Original Article

Repeated Starvation Leads Fat Accumulation in *Caenorhabditis elegans*.

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Abstract

Our aim is to define the effect of repeated-starvation on organism using nematode *Caenorhabditis elegans*. Adult worms were exposed to two cycles of 6 hr starvation-18 hr feeding protocol, and fat content in worms was analyzed by Nile Red staining and biochemical quantification. The expression of genes involved in fat synthesis (*pod-2*, *fasn-1*, *mboa-2*, *sbp-1*) and fat degradation (*hosl-1*, *lipl-4*, *cpt-1*, *cpt-2*, B03003.3, F53a2.7) was also analyzed by quantitative RT-PCR. Both Nile Red staining and biochemical quantification showed that fat content in worms that experienced repeated-starvation increased. There was no obvious change in the expression of genes involved in fat synthesis, but those of genes involved in fat degradation tended to decrease, which is consistent with the increment of fat in worms experienced repeated-starvation. The lifespan, fecundity and mobility of worms that experienced repeated-starvation did not show remarkable differences compared to those of the fed worms. However, the pharyngeal pumping increased upon experiencing starvation, indicating amount of food intake increased by starvation. Our results indicate that repeated-starvation caused metabolic and nutritional effect in worms. It is often mentioned that repeating weight loss leads to weight regain, sometimes referred as "weight rebound", and our data may also provide a molecular basis of weight regain.

Key words: repeated starvation, *Caenorhabditis elegans*, fat accumulation

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Introduction

It is an ideal situation for any organism if the organism could obtain adequate amount of food at appropriate timing. However, during the evolutionary course, it is almost sure that no organism had met such an ideal situation. Instead, during the evolutionary course, organisms, especially animals could only obtain food scarcely, irregularly and inadequately. Sometimes, animals had to spend certain length of time without any food, namely starvation. This had led the fasting and starvation the most universal and common stress for almost all organisms from higher mammalian, including *homo sapiens*, to nematode and protozoan.

Even in this modern world including advanced industrialized countries, starvation still exists due to poverty, climate instability, war and so on[1]. One of the characteristic aspects of nutritional problem in the advanced industrialized countries is unhealthy conditions derived from irregular lifestyle. The life in the countries has become to run under 24 hr cycles, and difference in lifestyle between day and night became vague. This situation made life activity such as eating, sleeping, exercising and working irregular and sporadic, and the impact of these irregularity in lifestyle has become evident, accelerating the risk of lifestyle-related diseases such as diabetes, cardiovascular disease and hypertension[2, 3]. It has previously been considered that once such irregular lifestyle was improved, the physical and mental conditions would also regain fitness. But how such irregular lifestyle affects physical and mental health in long-term is not well known.

Another example of irregular lifestyle is attempting weight loss, especially among young women. These weight loss attempts are often motivated not by the necessity from the medical point of view, but by the strong conscience to body weight and body shape driving people to attempt weight loss repeatedly, and one of the problems associated with the repeated weight loss is "weight regain". Weight regain, sometimes referred to as "weight rebound", is the phenomena that, when weight loss attempt was unsuccessful, the body weight increased more than the starting weight[4, 5]. Since attempting weight loss and keeping body weight is challenging, repeating weight loss is not uncommon among people attempting weight loss, resulting those attempters to experience irregular and repetitive eating/fasting cycle[6, 7]. This "irregular and repetitive eating/fasting cycle" can be considered

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as another example of irregular lifestyle which may contribute to the unhealthy condition among advanced countries. But again, if and how repetitive weight loss attempt affects our physiological and biochemical condition is largely unknown.

To clarify the effect of irregular eating/fasting cycle on organism, we employed nematode *Caenorhabditis elegans* as model organism, and treated the worm with repeated starvation, mimicking the irregular eating/fasting status. The worms have been shown to be an effective system to study development, nutrition, growth arrest and starvation[8–12]. One of the reasons for choosing the worm as model is that, because starvation study risks the subjects to malnutrition and nutrients deficiency, mammalian models such as rats and mice, not to mention human, may not be permitted to use from ethical standpoint. In addition, as described above, because starvation is considered as universal stress to many animals regardless of the taxonomical difference, results obtained in the worm may be expected to be applicable to mammals. Mammals are known to adapt to scarcity of food by metabolic-level such as promoting fat degradation, ketone body synthesis and gluconeogenesis, as well as by organ/tissue-level such as arresting growth, delaying sex maturation and suppressing reproductive activity[13–16]. Some, but not all, of these starvation adaptations are also observed in *C. elegans* such as promotion in fatty acid degradation[17], trehalose synthesis[18, 19], gluconeogenesis[20–21]. Suppression of translation[22–24], arrest in reproductive cells growth[25, 26] and modulation of locomotion[27–29] were also observed upon experiencing starvation, supporting worm can be a useful model in studying starvation responses.

