vials containing culture media. 3 vials per $rpd3^{def}$/+ and their controls, and 4 vials per $rpd3^{P\text{-}UTR}$/+ and $rpd3^{P\text{-}1.8}$/+ control were used. These vials were placed in mobility monitors that counted every time a fly passed the top, middle, or bottom of the vial. Data were taken at the start of the following light cycle to assure the flies had acclimated to the new environment [21].

2.3. Fecundity

Male $rpd3^{P\text{-}UTR}$*/TM3,Sb,Ser* or $rpd3^{P\text{-}1.8}$*/TM6,Tb* were crossed to *CS* virgin females. 1 female virgin of the same genotype (*CS*, $rpd3^{P\text{-}UTR}$*/CS* or $rpd3^{P\text{-}1.8}$*/CS)* was collected on CO_2 and placed in individual plastic vials containing 0.5 N or 1.5 N agar medium [22, 23]. The flies were passed to new vials

daily and the number of eggs was counted. 10 vials were used for each genotypes and each diet.

2.4. Starvation and paraquat resistance

Flies were collected as described above and aged until 10 or 40 days of age. They were separated into vials of 20 males or 20 females and transferred into new vials containing 2 filter papers with 300 μl of DI H_2O for starvation studies. For paraquat resistance 20 flies were transfer into a vial containing filter paper soaked with 300 μL of 20 mM paraquat following initial starvation for 6 hours [24]. The number of dead flies was counted hourly during the day and twice overnight. Stress resistance data were analyzed by log-rank tests using the JMP 12 program. The total number of flies per experiment is listed in Tables 3 and 4.

3. Results

3.1. The effect of rpd3 reduction on fly spontaneous locomotor activity

To test if the mechanism of *rpd3* reduction on fly longevity is similar to DR we investigated various aspects of *Drosophila* physiology in *rpd3* mutant flies and their genetic controls. We used two different heterozygous *rpd3* alleles due to embryonic lethality of homozygous *rpd3* mutant flies. We used *rpd3* deficient, an *rpd3* null line, ($rpd3^{def}$/+) and their genetic control, F1 progeny of $rpd3^{def}$/+ littermates. The levels of *rpd3* mRNA in $rpd3^{def}$/+ is 50% lower compared to controls. We also used $rpd3^{P\text{-}UTR}$/+ flies, an *rpd3* hypomorph, and $rpd3^{P\text{-}1.8}$/+, which are their genetic controls and have *rpd3* reduction only in the eyes [20]. The presence of one copy of a P-element in $rpd3^{P\text{-}UTR}$/+ flies results in 60% of *rpd3* mRNA levels compared to heterozygous $rpd3^{P\text{-}1.8}$/+ control flies [11]. Locomotor activity reflects the functional activity of the nervous system. There is an age-related decline in the locomotor activity of flies [21, 22]. One of the hallmarks of DR is increased spontaneous locomotor activity, and it is observed in multiple species [21, 22, 25, 26]. This is thought to be due to an increased scavenging for food. In mice and flies Sirt1 and dSir2 mediate the increased locomotor activity associated DR, respectively [25–27]. To test if there is an overlap in the mechanism of DR and *rpd3* mutation, we examined the spontaneous physical activity of *rpd3* mutants and their controls. We used population monitors, which allow continuous monitoring of spontaneous physical activity of the population of 10 flies during longer time periods [21]. At 10 days of age, we found an increase in peak activity in $rpd3^{def}$/+ male flies (Fig. 1A, Table 1). However, at an older age the peak activity in male $rpd3^{def}$/+ flies was not different compared to controls (Fig. 1B, Table 1). Total 24 hour activity was not different at age 10 or 40 (Fig. 1C). Females $rpd3^{def}$/+ flies showed little difference compared to their controls at 10 days of age (data not shown). A similar tendency toward increased peaks of spontaneous activity but no significant effect on the peak or on total 24 hour activity was observed in $rpd3^{P\text{-}UTR}$/+ male and female flies when compared to controls at 10 days of age (Fig. 1D-F).

