Original Paper

Starvation during L4-larval stage induces longevity in *Caenorhabditis elegans*.

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Abstract

The effects of experiencing starvation during adolescence, during which significant physical growth and sexual maturation occurs, on adulthood have remained largely unknown. In addition, the influence of nutritional status during adolescence on brain maturation and function are discussed on the viewpoints of young people's mental, psychology and behavior. In order to gain knowledge about the effects of starvation on adolescence, we starved the model organism *Caenorhabditis elegans* during its fourth larval stage (L4), and the lifespan and expression of genes in insulin/IGF-1 pathway, which is crucial to regulate lifespan, of the resulting adult worms were analyzed. The worm's gonadogenesis and spermatogenesis take place during the L4 stage, making the stage a possible equivalence to human puberty. We found that worms which experienced starvation during their L4 stage had significantly longer lifespan, especially in the latter half of their lifespan, namely the aged worms lived longer. The relative expression of *daf-16* in the insulin/IGF-1 pathway increased as worms become old, suggesting that the insulin/IGF-1 pathway functioned to extend lifespan in old worms. It was also observed that the locomotor ability (mobility) of L4-starved adult worms decreased. In addition, expression of *ser-4*, serotonin receptor, which was reported to be involved in lifespan regulation in worms, increased gradually toward late lifespan, suggesting that effect of experiencing starvation during L4 stage may also be regulated by serotonin. Our data posed a possibility that also in human, hunger and nutritional deficiencies in adolescence may affect health and activity in later adulthood.

Key words; starvation, adolescence, *Caenorhabditis elegans*, longevity

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Introduction

For organism to grow, mature, function and propagate, it is essential to obtain adequate amount and good quality of food. Especially in growth stage, during which physical structure and body function establish, good nutritional status is important. If good nutritional status was not provided during the growth stage, inadequate establishment in digestive and immune system could lead to growth malfunction and infectious disease contraction[1]. Previously and practically, the nutritional issue concerning young people has been largely focused on the aspect of proper growth. Recently, nutritional issue concerning adolescence has been attracting with the aspect of brain development, socioeconomy and epigenetics[2]. Adolescence is the specific stage which connects childhood and adulthood, and it was characterized by drastic physical growth to obtain adult body structure, and maturation of sexual gonads and external organs. In addition to physical and physiological change, profound change in their behavior and preference occurred due to brain development and changes in their social environment surrounding them. In fact, vast majority of eating disorder was reported to initiate when they were teens, which overlapped adolescent stage[3]. Therefore, experiencing extreme dieting and starvation during adolescence not only lead malfunction in physical and physiological condition in adult, but might also affect behavior and health status throughout lifespan.

The first consideration of the nutritional significance in adolescence is obviously the physical and physiological aspects. One indication of that is the global changes in the initiation age of puberty. The initiation age of puberty in girls in the advanced industrialized countries was reported to become lower than before, presumably due to establishment of good nutritional status in those countries[4]. The second is the effect of nutritional status on mental and behavior of adolescent. From criminal incidence report, inadequate nutrition and food are linked to mental, psychological and behavior status of adolescent[5]. It is

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also reported that supplementing vitamins and minerals to those individuals with criminal records significantly decreased the rate of repeating law-violating conducts, implicating the importance of good nutrition in adolescent conduct[5]. The third is the epigenetic aspect that inadequate nutritional status may establish long-term effect that last throughout adulthood due to epigenetic change. Epigenetic change during adolescence is not as well studied and established as those during embryogenesis[6–8]. However, as it is a common understanding that adolescence is one of the 4 important developmental stages during human life (embryogenesis, neonatal, early childhood, adolescence), it is not surprising that adolescence is the major stage for epigenetic change in human development.

