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Vitamin B₁₂ deficiency in *Caenorhabditis elegans* results in loss of fertility, extended life cycle, and reduced lifespan

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ABSTRACT

Vitamin B₁₂ (B₁₂) deficiency has been linked to developmental disorders, metabolic abnormalities, and neuropathy; however, the mechanisms involved remain poorly understood. *Caenorhabditis elegans* grown under B₁₂-deficient conditions for five generations develop severe B₁₂ deficiency associated with various phenotypes that include decreased egg-laying capacity (infertility), prolonged life cycle (growth retardation), and reduced lifespan. These phenotypes resemble the consequences of B₁₂ deficiency in mammals, and can be induced in *C. elegans* in only 15 days. Thus, *C. elegans* is a suitable animal model for studying the biological processes induced by vitamin deficiency.

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1. Introduction

Vitamin B₁₂ (B₁₂), the largest and most complex vitamin molecule, is exclusively synthesized by certain bacteria, and is most abundant in higher order predators in the natural food chain. After B₁₂ is taken up by living cells, it is converted into two coenzyme forms, 5'-deoxyadenosylcobalamin (Ado-B₁₂) and methylcobalamin (CH₃-B₁₂), which function as the coenzymes for methylmalonyl-CoA mutase (MCM; EC 5.4.99.2) [1] and methionine synthase (MS; EC 2.1.1.13) [2], respectively.

Food derived from animal products is the major dietary source of B₁₂; therefore, strict vegetarians and/or the elderly are at a high risk of developing B₁₂ deficiency [3]. The major symptoms of B₁₂ deficiency are developmental disorders, megaloblastic anemia, metabolic abnormalities, and neuropathy [4], although the underlying disease mechanisms are poorly understood [5,6]. Developing animal models of B₁₂-deficiency is essential for investigating the molecular mechanisms that are defective in this metabolic disorder. However, such animal models have proven difficult to generate because animals must be fed with a B₁₂-deficient diet for long periods to achieve B₁₂ deficiency

[7]. The lack of robust B₁₂-deficient animal models has limited investigations to the biochemical mechanisms induced by B₁₂-deficiency.

Caenorhabditis elegans offers several advantages for genetic and biochemical studies, including a short lifespan, a 3-day life cycle, a completely sequenced genome, <1000 somatic cells, and the ability to change reproductive rates, life cycle, and locomotive behavior [8]. In addition, many molecular and cellular processes are conserved between nematodes and mammals. Most human disease genes and pathways are present in the worm [9]. Thus, this animal has been widely used as a model organism for studying a variety of biological processes including apoptosis, cell signaling, cell cycle regulation, gene regulation, metabolism, and aging [8].

The enzymes responsible for human methylmalonic aciduria, a disease caused by B₁₂ deficiency, have been partially characterized in *C. elegans* using RNA interference techniques [10,11]. However, whether B₁₂ is an absolute requirement for normal growth and physiological function in *C. elegans* is unknown. If a method for creating viable B₁₂-deficient worms can be found, *C. elegans* could serve as a suitable model organism for studying the effects of B₁₂ deficiency.

In this study, we report a novel method for inducing B₁₂ deficiency in *C. elegans*, describe the effects of B₁₂ deficiency on various biomarkers, and characterize the physiological roles of B₁₂ in this model organism.

Abbreviations: Ado-B₁₂, 5'-deoxyadenosylcobalamin; B₁₂, vitamin B₁₂; *C. elegans*, *Caenorhabditis elegans*; CH₃-B₁₂, methylcobalamin; Hcy, homocysteine; MCM, methylmalonyl-CoA mutase; MMA, methylmalonic acid; MS, methionine synthase.