3.2. The effect of reduced rpd3 on fly fecundity

We have previously reported that *rpd3* reduction does not affect egg production in virgin $rpd3^{P\text{-}UTR}$ /+ compared to $rpd3^{P\text{-}1.8}$ /+ female flies aged on a regular diet [11]. DR reduces fly fecundity due to re-allocation of the resources from reproduction to maintenance and repair [22, 28]. Here we examined if feeding $rpd3^{P\text{-}UTR}$ /+ flies a high (1.5 N) or a low (0.5 N) calorie diet would affect their fecundity. We counted the number of eggs laid by flies heterozygous for wild type *Canton S* (*CS)* and $rpd3^{P\text{-}UTR}$ /+ or $rpd3^{P\text{-}1.8}$/+ (genetic control) daily during the first 10 days of life. *CS* female flies were included for an additional comparison since they share 50% of the genetic background, and to illustrate the negative effect of 0.5 N diet on female fecundity. If *rpd3* reduction mimics DR completely, we would expect $rpd3^{P\text{-}UTR}$/+ females to lay fewer eggs compared to controls on a 1.5 N diet, and even fewer on a 0.5 N diet. However, there was no difference in total egg number laid by $rpd3^{P\text{-}UTR}$/*CS* females compared to $rpd3^{P\text{-}1.8}$ on 1.5 N diet, and only a minor tendency toward a lower number on 0.5 N diet ($p = 0.053$) (Fig. 2A, B, Table 2). Furthermore, no differences in fecundity between $rpd3^{P\text{-}UTR}$/*CS* and *CS* were observed. Thus *rpd3* reduction does not affect female fecundity on either 0.5 N or 1.5 N diet.

3.3. Reduction in rpd3 increases starvation resistance of male and female flies

Increased starvation resistance is a phenotype found in many long-lived animals, yet its modification by DR remains a debatable topic. The first

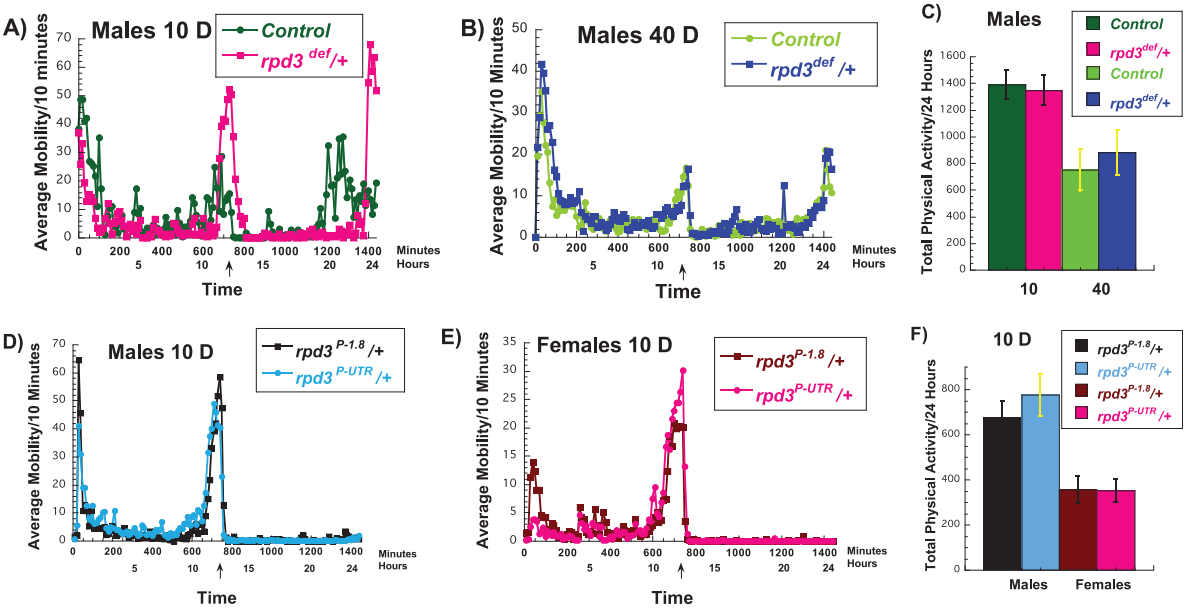

Fig. 1. Effects of *rpd3* mutations on fly spontaneous physical activity. (A-C) Average (A, B) and total (C) 24 hours spontaneous physical activity of $rpd3^{def}$/+ male flies and their genetic controls (+/+) at 10 (A) and 40 (B) days of age. (A-C) $rpd3^{def}$/+ male flies have increased peak spontaneous physical activity compared to their genetic controls but the total locomotor activity of $rpd3^{def}$/+ male flies over the course of 24 hours is simialr to their genetic controls at age 10 and 40 days. (D,E) Average spontaneous physical activity of $rpd3^{P\text{-}UTR}$/+ male (D) and female (E) flies and their genetic controls $rpd3^{P\text{-}1.8}$/+ at 10 days of age. (F) The total spontaneous physical activity of $rpd^{P\text{-}UTR}$/+male and female flies and their genetic controls $rpd3^{P\text{-}1.8}$/+ over the course of 24 hours at age 10 days. (A, B, D, E) The data are collected in 10 minutes bins and represent an average of sponteneous locomotor activity of three biological replicates of 10 female or 10 male flies (each replicate) of experimental ($rpd3^{def}$ /+ (A-C); $rpd3^{P\text{-}UTR}$ (D-F)) or control (+/+(A-C); $rpd3^{P\text{-}1.8}$ (D-F)) flies. An asterix on the x-axis marks the time when the light was switched off and the transition to dark cycle (6 : 00PM or 720 minutes).