Although the importance of nutrition in adolescence is attracting more interest, it is practically impossible to study the phenomena using human subject. The *caenorhabditis elegans* have been shown to be an effective system to study development, nutrition, growth arrest and starvation of eukaryotes[9–12]. One of the reasons for choosing the worm as the 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 easily permitted to use from ethical standpoint. In addition, because starvation is considered as the universal stress to many animals regardless of the taxonomical difference, results obtained in the worm system is expected to be applicable to other organisms including mammals. Mammals are known to adapt to scarcity of food by metabolic-level response such as promoting fat degradation, ketone body synthesis and gluconeogenesis, as well as organ/tissue-level response such as arresting growth, delaying sexual maturation and suppressing reproductive activity[13, 14]. During the larval stage 4 (L4) of *C.elegans*, maturation process of sexual organs, gonadogenesis and spermatogenesis, take place to become fully reproductive adult[11, 12]. Since human adolescence was also characterized by maturation of sexual organ, we hypothesized that worm L4 was equivalent to human adolescence, and therefore could be used as the model system to study adolescent nutrition.

Thus, we applied starvation to L4 worms and analyzed lifespan, mobility and fat accumulation in adulthood. We found that L4-starved adult worms had elongated lifespan, and that the elongation was evident at late lifespan, rather than overall lifespan. This can be interpreted that starvation in adolescence lead to longer lifespan at old stage in life. The insulin/IGF-1 pathway[15, 20–22] that regulate lifespan did not show remarkable change in its expression profile, but relative expression of *daf-16* and *daf-18* showed gradual increase, as well as increment in *sod-3* expression toward the latter stage of lifespan, which was consistent with the lifespan elongation at late lifespan of L4-starved adult worms. L4-starved adult worms showed significantly decreased mobility quantified by head-swinging movement, but no effect of L4-starvaton on fat accumulation was observed. Since serotonin is reported to be involved in fat accumulation, food intake[16, 17] and inhibition of *ser-4*, human ortholog of serotonin receptor 5-HT1, was reported to decrease the lifespan[18], we quantified the expression of *ser-4* in L4-starved adult worms and found that the expression was significantly increased in L4-starved adult worms compared to L4-fed adult worms (control).

Our data imply the possibility that nutritional status in adolescence may be an important factor in contributing adult health condition, particularly in later lifespan.

Materials & Methods

Nematode strains, maintaining and reagents

Caenorhabditis elegans strain Bristol 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[11, 19]. 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.

Starvation protocol

Gravid worms were destroyed with hypochlorous acid to obtain stage-synchronized eggs (day0). The eggs were allowed to grow for 2 days to become L4 on NGM plate seeded with OP50. On day2, L4 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 seeded with OP50 to grow to adult worms (L4-starved worms) (Figure 1A). The control worms were treated exactly the same, except that the control worms were kept on NGM plate seeded with OP50 for 6 hr during its L4 stage (L4-fed worms). On day5, the worms were collected for analysis of Nile Red staining (for fat accumulation) and mobility assay.

The reason why "6 hours" was employed for the starvation time was because longer the starved time was, more the worms were lost during starvation procedure, and "6 hours" was the longest starvation time in our experimental condition to obtain enough amount of worms to fulfill our aim and to maintain the experiments.

Lifespan assay

The adult lifespan of worms which experienced starvation during their L4 was analyzed as follows[23]. After the 6hr starvation in their L4 (day2), the worms were transferred to NGM plate containing 40 mM FUDR (5-fluoro-2'-deoxyuridine; Sigma-Aldrich, St. Louis, USA) and maintained at 20°C. FUDR is deoxyuridine analogue used to stop generating the offspring by inhibiting genome DNA replication[23]. Every worm was checked for its survival every two days until all worms in the plate were dead (Figure 1B). The lifespan was analyzed and plotted with Kaplan-Meier method, and the statistical difference was calculated with Log-Rank method. Both methods were calculated by the survival package in software R[24].