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2. Materials and methods

2.1. Organisms and growth conditions

The N2 Bristol wild-type *C. elegans* strain was maintained at 20 °C on nematode growth medium (NGM) plates using the *Escherichia coli* OP50 strain as the food source [12]. To induce B₁₂ deficiency, worms were grown on 1.7% (w/v) agar plates containing M9 medium (3 g/L KH₂PO₄, 6 g/L Na₂HPO₄, 0.5 g/L NaCl, 1 g/L NH₄Cl, 1 mmol/L MgSO₄, 50 μmol/L CaCl₂, 2 g/L glucose, 4 mg/L thiamine hydrochloride, and 5 mg/L cholesterol) in 1 L H₂O. Plates containing B₁₂-supplemented (100 μg/L cyanocobalamin) M9-medium each received one egg obtained from worms grown on NGM plates with B₁₂-deficient OP50 *E. coli* (described below). Eggs were allowed to hatch and develop into egg-laying adult worms. The adult worms were then removed from each plate, eggs were collected, and each egg was transferred onto a new control plate. After this procedure was repeated at least 10 times, the resultant worms were used as experimental controls.

2.2. Preparation of B₁₂-deficient *E. coli* cells

E. coli OP50 was grown in M9 medium (3 g/L KH₂PO₄, 6 g/L Na₂HPO₄, 0.5 g/L NaCl, 1 g/L NH₄Cl, 1 mmol/L MgSO₄, 50 μmol/L CaCl₂, 2 g/L glucose, and 4 mg/L thiamine hydrochloride) at 37 °C for 3 days. Cells were inoculated every 3 days into fresh M9 medium and used as a food source for *C. elegans*. The B₁₂ content (0.2 μg/g wet weight) of *E. coli* cells grown in the M9 medium was significantly reduced compared with cells grown in the standard Luria–Bertani medium (11.1 μg/g wet weight).

2.3. Preparation of B₁₂-deficient *C. elegans*

C. elegans were grown at 20 °C on B₁₂ (100 μg/L)-supplemented medium using B₁₂-deficient *E. coli* OP50 as a food source. An individual worm egg was transferred onto each plate, which contained fresh B₁₂-deficient medium seeded with the B₁₂-deficient *E. coli* OP50. After the eggs hatched, worms were allowed to grow until they became adults and had laid eggs (yielding the F1 generation). Individual eggs were removed from the plate and each was transferred onto a fresh plate containing B₁₂-deficient medium and grown to maturity under the same conditions (yielding the F2 generation). This process was repeated for five generations. After 3 days, adult worms of each generation (F1–F5) were used for experiments. This protocol is shown in Fig. 1.

2.4. Vitamin B₁₂ assay

F1–F5 worms grown under B₁₂-supplemented or B₁₂-deficient conditions were harvested and incubated for 1 h at 20 °C in fresh M9 medium to remove any residual *E. coli* cells. Worms (2 g wet weight) were then disrupted using a hand homogenizer (AS ONE Corp., Osaka, Japan) and sonicated (6 kHz for 60 s) three times. The worm homogenate was resuspended in 100 mL of 57 mmol/L sodium acetate buffer (pH 4.8) containing 0.05% (w/v) KCN and boiled for 30 min. The extract was centrifuged at 15,000 g for 15 min at 4 °C and the supernatant was used for assaying B₁₂ concentrations by standard microbiological methods utilizing *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830, as described previously [13].

2.5. B₁₂-related biomarker assays

F1–F5 worms grown under B₁₂-supplemented or B₁₂-deficient conditions were collected and washed in M9 medium as described above. The harvested worms were resuspended in 0.5 mL of 100

