

Received:
5 October 2018
Revised:
22 January 2019
Accepted:
26 February 2019

Cite as: Naijing Guo,
Jiayu Wang,
XiangMing Wang. Effect of
starvation and high-
carbohydrate diet on learning
ability of *Caenorhabditis
elegans*.
Heliyon 5 (2019) e01289.
doi: 10.1016/j.heliyon.2019.
e01289



Effect of starvation and high-carbohydrate diet on learning ability of *Caenorhabditis elegans*

Naijing Guo^a, Jiayu Wang^b, XiangMing Wang^{c,*}

^aThe High School Affiliated to Renmin University, China

^bChaoyang Experimental Primary School Affiliated to Capital Normal University, China

^cInstitute of Biophysics, Chinese Academy of Sciences, China

* Corresponding author.

E-mail address: xmwang@moon.ibp.ac.cn (X. Wang).

Abstract

Starvation and high-carbohydrate diet have a big impact on our health, while their effects on the learning ability are not so clear. Here, we used *C. elegans* as the model organism to investigate it. We starved the worms for 24 h or fed them with glucose since hatching, and then measured their learning ability at L4 stage using mechanosensory stimulation assay. The results showed that the learning ability was significantly decreased by starvation, while could be gradually recovered after 3 h normal feeding. After glucose treatment, the length-width ratio of worm was reduced and the learning ability was also significantly decreased. Interestingly, this effect could be passed down two generations probably through epigenetic inheritance. To understand the mechanism of these effects, *age-1* and *mec-3* mutants were used and they affected the learning ability differently under normal or adverse conditions. Therefore, we concluded that starvation and high-carbohydrate diet could modulate the learning ability of *C. elegans*, and they were regulated by different gene networks.

Keywords: Genetics, Biophysics, Metabolism

1. Introduction

Many people in developing countries face the problem of food shortage and starvation, which leads to changes in bodily functions. The effect of starvation on metabolism processes and the associated molecular mechanism are well studied [1, 2], but its effect on learning ability remains elusive. As the learning process needs high energy, we propose that starvation might lead to reduced learning ability, and it might be recovered by caloric intake.

On the other hand, obesity, cardiovascular diseases, hypertension, and type II diabetes, which are all induced by high-carbohydrate diet, have become a serious public issue [3]. Aside from causing these damages in peripheral organs and tissues, convincing evidence also shows that high-carbohydrate diet could cause dysfunction in the central nervous system [4, 5, 6]. Moreover, it is proved that the effect of high-carbohydrate diets can be epigenetically transmitted [7]. However, the effect of high-carbohydrate diet on learning ability is rarely studied. We hypothesize that high-carbohydrate diet would cause obesity and dysfunction in neurons, which would reduce the learning ability.

To test these two hypothesis above, we used *Caenorhabditis elegans* (abbreviated as *C. elegans*) as the model system to study it. *C. elegans* is a free-living nematode, which is about 1mm in length and has a rapid life cycle. It takes about 3.5 days to become sexual mature in 20 °C. The majority of them are self-fertilizing hermaphrodite, while males arise at a frequency of 0.2%. In 1965, Sydney Brenner used *C. elegans* as a model to study molecular biology and developmental biology. Since then, it has been widely used on embryonic development, sex determination, apoptosis, behavior, and neurobiology [8].

The learning ability of *C. elegans*, i.e., the change of behavior after certain experience, includes non-associative learning and associative learning. Non-associative learning is the change in the strength of response to a single stimulus; and associative learning is to make association between two stimuli, using one of those as a cue to avoid adverse environment or to find food [9]. In 1990, Rankin *et al.* established mechanosensory stimulation assay, and then it becomes a common assay to investigate the non-associative learning in *C. elegans* [10]. When a stimulus (such as a gentle touch of eyebrow) is applied on the head of a worm, it would instinctively move backward before normally moving forward. When the next stimulus is applied, the worm would still move backward. The distance of this reversal, however, is shorter than the previous one. The reversal distance would be shorter as more stimuli are applied until the worm completely stops moving backward, which means it has habituated to the stimulus. Therefore, the numbers of stimuli before the worms stop moving backward reflect their learning ability: the smaller the number is, the better the learning ability is. As this assay is easy and robust, it is widely used to measure

the learning ability of worms. In this study, the worms were starved or fed with high-carbohydrate diet to study the effect of starvation and high-carbohydrate diet on the learning ability.

