Investigating the Localisation and Action of Vitamin B_{12} in *C. elegans*

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Declaration

No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent, or any other University or Institution of learning.

University of Kent

Master's by Research in Cell Biology

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Abstract

Cobalamin, also known as vitamin B_{12}, is used as a coenzyme for key enzymes involved in the S-adenosyl methionine (SAM) cycle and branched-chain amino acid (BCAA) metabolism, which lead to many downstream targets. Thus, B_{12} is required for many physiological functions including DNA synthesis, cell division, erythrocyte production, and myelin sheath maintenance. Biologically active B_{12} is produced by a select group of bacteria, mainly by fermentation in the gastrointestinal tract of animals, and archaea. Humans must acquire B_{12} from animal products, and B_{12} deficiency is associated with pernicious anaemia and neural deterioration. However, deficiency is only acquired after several years, therefore studying B_{12} action and transport in an animal model where B_{12}-deficiency can be induced in a few weeks is both cost- and time-efficient. This project investigates the role and transport of B_{12} in the nematode *C. elegans,* with the outlook that it could be used as a whole animal model to fully understand its action.

B_{12}-deficiency was induced in *C. elegans* by growing them on media lacking this vitamin, and found that it caused delayed life cycle, reduced lifespan, and a reduction in progeny. After noticing that B_{12} deficiency had a negative impact on lifespan, and knowing that B_{12} is important for neuronal function in humans, the effects of B_{12}-deficency on *C. elegans* neuronal branching with age, which is related to neurodegeneration, were examined. We found that B_{12}-deficient worms had earlier-onset and increased neurite branching indicating the interesting possibility that this aspect of B_{12} function could be modelled in worms.

In conclusion, we found that B_{12}-deficiency had a detrimental effect on *C. elegans* health in general, and that this nematode is a good model for studying the action of vitamin B_{12} *in vivo*.

Acknowledgements

Over the course of my Master's I have had the pleasure to meet and learn from many wonderful people and great scientists.

First of all, a special thank you to Dr. Jennifer Tullet, for always keeping her door open and for all her support, guidance, and patience. In addition, to all the members of her laboratory: Dr. Isabel Gonçalves, Max Thompson, Dan Scanlon, Nikolaos Tataridas, Abbie King, and Jack Doolan, for your encouragement and friendship, and for making me feel welcome from the first day.

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I also want to thank Professor Iqbal Hamza for the opportunity to visit his laboratory in Maryland to support my project, with special thank you to Dr. Xiaojing Yuan and Ian Chambers for teaching me new techniques.

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Ana M. Humbert Camps

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Abbreviations

(L)-MM-CoA	L-methylmalonyl-CoA
'Ado'	5'-deoxyadenosyl
3-HP	3-Hydroxypropionate
ABC transporter	ATP-binding cassette transporter
ACDH-1	Acyl-CoA dehydrogenase (*C. elegans* protein)
AdoCbl	Adenosylcobalamin
ALML	Anterior lateral MT cell (left)
ALMR	Anterior lateral MT cell (right)
ATP	Adenosine triphosphate
AVM	Anterior ventral MT cell
B_{12}	Vitamin B_{12}
B_{12}+	M9 solid *C. elegans* media supplemented with 100 µg/L of cyanocobalamin
B_{12}-	Non-supplemented M9 solid *C. elegans* media
BCAA	Branched-chain amino acid
BoB_{12}	BODIPY-B_{12}
BODIPY	Boron-dipyrromethene - BODIPY® TR-X NHS Ester (Succinimidyl Ester)
bp	Base pairs
C. elegans	*Caenorhabditis elegans*
Cbl	Cobalamin
CDI	1,1'-Carbonyldiimidazole
CGC	Caenorhabditis Genetics Center
CN	Cyanide
CNCbl	Cyanocobalamin
-CoA	Coenzyme A
CZ10175	zdIs5[*mec-4p::GFP* + *lin-15*(+)] I. *C. elegans* strain
DMBI	5,6-dimethylbenzimidazole
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

dNTP	Deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
ESI-MS	Electrospray ionisation mass spectrometer
EtOH	Ethanol
FuDR	Fluorodeoxyuridine
GFP	Green fluorescent protein
HC	Haptocorrin
Hcy	Homocysteine
HPLC	High performance liquid chromatography
HPLC-MS	High performance liquid chromatography - Mass spectrometry
IF	Intrinsic factor
LB	Luria-Bertani media
MAP2	Microtubule-associated protein 2
mCeHR	Modified *C. elegans* Habituation and Reproduction media
MCM	Methylmalonyl-CoA mutase (Human)
MeCbl	Methylcobalamin
MMCM-1	Methylmalonyl-CoA mutase (homolog-1) (*C. elegans*)
MQ H_2O	Milli-Q water (ultrapure water)
MRP-5	Multidrug resistance-associated protein 5
MS	Methionine synthase
MT cell	Touch receptor neurons (containing large-diameter, 15-protofilament microtubules)
N, N-DIPEA/DIPEA	N,N-Diisopropylethylamine
N2	N2 Bristol *C. elegans* strain (wild-type)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NGM	Nematode growth media
NHS ester	N-hydroxysuccinimide ester
NR	Nerve ring
OP50	B-type *E. coli* strain OP50 - uracil auxotroph
PCR	Polymerase chain reaction
PLML	Posterior lateral MT cell (left)

PLMR	Posterior lateral MT cell (right)
Prop-CoA	Propionyl-CoA
PTL-1	Protein with tau-like repeats-1
PVM	Posterior ventral MT cell
RFP	Red fluorescent protein
ROS	Reactive oxygen species
SAM	S-Adenosyl methionine
Succ-CoA	Succinyl-CoA
TC	Transcobalamin
TFA	Trifluoroacetic acid
UV	Ultraviolet
VC1011	*acdh-1(ok1489)* I. – *acdh-1*-deletion *C. elegans* strain
WT	Wild-type

Introduction

This project examines the function and localisation of vitamin B_{12} in *C. elegans*. This will allow us to understand more about the role of this vitamin in this model organism, which should later inform studies in higher organisms.

Vitamin B_{12}

Vitamin B_{12} is usually used as an umbrella term for all forms of cobalamin that are bioactive in humans, with particular emphasis on the most commonly available commercial derivative, cyanocobalamin. Cobalamin is a water-soluble molecule comprised of a tetrapyrrole corrin ring, which is formed by a cobalt ion flanked by four nitrogen atoms, as well as a variable upper β-axial ligand, and 5,6-dimethylbenzimidazole (DMBI) base as its lower α-axial ligand (Fig. 1) (Gherasim *et al.*, 2013; Kumar and Kozlowski, 2017). There are multiple cobalamin analogues which can be produced by changing the upper β-ligand of the compound. In the case of the majority of industrially synthesised vitamin B_{12} the upper ligand is formed by cyanide, which forms cyanocobalamin. Cyanocobalamin is a stable, cost effective, and bioactive form of B_{12}, as well as being easy to crystallise, so it is the most common form available in supplements (Obeid *et al.*, 2015; Watanabe *et al.*, 2013). B_{12} is absorbed in the small intestine and converted to two active forms of vitamin B_{12} in the body: methylcobalamin (MeCbl), which has a methyl- group, and adenosylcobalamin (AdoCbl), which has a 5'-deoxyadenosyl (Ado) group, as their β-ligands.

Some bacteria and most cyanobacteria produce a form of B_{12} known as pseudocobalamin, which humans cannot convert to the active forms AdoCbl and MeCbl. In pseudocobalamin, the base of the α-axial position in the lower nucleotide loop is substituted with adenine (Fig. 1). Spirulina, which is often recommended as a vegan source of vitamin B_{12}, has been shown to mainly produce this non-bioactive cobalamin analogue (Watanabe *et al.*, 2007; Watanabe *et al.*, 1999). This is a problem for vegetarians, and vegans, in particular, as if they do not take B_{12} supplements they are far more susceptible to B_{12} deficiency than omnivores.

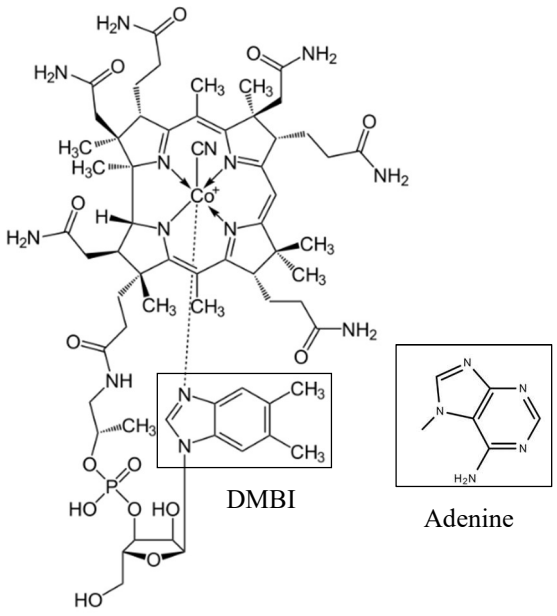


Figure 1. Structure of vitamin B_{12} (cyanocobalamin). Corrinoid tetrapyrrole ring with 5,6-dimethylbenzimidazole (DMBI) attached via the lower loop (box on the left). The upper β ligand in this case is cyanide (CN). The base in the box on the right is adenine. The unbound single bond is where the base binds to the lower α-axial loop of cobalamin (instead of DMBI), which forms pseudocobalamin.