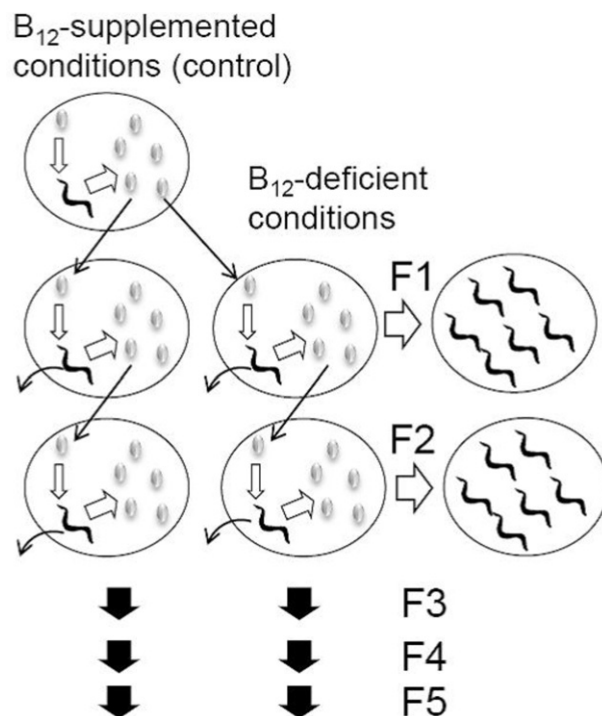


Fig. 1. Preparation of B₁₂-deficient *C. elegans*. Single eggs from *C. elegans* adults grown on NGM plate were transferred onto individual plates containing B₁₂-supplemented M9 seeded with B₁₂-deficient *E. coli* OP50 (control) and grown for 3 days. The eggs hatched and developed into adult worms, which then laid eggs. The adult worm was removed from each plate and individual eggs were transferred individually onto fresh identical plates. This procedure was repeated at least 10 times. These worms were used as experimental control worms. To prepare B₁₂-deficient worms, single eggs from the control worm were transferred onto individual plates containing B₁₂-deficient M9 medium seeded with B₁₂-deficient *E. coli* and allowed to reach maturity and lay eggs (F1 generation). F1 worms obtained from these eggs were used to generate F2 worms following the same procedures. This process was repeated to prepare F5 B₁₂-deficient worms.

mmol/L potassium phosphate buffer (pH 7.0) at 2 °C and homogenized using a hand homogenizer. The cell homogenate was centrifuged at 15,000 g for 15 min at 4 °C and the supernatant was used as a crude homogenate for subsequent biomarker assays.

Methylmalonic acid (MMA) and homocysteine (Hcy), two indices of B₁₂-deficiency, were assayed using the high performance liquid chromatography (HPLC) methods of Al-Dirbashi et al. [14] and Febriani et al. [15], respectively. MCM (methylmalonyl-CoA mutase)- [16] and MS (methionine synthase)- [17,18] activities were assayed at 37 °C as previously described. Total- and holo-enzyme activities were determined in the presence or absence of each B₁₂ coenzyme (Ado-B₁₂ for MCM and CH₃-B₁₂ for MS).

2.6. Analysis of egg-laying capacity, life cycle, and lifespan

Measurement of egg-laying capacity was based on the method of Byerly et al. [19]. Individual L4-stage worms grown under B₁₂-supplemented or B₁₂-deficient conditions were selected, transferred onto the fresh plates containing the same culture medium, and incubated for 1 day at 20 °C. After laying eggs, each worm was removed from the plate, and the eggs were counted. Egg counting was performed in triplicate.

The life cycle and lifespan of B₁₂-deficient F5 worms were determined at 20 °C using the synchronization method of Johnson and Wood [20]. Worms were scored as dead when they no longer responded to prodding with a pick. In each survival experiment, 100 worms were used.

2.7. Protein quantitation

Proteins were assayed by the method of Bradford [21] using ovalbumin as a standard.

2.8. Statistical analysis

The effects of B₁₂-deficiency on various *C. elegans* biomarkers were evaluated by one-way ANOVA, and a post-hoc analysis was performed using Tukey's multiple comparison tests. Analyses were performed with GraphPad Prism 3 for Windows version 2.01 (GraphPad software Inc., La Jolla, CA, USA). All data are presented as the mean ± SD. Differences were considered statistically significant when $p < 0.01$.

3. Results and discussion

3.1. Effects of B₁₂-deficient growth conditions on various B₁₂-related biomarkers in *C. elegans*

Nematodes grown on B₁₂-supplemented M9 medium with B₁₂-deficient *E. coli* OP50 as a food source (control) showed identical growth rates that were identical to that of worms grown under normal conditions (data not shown), indicating that the experimental control conditions were adequate for the normal growth of *C. elegans*. The control worms were able to ingest both B₁₂-enriched agar medium and B₁₂-deficient *E. coli* cells. Therefore, they would mainly absorb sufficient amount of free B₁₂ from the agar medium because of low B₁₂ content in *E. coli*.