2. Materials and methods

2.1. Maintenance of *C. elegans*

C. elegans were cultured as previously described [11], with little modification. Briefly, the worms were grown on nematode growth medium (NGM), fed with *E. coli* OP50, and kept at 20 °C. The wild type strain is Bristol N2 strain. The mutant strains used were as follow: *mec-3*(CB1338), *age-1*(TJ1052), *dpy-11*(TV51143). All strains are provided by *Caenorhabditis* Genetic Center.

2.2. Starvation and high-carbohydrate diet treatment of *C. elegans*

For the starvation treatment, synchronized worms are cultured at 20 °C several days. 24 hours after *E. coli* was consumed, L4 worms are picked to measure their learning ability. The worms do not enter dauer stage.

For the high-carbohydrate diet treatment, liquid culture of OP50 and 400 g/L glucose solution are mixed to make the final concentration of glucose to be 200 g/L, 40 g/L, 4 g/L. Then the solution is mixed well and seeded on NGM plates. Synchronized L1 are transferred to these plates to culture about 2–3 days until the worms are at L4 stage. Then they are used to measure the learning ability.

2.3. Mechanosensory stimulation assay

The assay is performed as described by Kitamura et al. [12] with some modification. One L4 hermaphrodite is picked to NGM plate streaked with *E. coli* OP50 and an eyebrow is used to gently touch its head. If it moves backward, count as the first stimulus. Normally, the worm would move forward after moving backward. During the time when it moves forward the next stimulus was performed. The stimulus is repeated until the worm stopped moving backward. The number of stimuli required is the index for learning ability. The smaller the number is, the better the learning ability is. If these numbers between two groups of treatment show significantly difference ($P < 0.05$), it is considered as improved or decreased learning ability.

The whole process is recorded by Motic Images Plus 2.0 with 25X magnification. At least 20 worms of different strains or under different treatments are recorded. Worms, which have already been recorded, should not be used again.

2.4. Fluorescence imaging

A drop of melted 3% agarose is added on a slide and another slide is put on the top of it. When the agarose is solidified, the top slide is removed and a drop of M9 buffer is added on the agarose pad. *ser-2P3::GFP* transgenic worms or *mec-3* mutants are picked into this drop of M9 buffer to observe using Zeiss fluorescence microscope. Zeiss CCD is used to take photos.

2.5. Statistical analyses

The difference between two groups is analyzed by two tail Student's *t* tests. Values are mean $\pm$ SEM. $n = 40\text{--}80$ for most cases. $P < 0.05$ means significant, and $P < 0.01$ means extremely significant. $P > 0.05$ means not significant. The data comparing multiple groups are analyzed by one way ANOVA and Tukey's multiple comparison test.

3. Results

3.1. The learning ability of normally fed worms

To explore the learning ability of worms under different adverse conditions, we need to measure the learning ability at normal condition firstly. So we used mechanosensory stimulation assay to measure the non-associative learning ability of normally fed worms. The result showed that most of the worms stopped moving backward after 10 stimuli, and the average backward distance decreased gradually (Fig. 1). Independent repeated experiments showed very similar data, indicating that this assay was robust enough to test the learning ability under starvation or high-carbohydrate diet conditions.

3.2. Starvation reduces the learning ability of *C. elegans*

Next, we used the same assay to measure the learning ability of starved worms (about 1 day starvation), and worms that were fed for 1h, 2h, and 3h after starvation. The times of stimuli for the starved, fed for 1h, 2h, and 3h animals after starvation to stop moving backward was about 27, 21, 15, and 10, respectively (Fig. 2 A). It indicated that starvation reduced the learning ability and could be gradually recovered by food intake.

To investigate the mechanism of starvation on learning ability, we used *age-1* and *mec-3* mutants to test if they could affect the learning ability under normal and starvation conditions. *age-1*, encoding a phosphoinositide 3-kinase, is essential in insulin-like pathway, which modulates the fat metabolism and longevity in *C. elegans* [13, 14]. Also, *age-1* mutant is reported to improve learning ability [15].