Based on these, we treated adult worms with two cycles of starving/feeding eating pattern, and analyzed its effect on fat accumulation, expression of genes associated with fat metabolism. The mobility, amount of food intake, fecundity and lifespan were also analyzed. We found that experiencing repeated-starvation induced fat accumulation in worms determined by Nile Red staining and direct biochemical quantification. The increment of fat storage was not associated with significant change in gene expression, but genes involved in fat degradation tended to be suppressed, which does not contradict with the increment of fat storage. Pharyngeal pumping, as an indicator of food intake, increased significantly but transiently after starvation. Lifespan of worms treated with repeated-starvation was significantly but only slightly longer than that of fed worms. No obvious difference was observed in fecundity and mobility.

Our results indicate that experiencing starvation repetitively may induce long term effect to the body through physiological and biochemical processes.

Material& Methods

Nematode strains, maintaining and reagents

Caenorhabditis elegans strain N2 and *Escherichia coli* OP50 were obtained from Caenorhabditis Genome Center (CGC: University of Minnesota, USA). The maintenance, reagents, manipulation of worms were performed as described[10, 30]. Worms were maintained at 20°C. General chemicals were purchased from Nacalai Tesque (Kyoto, Japan) and Wakenyaku Kogyo (Kyoto, Japan), and distilled water produced by the distiller WG510 (Yamato Scientific Co, Tokyo) was used in the study.

Repeated starvation protocol

Gravid worms were destroyed with hypochlorous acid to obtain stage-synchronized eggs (day 0). The eggs were allowed to grow to young adults on NGM plate seeded with OP50. On day 3, worms were collected and washed extensively with S-basal to remove OP50, and were maintained on NGM plate without OP50 for 6 hr. After the 6 hr starvation, the worms were transferred to NGM plate with OP50 to be fed for 18 hr. On day 4, the worms (starved worms) were treated with the same procedure as on day 3 for the second starvation cycle. The control worms (fed worms) were treated exactly the same, except that the control worms were kept fed with OP50 duirng 6 hr starvation procedure (Figure 1). At the end of 18 hr feeding of the second cycle, the worms were collected for analysis of Nile Red staining, biochemical triacylglycerol quantification, mobility, fecundity, lifespan and food intake.

The reason why "6 hr" was selected for the starvation period was because longer the starved period was, more worms were lost during two cycles of starvation procedure, and "6 hr" was the longest starvation period in our experimental condition to fulfill our aim and to maintain the experiments.

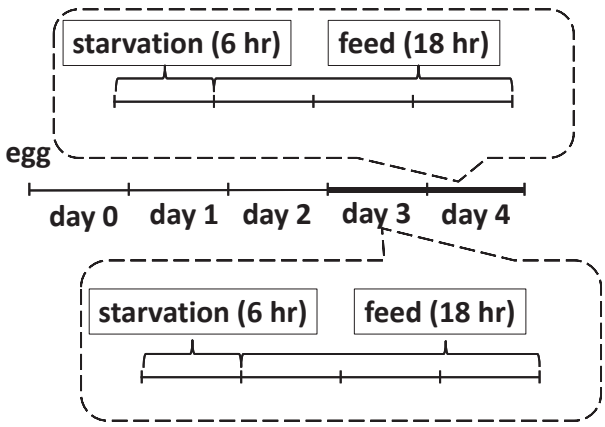

Figure 1. Starvation-Feeding cycle
Worms were treated with two cycles of starvation-feeding protocol on day 3 and day 4. At the end of 18 hr feeding on day 4, the worms were analyzed for Nile Red staining, biochemical triacylglycerol quantification, lifespan, fecundity and mobility.

Nile Red staining

The triacylglycerol accumulation in worms was analyzed using Nile Red staining as described[31] with modifications as follows. Concentrated Nile Red solution (500 μg/ml acetone) was diluted to 50 ng/ml PBS (10 mM sodium phosphate, 150 mM NaCl pH7.4) and a few drops of the diluted Nile Red solution were added to OP50-seeded NGM plates. These Nile Red-containing NGM was used in the repeated starvation experiments describe above. After the second starvation cycle, the recovered worms were placed on 2% agarose pad containing 0.1% NaN_3 in S-basal, and the worms were observed with SZ7 microscope equipped with RFP filter for Nile Red fluorescence (Olympus, Tokyo, Japan). The intensity of Nile Red fluorescence was calibrated against the background fluorescence intensity of area surrounding the worms. With sensitivity of CCD camera fixed to ISO200, five images with varying exposure time were taken for each worm and, after reviewing all the images, appropriate exposure time was selected for the whole sets of images to be analyzed for fluorescence quantification. In the fluorescence quantification using Adobe Photoshop Element (ver11), the area corresponding to worm intestine in an image was selected, and red channel histogram was obtained for the corresponding area to calculate average red fluorescence intensity. The average red fluorescence intensity was thought reflect the fat accumulation in the worm. The average fluorescence intensity was obtained from at least 30 worms in each experiment.

Biochemical quantification of fat content in C. elegans

To analyze fat content in worms experienced repeated-starvation, fat was directly extracted from worm and quantified with biochemical method. The worms treated with repeated-starvation were washed with S-basal buffer, and 9/10 volume of the worm suspension was used for fat extraction while remaining 1/10 volume was used for genome DNA extraction.