Table 1
Spontaneous physical activity of $rpd3^{def}$ and $rpd3^{P\text{-}UTR}$ heterozygous flies and controls

Gender	Genotype	Age	Peak activity						Total activity	(SE)
			1	*p*	2	*p*	3	*p*		
M	$rpd3^{def}$/+	10	141		388		192		1347	112
M	*Control*	10	223	0.3	171	0.004*	90	0.18	1390	305
M	$rpd3^{def}$/+	40	193	85	93	879	172			
M	*Control*	40	154	0.28	98	0.57	76	0.58	749	156
M	$rpd3^{P\text{-}UTR}$/+	10	116	328	5	775	93			
M	$rpd3^{P\text{-}1.8}$/+	10	137	0.6	331	0.95	6	0.08	677	79
F	$rpd3^{P\text{-}UTR}$/+	10	14	199	0	351		51		
F	$rpd3^{P\text{-}1.8}$/+	10	49	0.08	153	0.5	0	1.0	355	59

The peaks and the total spontaneous physical activity of $rpd3^{def}$/+ and their genetic controls+/+, $rpd^{P\text{-}UTR}$/+ and their genetic controls $rpd3^{P\text{-}1.8}$/+. The peak 1 is sum of the total activity between 6AM–7AM, peak 2 between 5PM–7PM, and 3 between 5AM–6AM. The total spontenaous activity is sum of the activity over the course of 24 hours at age 10 or 40 days. The data were collected for at least three biological replicates of 10 female or 10 male flies (each replicate) of experimental or control flies. Student *T* test analysis was used to determine the difference in the peaks of activity. *= a statistical significant result.

Drosophila DR experiment found that both genders of flies had increased starvation resistance on low-calorie food, yet the increase was not as great in males [28]. Reducing the calorie content in food, especially protein, increases starvation resistance in flies due to lipid accumulation [28, 29, 30]. Nevertheless, extended DR reduces starvation resistance in flies [28]. In addition, some studies indicate that genetic manipulation that mimics DR decreases starvation resistance because of the reduction in energy

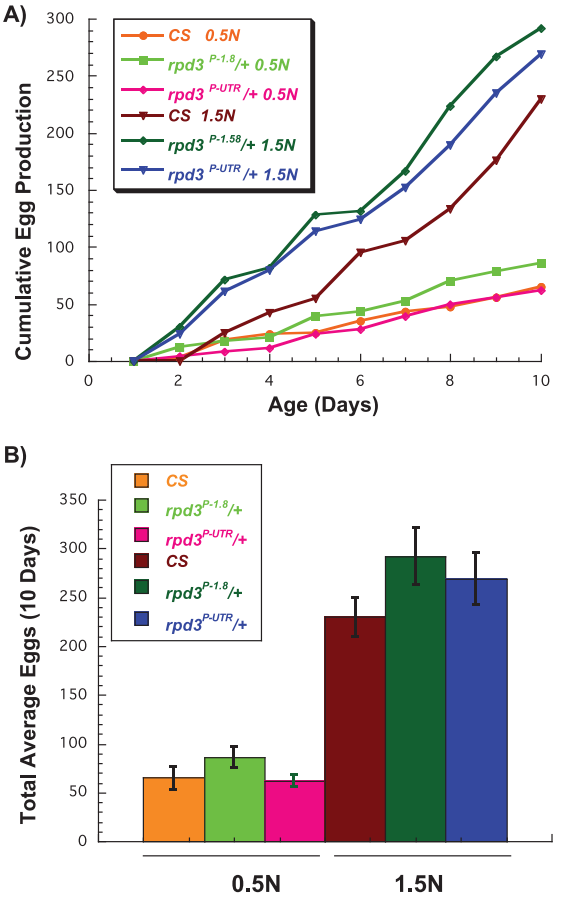

Fig. 2. Effect of *rpd3* reduction on fly fecundity. (A) The average number of eggs per day laid by wild type *Canton-S (CS)*, $rpd3^{P\text{-}UTR}$/*CS*, and genetic controls $rpd3^{P\text{-}1.8}$/CS on 0.5 N and 1.5 N diets. (B) The total number of eggs per 10 day period laid by *CS*, $rpd3^{P\text{-}UTR}$/*CS*, and $rpd3^{P\text{-}1.8}$/CS on 0.5 N and 1.5 N diets. $rpd3^{P\text{-}UTR}$/*CS* flies laid similar numbers of eggs on 1.5 N but lower numbers on 0.5 N compared to the controls, a difference that did not reach significance ($p = 0.053$). 10 vials with a single male and a single female were used per genotype and diet.