B_{12} is synthesised only by a select group of bacteria and archaea, either via an aerobic or anaerobic pathway (Fang *et al.*, 2017). B_{12} biosynthesis is mediated by a select but broad group of prokaryotes, but in all cases, cobalt is essential for the synthesis of the molecule. Industrial production of B_{12} is usually carried out by *P. denitrificans, P. shermanii,* and *S. meliloti*, although industrial production of B_{12} using recombinant *E. coli* is also being studied (Martens *et al.*, 2002). There are many species of *E. coli*, one of which (called OP50) is used as a food source for the *C. elegans* in this project. OP50 does not produce vitamin B_{12}, which allows us to accurately control the amount of this compound administered to the nematodes via their growth media.

Vitamin B_{12} deficiency in humans

In humans, the uptake of vitamin B_{12} is mediated by haptocorrin (HC) and intrinsic factor (IF), each of which carry a single B_{12} molecule. First, B_{12} is released from the food during chewing and in the stomach due to gastric acid and pepsin. It then binds to HC, a glycoprotein secreted in saliva and the parietal cells of the stomach, which binds all cobalamin analogues indiscriminately. Once in the duodenum (beginning of the small intestine), the higher pH causes HC to be degraded by pancreatic proteases, allowing IF to bind the B_{12}. In the ileum (distal small intestine), the IF-B_{12} complex enters the mucosal cells by specific receptor-mediated endocytosis, which degrades the IF, subsequently freeing the B_{12}. The free B_{12} enters the systemic circulation, where it binds to transcobalamin (TC) (Kozyraki *et al.*, 2013). The B_{12}-TC complex is known as holotranscobalamin, and is the bioactive form of B_{12} that is delivered to the cells. The fact that humans need a very small amount of vitamin B_{12}, and that B_{12} is also stored in the liver, means that in healthy humans with no uptake disorders, the onset of B_{12} deficiency can take months to years.

Humans also have B_{12}-producing bacteria as part of their gut microflora, however they are found in the large intestine. As this vitamin is absorbed in the small intestine, humans cannot absorb the B_{12} produced in our own guts and must obtain it from our diet. The daily recommended amount of vitamin B_{12} for adults is determined at around 1.5 µg in the UK (Wiseman, 1992), and 2.4 µg in the USA. Foods containing utilisable B_{12} analogues include meat, fish and dairy products, where it is synthesised by their gut microbiota and stored in the muscles and liver, or secreted in milk.

Due to the fact that crop-based foods do not contain cobalamin, strict vegetarians and vegans are more prone to vitamin B_{12} deficiency (Antony, 2003; Pawlak, 2015; De Rosa *et al.*, 2012). Vitamin B_{12} deficiency is rare in developed countries, however, people in developing countries that have a predominantly plant-based diet (e.g. India) are at higher risk, with rates rising to up to 80% (Naik et. al., 2018). In addition, the rate of B_{12}-deficiency among the elderly rises up to 20%, as they are predisposed due to reduction in the efficiency of absorbance due to diseases such as atrophic gastritis, which increases with age, and reduces the amount of gastric acid and IF produced (Andres, 2004). In the elderly, vitamin B_{12} deficiency is strongly associated with increased oxidative stress and cognitive decline (Solomon, 2015).

Vitamin B_{12} deficiency can also be caused by malabsorption disorders or infections. Gastric intrinsic factor (IF) is necessary for B_{12} uptake and vitamin B_{12} deficiency caused by lack of this transport factor causes pernicious anaemia. This type of anaemia is caused by a shortage of red blood cells, due to inhibition of DNA synthesis during the production and maturation of red blood cells in the bone marrow. Vegetarians and vegans that ingest high quantities of folate (vitamin B9) from dark green leafy vegetables are protected from this disease, however this masking effect of folate in pernicious anaemia may prevent the diagnosis of B_{12} deficiency, which leads to other complications (Herrmann and Obeid, 2011). B_{12}-deficiency by infection can be caused by *Helicobacter pylori*, which causes gastric atrophy and lack of gastric acid, leading to impaired absorption of B_{12}. Infection by parasitic nematodes, such as fish tapeworm (*Diphyllobothrium latum*), can also cause deficiency due to competition for vitamin B_{12} in the intestine (Allen, 2008; Bonsdorff and Gordin, 2009).

Vitamin B_{12} is needed for DNA synthesis (which requires tetrahydrofolate), cell maintenance and division, neurotransmitter synthesis, and erythrocyte production (Fenech, 2001; Dror, 2008; Battaglia-Hsu, 2009; Greer, 2014). It is also involved in normal neural function, as methionine is necessary in myelin repair and maintenance in the central and peripheral nervous system. B_{12} acts as a cofactor for methionine synthase, which recycles methionine from homocysteine (Miller, 2005; Scalabrino, Veber and Tredici, 2014). B_{12} deficiency causes the accumulation of homocysteine and lower amounts or methionine, which subsequently causes a variety of symptoms downstream of the SAM cycle.

In adults, initial B_{12} deficiency symptoms include fatigue, reduced sensitivity to touch, tremors, and blurred vision. As B_{12} becomes depleted from the liver and the deficiency increases, symptoms become more severe, and can include memory loss, mood swings, depression, seizures, and permanent neurological disorders such as multiple sclerosis and dementia (Savage, 1995; Kocer, 2009; Chien Tu *et al.*, 2017; Irevall, 2017). In infants, it can cause hypotonia (low muscle tone) and neurodevelopmental retardation, however, if diagnosed early on, the symptoms can be reversed with B_{12} supplementation (Taskesen *et al.*, 2012).

The onset of severe vitamin B_{12} deficiency can take years, which is why it is usually diagnosed in time to be reversed with B_{12} administration. When B_{12} deficiency is in its

advanced stages, or if the patient has a disease which impairs the absorption of this vitamin, B_{12} is administered via intramuscular injections, however oral B_{12} supplements are enough to replenish B_{12} to normal levels in the beginning (Morita *et al.*, 2003; Shyambabu *et al.*, 2008). Vitamin B_{12} deficiency is usually first tested by measuring total serum cobalamin because of its low cost compared to other tests. According to this test, B_{12}-deficiency is defined as having less than 200 pg/ml of B_{12} in the serum (Green, 1995; Herrmann *et al.*, 2000). B_{12} insufficiency diagnosis is important as it can be simply reversed by the administration of oral supplements, which is easier and cheaper for the patient than injections, and it avoids reaching the point of severe neurological deficiency symptoms.

Only two enzymes are B_{12}-dependent in mammalian cells: L-methylmalonyl-CoA mutase (MCM) and methionine synthase (MS). AdoCbl is a coenzyme of methylmalonyl-CoA mutase (MCM), which is involved in the breakdown of branched-chain amino acids (BCAAs), cholesterol, and fatty acids in the mitochondria by catalysing the conversion of methylmalonyl-CoA to succinyl-CoA. MeCbl is a cofactor of methionine synthase, which catalyses the conversion of homocysteine to methionine in the cytoplasm as part of the S-Adenosyl methionine (SAM) cycle, which recycles methionine. B_{12}-deficiency causes the accumulation of homocysteine (Hcy), as well as lower amounts of methionine in the SAM cycle. The accumulation of Hcy causes hyperhomocysteinemia, leading to the activation of NADPH oxidase, which generates reactive oxygen species (ROS) and, consequently, superoxide anions, hydrogen peroxide, and hydroxyl radicals (Labuschagne and Brenkman, 2013). This can lead to vascular dysfunction, neurodegenerative diseases (e.g. Alzheimer's, seizures), neural tube defects in infants (Obeid and Herrmann, 2006; Herrmann and Obeid, 2011; Edirimanne *et al.*, 2007). While only two enzymes are B_{12}-dependant in humans, B_{12} deficiency causes a wide variety of downstream detrimental effects. Thus, it is important to study B_{12} deficiency in a model that is effective and time-efficient, such as *C. elegans.*

C. elegans as a model organism

C. elegans has only recently been studied and as a model for B_{12}-deficiency. These nematodes are hermaphroditic and have short life cycles, which allows for maintenance of strains without genomic variability. The short life cycle of these nematodes also allows rapid depletion of B_{12}, which allows studies which would not be possible to carry out with higher organisms (as it can take several years for a mammal to acquire vitamin B_{12} deficiency). In addition, their short lifespans also allow ageing studies which may take months or years in other model organisms (Lian Chew *et al.*, 2013). Their growth media and environment can be specifically modified and controlled, enabling control of the exact amount of vitamin B_{12} administered. The transparency of these nematodes enables us to carry out B_{12} localisation studies *in vivo* by using fluorescent cobalamin analogues. Whilst *C. elegans* cannot be used as a model for human B_{12} intestinal transport, as they lack the equivalent human transport proteins, they can be used as a model to study the absorbance of B_{12} in parasitic nematodes. This could elucidate a drug target for the B_{12}-deficiency causing intestinal parasites. On the other hand, *C. elegans* can be used as a model for the neural effects of B_{12}-deficiency in humans. Their neuronal network is composed of 302 neurons and has been extensively studied and mapped, and markers for neural ageing have been established, such as neurite branching (White *et al.*, 1986). In addition, they have been used as models for human Alzheimer's, Parkinson's, and other tauopathies and neurodegenerative diseases (Li and Le, 2013; Alexander, Marfil and Li, 2014; Wang *et al.*, 2018). They are comparable to humans as they also require vitamin B_{12} for their growth and development, and the enzymes requiring B_{12} as well as the metabolic pathways are conserved.

The life cycle of *C. elegans* (Fig. 2), from egg to adult worm, lasts approx. 3 days. N2 Bristol strain worms were used in this study as wild type (WT), which have an approximate lifespan of 12 to 20 days (Gems and Riddle, 2000). *C. elegans* were maintained on nematode growth medium (NGM) with the uracil auxotroph B strain OP50 grown in LB as a food source for the control condition (Yen and Curran, 2016).