Under B₁₂-deficient conditions, the B₁₂ content of the worms decreased gradually over four generations (Fig. 2). The B₁₂ concentration in F5 generation worms was only 4% compared with that in the control worms. These results indicate that dietary B₁₂ deprivation over five generations leads to a significantly decreased B₁₂ status in *C. elegans*. The MMA and Hcy indices of B₁₂ deficiency were assayed in *C. elegans* grown under both control and B₁₂-deficient conditions. There was a significant increase in the levels of both compounds between F3 and F5 generation worms (Fig. 3A and B). Hcy and MMA levels were approximately four and five times greater, respectively, in F5 worms grown under B₁₂-deficient conditions than in the control worms. Although holo-MCM activity significantly decreased in F4 and F5 worms grown under B₁₂-deficient conditions (Fig. 3E), the total MCM activity (holo- and apo-enzymes) increased (Fig. 3C), which indicates that apo-MCM activity is significantly increased by B₁₂ deficiency. Both total- and holo-MS activities significantly decreased with each generation until the F4 generation and was maintained at a constant level thereafter (Fig. 3D and F). These results indicate that F5 generation worms grown under B₁₂-deficient conditions develop severe B₁₂ deficiency. However, one day after transfer of B₁₂-deficient worms onto the B₁₂-supplemented medium, the level of these B₁₂-related biomarkers recovered considerably (data not shown). These results indicate that B₁₂ functions as a cofactor for both MCM and MS in *C. elegans* and that B₁₂-dependent changes in both MCM and MS enzyme activities occur in *C. elegans* and mammals [22,23]. However, using the nematode model, the time needed to produce a severely B₁₂-deficient animal is only 15 days (i.e. five generations).

Holo-MS activity was rapidly decreased by F1 generation under B₁₂-deficient conditions (Fig. 3F), but holo-MCM activity was not changed until the F4 generation (Fig. 3E), which indicated that MS is more sensitive to cellular B₁₂ concentrations than MCM. Yamada et al. have demonstrated that most MS activity is derived from holo-enzyme in B₁₂-sufficient or -deficient mammals because the apo-enzyme is very unstable [22]. In contrast, Nakao et al. have indicated that holo-MCM activity was less than 5% of the total enzyme activity in B₁₂-sufficient rats and that a marked increase in the apo-enzyme activity occurred under B₁₂-deficient conditions [23]. In this *C. elegans* study, holo-MCM activity was 97% of the total enzyme activity in the

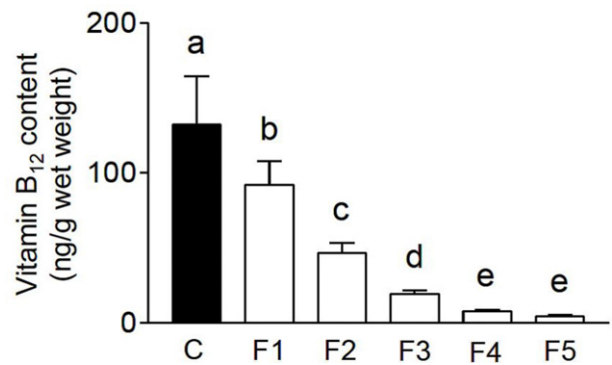


Fig. 2. B₁₂ content is reduced in worms grown under B₁₂-supplemented and -deficient conditions. The B₁₂ content of F1–F5 worms grown under control (black bar) and B₁₂-deficient (white bars) conditions was assayed using microbiological methods. Data represent the mean ± SD of 10 independent experiments. Different letters (a–e) indicate values that are significantly different ($p < 0.01$); identical letters indicate values that are not significantly different.