Table 2
Effect of *rpd3* reduction on female fecundity

Genotype	Food	Total eggs	SE
$rpd3^{P\text{-}UTR}$/+	0.5 N	62	6.2
$rpd3^{P\text{-}1.8}$/+	0.5 N	86	10.9
CS	0.5 N	65	11.6
$rpd3^{P\text{-}UTR}$/+	1.5 N	269	26.1
$rpd3^{P\text{-}1.8}$/+	1.5 N	292	28.7
CS	1.5 N	230	20.0

The total number of eggs per 10 day period laid by *CS*, $rpd3^{P\text{-}UTR}$/*CS*, and $rpd3^{P\text{-}1.8}$/*CS* flies aged on 0.5 N or 1.5 N diets. 10 vials with a single male and a single female were used per genotype and diet.

stores [31]. $rpd3^{def}$/+ and control (+/+) flies were raised on different food levels until 10 or 40 days of age and then exposed to starvation. We used 0.7 N as a DR diet and 1.5 N as a high calorie diet. We first compared the starvation resistance of aging control flies on 0.7 N to controls aged on 1.5 N food. At 10 days of age male and female control flies aged on 1.5 N food had a higher starvation resistance compared to flies kept on 0.7 N food (Fig. 3A,B, Table 3). However, at 40 days of age, there were no differences in starvation resistance for flies aged on 0.7 N or 1.5 N food (Fig. 3C,D, Table 3). Another interesting observation was that control male flies aged on 0.7 N or 1.5 N had a much lower starvation resistance compared to control females aged on the same food at 10 days of age. However, both male and female control flies had an almost identical starvation resistance at 40 days of age regardless of the content of the food on which they were aged (Fig. 3E). Male and female $rpd3^{def}$/+ flies were more resistant to starvation than +/+males at 0.7 N and 1.5 N (Fig. 3A-D, Table 3). In addition, starvation resistance in female $rpd3^{def}$/+ was much higher compared to males at both 10 and 40 days of age. Thus *rpd3* reduction increases starvation resistance in $rpd3^{def}$/+ flies when aged on a standard, a low, or a high calorie diet.

3.4. *The effect of reduced rpd3 mRNA levels on paraquat resistance*

Increased resistance to oxidative stress is often associated with increased longevity and it has been suggested that improved stress response could be one of the contributors to longer life [24]. DR does not affect oxidative stress resistance of young flies but has a negative effect on aged flies [28]. We have reported that *rpd3* reduction increases resistance to hydrogen peroxide, but this resistance depends on fly age and sex. Resistance is higher in females compared to males, and more pronounced at older ages [18]. Increased resistance to paraquat is also observed in flies with heart-specific *rpd3* reduction, as well as whole body reduction in 2 days old flies [15]. However, the effect of whole body *rpd3* reduction on paraquat resistance during aging has not been determined. There is an age-associated decline in resistance to paraquat in wild type flies, thus we examined if *rpd3* reduction could postpone the known age-associated decline in resistance [32]. The response of $rpd3^{def}$/+ and $rpd3^{P\text{-}UTR}$/+ flies to paraquat depends on fly sex, age, and allele.