The worms in 9/10 portion were suspended in 3 ml 0.9% KCl and homogenized to obtain lysate. The lysate was extracted with 2 ml chloroform-methanol (2:1). The extraction was repeated three time, and the lipid fractions were merged. The chloroform and methanol were evaporated by blowing N_2 gas into the tube to obtain total lipid fraction followed by dissolving with 300 μl in isopropylalcohol supplemented with 10% Triton-X (solvent IPTX). Triacylglycerol in IPTX was quantified using Triacylglycerol E-Test Wako Kit (Wakenyaku Kogyo, Kyoto, Japan) according to the manufacture's instruction. The kit utilizes lipoprotein lipase, glycerol kinase and glycerol-3-phosphate oxidase to generate H_2O_2 from triacylglycerol, and this H_2O_2, corresponding to the triacylglycerol amount, was quantified using peroxidase[32, 33]. It was confirmed that IPTX used in our fat quantification was compatible with the kit.

The worms in 1/10 portion were suspended in 0.5 mg Proteinase K/ml NTES (50 mM Tris·HCl (pH8.0), 20 mM EDTA, 100 mM NaCl, 1% sodium dodecylsulfate) and incubated for 60 min at 65°C with constant rotation. The lysate was extracted once with 0.1% oxyquinoline-containing phenol saturated with TE (10 mM Tris·HCl (pH8.0), 1 mM EDTA). The resulting aqueous and phenol fraction were respectively extracted again with TE-saturated phenol and NTES. The resulting two aqueous fraction were combined, and genome DNA was insolubilized by ethanol precipitation followed by 70% ethanol wash. The genome DNA was dissolved in TE and quantified by spectrometry.

After normalized to genome DNA (mg fat/μg genome DNA), the fat contents in worm were compared and analyzed by the Student's *t*-test.

Gene expression analysis by quantitative RT-PCR

The gene expression was analyzed by real-time quantitative RT-PCR. The worms treated with repeated-starvation were washed with S-basal and sonicated in RNA Extraction Reagent Sepasol II (Nacalai Tesque, Kyoto, Japan). The lysate was then treated with TE-saturated phenol followed by chloroform/isoamylalcohol according to the manufacturer's instruction. The total RNA (300 ng) was used as template for cDNA synthesis using ReverTra Ace qPCR RT Master Mix with gDNA Remover cDNA (Toyobo, Osaka, Japan). The cDNA was then applied to real-time PCR using SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) in Thermal Cycler Dice (Takara, Kyoto, Japan). Following primers were used in RT-PCR; fatty acid synthase ortholog *fans-1* (F32H2.5), forward: 5'-TTCAAGAGTCCGACGGAAGA-3', reverse: 5'-CGTTACTCCAAAGGCCATAC-3'; acetyl-CoA carboxylase ortholog *pod-2* (W09B6.1), forward: 5'-CCAAAGTTGGAATGGCAGAG-3', reverse: 5'-CGGTTGCACATATGATCGGA-3'; SREBP-1 ortholog *sbp-1* (YD473B.7) forward: 5'-ACAAGGGGAGATTGTTGGAG-3', reverse: 5'-TTTGTAGATCGAACGCGTCG-3'; acyl-CoA transferase ortholog *mboa-2* (H19N07.4), forward: 5'-CGATTGTGGTCCTACTATGG-3', reverse: 5'-ATCAGTGAGAGCCAGACGAT-3'; carnitine palmitate transferase I ortholog *cpt-1* (Y46G5A1.7), forward: 5'-GGATTCGCGAAGAAGTTGAA-3', reverse: 5'-ATTGAGGCTTCGTAGGTCAG-3'; carnitine palmitate transferase II ortholog *cpt-2* (R07H5.2a), forward: 5'-GGAAGAACTGAATGCATGAG-3', reverse: 5'-GTGGAGCAATCAGTCAATAG-3'; β-oxidation thiolase ortholog B0303.3, forward: 5'-GCTCTTGCCAACGGATACAA-3', reverse: 5'-AGCTTTGGAATGACGTAGGC-3'; β-oxidation thiolase ortholog F53A2.7, forward: 5'-ATGTCAATGGTGGAGCCATC-3', reverse: 5'-GATTCCGTACTTGACGTTGC-3'; hormone-sensitive triacylglycerol lipase ortholog *hosl-1* (V46C11.1a), forward: 5'-GCTCCATCTACGCCATTACA-3', reverse: 5'-GTGTCCGCCAAACTTTGCGA-3'; triacylglycerol lipase ortholog *lipl-4* (K04A8.5), forward: 5'-AGAAACGTTGTTCGCGCAGT-3', reverse: 5'-AAACTTGGCTGGCTGCATTT-3'; myosin heavy chain ortholog *myo-4* (F11C3.3) forward: 5'-CCAACAGAAGTTGAAGACCC-3', reverse: 5'-CCTCAGCATCTTCAAGTTGG-3'. *myo-4* was used as internal reference to calibrate the expression of analyzed genes. The relative expression (to that of *myo-4*) was calculated with the

following formula: The relative expression of gene of interest = $10^{0.301 \times (n1-n2)}$: $n1$ and n_2 represent the cycle numbers in PCR when amplification curve started to elevate for *myo-4* and gene of interest, respectively.