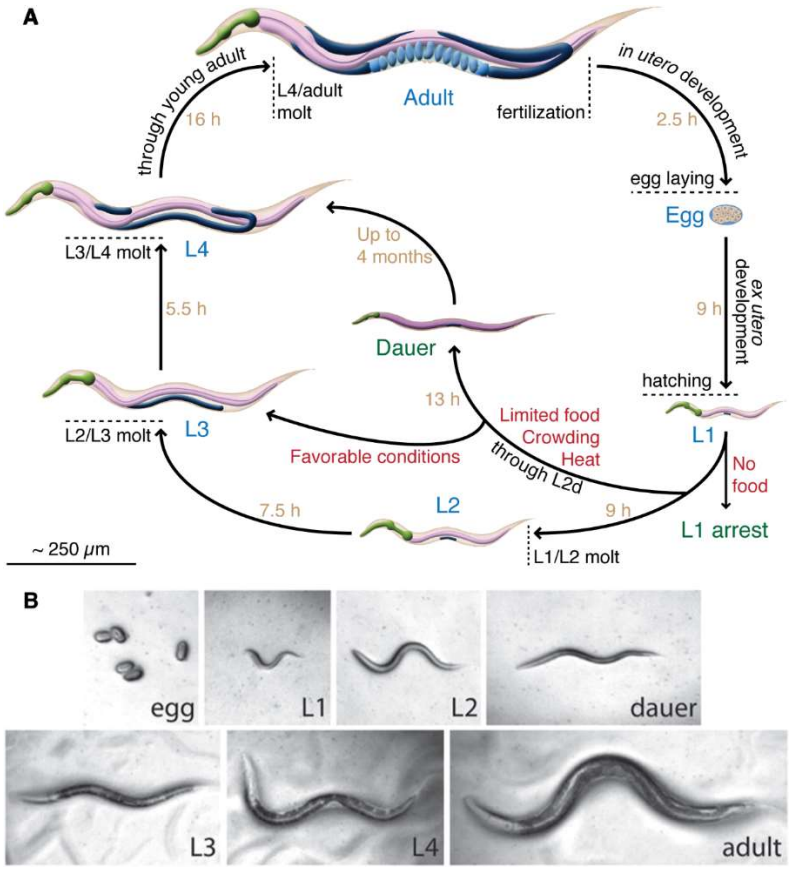


Figure 2. *C. elegans* life cycle at 25°C. A. Diagram of each stage of the life cycle at 25°C, including average time between stages (Modified from Altun and Hall, 2012). **B.** Example images of each stage of the life cycle (Fielenbach and Antebi, 2008).

Vitamin B_{12} in *C. elegans*

C. elegans require vitamin B_{12} as AdoCbl and MeCbl, and have conserved metabolic pathways and enzymes with humans. However, *C. elegans* can rewire their metabolic network to a secondary pathway that does not require vitamin B_{12} in order to survive when vitamin B_{12} is not readily available (Watson *et al.*, 2016). This may have evolved as a survival mechanism, as in the wild they may encounter a variety of environments, some with B_{12}-producing bacteria (e.g. soil containing faecal matter), and others with low amounts of B_{12}-synthesising bacteria. The B_{12}-dependent pathway is known as the carboxylation pathway and is conserved in both humans and nematodes, however *C. elegans* can convert to the secondary B_{12}-independent pathway, known as the β-oxidation-like pathway, in environmental conditions lacking B_{12} (Fig. 3). The breakdown of odd-chain fatty acids and branched-chain amino acids (BCAA)

produces propionate is interconverted with propionyl-CoA, a toxic intermediate if it accumulates in the cell. Vitamin B_{12} acts as a cofactor of methylmalonyl-CoA mutase (mmcm-1, the *C. elegans* ortholog of human MCM), which catalyses the conversion of L-methylmalonyl-CoA ((L)-MM-CoA) to succinyl-CoA (Succ-CoA) in the carboxylation pathway. B_{12} deficiency and propionic acidemia prevent the catabolism of propionate, which causes toxic intracellular build-up (La Marca *et al*., 2007).

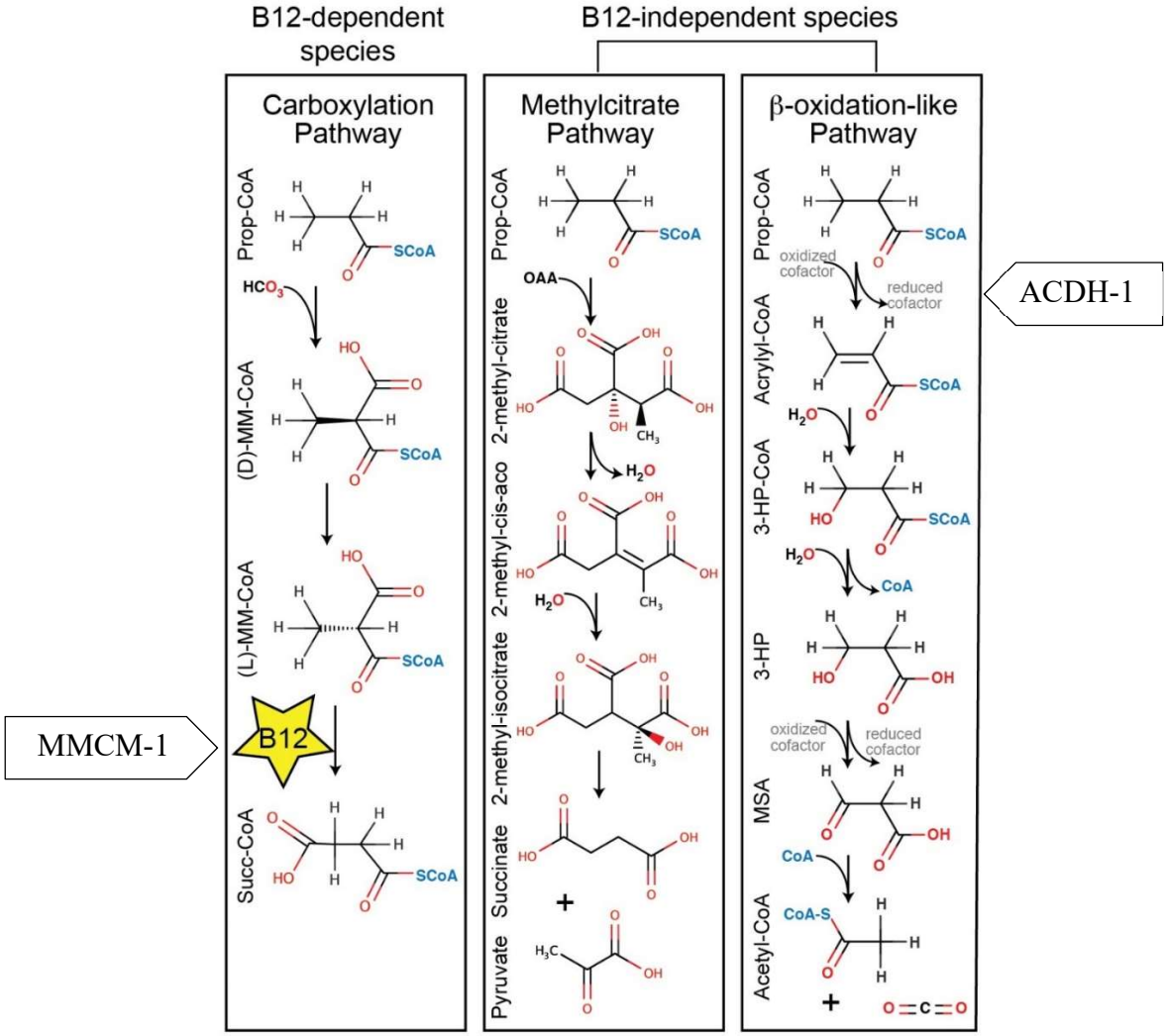


Figure 3. Propionate breakdown pathways. Vitamin B_{12}-dependent species use a carboxylation pathway to catabolise propionate. B_{12}-independent species use either the methylcitrate pathway or the β-oxidation-like pathway. *C. elegans* uses the carboxylation pathway in the presence of B_{12} and the β-oxidation-like pathway in B_{12}-deficient environments. MMCM-1: methylmalonyl-CoA mutase-1 (*C. elegans* ortholog of MCM); ACDH-1: acyl-CoA dehydrogenase; 3-HP: 3-hydroxypropionate. (Watson *et al.*, 2016)

Whilst *C. elegans* can use either pathway depending on vitamin B_{12}-availability, other organisms are thought to have a preference of one over the other, or just one pathway available. B_{12}-dependent species include humans and most animals, while B_{12}-independent species that use the methylcitrate pathway include *S. cerevisiae* and ones that use the β-oxidation-like pathway include plants and *C. albicans* (Otzen *et al.*, 2014). In humans, a marker of propionic acidemia is elevated levels of 3-hyroxypropionate (3-HP), which suggests that the secondary B_{12}-independent β-oxidation-like pathway of propionyl-CoA (prop-CoA) catabolism may be conserved in some manner (Watson *et al.*, 2016).

Vitamin B_{12} deficiency in *C. elegans* has been shown to cause elongated life cycle, reduced lifespan, and reduced fertility (Bito *et al.*, 2013; Bito and Watanabe, 2016). *Comamonas aquatica*, which naturally produces vitamin B_{12}, has been shown to increase developmental rate when used as a food source for the worms (Watson *et al.*, 2014; MacNeil *et al.*, 2013). B_{12} deficiency has also been shown to cause oxidative stress, which impacts the nervous system by causing a memory retention decreased of 42% (Bito *et al.*, 2017). *C. elegans* can also acquire B_{12} deficiency via the introduction of the cobalamin analogue cyanocobalamin dodecylamine, which has a higher affinity for the B_{12}-dependent enzymes than AdoCbl and MeCbl (Bito *et al.*, 2014).