Gene expression analysis by quantitative real time RT-PCR

The gene expression was analyzed by quantitative real-time RT-PCR. The worms were washed with S-basal and sonicated in RNA Extraction Reagent Sepasol II (Nacalai Tesque, Kyoto,

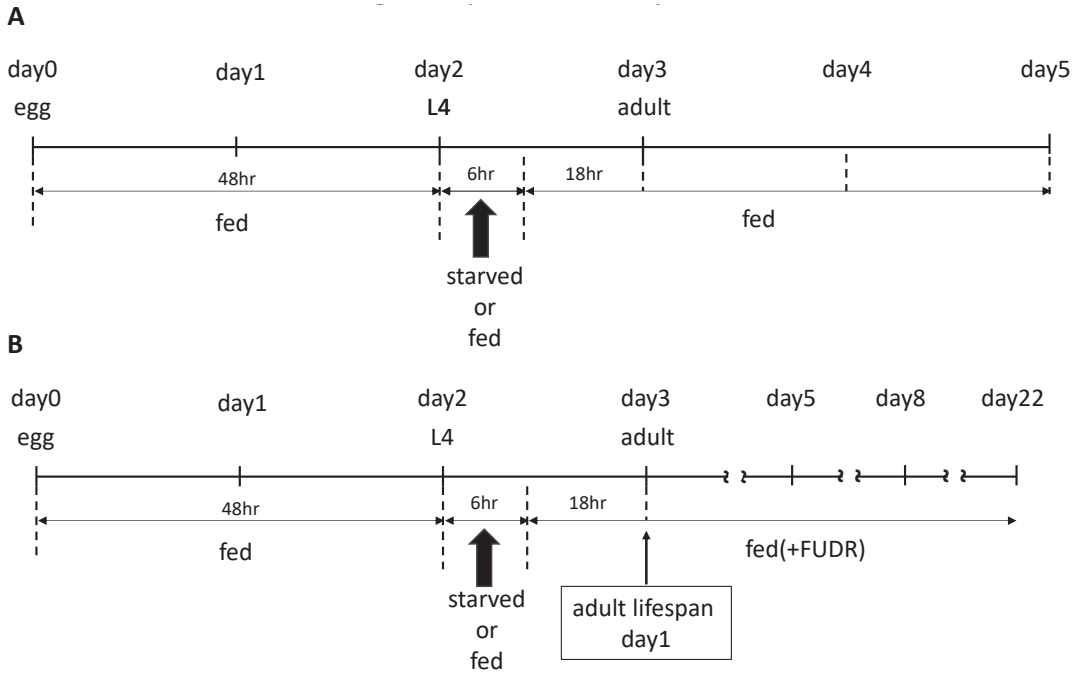

Figure 1. Experimental protocol

(A): starvation protocol: Synchronized eggs were placed on NGM (day0) to grow and on day2 the resulting L4 larvae were transferred to NGM plate without OP50 to starve for 6 hours. The larvae were then collected to NGM plate with OP50 to grow to adult. On day5, the mobility and fat accumulation (Nile Red staining) were analyzed. (B) lifespan protocol: Starvation was applied to the worm as described in (A) except that worms were bred on the NGM plate that contained FUDR after starvation to eliminate generation of progenies. On day5, 8 and 22, the worms were collected, and total RNA was prepared for gene expression analysis by real time RT-PCR.

Japan). The lysate was then treated with TE-saturated phenol followed by chloroform/iso-amylalcohol 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; *daf-2* (Y55D5A.5a), forward: GTTCGGACGTATGCTGGTGA, reverse: CCACCACCTGTGTAACTTGA; *daf-18* (T07A9.6), forward: CTGGCAAAGACACTCAGAAG, reverse: ATCCTCTGGATGTAGCTCCG; *sod-3* (C08A9.1), forward: TTGGAATTGACGTTTGGGAG, reverse: TCTCTCGCTGATATTCTTCC; *age-1* (B0334.8), forward: TGTGGATGGAAATTCGCATG, reverse: GCATCAAGGTGAACAAGGAA; *daf-16* (R13H8.1h), forward: GTGCCAAGCACTAACTTCAA, reverse: AGTGGAGATGAGTTGGATGT; *ser-4* (Y22D7AR.13.1), forward: ATTGTAAAGAGGAGGCGGCG reverse: GGTCCAACACGCAACGAATG; myosin heavy chain ortholog *myo-4* (F11C3.3), forward: CCAACAGAAGTTGAAGACCC, reverse: CCTCAGCATCTTCAAGTTGG. *myo-4* was used as internal reference to calibrate the expression of genes. The relative expression (to that of *myo-4* expression) was calculated with the following formula: The relative expression of the gene of interest = $0.301 \times (n_1\text{-}n_2)$: n_1 and n_2 represent the cycle numbers in PCR when amplification curve started to elevate for *myo-4* and the gene of interest, respectively.