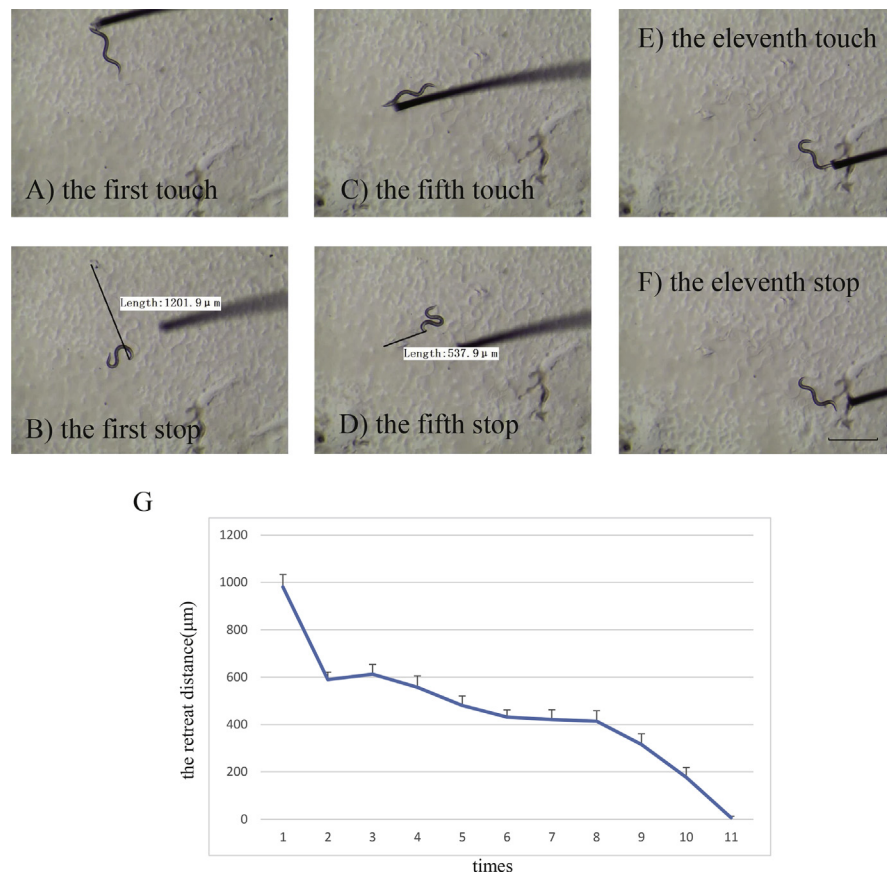


Fig. 1. The non-associative learning ability of *C. elegans* measured by mechanosensory stimulation assay. A-F) the serial pictures of worm under stimulations. Scale bar is 500 μm . A-D showed that the worm moved backward after the stimulus, and the reversal distance (black lines) is also presented in figure. E and F showed that the worm moved forward instead of reversal after the stimulus, demonstrating that this worm habituated to stimulus. G) The abscissa presents the times of the mechanosensory stimulation. The ordinate presents the average length that the worm moved backward after stimulation. Values are mean $\pm$ SEM. $n = 80$. Four times independent experiments.

mec-3 regulates the differentiation of ALM (anterior lateral microtubule cells), AVM (anterior ventral microtubule cell), PVM (posterior ventral microtubule cell), PLM (posterior lateral microtubule cells), FLP (Sensory neuron, Name originally derived from "FLAP".) and PVD neurons (Sensory neuron, Postembryonically born)), which might be involved in the learning ability of *C. elegans* [16]. Here, we used PVD morphology as intuitive illustration of the difference between wild type and *mec-3* mutants (Fig. 2 C). The highly branched dendrites were disappeared in *mec-3* mutants (Fig. 2 C).

The result showed that *age-1* mutant significantly reduced the learning ability when compared with wild type ($P < 0.001$), indicating blockage of insulin-like pathway was adverse for the learning process. Furthermore, the phenotype of *age-1* mutant