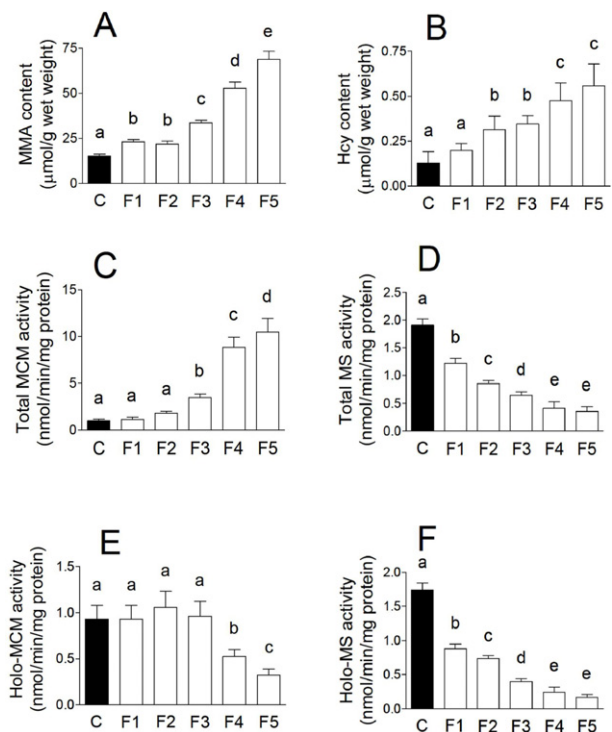


Fig. 3. Vitamin B₁₂ deficiency changes the concentration of various B₁₂-related biomarkers. (A) Methylmalonic acid (MMA) and (B) homocysteine (Hcy) content, (C) total- and (E) holo-methylmalonyl-CoA mutase (MCM) activity, and (D) total- and (F) holo-methionine synthase (MS) activity were measured in extracts of worms grown under B₁₂-supplemented (black bar) and B₁₂-deficient conditions (white bars) for up to five generations. For (E) holo-MCM and (F) holo-MS activities, measurements were made in the absence of specific coenzymes. Data represent mean ± SD of 10 independent experiments. Different letters (a–e) indicate values that are significantly different ($p < 0.01$); identical letters indicate values that are not significantly different.

control worms and holo-enzyme activity gradually decreased in the F1 (92%), F2 (50%), and F3 (24%) generations, under B₁₂-deficient conditions. However, the specific activity of holo-MCM did not change until the F4 generation even if the B₁₂ content of the worms significantly decreased along with increased MMA content. The details of the occurrence of this MCM-independent increase in MMA concentration soon after the onset of B₁₂-deficiency remain to be elucidated.

3.2. B₁₂ deficiency affects *C. elegans* egg-laying capacity, life cycle and lifespan

Egg-laying rates significantly decreased in B₁₂-deficient worms (Fig. 4A), which also showed a significantly prolonged life cycle compared with the control worms (Fig. 4B). Similarly, B₁₂-deficient rats have been reported to show severe growth retardation [24] and infertility [25].

The lifespan of B₁₂-deficient F5 worms were significantly decreased by B₁₂ deficiency (Fig. 4C). The maximal lifespan of B₁₂-deficient worms was reduced to 18 days, compared with a lifespan of 24 days in the control worms (Fig. 4C). These data demonstrate for the first time that B₁₂ deficiency significantly reduces the lifespan of animals.

B₁₂ deficiency causes severe growth retardation and various metabolic disorders in mammals [24]. The B₁₂ coenzyme Ado-B₁₂ functions as a coenzyme of MCM, catalyzing the isomerization of R-methylmalonyl-CoA to succinyl-CoA in the mitochondria. Odd-numbered fatty acids, branched chain amino acids, and cholesterol are metabolized by methylmalonyl-CoA to the tricarboxylic acid (TCA) cycle intermediate succinyl-CoA by MCM [1]. When MCM activity was significantly decreased by B₁₂ deficiency, MMA abnormally accumulated in the cells [24]. The elevated concentration of MMA mainly inhibits mitochondrial respiration because of competitive inhibition of succinate dehydrogenase (EC 1.3.99.1) by MMA [25]. TCA cycle inhibition by MMA accumulation contributes to various metabolic disorders associated with B₁₂ deficiency [24], including severe growth retardation (prolonged life cycle) in the B₁₂-deficient worms.