A marker for ageing in *C. elegans* is neuronal branching, which has been shown to be influenced by oxidative stress, the Jun kinase pathway, and the insulin signalling pathway (Tank, Rodgers and Kenyon, 2011; Toth *et al.*, 2012). This phenotype of neurite branching and blebbing (beading of axons) with age is typically studied in the mechanosensory "touch receptor" neurons, visualised by labelling with a *mec-4p::GFP* reporter, which fluorescently tags the MEC-4 ion channel (required for gentle touch sensation) (Lian Chew *et al.*, 2013; Suzuki *et al.*, 2003). These morphological defects are accompanied by a decline in response to touch, indicating functional deterioration (Pan *et al.*, 2011). Neurite branching has also been observed in humans with Alzheimer's disease (Masliah *et al.*, 1993; Hashimoto and Masliah, 2003; Gordon, Hegedus and Tam, 2004).

The uptake mechanism of B_{12} and the effects of vitamin B_{12} deficiency are still not completely elucidated, particularly in the model organism *C. elegans*. This project aims to shed light on the phenotypic effects of vitamin B_{12} deficiency, as well as the

uptake and localisation of B_{12} in these worms. As part of an ageing laboratory, we will be particularly focusing on the effect of this deficiency on ageing phenotypes, such as development, lifespan, and neurodegeneration. In addition, we will study B_{12} uptake *in vivo* by using fluorescent cobalamin analogues. This investigation will expand our knowledge of the phenotypic effects of this deficiency and establish the use of *C. elegans* as a suitable model for B_{12} deficiency regarding development, fertility, and neurodegeneration. In addition, the study of fluorescent B_{12} localisation in these nematodes may determine a method for investigating molecular trafficking *in vivo*, as well as revealing a target for B_{12}-deficiency-causing parasitic worms.

Materials and Methods

Resources

Strains

E. coli OP50 uracil auxotroph: gift from J. Tullet. *E. coli*: OP50 (pET-BAD-*btuBF*): gift from A. Lawrence. Bristol N2 *C. elegans* strain: gift from J. Tullet at the University of Kent. VC1011 *acdh-1(ok1489)* I. *C. elegans* strain: purchased from CGC, University of Minnesota, MN. CZ10175 (zdIs5[*mec-4p::GFP + lin-15(+)*]I.) *C. elegans* strain: gift from B. Schafer's lab at the LMB Cambridge.

Reagents

Reagents were purchased from Sigma Aldrich (now Merck), or Thermo Fischer Scientific Inc., when specified. BODIPY® TR-X NHS Ester (Succinimidyl Ester) was purchased from ThermoFischer.

Methods

Chemical Methods

10x M9 solution

60 g Na_2HPO_4, 30 g KH_2PO_4, 10 g NH_4Cl, 5 g NaCl, make up to 1 L with MQ water and autoclave.

M9 buffer

6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, make up to 1 L with MQ water and autoclave. After solution cools down, add 1 ml autoclaved/sterile 1 M MgSO4.

BODIPY® TR-X ribose-linked cyanocobalamin (BoB_{12})

BoB_{12} was synthesised as described previously (Lawrence *et al.*, 2018). 1,1'-carbonyldiimidazole (CDI) was used to activate the 5' ribose hydroxyl group of cyanocobalamin, and was then coupled with 1,2-Diaminoethane as described previously, forming a primary amine (McEwan *et al.*, 1999; Lee and Grissom 2009). BODIPY® TR-X dissolved in anhydrous DMSO was added in excess (1.5x), along with N, N-DIPEA as an organic base. The reaction was incubated on a shaker in the dark for 24 hours at room temperature. The product was precipitated with a solution

of 50% acetone and 50% ethyl acetate, centrifuged, and dried down using pressurised air. The BoB_{12} was then resuspended in 50% methanol and purified and confirmed by HPLC-MS (Agilent 1100 series HPLC coupled to a micrOTOF-Q (Bruker) mass spectrometer).

The initial semi-preparative HPLC separated large amounts of impurities from our sample by identifying the elutions using the UV spectra. 200 µl of our sample was injected at a time into a 'CS SIL C18' column (250 mm x 10 mm, 5 µm; Charlton Scientific) column on an Agilent 1100 series HPLC. The sample was run at a 4.5 ml/min flow rate using 0.1 % (v/v) TFA (solvent A) and 100 % acetonitrile (solvent B) running from 5% to 95% in 50 min (and back again to restore the column), with the column kept at 25 °C.

For the preparative HPLC-MS, the previous HPLC purified sample was mixed with one-part ultrapure water. 20 µl of sample was injected at a time, and the protocol was run at a 0.2 ml/min flow rate using 0.1 % (v/v) TFA (solvent A) and 100 % acetonitrile (solvent B), with the Ace 5 AQ column (2.1 x 150 mm, 5 µm, 100 Å, from Advanced Chromatography Technologies) column kept at 25 °C. The product was confirmed using positive mode electrospray ionisation on an electrospray ionisation mass spectrometer (ESI-MS) (Bruker micrOTOF II-MS) (Fig. S5 of supplemental data).

Bacterial methods

OP50 uracil auxotroph *E. coli* was used as a food source for *C. elegans*. The *E. coli* were grown in LB media for the NGM plates, and in minimal M9 media for the M9 plates, as this has been shown to reduce the amount of vitamin B_{12} contained in them (0.2 µg/g wet weight in M9 media vs. 11.1 µg/g wet weight in LB media) (Bito *et al.*, 2013). The *E. coli* was grown in a shaker overnight at 37°C (for all conditions), then used to seed the plates, which were left overnight to dry before using.

LB media

2 g LB powder, 80 ml dH_2O, dissolve and autoclave.

M9 media

Autoclave 900 mL MQ H_2O and cool. Add 100 mL 10x Salt Solution. Add (sterile): 1 mL 1M $MgSO_4$ solution, 0.5 mL 1M $CaCl_2$ solution, 1 mL 4 g/L thiamine

hydrochloride solution, 10 mL 20% w/v glucose solution, 10 ml 2 g/L uracil solution, mix and store.

Cobalamin analogue uptake in *E. coli*

Cobalamin analogue brood size rescue assay:

Inoculate 5 ml of media (LB or M9) with OP50 *E. coli* containing the pET-TBAD *btuBF* plasmid (Fig. S6 of supplemental data). Incubate overnight at 37°C and then take 200 µl of the starter culture and inoculate 5 ml of media (again either LB or M9). Grow for 4 hours at 37°C, then add 10 µl of 5% arabinose and the respective cobalamin analogues to a concentration of 1 µM. Grow overnight at 28°C, then seed the plates (NGM plates for LB cultures, M9 plates for M9 cultures). The conditions we used were: LB, M9, M9 with cyanocobalamin, M9 with pseudocobalamin, and M9 with BoB_{12}.

BoB_{12} localisation assay:

BoB_{12} was introduced into the *E. coli* as described previously. However, before seeding the plates, the culture was spun down and resuspended in M9 media three times to get rid of extracellular BoB_{12} that had not been internalised. To verify that the BoB_{12} had been taken up, the *E. coli* was imaged using an Olympus IX-81 inverted fluorescence microscope at 100x magnification using an RFP 580 filter (BODIPY® TR-X fluorophore has an excitation of 588 nm and an emission of 620 nm). A drop of the washed OP50 pET-TBAD *btuBF E. coli* incubated with BoB_{12} was placed on a glass slide, a cover slip was placed on top and sealed with clear nail polish to prevent drying out, and imaged. Brightfield images were then overlaid with the fluorescence images to confirm colocalisation of BoB_{12} and *E. coli,* verifying the uptake of BoB_{12}.

C. elegans methods

C. elegans strains were maintained either on nematode growth media (NGM) seeded with *E. coli* OP50 LB culture, on M9 agar seeded with *E. coli* OP50 M9-media culture, or on B_{12}-supplemented M9 agar seeded with *E. coli* OP50 M9-media culture.

Nematode Growth Media (NGM)

3 g NaCl, 17 g Agar, 2.5 g Bactopeptone, 1 L H_2O, autoclave and cool to 55°C. Add (sterile): 25 mL 1M KH_2PO_4 (pH 6), 1 mL 1M $MgSO_4$, 1 mL 1M $CaCl_2$, 1 mL 5 mg/ml Cholesterol in EtOH, pour plates, let set, and store.

M9 agar

17 g agar, add 900 mL dH_2O, autoclave and cool to 55°C. Add 100 mL 10x Salt Solution. Add (sterile): 1 mL 1M $MgSO_4$ solution, 0.5 mL 1M $CaCl_2$ solution, 1 ml 4 g/L Thiamine hydrochloride solution, 10 ml 20% w/v Glucose, 10 ml 2 g/L Uracil, 1 ml 5 g/L Cholesterol in EtOH (for B_{12}-supplemented plates, add 100 µL of 1 g/L cyanocobalamin). Pour plates, let set, and store.

Axenic liquid media (mCeHR-2 media)

The modified *C. elegans* habituation and reproduction media (mCeHR-2) was produced as described previously (Samuel *et al.*, 2014), with the protocol being provided by I. Hamza at the University of Maryland. Using sterile technique and a 1L (0.2 µm) vacuum filter unit, filter the following volumes of stock solutions and water in the order described. 10 ml choline (100x), 10 ml vitamin mix (100x), 10 ml i-Inositol (100x), 20 ml nucleic acid mix (50x), 280 ml dH_2O. Vacuum filter. Add 20 ml salt solution (50x), 20 ml lactalbumin (50x), 20 ml essential amino acid mix (50x), 10 ml non-essential amino acid mix (100x), 20 ml KH_2PO_4 (50x), 50 ml d-glucose (20x), 10 ml HEPES (100x), 319 ml dH_2O, and vacuum filter again. Remove filter unit and add (sterile) 1 ml 5 mg/ml cholesterol, and 200 ml high temperature ultra-pasteurized skim milk.