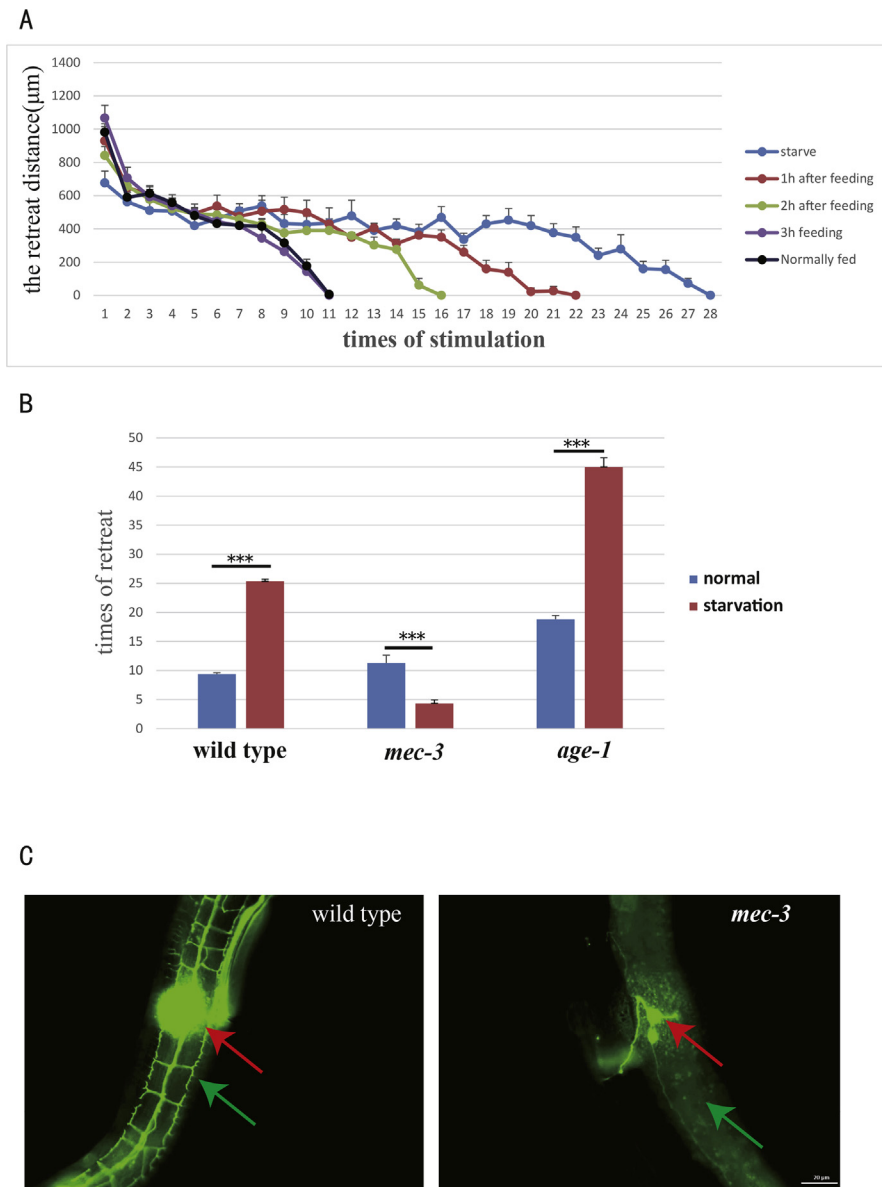


Fig. 2. The effect of starvation to learning ability of *C. elegans*. A) The learning ability in starved worms and worms that were fed for a 1, 2, and 3 h after starving. The abscissa presents the times of the mechanosensory stimulation. The ordinate presents the average length that the worm moved backward after stimulation. Values are mean $\pm$ SEM. $n = 80$. Four times independent experiments. B) The learning ability in starved worms, starved *age-1* mutants and starved *mec-3* mutants. Values are mean $\pm$ SEM. $n = 80$. Four times independent experiments. *** $p < 0.001$. C) The PVD morphology of wild type and *mec-3* mutant. The wild type had highly branched PVD dendrites (green arrow), while *mec-3* mutant showed severely defective PVD morphology (green arrow). Red arrows show the PVD cell body. Scale bar is 20 μm.

could be enhanced after starving ($p < 0.001$) (Fig. 2 B), suggesting that starvation might reduce learning ability through a pathway that was in parallel with insulin-like pathway. However, there was no significant difference in the learning ability of normally fed wild type and *mec-3* mutant ($P < 0.5$), although the backward

distance after stimulus was reduced in *mec-3* mutant, indicating *mec-3* was not involved in the learning process under normal condition. Furthermore, the starved *mec-3* mutant needed fewer stimuli before stopping moving backward ($P < 0.001$), indicating it improved the learning ability and functioned in learning process under starvation specifically (Fig. 2 B).

3.3. High-carbohydrate diet reduces the learning ability of *C. elegans*

Starvation showed big influence on learning ability, next we'd like to know if high-carbohydrate diet, the opposite of starvation, could also affect learning. Firstly, we test the glucose concentration with highest significance. It was reported that glucose treatment could cause worms to get "fatter". Then we measured the length-width ratio of worms fed with 0 g/L, 4 g/L, 40 g/L and 200 g/L glucose. The result showed that all three concentrations decreased the length-width ratio, when compared with the 0 g/L group (meant they got "fatter") (Fig. 3 A-C). Since the decrease of the ratio is most significant at the concentration of 200 g/L, we used this concentration for all the subsequent experiments. Then, mechanosensory stimulation assay was used to measure the non-associative learning ability of worms fed with 200 g/L glucose. The result showed that most worms needed 18 stimuli before stopping moving backward, indicating high glucose decreased learning ability ($p < 0.001$) (Fig. 3 D). Similar with wild type worms, *mec-3* mutants decreased length-width ratio (Fig. 3 E) and learning ability ($p < 0.001$) (Fig. 3 F) after fed with 200 g/L glucose, suggesting *mec-3* did not affect learning process under high sugar condition. Unlike wild type worms, however, *age-1* mutant did not change the length-width ratio when fed with the same concentration of glucose ($p > 0.5$) (Fig. 3 E), and their learning ability was significantly improved ($p < 0.001$) (Fig. 3 F). It suggested that blockage of insulin pathway resisted the high sugar effect to learning ability, and even exceeded the adverse effect of high sugar.