Lifespan assay

The lifespan of adult worms treated with repeated-starvation was analyzed as follows[31]. After the second 6 hr starvation, the worms were transferred to OP50-seeded NGM plate containing 40 mM FUDR (5-fluoro-2'-deoxyuridine; Sigma-Aldrich, St. Louis, USA) and maintained at 20°C. FUDR is the deoxyuridine analogue used to stop generating the offspring by inhibiting genome DNA replication[31]. Every worm was checked for its viability everyday until all worm in the plate were dead. The lifespan was analyzed and plotted with Kaplan-Meier method, and the statistical difference and standard error mean (SEM) were calculated with Log-Rank method. Both methods were calculated by the survival package in software R[34].

Fecundity and mobility assay

To analyze the fecundity of worms which experienced repeated-starvation, after treating the worms with repeated-starvation, worms were singled in NGM plate. All offspring that derived from the singled worm throughout the starvation protocol and thereafter were counted. The fecundity was compared, and statistical analysis was performed with Student's *t*-test.

For mobility assay, worm treated with repeated starvation-protocol was singled in OP50-seeded NGM plate and left for 5 min to rest. Under microscope, the movement of the worm was observed. When the worm made a clear head bending during its sigmoidal crawling, one count was added. The worms were observed for 3 min. When the worm stopped moving or escaped form bacterial lawn for 10 sec, the assay was terminated, and the data was abandoned. At least 20 worms were analyzed, and the counts were determined for statistical significance by Student's *t*-test.

Food intake analysis

Food intake of worm was determined by counting pharyngeal pumping because it was reported to reflect the amount of food, in this case OP50, that worm swallowed into its intestine[35, 36]. The worm grown to young adult on OP50-seeded NGM plate were starved on non-seeded NGM plate for 6 hr at 20°C, and pharyngeal pumping was counted by direct observation under microscope for 20 sec. The count was expressed as counts/minutes for comparison. The worms were further maintained on OP50-seeded NGM for 24 hr, and pharyngeal pumping was counted again. The pumping of 20–30 worms were counted to obtain average data, which was further analyzed by Student's *t*-test.

Results

Fat accumulates after experiencing repeated-starvation

To determine the effect of irregular eating habit on animals, fat content in worms which experienced repeated-starvation (6 hr starvation/18 hr feeding) was analyzed by Nile Red staining and biochemical quantification. Nile Red is lipophilic compound which emits fluorescence upon excitation in the presence of lipid including triacylglycerol[31]. When worms were treated with repeated-starvation (starved worms) in the presence of Nile Red, the fluorescence intensity of the compound showed significant increase compared to that of worms which did not experience any starvation (fed worms), indicating that repeated-starvation induced fat accumulation (average intensity; 35.41 ± 7.91 (standard deviation: SD) for starved worms, 27.97 ± 6.58 (SD) for fed worms, $p = 1.47 \times 10^{-4}$) (Figure 2).

Although Nile Red is a useful compound which can stain lipid in live worms, it has been reported that its staining was not so specific to triacylglycerol unless worms were fixed prior to staining procedure[37, 38]. To gain more specificity, triacylglycerol fraction in worms was directly extracted and quantified by biochemical method. The commercial kit originally designed for serum triacylglycerol quantification utilizing combination of several enzymes (lipoprotein lipase, glycerol kinase, glycerol-3-phospate oxidase peroxidase)[32, 33], was modified for our experiment. We designed solvent IPTX, consisted of isopropylalcohol and Triton-X, to dissolve triacylglycerol extracted from worms. The sensitivity and specificity in detecting triacylglycerol using IPTX was confirmed to be unaffected prior to the analysis (data not shown). For comparison, the amount of triacylglycerol was normalized to the amount genome DNA extracted from worms. The biochemical quantification revealed that the triacylglycerol content in worms experienced repeated-starvation was significantly higher than that of fed worms (average triacylglycerol amount; 0.010 ± 0.0022 (SD) mg triacylglycerol/μg genome DNA for starved worms, 0.0059 ±

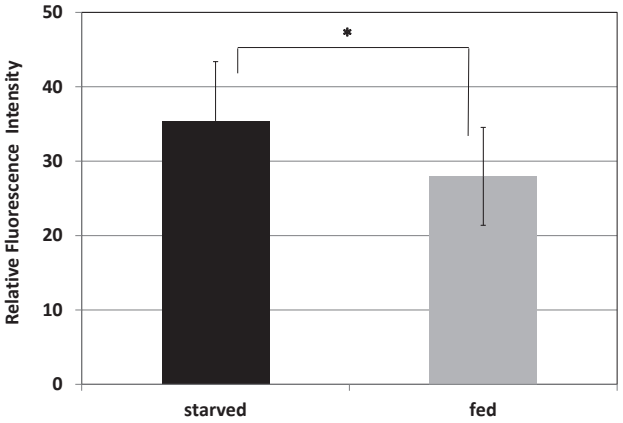

Figure 2. Fat accumulation in worms treated with repeated-starvation (Nile Red staining).
The relative intensity of Nile Red fluorescence in worms was analyzed as described in the text. The horizontal axis represents Nile Red fluorescence intensity. The black and grey bar represent respectively the average fluorescence intensity in the worm treated with the repeated-starvation (starved: n =31) and without starvation (fed: n =32). The error bars represent standard deviation of the data. The p-value calculated by Student's t-test was 1.47×10^{-4}, which showed the fat accumulation was significantly increased in the worm experienced repeated starvation, as indicated with an asterisk mark. The data shown is the representative of five experiments.