Mobility assay

For mobility assay, a single adult worm was placed 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 1 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 35 worms were analyzed, and the counts were determined for statistical significance by Student's *t*-test.

Results

Elongation of lifespan in worms that experienced starvation in L4

To investigate how starvation in adolescence affect adulthood conditions, we analyzed lifespan of L4-starved adult worm. As shown in Fig.1, L4 worms were starved for 6 hours and subsequently allowed to grow to adult in the presence of OP50. The lifespan of L4-starved adult worms was significantly longer than control L4-fed adult worms (average lifespan: L4-starved;17.9 days (n = 120) , L4-fed; 14.9 days (n = 180), $p = 6.2 \times 10^{-8}$) (Fig.2). It is noteworthy that there was little difference in their survival pattern until mid-lifespan, but the difference became evident at late lifespan that L4-starved adult worms showed higher survival at late lifespan. This means that L4-starved adult worms lived longer at later stage of their life, namely old worms lived longer.

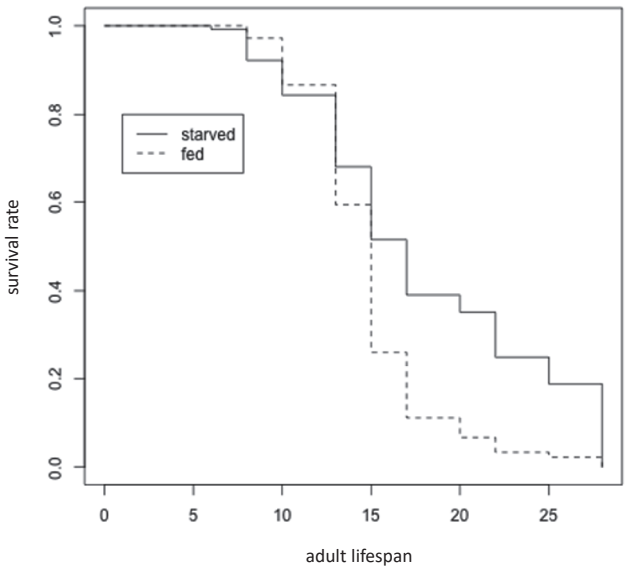

Figure 2. Effect of starvation during L4-stage on adulthood lifespan

The effect of starvation during L4-stage on adult lifespan 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 17.9 days for L4-starved worms (solid line: n = 120), and 14.9 days for L4-fed worms (dashed line: n = 180). The statistical significance was calculated using Log-Rank method, which is one of the chi-squared test commonly used to compare the overall survival distributions of two samples, that lifespan of L4-starved worms to be significantly longer compared to that of control worms ($p = 6.2 \times 10^{-8}$). Kaplan-Meier plotting and Rog-Lank methods were performed by the survival package provided in software R. The figure is the representative of 6 experiments.

Effect of L4 starvation on adult mobility and fat accumulation.

We also analyzed mobility function and fat accumulation of worms that experienced starvation during their L4 stage. We evaluated the mobility of worms by counting their head swinging during certain time. The number of head-swinging in L4-starved worms was significantly small compared to that of L4-fed worms. This indicates that experiencing starvation during its L4 stage could damage the mobility of adult. However, although the result was reproduced twice in our experiment, we should be cautious to draw the conclusion because the significance was close to borderline (p = 0.0450). There was no difference in fat accumulation between L4-starved and L4-fed worms (data not shown).

Expression of insulin/IGF-1 pathway genes in worm that experienced starvation during L4.