The data above suggested a correlation between obesity and decreased learning ability. Then, we used mechanosensory stimulation assay to measure the learning ability of *dpy-11* mutant, which was much "fatter", to further understand the effect of obesity on learning ability. *dpy-11*, encoding a thioredoxin-like protein, expresses exclusively in hypodermis. And the mutation of *dpy-11* gene has a significant impact on body morphogenesis, causing the worm to be "fat" (Fig. 4 A and B) [17]. The result showed that *dpy-11* mutant decreased the learning ability significantly when compared with wild type ($p < 0.01$) (Fig. 4 C), supporting the hypothesis that fat reduced the learning ability.

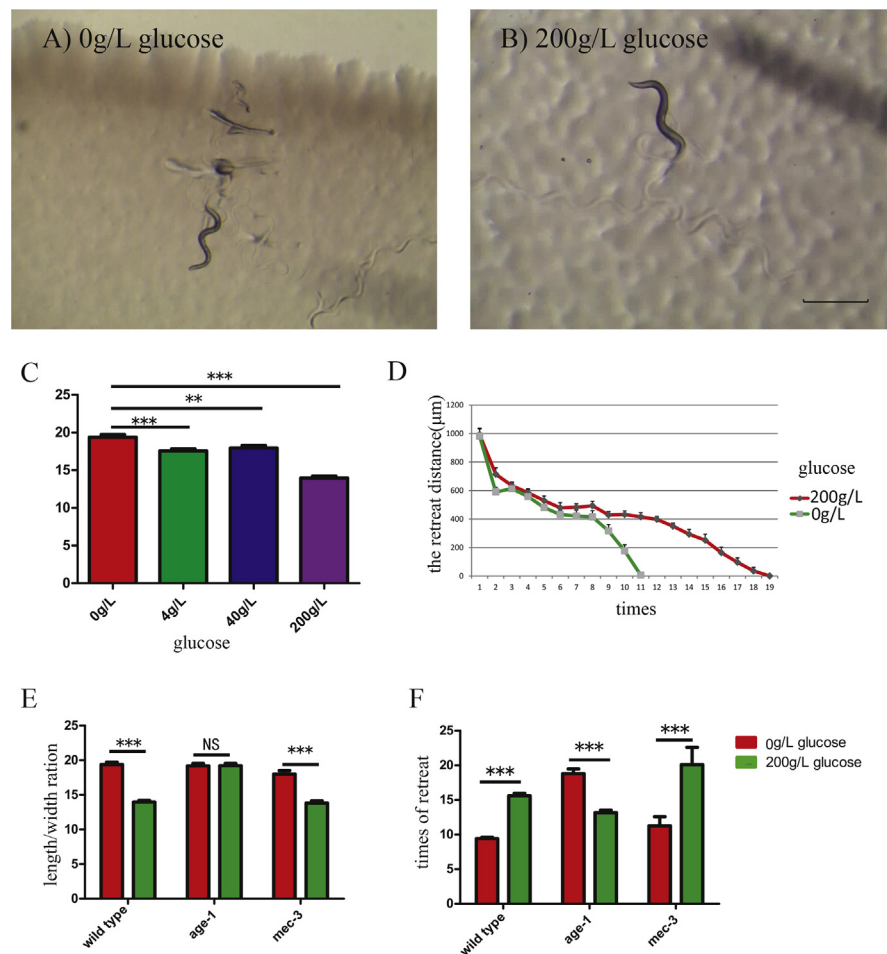


Fig. 3. High-carbohydrate diet caused “obesity” of worm. A) Normal feeding worm. B) 200 g/L glucose feeding worm. Scale bar is 500μm. C) The length-width ratio of worms fed with different concentration of glucose. The abscissa presents the length-width ratio. Values are mean ± SEM. n = 40. ***p < 0.001, **p < 0.01. Two times independent experiments. D) Using mechanosensory stimulation assay to measure the non-associative learning ability in normally fed worms and worms on high-carbohydrate diet. The abscissa presents the number of times of the mechanosensory stimulation. The ordinate presents the average length that the worm moved backward after stimulation. Values are mean ± SEM. n = 80. Four times independent experiments. E) The effect of high-carbohydrate diet on the length-width ratio of wild type worms, *age-1* mutants and *mec-3* mutants. The abscissa presents the length-width ratio. Values are mean ± SEM. n = 40. Two times independent experiments. ***p < 0.001. ns stands for no significant difference. F) Using mechanosensory stimulation assay to measure the non-associative learning ability in wild type worms, *age-1* and *mec-3* mutants on high-carbohydrate diet. Values are mean ± SEM. n = 40. Two times independent experiments. ***p < 0.001.