Single worm lysis and PCR

Single worm lysis: 10 µl of Single Worm Lysis Buffer (50 mM KCl, 10 mM Tris-HCL [pH 8.3], 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween 20, 0.01% Gelatin) and 1 mg/ml of Proteinase K (*For 1 ml:* 20 mg Proteinase K, 949 µl dH_2O, 50 µl 1 M Tris [pH 8], 1 µl 1 M $CaCl_2$) were pipetted into the cap of a 0.2 ml PCR tube. A single worm was transferred to the buffer and the tube was spun in a microcentrifuge (1 G for 1 min). The worm(s) were frozen at -80°C for 2 hours, then incubated at 65°C for 1 hour, and then at 95°C for 30 min. *PCR:* Each sample (single worm lysed in 5 µl of buffer), was divided into two tubes (2.5 µl each), and one was stored at -20°C. To the other tube (containing 2.5 µl of sample DNA), we added 11 µl ultrapure water, 2.5 µl 10x Taq buffer, 2.5 µl 25 mM $MgCl_2$, 2 µl 2.5 mM dNTPs mix, 2 µl 10 µM 5' (forward) primer, 2 µl 10 µM 3' (reverse) primer, and 0.5 µl Taq (25 µl total).

Making B_{12}-deficient *C. elegans*

To make the *C. elegans* B_{12}-deficient, they were grown on 1.7% (w/w) M9 agar plates containing OP50 *E. coli* grown in M9 media as a food source, and were maintained at 20°C. First, the worms were grown on the M9 plates supplemented with 100 µg/L of cyanocobalamin using the B_{12}-deficient *E. coli* (grown in M9 media) as a food source. An individual egg was transferred on to each B_{12}-supplemented M9 plate and allowed to develop into an egg-laying adult. Another egg was then transferred to a new plate. This was repeated at least 10 times before experiments were carried out, and these worms were then used as the "B_{12}+" condition. To make the worms B_{12}-deficient, eggs from the B_{12}-supplemented M9 plates were taken and each placed on to an individual non-supplemented M9 plate seeded with B_{12}-deficient *E. coli*. They were maintained at 20°C and allowed to lay eggs. As with the previous condition, each egg was placed on to a new M9 plate and allowed to grow into an egg-laying adult. This process was repeated for at least five generations before the worms were used in experiments, as this is how long it has been shown to take to make the worms B_{12}-deficient (Bito *et al.*, 2013).

Assays

Developmental assay

Several adult worms from each condition were transferred on to fresh plates of the same medium and left to lay eggs for 5 hours. The adult worms were removed and the plates were incubated at 20°C. 24, 48, 60, and 84 hours later, the worms were imaged on the Leica DMR microscope at either 10x (for L3, L4, and adult worms) or 20x (for L1 and L2 stage worms) magnification. The worms were then measured from the images using the Leica Application Suite X (LAS X) software by drawing a continuous line from the mouth to the end of the tail through the midline of the worm, which the software used to convert pixels to µm.

Lifespan assay

The lifespan assay was carried out by placing L4-stage worms (100 worms per condition) from each condition onto fresh plates containing the same culture media as well as FuDR to prevent egg-hatching, and incubated at 20°C. Worms were scored as dead when they no longer responded to prodding with a pick. Worms dead from

unnatural causes (internal hatching, gut explosion, etc.) and escaped worms were censored.

Brood size assay

Fecundity was measured via brood size assays, which count the number of eggs laid by each worm (that hatch). Individual worms at stage L4 grown on NGM, B_{12}-supplemented M9 media, or B_{12}-deficient M9 media, were transferred on to fresh plates containing their same respective media. After incubation at 20°C for 24 hours, each adult worm was transferred to a new empty plate. The plates they were moved from were incubated at 20°C for a further 24-36 h and the hatched progeny was counted.

Neurite branching assay

Young adult worms were placed on NGM, B_{12}-supplemented M9, and M9 plates during 5 hours for a timed egg lay, after which they were picked off. The offspring were allowed to hatch and develop at 20°C for three days, after which they were placed on corresponding plates containing FuDR. On adult days 1, 8 and 15 they were imaged on the Leica DMR microscope at 10x and 20x magnification using a GFP filter. 2% w/v agarose pads with 5 µl of 0.06% w/v tetramisole hydrochloride were used to immobilise the nematodes. Worms were scored as either positive or negative for neurite branching.

BoB_{12} localisation

BoB_{12} was synthesised and introduced into *E. coli* as described previously (Lawrence *et al.*, 2018) and localisation was studied in two different medias: liquid and solid. *Liquid media:* Eggs from a bleach drop were placed in modified *C. elegans* habituation and reproduction media (mCeHR) supplemented with 3.3 µM of BoB_{12} and incubated in a shaker at 20°C until adult stage. Worms were then washed three times in M9 to remove BoB_{12} that had not been taken up and imaged. *Solid media:* Adult worms were placed on B_{12}-deficient M9 media seeded with the OP50 *BtuBF E. coli* containing 1 µM BoB_{12} and allowed to lay eggs. The adults were removed and the eggs were incubated at 20°C for three days. The adult-stage worms were then imaged on 2% agarose pads with 0.06% tetramisole hydrochloride on an Olympus IX-81 inverted fluorescence microscope at 60x magnification using an RFP 580 filter. Brightfield

images were then overlaid with the fluorescence images to confirm the localisation of the BoB_{12} to the coelomocytes.

Cobalamin analogue brood size rescue assay

Brood size was carried as described previously, but B_{12}-deficient L4-stage worms were placed on each condition to test the effectiveness of each condition to rescue the reduced brood size B_{12}-deficiency phenotype. The conditions used were: NGM plates seeded with OP50 pET-BAD *btuBF E. coli* grown in LB media, M9 plates seeded with OP50 pET-BAD *btuBF E. coli* grown in M9 media, M9 plates seeded with OP50 pET-BAD *btuBF E. coli* grown in M9 media supplemented with 1 μM of cyanocobalamin, M9 plates seeded with OP50 pET-BAD *btuBF E. coli* grown in M9 media supplemented with 1 μM of pseudocobalamin, and M9 plates seeded with OP50 pET-BAD *btuBF E. coli* grown in M9 media supplemented with 1 μM of BoB_{12}. The plates containing BoB_{12} were kept in the dark.

Results

The aims of this study were to determine the action and localisation of vitamin B_{12} in *C. elegans*. This was broken down into a series of smaller goals, the first being to make the worms B_{12}-deficient. Once the worms were depleted of B_{12}, several experiments studying the overall health and ageing of the worms were carried out. This was measured by studying larval development and life cycle, lifespan, fecundity, and neuronal health. The ability of various cobalamin analogues to rescue the B_{12}-deficient worms was also tested. B_{12} localisation was studied by synthesising fluorescent cyanocobalamin and inserting it into the worms indirectly via *E. coli* OP50.

Making B_{12}-deficient *C. elegans*

To understand the physiological role of B_{12} in *C. elegans* it was necessary to make N2 *C. elegans* B_{12}-deficient and compare these with those that were not. *C. elegans* are usually maintained on nematode growth media (NGM), which is seeded with *E. coli* grown in LB media. Worms grown on this condition were used as a control, as most published data on ageing uses this media, which allowed us to compare our results to the expected, verifying that our experiments had been carried out correctly. Whilst *E. coli* cannot synthesise B_{12} de novo, NGM and LB contain trace amounts of B_{12}. To make the worms B_{12}-deficient we grew them on M9 agar plates seeded with *E. coli* grown in M9 media so that no trace of B_{12} was present (M9 B_{12}- plates). As these growth conditions were considerably different to the standard NGM one, we also compared with the M9 B_{12}- protocol with M9 plates where B_{12} was supplemented back into the agar (100 µg/L cyanocobalamin) and seeded with the B_{12}-deficient *E. coli* (M9 B_{12}+ plates). This allowed us to determine to what degree the effect observed was due to the minimal growth conditions (M9 media) or due to the lack of vitamin B_{12}.

One egg from the NGM plates was placed on a M9 B_{12}+ plate (3 repeats) and allowed to develop and lay eggs, of which one was taken and placed on to another M9 B_{12}+ plate. This was repeated for ten generations, after which the worms were used as controls. This process made sure that the worms were completely acclimatised to the condition before carrying out any experiments, which made our results less variable and more reliable. To make the worms B_{12}-deficient, an egg was taken from this control group and placed on to an B_{12}-deficient plate seeded with B_{12}-deficient *E. coli*. The same procedure of allowing the egg to develop into an adult worm, and taking an

egg and placing it onto another B_{12}- plate, was carried out for five generations (Fig. 4). After the five generations the worms were B_{12}-depleted and were kept on the B_{12}-deficient media to be used as the experimental group (Bito *et al.*, 2013). According to previously published data it takes five generations to make *C. elegans* B_{12}-deficient because vitamin B_{12} is transported from mother to offspring via the ABC transporter MRP-5 (Na *et al.*, 2018).