C.elegans has been a major model organism to study the mechanism of lifespan, and from that background, it has been reported insulin/IGF-1(insulin-like growth factor1) pathway, mTORC1 (mammalian target of rapamycin complex 1) pathway, and AMPA (AMP-dependent protein kinase) pathway were identified to be involved in lifespan regulation[15, 20–22]. Insulin/IGF-1 pathway has been well preserved among species from worm, fruit fly and mammal including human, and was involved in regulation of lifespan, growth, propagation, and dauer formation in response to food availability. Insulin/IGF-1 pathway was activated by binding of insulin-like peptides to their receptor

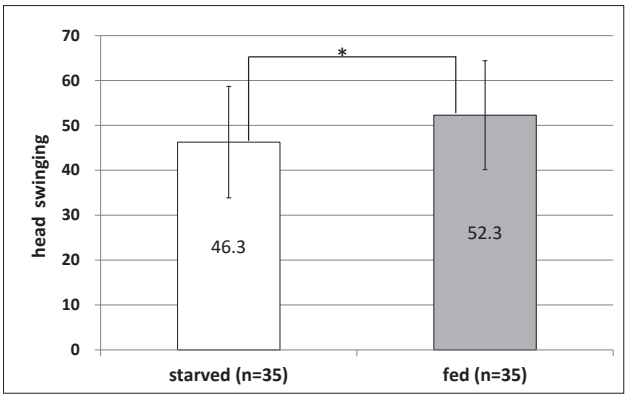

Figure 3. The effect of starvation during L4-stage on adulthood mobility.

The average number of head-swinging action of worms that experienced starvation during L4-stage (white bar) and worms without starvation in their L4-stage (grey bar) are shown (L4-starved worms; 46.3/min, fed worms (control) ; 52.3/min). Error bars represent standard deviation of average data. Asterisk mark (*) represents significant difference determined by Student's t-test (p = 0.045). The figure is the representative of 2 experiments.

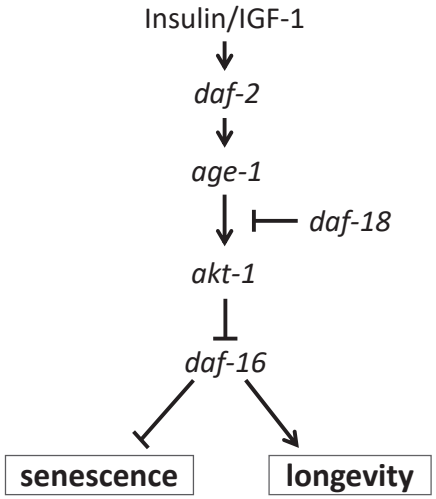

Figure 4. Schematic diagram of insulin/IGF-1 pathway

Insulin/IGF-1 pathway is the major pathway (but not the only) regulating lifespan. Activation of *daf-2* and *age-1* leads to suppression of *daf-16* (homologous to human nuclear receptor FOXO1) which then drives worms to reproductive phase that live short. When *daf-2* and *age-1* was suppressed, *daf-16* was enhanced resulting longevity by activating genes that favor longevity such as *sod-3*.

DAF-2, subsequently activating PI3 kinase AGE-1. The activation of insulin/IGF-1 pathway results in the suppression of nuclear receptor DAF-16, homologous to human nuclear transcription factor FOXO, to drive the organism to reproductive phase thereby suppressing the longevity. On the other hand, suppression of *daf-2* and *age-1* or activation of *daf-16* lead to longevity. The *sod-3*, whose expression was regulated by DAF-16, is thought to contribute to longevity by its antioxidation activity[25] (Fig.4).