3.4. *age-1* mutant keeps thinner on high-carbohydrate diet through low pharyngeal pumping rate

Since *age-1* mutant showed no significant change in length-width ratio when fed with glucose, we speculated that these mutants ate less food than wild type on high-carbohydrate diet. To test this hypothesis, we counted the pharyngeal pumping time in wild type and *age-1* mutant within 30 s. The result showed that *age-1* mutant

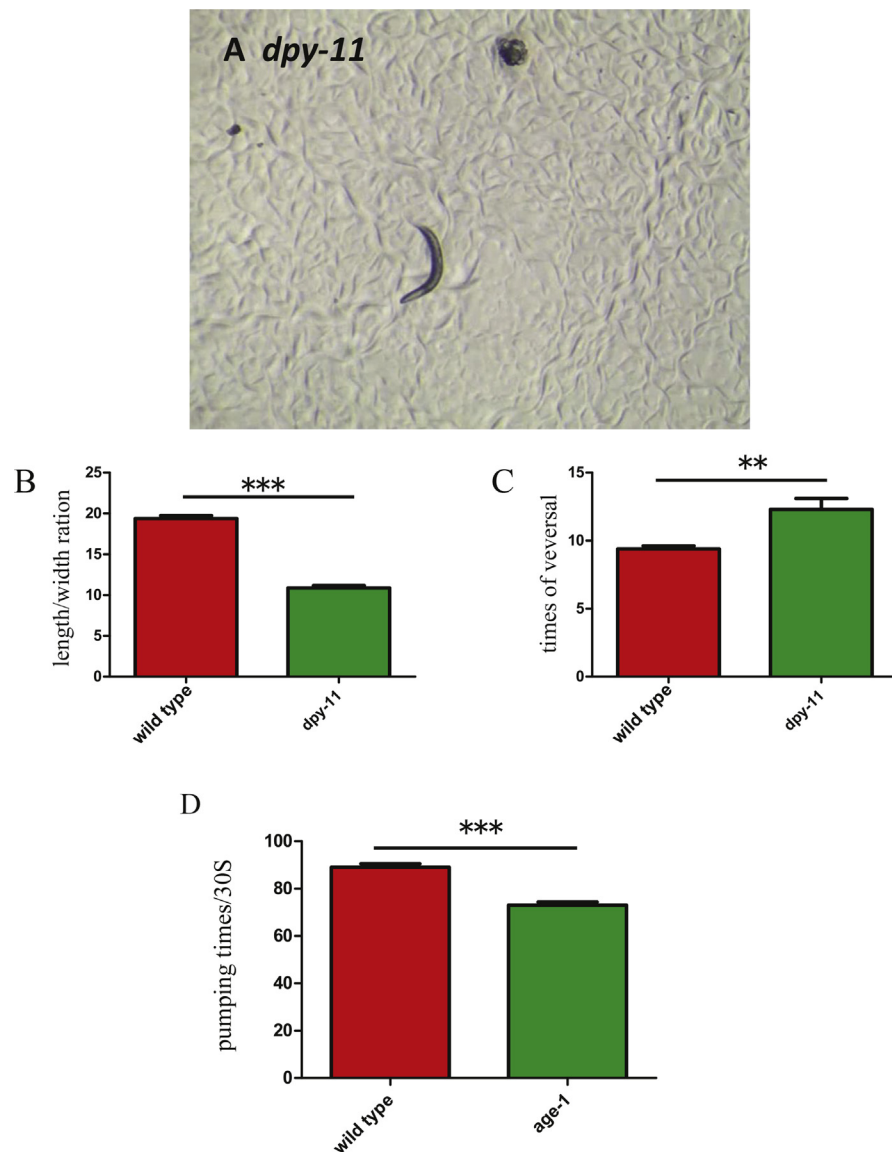


Fig. 4. High-carbohydrate diet's effect on learning ability of worm. A) *dpy-11* mutant. B) The length-width ratio of *dpy-11* mutant. C) Using mechanosensory stimulation assay to measure the non-associative learning ability in *dpy-11* mutant. Values are mean $\pm$ SEM. $n = 40$. Two times independent experiments. ** $p < 0.01$. D) Pharyngeal pumping rate in wild type worms and *age-1* mutants on high-carbohydrate diet. Values are mean $\pm$ SEM. $n = 40$. Two times independent experiments. *** $p < 0.001$.

showed a significantly lower pumping rate when compared with wild type ($p < 0.001$) (Fig. 4 D), suggesting that it indeed ate slower and thus less food than wild type.