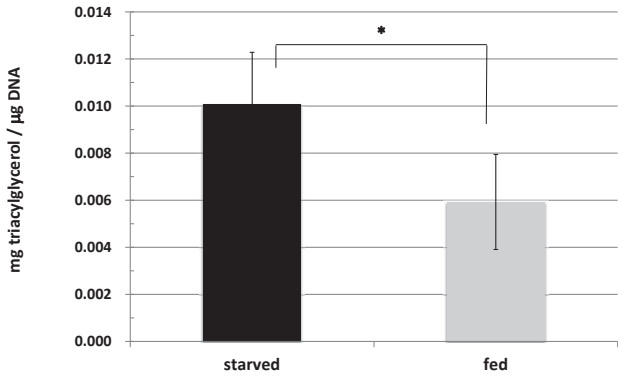

Figure 3. Fat accumulation in worms treated with repeated-starvation (biochemical quantification).
Fat in worms was extracted and quantified by biochemical method as described in the text. The horizontal axis represents amount of fat normalized to the amount of genome DNA (mg triacylglycerol/μg genome DNA). The black and grey bar represent respectively the average amount of triacylglycerol in the worm treated with repeated-starvation (starved) and without starvation (fed) from 4 independent quantification assay. The error bars represent standard deviation. The *p*-value calculated by Student's *t*-test was 0.032, showing the fat accumulation was significantly increased in the worm which experience repeated-starvation, as indicated with an asterisk mark.

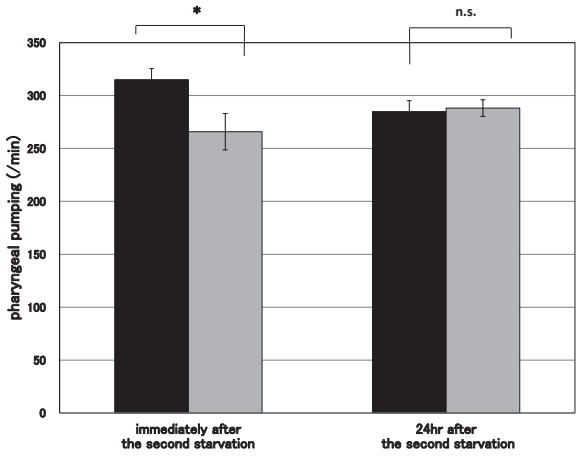

Figure 4. The effect of starvation on food intake.
The food intake of worm was measured by counting pharyngeal pumping as described in the text. The pumping was counted twice, immediately after and 24 hr after starvation. The black and grey bars represent the pumping number of worms treated with and without starvation, respectively. The number of worms used in this experiment was 20 for all experiments. Immediately after 6 hr starvation, the food intake was significantly high in starved worms compared to that of fed worms (indicated as asterisk; $p = 3.06 \times 10^{-12}$) but reached almost same level after 24 hr (indicated as n.s.). The data shown is the representative of 3 experiments.

0.0020 (SD) mg triacylglycerol/μg genome DNA for fed worms, $p = 0.032$) (Figure 3). These Nile Red staining and biochemical quantification showed that repeated-starvation increased fat accumulation in worms.

Food intake increased transiently upon experiencing starvation

Among possible reasons that drove the increment of fat accumulation in worms experienced repeated-starvation, one hypothesis is that worms merely took more food after starvation. To test the hypothesis, the pharyngeal pumping was counted. When *C. elegans* eat bacteria food, its pharynx works as a grinder to smash bacteria body to deliver the bacterial debris to intestine, and its activity is known to increase as worm eats more food making pharyngeal pumping rate a good indication of amount food intake. We measured the pharyngeal pumping rate of worms immediately after, and 24 hr after the starvation. The pharyngeal pumping rate at immediately after the starvation was significantly higher compared to the fed worms, indicating starved worms became to eat more food (average pumping rate; 315 ± 10.5 (SD) for starved worms, 266 ± 17.2 (SD) for fed worms, $p = 3.06 \times 10^{-12}$). However, no statistical difference was observed in pharyngeal pumping rate 24 hr after the starvation, indicating the increment of food intake in starvation-experienced worms was transient (average pumping rate; 285 ± 10.3 (SD) for starved worms, 288 ± 7.9 (SD) for fed worms) (Figure 4). Since the increment of food intake observed in in starvation-experienced worms seemed to be transient, it was presumed not enough to explain increment of fat accumulation in worms that experienced repeated starvation.