Many studies have demonstrated that there is a relationship between B₁₂ deficiency and infertility in males and females [26,27]. However, the mechanism whereby B₁₂ deficiency causes infertility is poorly elucidated. CH₃-B₁₂ functions as a coenzyme of MS, which catalyzes the methyl transfer from methyltetrahydrofolate to homocysteine, resulting in the donation of a methyl group to homocysteine, forming methionine [2]. MS is important to re-synthesize methionine and to metabolize methyltetrahydrofolate. Tetrahydrofolate is the precursor for the methylene derivative of folate, which is essential for thymidine supply and normal DNA replication in cells [28]. Furthermore, methionine is one of the amino acid building blocks of protein and acts as the universal methyl group donor (*S*-adenosylmethionine) for a large number of methylation reactions. Yamada et al. [29] reported that reduced testicular MS activity is the primary cause of pathological impairment of spermatogenesis owing to B₁₂ deficiency and that methionine supplementation to the diet can reduce this impairment. Bennet [30] has reported that B₁₂ deficiency may lead to recurrent fetal death owing to the elevated Hcy levels. The epigenetic regulation of gene expression involves remodeling of chromatin by either the addition of methyl group to DNA and/or the post-translational modification of histone amino acid residues. *S*-adenosylmethionine is a critical substrate for histone methyltransferases, whereas *S*-adenosylhomocysteine is a potent inhibitor of the enzymatic reaction [31]. It has been observed that the concentration of *S*-adenosylmethionine is reduced, with a concomitant increase in *S*-adenosylhomocysteine concentration, in B₁₂-deficient animal models or humans [32]. Our preliminary experiments indicated that *S*-adenosylmethionine/*S*-adenosylhomocysteine ratios significantly decrease in B₁₂-deficient *C. elegans* relative to control worms (data not shown). The decreased *S*-adenosylmethionine/*S*-adenosylhomocysteine ratios may lead to abnormal epigenetic regulation of gene expression, including gene relevant to fertility.

These observations suggest that decreased egg-laying and prolonged life cycle found in B₁₂-deficient worms are because of various B₁₂-associated metabolic disorders, which results in abnormal epigenetic regulation of the expression of certain genes.

Approximately, 1% of B₁₂-deficient worms showed a specific morphological abnormality (Fig. 5A), similar to the short and plump