3.5. The effect of high-carbohydrate diet could be passed down to several generations

To study whether the decreased learning ability of high-carbohydrate diet has the epigenetic phenomenon, synchronized L1 worms (defined as P0) were treated

with 200 g/L glucose. Then, they were transferred to normal plate at L4 stage and the progenies (F1, F2, F3) were also fed on normal food. We measured the width-length ratio and learning ability of F1, F2, and F3. The result showed that F1 and F2 significantly decreased width-length ratio ($P < 0.001$) and learning ability ($P < 0.001$), when compared with normally fed worm, although a lesser extent when compared with P0. F3 had no significant change in width-length ratio and learning ability when compared with normally fed worm ($P > 0.05$) (Fig. 5). It indicated that the effect of high-carbohydrate to learning ability could be passed down two generations and it might be caused by epigenetic factors, as high sugar could not cause lesion to DNA sequence.

4. Discussion

In this study, we showed that starvation led to a significantly decreased non-associative learning ability, while the study of Vohra et al. shows that starvation for 1 h causes an improved associative learning ability [18]. These data indicated that mild starvation could improve learning ability, while acute starvation would reduce learning ability, suggesting that different degree of starvation exerts different effect on learning ability. This assumption is also consistent with Mita et al.'s observation on taste aversion of pond snail *lymnaea stagnails* [19] and the mechanism needs further investigation.

It is already known that *mec-3* is essential for the differentiation of ALM, AVM, PVM, PLM, FLP, PVD mechanoreceptor neurons. When *mec-3* mutants were normally fed, although the worms reversed shorter distance after stimulus, they had no significant change in their learning ability when compared with wild type, suggesting that those mechanoreceptor neurons are involved in touch sensing but not non-associative learning. However, when the mutant is starved, it shows a significantly improved learning ability, suggesting that those neurons function as negative regulators in the learning progress when starved. Therefore, our data suggest that different neural pathways regulate the learning process when normally fed and starved, and the mechanism requires further study.

High-carbohydrate diet significantly decreases the length-width ratio as well as the learning ability in wild type. On the other hand, *age-1* mutant shows neither decreased length-width ratio nor decreased learning ability, suggesting that obesity would affect learning ability. Moreover, *dpy-11* mutant, which is fatter than wild type, also shows significantly decreased learning ability, supporting the correlation between fat and decreased learning ability. Based on the research from Agrawal et al., fructose consumption would lead to decreased functional mitochondria bioenergetics, causing a decreased learning ability in mice [20]. We propose that the decreased learning ability of high-carbohydrate diet worms might be caused by