Genes involved in fat degradation tended to decrease upon experiencing starvation

Since the transient increment of food intake in starvation-experienced worms may not be enough to explain the increment of fat accumulation, the expression of genes involved in fat metabolism were analyzed to test whether repeated-starvation affected fat metabolism. The following genes were analyzed: for fat synthesis; *pod-1* (worm ortholog of acetyl-CoA carboxylase catalyzing conversion of acetyl-CoA to malonyl-CoA), *fasn-1* (worm ortholog of fatty acid synthase catalyzing synthesis of fatty acid form malonyl-CoA), *mboa-2* (worm ortholog of acyl-CoA transferase catalyzing synthesis of triacylglycerol), *sbp-1* (worm ortholog of SREBP (sterol regulatory element-binding protein) transcriptionally promoting genes involving cholesterol and fatty acid synthesis), for fat degradation; *lipl-4* (worm ortholog of triacylglycerol lipase catalyzing triacylglycerol degradation), *hosl-1* (worm ortholog of hormone-sensitive triacylglycerol lipase catalyzing triacylglycerol degradation), *cpt-1 and cpt-2* (worm ortholog of carnitine palmitate transferase I and II transporting acylcarnitine into mitochondria), B030.3·F53A2.7 (worm orthologs of β-oxidation thiolase catalyzing fatty acid degradation). Total RNA was extracted from starvation-experienced worms followed by cDNA synthesis, and expression of the genes were analyzed by real-time quantitative RT-PCR using specific primers. The expression of the genes was normalized to that of *myo-4*, the ortholog of myosin heavy chain which was a common internal standard in worms. No obvious change was observed in the expression of these genes (Figure 5). However, although statistically not significant, the expression of *cpt-1*, *cpt-2*, B030.3 and F53A2.7, the genes involved in fat

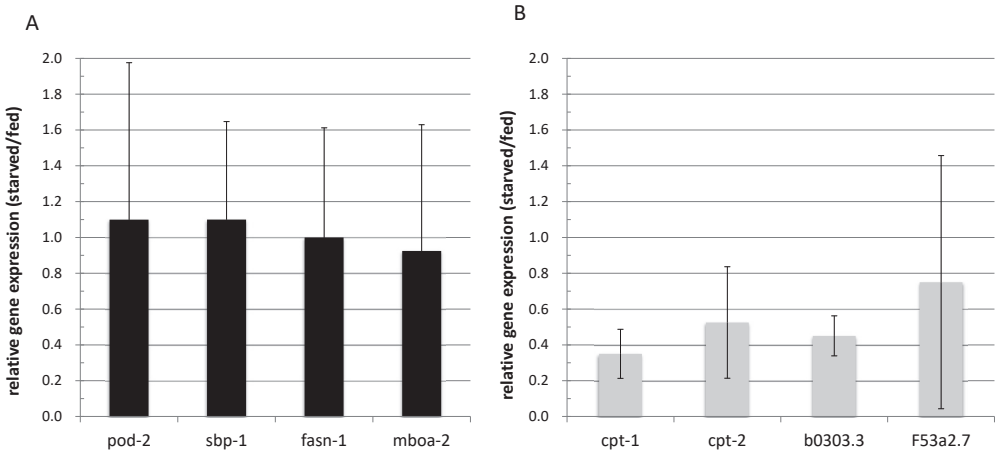

Figure 5. Expression of genes involved in fat metabolism in worm treated with repeated starvation.
The expression of genes involved in triacylglycerol synthesis (A) and degradation (B) was analyzed by real-time quantitative RT-PCR as described in the text. The horizontal axis represents the ratio of gene expression in worm treated with repeated-starvation (starved worms) to that of worms never experienced starvation (fed worms). The ratio larger than 1 indicates that the expression of the gene increased in starved worm compared to fed worms, whereas less than 1 indicates that the expression of the gene decreased. The figures are the average of 5 experiments and the error bars represent the standard deviation.

degradation, tended to decrease upon starvation experience (Figure 5B). Since the decrease of fat degradation activity could lead, in theory, to the accumulation of fat, the expression pattern of the degradation genes does not seem to contradict with the fat accumulation observed in starvation-experienced worms.

The expression of *lipl-4* and *hosl-1* was too low to assess (data not shown).

No remarkable change was observed in lifespan, fecundity and mobility in starvation-experienced worms.

To gain more information about the long-term effects of repeated-starvation, the lifespan, fecundity and mobility were analyzed. The worms that experienced repeated-starvation showed statistically longer lifespan, but only in small extent, compared to that of fed worms (average lifespan; 19.2 ± 0.37 (SEM) days for starved worms (n = 219), 19.1 ± 0.40 (SEM) days for fed worms (n = 204), $p = 1.74 \times 10^{-4}$). Although the difference was statistically significant, we should be cautious to interpret its biological meaning since the difference was very small.

The repeated starvation showed little effect on fecundity and mobility (average brood size; 300.0 ± 75.9 (SD) for starved worms, 323.6 ± 36.8 (SD) days for fed worms: average head-swinging action; 48.0 ± 7.16 (SD) for starved worms, 47.9 ± 6.10 (SD) days for fed worms) (Figure 7).