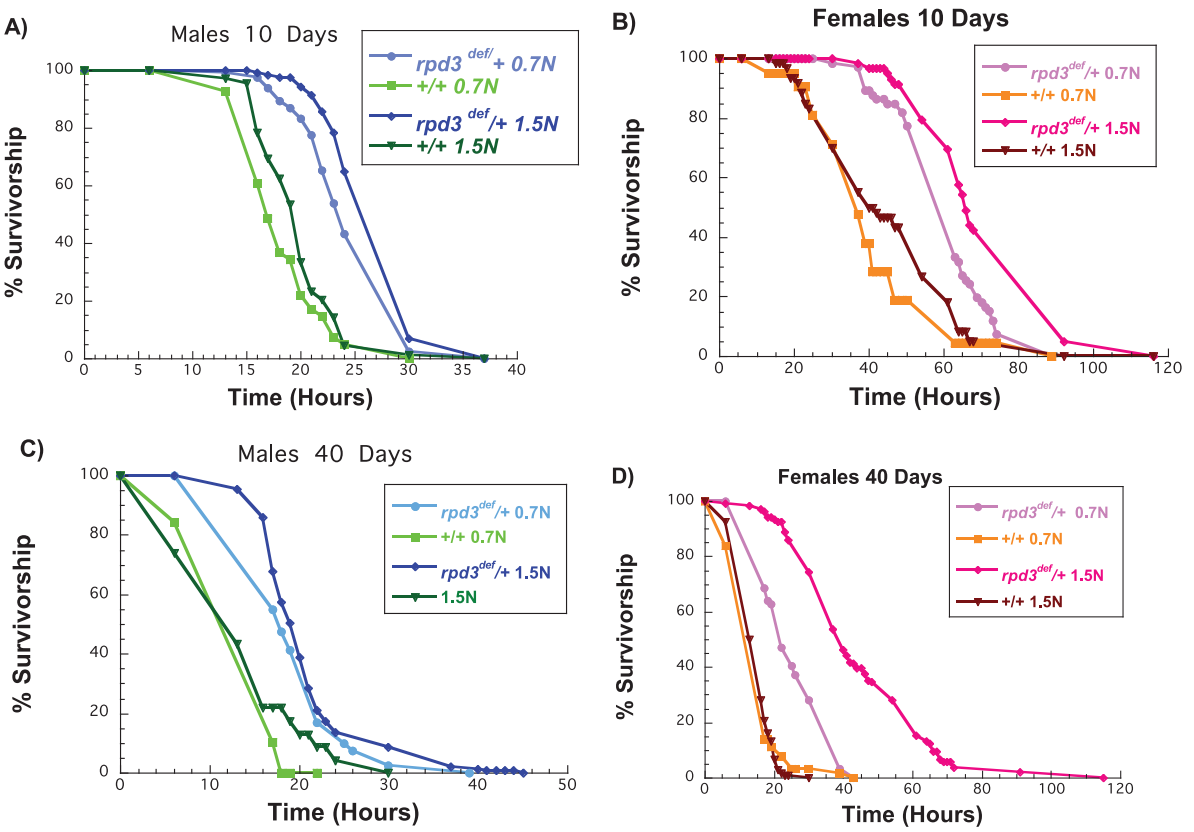

Fig. 3. Reduction of *rpd3* increases resistance to starvation of male and female flies on a low and a high calorie diet. (A, B) Survival curves of $rpd3^{def}$/+ and +/+ male (A) and female (B) flies aged on 0.7 N or 1.5 N food levels and exposed to starvation at 10 days of age. (C, D) Survival curves of $rpd3^{def}$/+ and+/+male (C) and female (D) flies aged on 0.7 N or 1.5 N food levels and exposed to starvation at 40 days of age.

$rpd3^{P\text{-}UTR}$/+ females had increased resistance to paraquat at both 10 and 40 days, while males had no difference compared to controls (Fig. 4A, B). In contrast, $rpd3^{def}$/+ females were less resistant to paraquat at 10 days while there was no difference at age 40. There was no difference in resistance to paraquat in $rpd3^{def}$/+ males at both ages (Fig. 4C, D). These data illustrate how genetic background, level of *rpd3* reduction, age, and sex affect fly resistance to paraquat.

4. Discussion

DR has multiple effects on physiology. In flies, DR reduces fecundity, increases fly spontaneous physical activity, increases mitochondrial biogenesis, and slows down age-related mortality [22, 28, 33]. The effects of *rpd3* reduction on lifespan in flies appear to at least partially overlap with the effects of DR, as DR and *rpd3* whole body under-expression share a key effect on dSir2, *takeout*, *chico*, *InR,* and *dFoxo* levels of transcription [11, 13–15, 17–19]. Heart-specific *rpd3* reduction increases the levels of *Sod2, dSir2, dFoxo, Thor*, and *DptB* in 2 day old flies [15]. dSir2 has been thought to mediate longevity extension in DR flies, illustrated by a lack of DR longevity effects in *dSir2* mutant flies. Flies double mutant for *dSir2* and *rpd3* live shorter compared to single *rpd3* mutant flies suggesting that dSir2 mediates at least partially the longevity effect in *rpd3* flies [13]. In addition, *rpd3* reduction and DR are not additive in their effects on lifespan [11]. Therefore, it was hypothesized that the mechanism of longevity extension in DR and *rpd3* flies is the same or related [11, 13]. However, *rpd3* mutants live longer under conditions close to starvation and during starvation, suggesting that DR and *rpd3* may have distinct but interacting or overlapping effects on fly longevity [14, 18]. To gain further insight into the mechanism of lifespan extension observed in *rpd3* flies, we determined if similar

Table 3
Starvation resistance of $rpd3^{def}$/+ heterozygous flies and genetic controls