We are interested if insulin/IGF-1 pathway was involved in the longevity caused by L4-starvation, so expression of insulin/

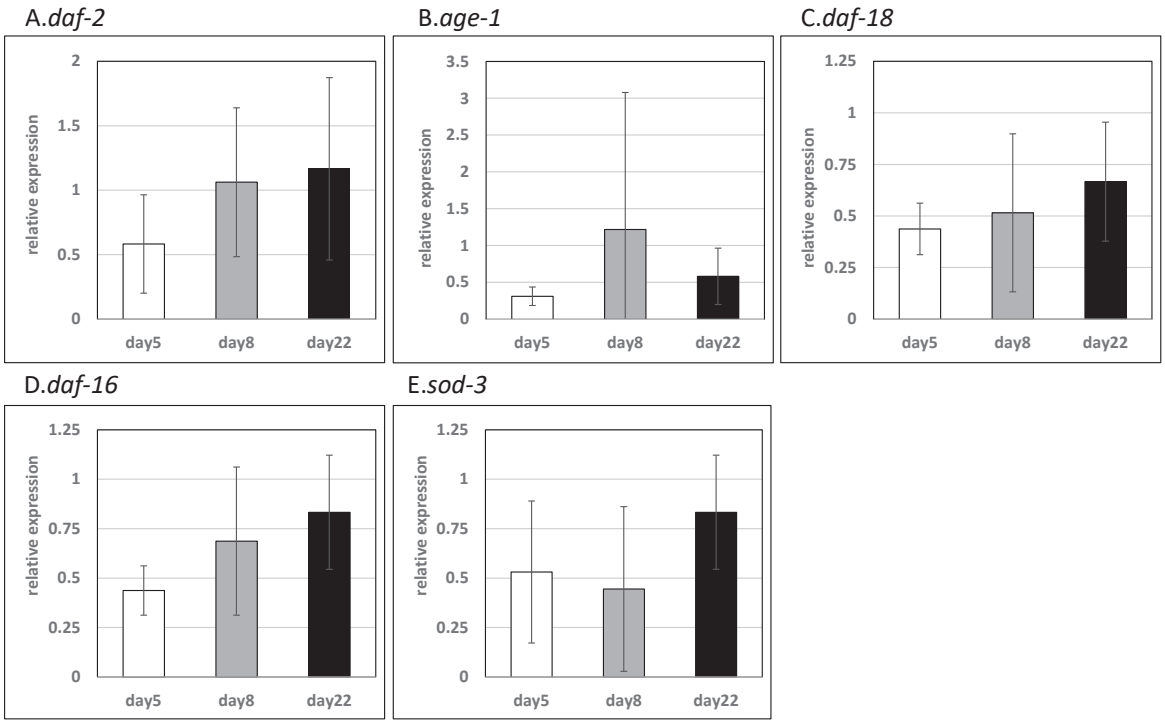

Figure 5. Effect of starvation during L4-stage on expression of age-related genes.
The expression of genes involved in lifespan regulation was analyzed by quantitative real-time RT-PCR as described in the text. The panel A, B, C, D, E represent the expression of *daf-2*, *age-1*, *daf-18*, *daf-16*, *sod-3* respectively. The horizontal axis represents adulthood days (see Figure 1B). Expression of each gene was normalized to that of *myo-4*, and the ratio of the normalized gene expression in L4-starved adult worms to that of control worms were averaged. No significance was observed between the gene expression profile. Error bars represent standard deviation of average data. The data is the average obtained from 3 independent experiments.

IGF-1 pathway genes was analyzed by real time RT-PCR at day5 (end of reproductive stage), day8 (mid lifespan), and day22 (late lifespan) (Fig.5). Throughout the lifespan, the relative expression level ((expression of the gene of interest in L4-starved worm normalized to that of *myo-4*) / expression of the gene of interest in L4-fed worms normalized to that of *myo -4*)) of *daf-2* and *age-1* was mostly below 1 (Fig.5A, B), indicating insulin/IGF-1 pathway in L4-starved worms are somewhat suppressed compared to that of L4-fed worms. However, since the expression of *daf-16* was not upregulated (Fig.5 D), which was anticipated if *daf-2* and *age-1* was suppressed, the suppression of insulin/IGF-1 pathway was presumed to be not remarkable, if not zero. On the other hand, the relative expression of *daf-18* (PTEN), which inhibits insulin/IGF-1 pathway, and *daf-16* increased gradually as worms became older (Fig.5C, D). The increment pattern of *daf-16* and *daf-18* expression could explain the longevity in L4-starved adult worm observed in late lifespan. The relative increment of *sod-3* expression, a gene coding antioxidant superoxide dismutase, was also observed, consistent with the idea that L4-starvation longevity was regulated by insulin/IGF-1 pathway (Fig.5E).