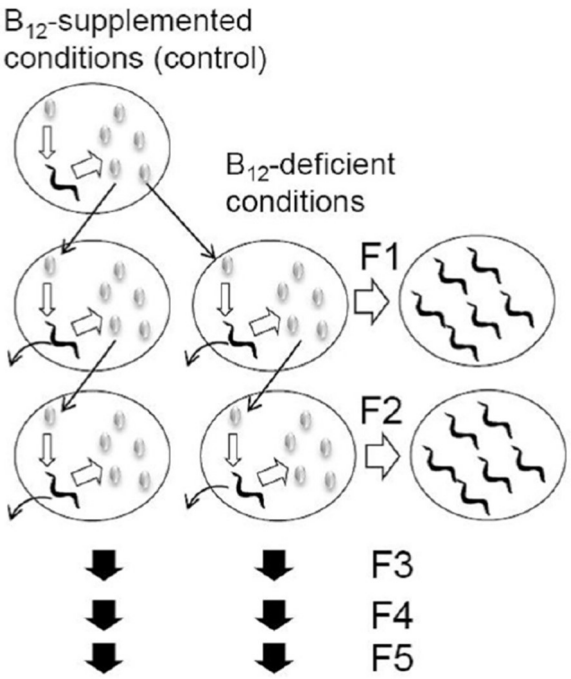
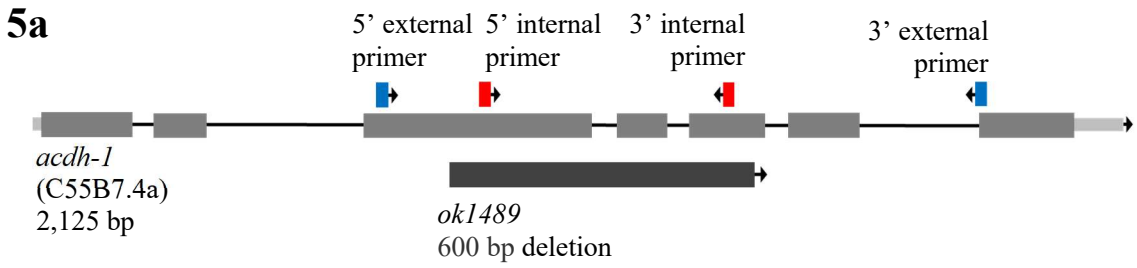


Figure 4. Process of making the *C. elegans* B_{12}-deficient. The B_{12}+ condition was made by taking one egg from N2 *C. elegans* grown on NGM and placing it on to a M9 B_{12}-supplemented (B_{12}+) plate. It was allowed to develop into an egg-laying adult and one of its offspring was taken and placed on to a new B_{12}+ plate. After 10 generations these worms were used as controls. To make B_{12}-deficient worms, we took one egg from the B_{12}-supplemented control condition and placed it on to a non-supplemented M9 plate (B_{12}-). After five generations these worms were considered B_{12}-deficient and were used for experiments. The worms were kept at 20˚C on each of the three conditions used in the experiments (NGM, B_{12}+, and B_{12}-) and were not mixed at any time during the duration of the study, except when specifically indicated. (Bito *et al.*, 2013)

To confirm that we had successfully made the N2 *C. elegans* strain B_{12}-deficient by growing them on the B_{12}- condition, we used a superficially wild-type *C. elegans* strain that had the acyl-CoA dehydrogenase-encoding (*acdh-1*) gene (located on chromosome 1) knocked out (VC1011 *acdh-1(ok1489)* I.). This gene encodes the first enzyme in the β-oxidation-like propionate breakdown pathway (ACDH-1), which does not require vitamin B_{12}. The *acdh-1* promoter is induced when vitamin B_{12} is not available, demonstrating the metabolic network rewiring mechanism found in *C. elegans* (Watson *et al.*, 2014). The *acdh-1* deletion mutant *C. elegans* strain cannot survive without vitamin B_{12}, as propionate accumulates to toxic concentrations intracellularly (Watson *et al.*, 2016).

We tested whether the media was effectively depleting the N2 worms of vitamin B_{12} by using this VC1011 *acdh-1*-deletion strain. To make sure that the strain had the correct gene knocked out, several worms were genotyped. To do this, we designed primers for the *acdh-1* gene sequence (Fig. 5a and Fig. S1 of supplemental data) and then carried out single-worm PCR of the *acdh-1* deletion mutant strain (VC1011) and the N2 strain, which was used as the control. The primers were designed based off of the gene nucleotide sequence for the wild-type and the *acdh-1(ok1489)*-deletion strain (Fig. 5a). The expected PCR products for the N2 strain are 1190 bp with the external primers and 500 bp with the internal primers. For the VC1011 strain, the expected PCR products are 590 bp with the external primers and no product with the internal primers (as the 600 bp sequence of the *acdh-1* gene is deleted). The results of this are seen in Fig. 5b, which show that our strain indeed has the correct gene knockout.



5b

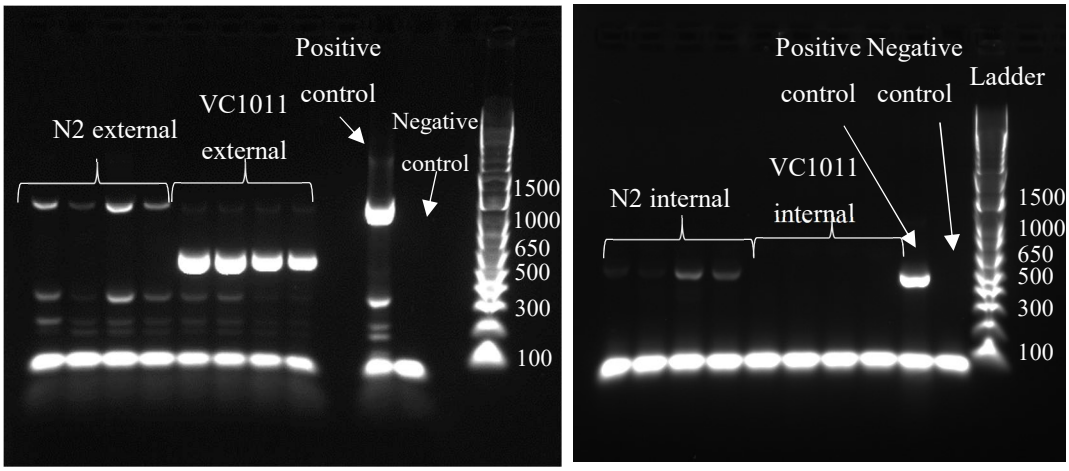


Figure 5a and b. VC1011 (*acdh-1*-deletion) strain genotyping using single-worm PCR. a. Diagram of the *achd-1* gene (C55B7.4a: ACDH-1, isoform A), the *ok1489* deletion allele, and primers (to scale). Grey rectangles indicate exons, and the black lines indicate introns. The dark grey rectangle indicates the position of the 600 bp deletion found in the VC1011 (*acdh-1(ok1489)* I.) *C. elegans* strain. The blue rectangles indicate the external primers and the red ones represent the internal primers, with arrows indicating direction. **b. Images of PCR product gels.** *Gel on the left:* Gel of external primer PCR products. Columns 1-4: N2 DNA with external primers. Columns 5-8: VC1011 DNA with external primers. Column 9: Blank. Column 10: WT DNA with external primers (positive control). Column 11: Water (ultrapure) with external primers (negative control). Column 12: Blank. Column 13: 1 Kb Plus DNA Ladder. *Gel on the right:* Gel of internal primer PCR products. Columns 1-4: N2 DNA with internal primers. Columns 5-8: VC1011 DNA with internal primers. Column 9: WT DNA with internal primers (positive control). Column 10: Water (ultrapure) with external primers (negative control). Column 11: 1 Kb Plus DNA Ladder.

The expected PCR products for the N2 strain were 1190 bp with the external primers and 500 bp with the internal primers. For the VC1011 strain the expected products were 590 bp with the internal primers and no product with the internal primers. The results obtained were conclusive with the expected results.

To test whether the conditions used made our N2 worms B_{12}-deficient we placed the VC1011 strain on the same conditions and treated them the same as our N2 worms. After five generations all of the VC1011 worms were dead, whilst the N2 worms were still viable (data not shown). Thus, we are confident that we have established a protocol to make worms B_{12} deficient.

The physiological effects of B_{12} deficiency *in vivo*

We then profiled the physiological effects of B_{12} deficiency in whole, live worms. There are several well characterised outputs of worm-health, out of which we examined those particularly focused on ageing (larval development, lifespan, and neurite branching, which indicates neuronal health), and fecundity.

B_{12} deficiency slows larval development

The time it takes for a worm to develop from embryo to adult is a good read out of overall health and fitness, and under normal growth conditions, at 20˚C, this takes approximately 3 days. To examine the effect of vitamin B_{12} deficiency on larval development, we synchronised a population of embryos by carrying out a timed egg lay, and monitored their growth over a 4-day period by measuring the length of the animals using light microscopy and image quantification software every 24 hours (Fig. 6). We found that worms lacking B_{12} (B_{12}-) grew slower and remained slightly smaller as adults that worms grown in either B_{12}+ or NGM conditions. Thus, we concluded that B_{12} is important for normal larval development.