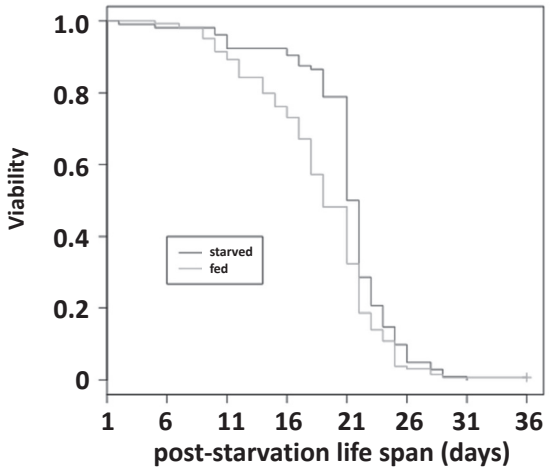

Figure 6. The effect of repeated starvation on the lifespan.
The post-starvation lifespan (lifespan after the second starvation) in the presence of FUDR was analyzed as described in the text. The result was plotted by Kaplan-Meier method. The mean lifespan was calculated as 19.2 days for repeated-starved worms (starved: n=219) and 19.1 days for fed worms (fed: n=204). The statistical significance was calculated using Log-Rank method, that lifespan of starved worms to be significantly longer compared to that of fed worms ($p = 1.79 \times 10^{-4}$). Both analysis were performed by the survival package provided in software R.

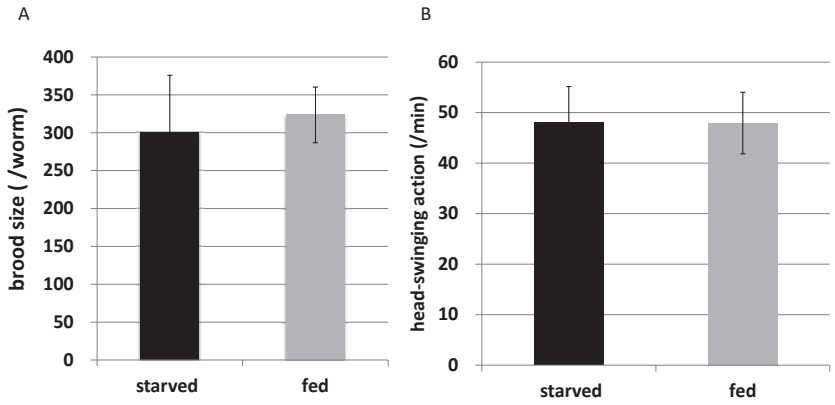

Figure 7. The effect of repeated-starvation on the fecundity and mobility.
Effect of repeated-starvation on fecundity (A) and mobility (B) was analyzed as described in the text.
The black and grey bar in A panel represent respectively the average brood size of worms treated with the repeated-starvation (starved: *n*=6) and worms never experienced starvation (fed: *n*=5). No significant difference was observed. The data shown is the representative of 3 experiments.
The black and grey bar in B panel represent respectively the average number of head-swinging action, an index of the mobility, of worms treated with the repeated starvation (starved: *n*=15) and worms never experienced starvation (fed: *n*=15). No significant difference was observed. The data shown is the representative of 3 experiments.

Discussion

It is a challenging work to clarify the long-term effect of irregular lifestyle such as skipping meal and occasional weight loss attempt using human as subjects. Therefore, we employed well defined *C. elegans* as model organism to address the problems. *C. elegans* is a small, free-living nematode commonly found in soil, rotten fruits and leaves. It takes only 3 days at 25°C to grow from eggs to adults and have lifespan of about 2–3 weeks. They mostly exist as self-fertilizing hermaphrodite, which make the nematode a very useful model for eukaryotic genetic studies. Due to these useful features, *C. elegans* became the first multicellular organism for its genome sequence completed[39]. In addition, the similarities between the molecular processes present in *C. elegans* and other animals such as metabolism, organelle structure, gene regulation and protein biology have made *C. elegans* an excellent model organism for studying general metazoan biology[39]. About 38% of the *C. elegans* genes were found to have orthologs in the human genome[40], and 60–80% of human genes have an ortholog in the C. elegans genome[42], and aa many as 40% of genes associated with human diseases have orthologs in the *C. elegans* genome[42]. We treated worms with repeated cycles of starvation/feeding regimen to mimic the repeated starvation that young women often experienced during their weight loss attempt. Although *C. elegans* is structurally, functionally and evolutionally far different from mammals including *homo sapiens*, the worm had been extensively used in the field of nutrition, development and growth, and provided considerable amount of information in this field[8]. The basis of *C. elegans* being a good model is the assumption that food scarcity is considered to be the universal stress to almost every animal, so information obtained in worms would be expected to be useful to mammals. However, there are few reports that *C. elegans* was used as a model of the repetitive starvation study, so the 6 hr starvation/18 hr feeding cycle adopted in this study should be evaluated. How does the "6 hr" starvation in worms correspond to the mammal, especially to human, may be the key question for the evaluation. As described in Materials and Methods, worms climbed up the plastic dish wall to die during starvation procedure, so longer starvation time resulted in losing too many worms making subsequent analysis difficult. The starvation length of "6 hr" had been chosen to minimize the loss of worms during starvation procedure. As it was adopted from the experimental technical point of view, the "6 hr" may not represent the actual irregular eating habit observed in human lifestyle. In addition, because lifespan and time required for sexual maturation between human and the worm are so different, simple ratio (length of starvation time/lifespan) makes less meaningful comparison (for example, 6 hr starvation in worm's 21 days lifespan corresponds to 0.95 years starvation in human provided 80 years lifespan). Therefore, comparison based on physiological change rather than actual length of time would probably be better to understand the impact of starvation. During our 6 hr of starvation protocol, the worms almost halt laying eggs presumably due to oocyte development suppression. Based on the notion that moderate level of PEM (protein-energy malnutrition) in human leads to the halt in ovulation and spermatogenesis, and that 30–40 days fasting was required to induce that level of PEM[43], the 6 hr starvation in worms in our study may correspond to 30–40 days fasting in human. It is noteworthy that the 30–40 days of fasting could be life-threatening stress.