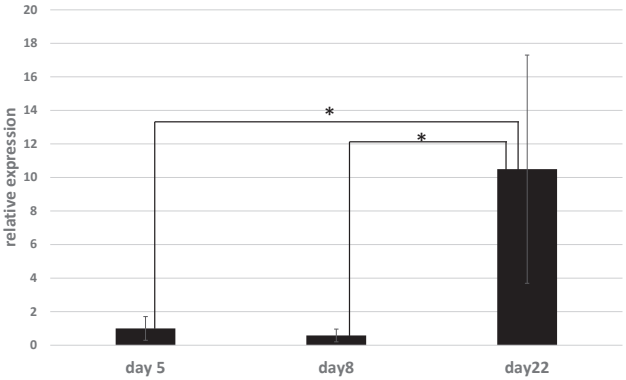

Figure 6. The effect of starvation during L4-stage on *ser-4* expression.
The expression of serotonin receptor *ser-4* in adult worm was analyzed by real-time quantitative RT-PCR as described in the text. The horizontal axis represents adulthood days (see Figure 1). Expression of *ser-4* was normalized to that of *myo-4*, and the ratio of normalized gene expression in worm that experienced starvation during L4 to that of control worms were averaged. Error bars represent standard deviation of average data. Asterisk mark (*) represents significant difference determined by Tukey-Kramer method using software R (day5 vs day22; $p = 0.030$, day8 vs day22; $p = 0.035$). The data is the average obtained from 4 independent experiments.

Upregulation of serotonin receptor ser-4 in worms that experienced starvation during L4.

Murakami H *et al*. investigated effect of serotonin receptors on longevity in *C.elegans* and reported that deletion mutant of *ser-4*, an ortholog of human 5-HT1 receptor, showed shortened lifespan during its mid lifespan [18]. Since L4-starved adult worms showed elongated lifespan in its late lifespan, which seemed stage-specific, it was interesting to know if *ser-4* take part in the elongation. We analyzed the expression of *ser-4* by real time RT-PCR and found that *ser-4* expression increased significantly at day22 (Fig.6). This suggests the possibility that L4-starvation may drive adult worms to live longer at the later life stage through

serotonin receptor *ser-4* activation.

Discussion

It is impossible to clarify the long-term effect of starvation during adolescent or teenage individuals using human. Therefore, we employed well defined *C.elegans* as model organism to address the hypothesis. Although *C.elegans* is structurally, functionally and evolutionally far different from mammals, the worm had been extensively used in the field of nutrition, development and growth, and provided considerable amount of information in this field[26]. We treated L4 worms with starvation to mimic the starvation in adolescent individuals, because L4 was the larval stage when spermatogenesis and gonadogenesis occurred. This developmental similarity could make L4 the comparable stage to human adolescence.

We first analyzed the effect of L4-starvation on adulthood lifespan, and found that late lifespan was significantly elongated in worms that experienced starvation in their L4 (Fig.2). There are two points that should be discussed. The first is that starvation experience in its adolescence could influence later in life. This is important because we tend to consider the nutritional problems in adolescence, such as eating disorder and unhealthy weight controls, would have been overcome once the normal nutritional condition regained. But our data indicate the effect of starvation last longer than we imagine, and that we should be kept concerned for much longer term. The second is that because starvation in young stage affecting not only the overall lifespan, but specifically on later life, it could be a crucial factor for our "health lifespan". It is obvious that people do not wish to live longer just simply, but longer in good health. We did not analyze the fitness of old worms that experienced starvation in their L4, so we don't know whether L4-starvation led to "healthy old worms" or "long-lived old worms", but our data may provide new viewpoint in controlling our health lifespan.