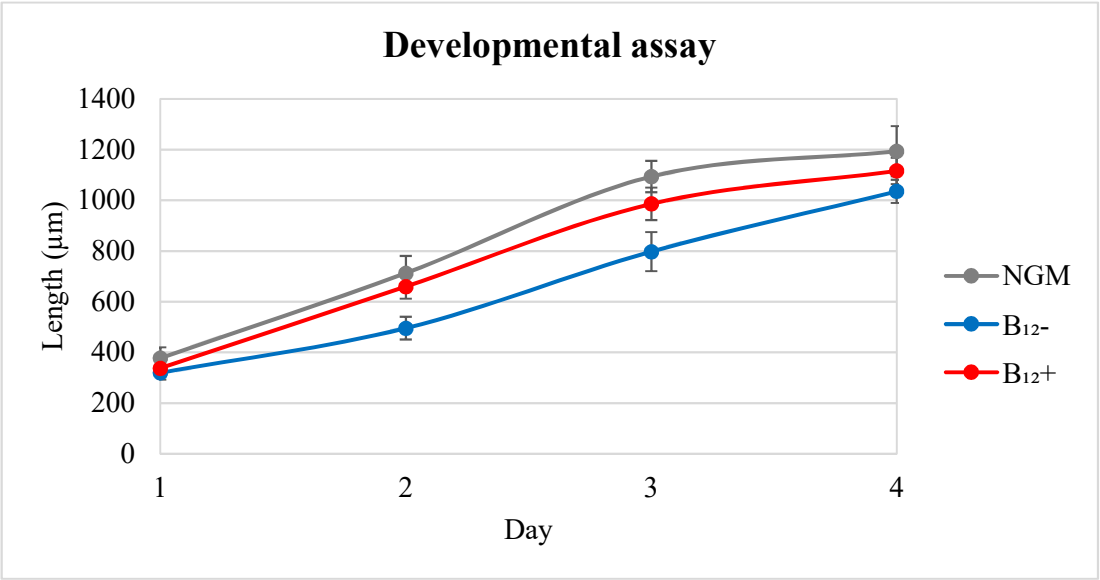


Figure 6. Developmental assay. Experiments were carried out at 20°C. Using a two-tailed T-test, all P-values were significant: Day 1: B_{12}- vs B_{12}+ (p=6.76498E-08), B_{12}+ vs NGM (p=1.89498E-18), B_{12}- vs NGM (p=5.1683E-32). Day 2: B_{12}- vs B_{12}+ (p=1.77042E-65), B_{12}+ vs NGM (p=6.70488E-06), B_{12}- vs NGM (p=1.33606E-78). Day 3: B_{12}- vs B_{12}+ (p=1.72473E-51), B_{12}+ vs NGM (p=0.000774796), B_{12}- vs NGM (p=2.52165E-66). Day 4: B_{12}- vs B_{12}+ (p=2.46627E-20), B_{12}+ vs NGM (p=0.03316559), B_{12}- vs NGM (p=2.38518E-24). Worms were imaged and measured using a Leica DMR microscope and measured using Leica Application Suite X (LAS X) microscopy software. The experiments were performed in triplicate and the individual trial information can be found in Fig. S2 of the supplemental data.

B_{12} deficiency decreases lifespan

Lifespan is also a well characterised attribute of nematode health, with several studied factors which influence it (e.g. food availability, insulin pathway signalling, and temperature). A healthy N2 worm grown at 25˚C on NGM with *E. coli* OP50 as a food source will typically live approximately 20 days, and a reduced lifespan indicates poor growth conditions (with the exception of elongated lifespan due to the stress-induced dauer state) (Zhang *et al.*, 2015). To measure the lifespan of the worms on our three conditions, we conducted a lifespan assay, with 100 worms per assay (Fig. 7). L4 worms grown at 20˚C from each condition were placed on to corresponding fresh

plates containing FuDR to prevent the eggs from hatching (a standard technique used for this type of assay) and incubated at 25˚C. The worms were counted every other day and scored as alive, dead (when no longer responding to light touch from a worm pick), or censored (escaped worms or unnatural deaths, such as internal hatching). Worms grown on the nutrient-rich NGM media had ~20-day lifespans, as expected for this temperature, which indicated that the experiment had been carried out correctly. Worms grown on the B_{12}+ and B_{12}- conditions had reduced lifespans compared to the ones grown on the NGM condition, which was also to be expected as they were grown on media containing only the bare essential nutrients. However, the B_{12}+ condition was significantly better off than the B_{12}- condition, with the only difference being the presence of vitamin B_{12} in the media.

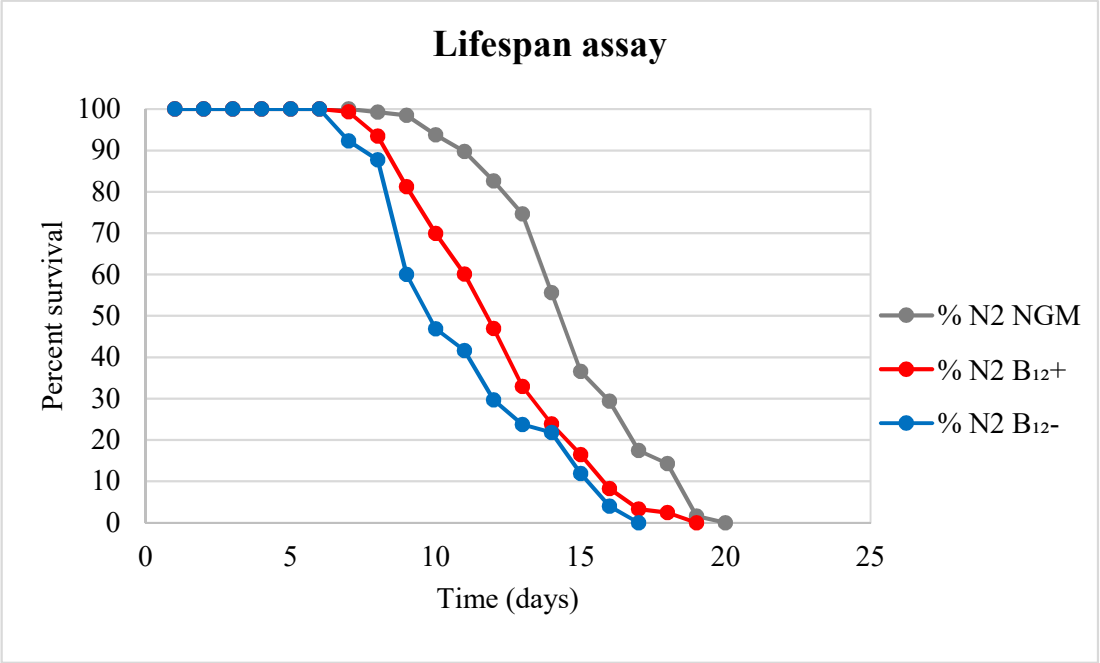


Figure 7. Lifespan assay. Experiments were carried out at 25°C with FuDR to prevent internal hatching and progeny development. The experiments were carried out as described above and performed in triplicate. A two-tailed T-test was carried out to determine the statistical significance: for the NGM vs B_{12}+ and the NGM vs B_{12}- conditions p=0, and for the B_{12}+ vs B_{12}- p=0.0036. This indicates a significant difference between all three conditions. The individual trial information can be found in Fig. S3 of the supplemental data.

The combined data shows that there is a significant difference between all three conditions, with the B_{12}-deficient worms having the shortest lifespan. This is consistent with previously published data, although our experiments were carried out at 25°C vs. 20°C, and we had worms grown on NGM as a control to compare the M9 media to the standard nutrient-rich NGM (Bito *et al.*, 2013). Having the NGM control allowed us to verify whether the overall experiment was carried out correctly. The lifespan of the worms grown on NGM (control) correlated with previously published data, indicating that the assay was performed appropriately (Zhang *et al.*, 2015). We concluded from these experiments that vitamin B_{12}, is important for general health and lifespan, and that nutrient availability plays a large role in lifespan.

B_{12} deficiency reduces fecundity

Fecundity is another measure of nematode health, with N2-strain worms laying between 250 and 300 eggs at 20˚C on NGM (Hodgkin *et al.,* 1991). To determine the effect of vitamin B_{12} deficiency on this attribute, we carried out a brood size assay. We transferred individual L4-stage worms to fresh plates of their corresponding growth medias and incubated them at 20˚C (10 per condition), transferring them each day on to fresh plates. This allowed us to measure not only the total amount of eggs laid (which hatched) (Fig. 8), but also the amount of eggs laid per day (Fig. S4 of supplemental data).

A two-tailed T-test analysing the combined data shows that there is a significant difference between both the NGM and the B_{12}- and the B_{12}+ and the B_{12}- conditions, but not between the NGM and the B_{12}-supplemented condition. The result of this test for NGM vs B_{12}+ is p=0.346569706, for NGM vs B_{12}- it is p=8.60383E-07, and for B_{12}+ vs B_{12}- it is p=2.236E-09. From these results we can conclude that there is a decrease in fecundity in B_{12}-deficient *C. elegans*, and that it can be completely rescued by growing them on media supplemented with cyanocobalamin.

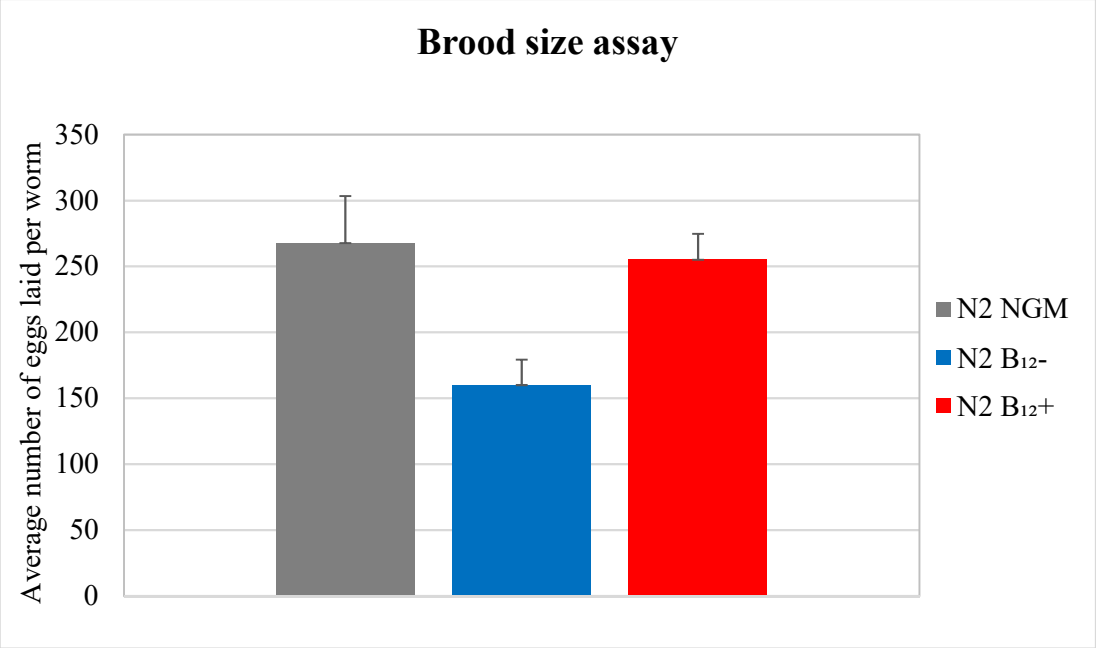


Figure 8. Brood size assay. Graph shows the average total amount of eggs laid by an individual worm at 20°C on each condition. The experiments were performed in triplicate and the individual trial information can be found in Fig. S4 of the supplemental data.