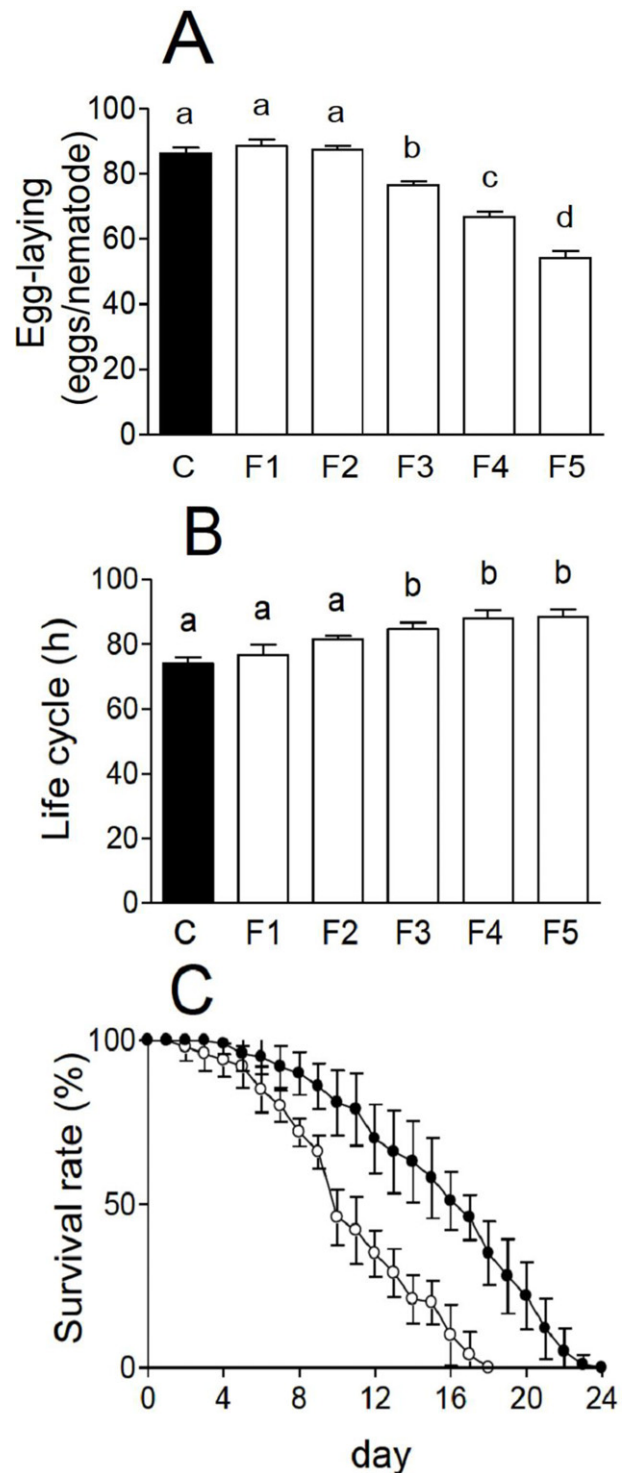


Fig. 4. Vitamin B₁₂ deficiency reduces egg-laying capacity and lifespan, and increases the length of the life cycle in *C. elegans*. (A) Total number of eggs per worm, (B) the length of the life cycle (h), and (C) the lifespan were determined in the control (black bar) and B₁₂-deficient (white bars) F5 worms. Data represent mean \pm SD of 10 independent experiments. Different letters (a–d) indicate values that are significantly different ($p < 0.01$); identical letters indicate values that are not significantly different.

“dumpy” mutant phenotype that is formed because of disordered cuticle collagen biosynthesis [33,34]. However, there is no information available on the relationship between B₁₂ deficiency and collagen biosynthesis. Hcy, which is significantly increased by B₁₂ deficiency,

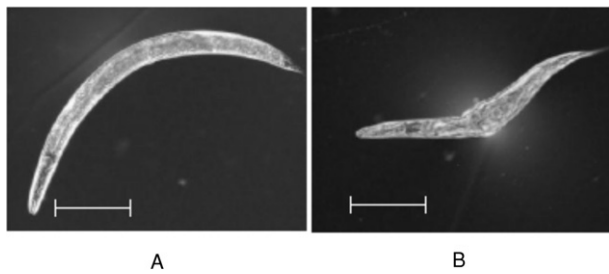


Fig. 5. B₁₂-deficient *C. elegans* show morphological changes. Differential interference microscopy images of the (1) control and (2) B₁₂-deficient worms were obtained using an IX71 microscope (OLYMPUS Corp., Tokyo, Japan). The length of individual worms was measured using Image J software, bar = 200 μm.

has been shown to interfere with post-translational modifications of collagen directly by inhibiting lysyl oxidase (EC 1.4.3.13), which is involved in collagen cross-linking [35]. This observation and our data indicate a possible link between B₁₂ deficient and collagen biosynthesis including post-translational modifications of collagen cross-linking.

Our results indicate that B₁₂ is essential for *C. elegans* growth and that prolonged B₁₂ deficiency induces a number of phenotypes, including decreased egg-laying capacity (infertility), prolonged life cycle (growth retardation), and a reduced lifespan. Therefore, we propose that *C. elegans* is an ideal model organism for investigating the mechanisms driving such B₁₂-deficient phenotypes, as B₁₂ deficiency can be induced in this animal in only 15 days. However, there are some limitations to this animal model; for example, *C. elegans* does not have any blood corpuscle systems. Moreover, bioinformatic analyses indicate that *C. elegans* also does not have any orthologs of three B₁₂-transport proteins (haptocorrin, intrinsic factor, and transcobalamin II) involved in human gastrointestinal absorption and subsequent blood circulation of B₁₂. Thus, *C. elegans* is not suitable for use as a model organism to study the mechanisms of some human B₁₂-deficient disease phenotypes, such as megaloblastic anemia and dysfunctions of intestinal absorption and transport of B₁₂. However, this animal is widely used as a model organism for studying the mechanisms of fertilization [36] and embryonic cell division [37]. Moreover, *C. elegans* is often used for understanding human brain and neuronal disorders [38] in addition to the effects of certain molecules on learning and memory [39]. *C. elegans* may become a suitable organism and a powerful new tool for the study of B₁₂-deficient human diseases such as infertility [26,27,29], fetal death [30,40], neuropathy [41], and cognitive impairment [42].

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