Because suppression of insulin/IGF-1 pathway enhances longevity[15, 20–22, 25], we were interested if insulin/IGF-1 pathway contributed to the L4-starvation-driven lifespan elongation. Although we found that relative expression of *daf-2* and *age-1* tended to be lower in L4-starved adult worms than in L4-fed worms, the decrement of their expression was not significant enough to say the pathway was suppressed (Fig.5A, B). On the other hand, the relative expression of *daf-18*, which suppress the pathway by inhibiting *akt-1*, and *daf-16*, whose expression increased upon suppression of the pathway, increased as worms became old (day22, Fig.5C,D). This indicated a possibility that the pathway had shifted to the state of suppression, possibly contributing to the longevity in late lifespan of L4-starved adult worms. That the gradual increment of *sod-3* expression was observed, known to be associated with longevity[22], may be supportive to the idea (Fig.5E).

L4-starvation seemed to have affected the mobility of adult worms (Fig.3), but the statistical difference was not evident, so we should be cautious in evaluating the effect of L4 starvation on mobility function. Since there has been reported that experiencing starvation during embryogenesis and early childhood could lead to adult diseases including obesity[5–8], we were interested whether starvation in adolescence could lead to obesity in adult. However, we could not detect any influence of L4-starvation on adult fat accumulation using Nile Red fluorescent staining technique. Although this data indicates nutritional status in adolescence may have little effect on adult obesity, it has been reported that Nile Red staining was not so specific to triacylglycerol unless worms were fixed prior to staining procedure[27, 28]. Therefore, the effect of L4-starvation on fat accumulation should be analyze with different method, such as direct quantification of triacylglycerol and GC-MS quantification of fat.

Serotonin has been implicated in longevity in *C.elegans*[16–17, 29], and Murakami *et al.* showed that deletion mutant of *ser-1* and *ser-4*, both encoding seven-transmembrane G-protein-coupled receptors that have properties of 5-HT1 and 5-HT2 receptors[30], showed an opposite effect on lifespan[18]. The deletion mutant of *ser-1* (*ok345*) showed prolonged lifespan. Insulin/IGF-1 pathway seemed to be involved in this *ser-1*-regulated lifespan elongation, because the life-extension effect of *ser-1* mutation was suppressed by *daf-16* knockdown. On the other hand, deletion mutant of *ser-4* (*ok512*) showed shortened lifespan in lifestage-specific manner, and interestingly this lifespan-shortening effect was evident in mid-lifespan. Although *ser-4* has been reported to be involved in locomotion and chemosensation[31] involvement of insulin/IGF-1 pathway in life-shortening effect of *ser-4* is not well understood. Since lifespan of L4-starved adult worms was also stage-specifically elongated at late stage, we were interested in the expression of *ser-4* in L4-starved adult worms. Expression of *ser-4* was significantly increased at late lifespan (day22), supportive to the idea that *ser-4* was involved in L4-starvation lead lifespan elongation (Fig.6).

Our results showed that starvation during the young stage, especially the adolescence, may have profound effect on later life. However, several limitations have to be pointed out in our study. For the first, although the usefulness of worms in mammalian nutrition was addressed[32], there exist distinctive metabolic differences between worms and mammals, such as ability to synthesize *n*-3 and *n* -6 fatty acid in worms. The existence of glyoxylate cycle in worms is also noteworthy because worms can synthesize glucose from fat[33–34]. Therefore, it should be cautious when applying data obtained using worm to human. For the second, although there is developmental similarity that sexual organ maturation occurs in both worm L4 and human adolescence, the hypothesis that comparing worm L4 stage to human adolescence stage should be further tested. More developmental, biochemical and genetic information on the similarity between worm L4 and human adolescence was required to evaluate and reinforce the hypothesis. Lastly, the role of *ser-1* was not tested in this study due to technical issue. Since lifespan elongation effect of *ser-1* deletion was reported to be linked to insulin/IGF-1 pathway, association of *ser-1*, together with *ser-4*, with the pathway may provide important information in the lifespan elongation effect of adolescence starvation.

Author contributions

SM conceived and designed the experiments. MS, MD, FI, MT and MI performed the experiments and analyzed the data with the help of SM. SM prepared the manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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