From the Nile Red staining and direct biochemical quantification, it was shown that fat accumulated in the worms that experienced repeated-starvation compared to the fed worms (Figure 2, 3). Because Nile Red staining was less specific to triacylglycerol when used to stain live worms[37], it was necessary to confirm the data with the different method. The biochemical quantification using combination of enzymes did show the increment of fat accumulation in worms experienced

repeated-starvation (Figure 3). To explain the reason of the fat accumulation, amount of food intake was analyzed by counting the pharyngeal pumping rate[36]. It was shown that food intake in worms that experienced repeated-starvation was indicated to increase determined by significant increment in pharyngeal pumping rate (Figure 4). However, the difference in pumping rate between starvation-experienced and fed worm diminished 24 hr after the starvation, indicating the increment in food amount in starvation-experienced worms was transient. This result does not necessarily exclude the contribution of increased food intake to fat accumulation observed in starvation-experienced worms but evoked the question if such transient increment in food intake was enough to explain the fat accumulation.

The expression pattern of genes involved in fat metabolism may provide the information to the question. As shown in Figure 5, the genes involved in fat degradation, such as *cpt-1*, *cpt-2*, B030.3, F53A2.7 tended to be suppressed in worms that experienced starvation, while that of genes involved in fat synthesis (*pod-1, fasn-1, mboa-2, sbp-1*) did not change. Although the expression of fat degradation genes was not statistically significant, its pattern does not contradict, if not supportive, with the increment of fat accumulation observed in worm experienced starvation. The pattern also implied that repeated-starvation may have induced the worms physiologically and metabolically to "fat storage-prone" direction. When *C. elegans* larvae is exposed to environmental stress including food scarcity, the larvae undergoes the physiological, morphological and metabolic change to become special diapause state called dauer, and lipid store was reported to be enhanced with numerous genes involved[8]. Therefore, similar physiological and metabolic shift might have been induced by repeated starvation to make the worm more prone to store fat.

Our results may provide some aspects to the weight loss attempt that many people conduct. Since losing weight and maintaining the reduced weight is challenging, it is common that people regain weight[44–46]. Although the precise mechanism of the weight regain after weight loss is not fully understood, there are increasing evidence that the weight regain was driven by cellular and metabolic process[6]. It might be interesting to see if the expression of fat degradation genes was also suppressed in individuals of weight regain status as observed in our result.

The average lifespan of worm that experienced repeated-starvation was longer than that of fed worm (Figure 6). But the difference was so small (0.1day), so it may be difficult to find biological significance in the lifespan extension. In fact, there had been several reports that lifespan was not affected in duaer and L1 growth arrest (growth arrest induced by food scarcity during first larval stage in worm development), which seems supportive with our result[25, 44, 45]. There was no obvious change in fecundity and mobility (Figure 7).

Our results showed that repeated starvation induced fat accumulation in worms, and also indicated the possibility that the increment may be derived from the change in gene expression profile. However, several limitations have to be pointed out in our study. For the first, the genes analyzed was not enough in number and comprehensiveness. In addition to those genes directly involved in fat metabolism, transcriptional factors known to control overall metabolism such as *pha-4* (*Fox*A ortholog), *nhr-49* (NHR ortholog), and *daf-12* (vitamin D receptor ortholog) should be included together with genes involved in insulin signal pathway (*daf-2* pathway) that regulate growth/nutrition and longevity in worm[45]. The second limitation in our study is that metabolic properties specific to worm must be carefully considered when applying to mammals. Although the usefulness of worms in mammalian nutrition was addressed[8], there exist distinctive metabolic differences between worms and mammals, such as ability to synthesize ω-3 and ω-6 fatty acid in worms. The existence of glyoxylate cycle in worms is also noteworthy because worms can synthesize glucose from fat[14, 25, 26]. Therefore, it should be cautious when applying data obtained using worm to human.

Author contributions

S.M. conceived and designed the experiments. R. Y., K. Y., N. S., S. S., S. Y. and A. S. performed the experiments and analyzed the data with the help of S.M. S.M. prepared the manuscript.

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Disclosure statement

No potential conflict of interest was reported the authors.

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