Gender	Genotype	N	Age	Food	Mean LS (% change)	X^2	p
M	$rpd3^{def}$/+	115	10	0.7 N	25	66.054	<0.0001*
M	*Control*	41	10	0.7 N	19(–31.5)		
F	$rpd3^{def}$/+	104	10	0.7 N	45	15.075	<0.0001*
F	*Control*	8	10	0.7 N	38(–18.4)		
M	$rpd3^{def}$/+	126	10	1.5 N	28	124.6	<0.0001*
M	*Control*	69	10	1.5 N	20 (–40)		
F	$rpd3^{def}$/+	117	10	1.5 N	75	95.77	<0.0001*
F	*Control*	100	10	1.5 N	46 (–63)		
M	$rpd3^{def}$/+	82	40	0.7 N	20	23.267	<0.0001*
M	*Control*	19	40	0.7 N	15 (–33)		
F	$rpd3^{def}$/+	89	40	0.7 N	26	44.512	<0.0001*
F	*Control*	63	40	0.7 N	17 (–52)		
M	$rpd3^{def}$/+	137	40	1.5 N	21	23.01	<0.0001*
M	*Control*	23	40	1.5 N	14 (–50)		
F	$rpd3^{def}$/+	136	40	1.5 N	45	261.77	<0.0001*
F	*Control*	106	40	1.5 N	15 (–200)		

The mean lifespan of $rpd3^{def}$/+ and genetic control (+/+) heterozygous male (M) and female (F) flies after exposure to starvation at 10 or 40 days of age. The flies were aged on 0.7 N or 1.5 N food levels before being exposed to starvation as indicated. Control values are compared to either male or female $rpd3^{def}$/+ groups to determine the percent change in mean lifespan. Mean lifespans are in hours. Log-rank analyses were performed using the JMP 12 program. M = Males, F = Females, N = number of flies in the experiment. *= a statistical significant result.

Table 4
$rpd3^{P\text{-}UTR}$ but not rpd3^{def} heterozygous female flies have higher paraquat resistance compared to the control flies in the same genetic backgrounds

Gender	Genotype	N	Age	Mean (% change)	X^2	p	Maximal lifespan (% change)
M	$rpd3^{def}$/+	71	10	24	8.7859	<0.0030*	43
M	*Control*	51	10	20 (–20)			25 (–72)
F	$rpd3^{def}$/+	70	10	31	9.0113	<0.0027*	67
F	*Controls*	94	10	39 (21)			70 (4)
M	$rpd3^{def}$/+	49	40	11	0.5829	0.4633	17
M	*Control*	17	40	12 (0.8)			17 (0)
F	$rpd3^{def}$/+	28	40	16	0.0192	0.8898	24
F	*Controls*	55	40	17 (0.5)			25 (0)
M	$rpd3^{P\text{-}UTR}$/+	172	10	32	7.0464	0.0079*	54
M	$rpd3^{P\text{-}1.8}$/+	107	10	36 (11)			66 (18)
F	$rpd3^{P\text{-}UTR}$/+	172	10	52	29.548	<0.0001*	84
F	$rpd3^{P\text{-}1.8}$/+	121	10	42 (–24)			66 (–27)
M	$rpd3^{P\text{-}UTR}$/+	300	40	16	7.757	<0.0053*	18
M	$rpd3^{P\text{-}1.8}$/+	306	40	17 (0.5)			19 (0.5)
F	$rpd3^{P\text{-}UTR}$/+	160	40	24	94.416	<0.001*	42
F	$rpd3^{P\text{-}1.8}$/+	169	40	17 (–41)			24 (–75)

The mean and maximal lifespan of $rpd3^{def}$/+ and genetic control (+/+), and $rpd3^{P\text{-}UTR}$/+ and their genetic control ($rpd3^{P\text{-}1.8}$/+) heterozygous male (M) and female (F) flies after exposure to paraquat at 10 or 40 days of age. Control values are compared to either male or female $rpd3^{def}$/+ groups or $rpd3^{P\text{-}UTR}$ to determine the percent change in mean and maximal lifespan. Mean and maximal lifespan are in days. N = number of flies in the experiment. Log-rank analyses were performed using the JMP 12 program. *= a statistical significant result.

changes in physiology associated with DR can be observed in flies with reduced *rpd3* levels. Particularly, we investigated the effects of decreased levels of *rpd3* on spontaneous physical activity, fecundity, and stress resistance. $rpd3^{def}$/+ male flies had an increased peak in spontaneous activity at a younger age most likely mediated by increased dSir2 levels. Fly spontaneous physical activity decreases with