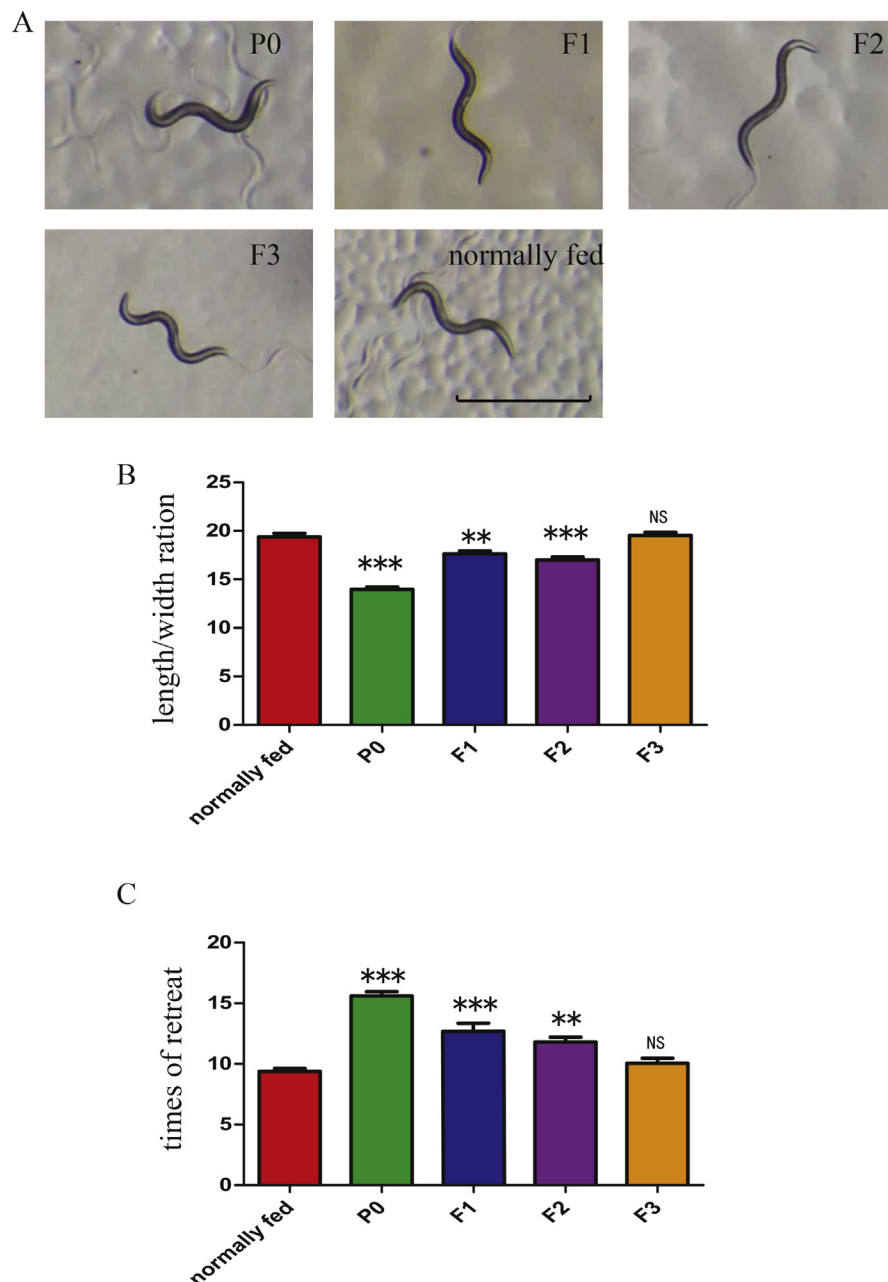


Fig. 5. High-carbohydrate diet effect on learning ability passes several generations through epigenetics. A) Pictures of worms fed with glucose, progenies of those worms and normally fed worms. Scale bar is 500 μ m. B) The length-width ratio of worms fed with glucose, progenies of those worms and normally fed worms. The abscissa presents the length-width ratio. Values are mean $\pm$ SEM. $n = 40$. Two times independent experiments. ** $p < 0.01$, *** $p < 0.001$. C) The non-associative learning ability in worms on high-carbohydrate diet, their progenies and normally fed worms. Values are mean $\pm$ SEM. $n = 40$. Two times independent experiments. ** $p < 0.01$, *** $p < 0.001$. NS: not significant.

the similar mechanism. Also, we found that normally fed progenies of high-carbohydrate diet parent showed decreased width-length ratio and learning ability, suggesting that epigenetic factors function to pass down the effect to several generations. The mechanism might be associated with histone modification, non-coding RNA, and so on.

When normally fed, *age-1* mutant has a reduced learning ability compared to wild type. This might be caused by failure to inhibit the activity of downstream DAF-16, allowing this transcription factor to transport to the nucleus to activate the expression of several genes [21], and these genes might act as negative regulators in the learning process. Also, *age-1* functions as a positive regulator. However, after fed with high-carbohydrate diet *age-1* mutant increases the learning ability when compared with normal fed animals, which might be caused by the decreased pumping rate in *age-1* mutant. Therefore, we speculate that *age-1* mutant could reduce their food consumption when treated with high-carbohydrate diet, leading to the fact that they do not get fatter or reduce learning ability as the wild type. The reason why *age-1* mutant shows an improved learning ability could also be that the high-carbohydrate induced decrease of learning ability is inhibited by some genes under the control of *daf-16* gene.

5. Conclusion

Our results show that starvation decreases the learning ability of *C. elegans* and could be gradually recovered by food intake. High-carbohydrate diet decreases the learning ability of *C. elegans* and this effect could be passed down two generations through epigenetic inheritance. The mechanism study shows that insulin-like pathway affects the learning ability of *C. elegans* and MEC-3 governing sensory neurons are associated with the learning ability of *C. elegans* under starvation situation.

Declarations

Author contribution statement

Naijing Guo, Jiayu Wang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Xiangming Wang: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

Xiangming Wang was supported by grants from the Natural Science Foundation of China (31571061, 31771138) and Youth Innovation Promotion Association of Chinese Academy of Sciences funding.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

We are grateful to the *Caenorhabditis* Genetics Center for strains.

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