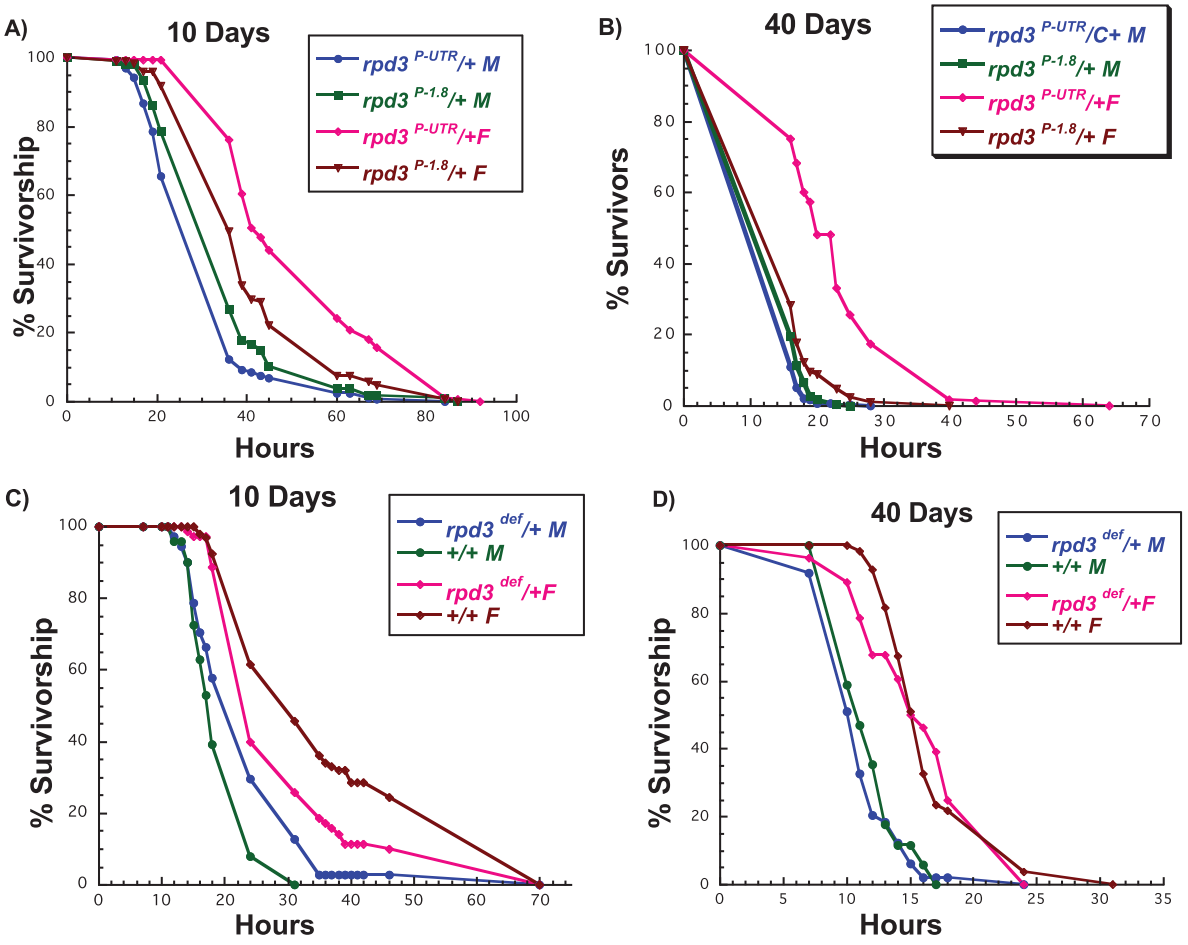

Fig. 4. *rpd3* reduction affects stress resistance in flies. (A, B) Survival curves for male and female $rpd3^{P\text{-}UTR}$/+ (experimental) and $rpd3^{P\text{-}1.8}$/+ (control) flies exposed to 20 mM paraquat at age 10 (A) or 40 (B) days. $rpd3^{P\text{-}UTR}$/+ females are more resistant to paraquat at 10 and 40 days, but no changes in resistance were observed in male $rpd3^{P\text{-}UTR}$/+ flies. (C, D) Survival curves for male and female $rpd3^{def}$/+ and control flies exposed to 20 mM paraquat at age 10 (C) or 40 (D). Male $rpd3^{def}$/+ flies are more resistant and female $rpd3^{def}$/+ are less resistant compared to control flies at age 10. No difference in paraquat resistance was observed between $rpd3^{def}$/+ male and female and control flies at 40 days.

age, and so do the differences in the peak activity. No difference was observed in $rpd3^{def}$/+ males at age 40. Although peak activity appears to be associated with *rpd3* reduction in male $rpd3^{def}$/+ flies at a younger age, no clear difference in peak or total spontaneous physical activity was associated with *rpd3* reduction in $rpd3^{def}$/+ females or $rpd3^{P\text{-}UTR}$/+ male and female flies. The two *rpd3* alleles have different levels of *rpd3* mRNA that could explain difference in the peak of spontaneous physical activity or resistance to paraquat. While *rpd3* mRNA expression is reduced to 50% in the $rpd3^{def}$/+ heterozygous flies compared to controls, the level of *rpd3* mRNA is reduced by 40% in $rpd3^{P\text{-}UTR}$/+ compared to $rpd3^{P\text{-}1.8}$/+ flies. The levels of *rpd3* mRNA are linked to different physiological consequences, as well as longevity effects. For instance, while males of both *rpd3* alleles and female $rpd3^{P\text{-}UTR}$/+ flies have dramatically extended longevity, $rpd3^{def}$/+ females only have extended maximal lifespan but not median lifespan [11]. Similarly, different effects on starvation resistance were observed in flies with whole body *rpd3* reduction using *RNAi* mediated silencing compared to reduction only in fly heart tissue [15]. Likewise, the two *rpd3* alleles respond differently to paraquat. However, we show that H_2O_2 and starvation resistance were similar in both *rpd3* alleles (discussed below).

DR has a negative effect on egg production in female flies due to a shift in use of available

resources from reproduction to maintenance [25, 28]. If *rpd3* reduction mimics DR we would expect lower fecundity in *rpd3* females aged on a DR diet. However, no difference in fecundity was associated with *rpd3* reduction. No difference in fecundity could be explained by higher energy storage and/or increased adaptation to DR found in *rpd3* flies, as discussed below [18].

The effects on starvation and oxidative resistance caused by DR of wild type flies changes with age [28]. While DR increases starvation resistance early in life, the opposite was found in aged DR flies [28]. Increased resistance to starvation of young flies is associated with increased lipid content. However, this increased lipid storage is not sustained over longer time, which results in lower starvation resistance in older flies [28]. We previously reported that $rpd3^{def}$/+ and $rpd3^{P\text{-}UTR}$/+ flies have increased starvation resistance [18]. Heart-specific *rpd3* reduction also increases starvation resistance in flies at 2 days of age [15]. Nevertheless, the same authors did not find increased starvation resistance in flies when *rpd3* was reduced in other tissues. Here we examined how aging *rpd3* or control flies on 0.5 N or 1.5 N affect their starvation resistance. Our control flies aged on 1.5 N food had a small increase in starvation resistance compared to flies aged on 0.5 N food at an early age. However, no difference in starvation resistance between control male or female flies was observed at age 40 days, and diet did not have an effect at this age either. We observed increased starvation resistance in both *rpd3* alleles, with females showing a larger resistance compared to male *rpd3* flies. An increase in lipid storage caused by an increase in the activity of the enzymes associated with lipid biogenesis is often associated with increased starvation resistance [28]. Increased metabolic reserves in the form of both lipids and carbohydrates are associated with increased starvation resistance in an outbred population of *Drosophila melanogaster* [34]. We recently reported that *rpd3* reduction is associated with increased levels of triglycerides before and after starvation, which is at least partially mediated by reduced IIS [18]. Consistently, flies with ablated insulin-producing cells have increased levels of lipids and starvation resistance [35]. Flies with reduced *rpd3* levels have also increased levels of glucose, glycogen, and trehalose at older ages, illustrating increased energy storage [18]. It is also possible that increased resistance to starvation observed in *rpd3* flies results from metabolic adaptation to nutrient availability. Such changes could be mediated by changes in acetylation of metabolic enzymes, which could affect their enzymatic activity allowing a shift from one metabolic pathway to another [36, 37]. It was shown that histone modifying enzymes link changes in nutrient availability to changes in intermediary metabolism by affecting the activity of the enzymes involved in glycogenesis, glycolysis, gluconeogenesis, and β-oxidation through acetylation [36, 37].

DR has no effect on oxidative stress resistance early in life but decreases resistance to paraquat as flies age [28]. We showed that *rpd3* reduction increases resistance to oxidative stress, as measured by increased resistance to H_2O_2. This resistance is mediated by reduced IIS, illustrated by increased *dFoxo* mRNA levels found in *rpd3* mutant flies, and lack of increased resistance to H_2O_2 and shorter lifespan observed in flies double mutant for *dFoxo* and *rpd3,* compared to single *rpd3* mutant flies [18]. This is in agreement with findings that overexpression of nuclear localized dFoxo mediates increased resistance to oxidative stress in flies [38]. Furthermore, flies treated with PBA, a HDAC1 inhibitor, have increased expression of genes that have been implicated in response to oxidative stress such as glutathione S-transferase and SOD [16]. PBA-treated flies have increased climbing activity, increased resistance to starvation and to paraquat, and extended lifespan [16].

Several findings presented here further confirm that *rpd3* reduction extends longevity by a mechanism that partially overlaps with DR but has also distinct characteristics. $rpd3^{def}$ male flies have an increased peak of spontaneous physical activity. However, *rpd3* flies have no changes in fecundity, total spontaneous physical activity, and are more resistance to starvation and oxidative stress compared to controls. Our data illustrates how complex interactions between reduced *rpd3* levels and its downstream targets results in various phenotypic changes at the organismal level.

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