Vitamin B_{12} deficiency increases neurite branching in *C. elegans*

Neurite branching in *C. elegans* has been previously used as a marker for neural health and neurodegeneration. The increase in branching of mechanosensory neurons as worms age is associated with a decrease in response to touch, indicating the deterioration of these neurons. In addition, *C. elegans* is an excellent model for human neurodegenerative diseases, as its neural network has been completely mapped, and changes can be observed *in vivo* due to the natural transparency of these worms.

The effect of several conditions on neurite branching in *C. elegans* has been studied, such as ageing, oxidative stress, and insulin signalling, but the effect of vitamin B_{12} deficiency had not been studied until now. To determine the effect of B_{12} deficiency on neurodegeneration in these nematodes, we carried out a neurite branching assay using worms with GFP-tagged MEC-4 channels (CZ10175 strain). MEC-4 sodium ion channels are only found in the six mechanosensory neurons, which makes it easy to visualise and determine whether they are branched or not. The physiology of healthy, unbranched mechanosensory neurons with GFP-tagged *mec-4* sodium ion channels

has been previously determined, and they appear linear and smooth (Fig. 9) (Suzuki *et al.*, 2003). As worms age, the neurons develop random 'blebbing' (which appears as dots along a thinner axon), zig-zagging axons, and neuronal branching, which especially occurs at the cell body and at 'blebs'.

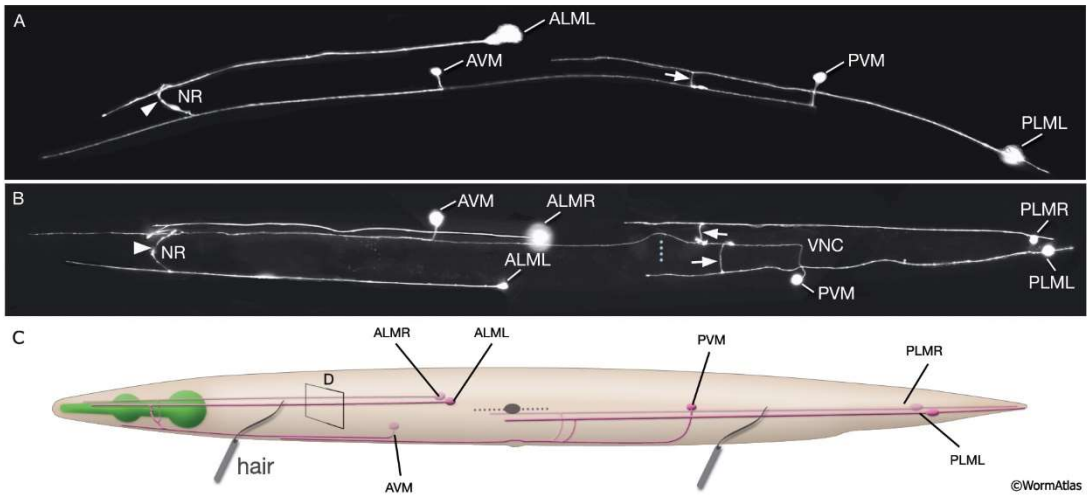


Figure 9. Images of fluorescent mechanosensory neurons of an adult *C. elegans* expressing the reporter gene *mec-4p::GFP*. A. Left lateral view. **B.** Ventral view. ALML: anterior lateral MT cell (left). ALMR: anterior lateral MT cell (right). AVM: anterior ventral MT cell. PLML: posterior lateral MT cell (left). PLMR: posterior lateral MT cell (right). PVM: posterior ventral MT cell. NR: nerve ring. (Altun and Hall, 2011)

A timed egg lay was carried out to synchronise the worms, which were then aged at 20˚C and imaged on days 1, 8, and 15 of adult-stage on a Leica DMR fluorescent microscope to observe their rate of ageing. Worms were categorised as either positive or negative for neuronal branching each day, and the percentage of worms with neuronal branching was calculated, which gave us an indication of the degree of overall neural health in each population at each timepoint. Representative images of the structure of the neurons on each day for each condition can be found below (Fig. 10). As can be observed, the B_{12}-deficient worm neurons are significantly more deteriorated by adult day 8 compared to the neurons of the worms on the NGM or the B_{12}+ conditions. Moreover, by day 15, many of the worms are dead by this age, and most of the B_{12}-deficient worms which are alive have severe, widespread neurite branching.

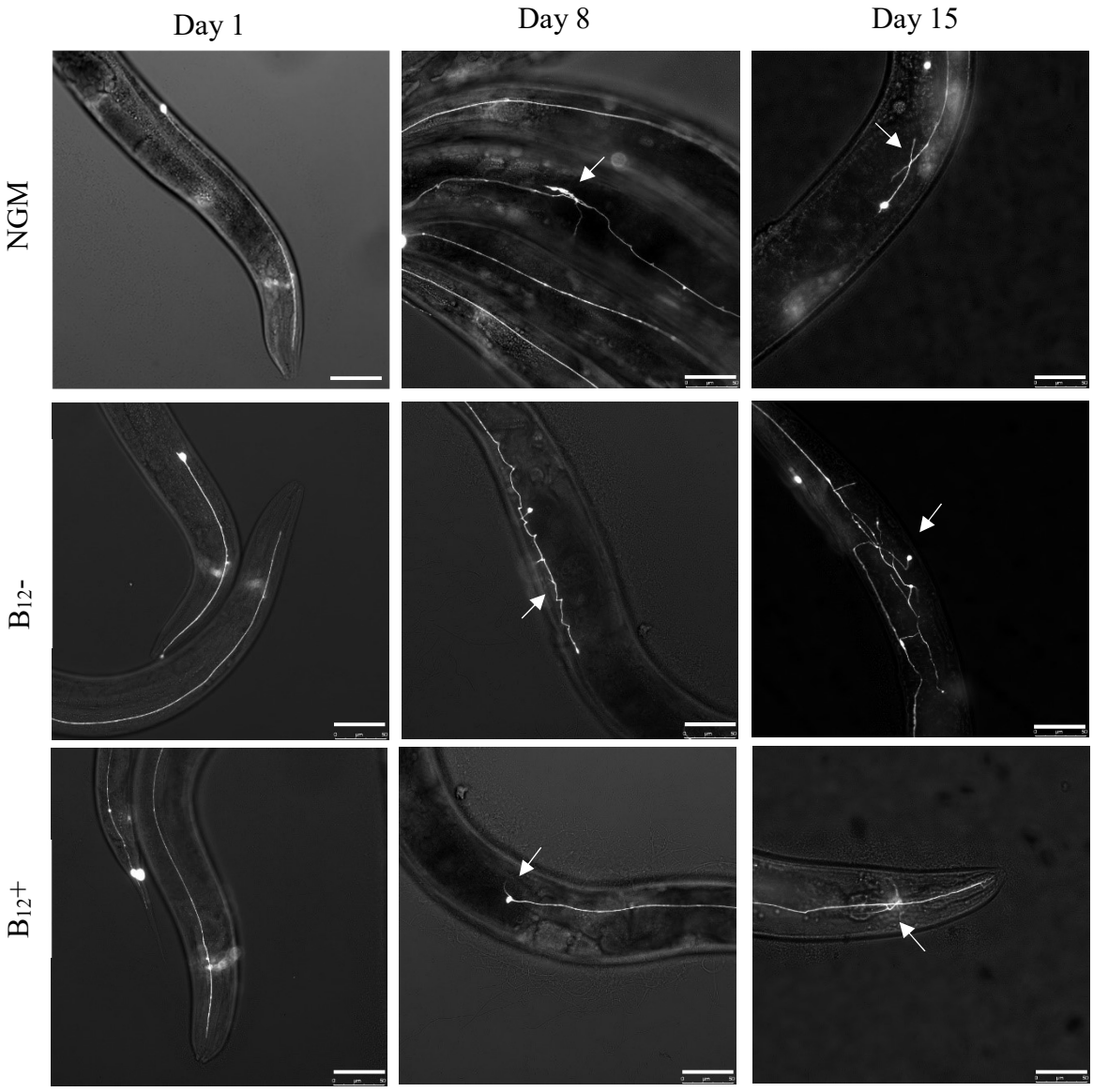


Figure 10. Neurite branching assay images. Worms were imaged on a Leica DMR fluorescent microscope (GFP filter) at 20x magnification at day 1, day 8, and day 15 of adult stage. White arrows indicate neuronal branches. Worms were first observed at 10x magnification. Worms that had visible neuronal branching at this magnification were scored as positive for neurite branching, and those that did not were scored as negative. Branches were then imaged at 20x magnification, showed above. 'Blebbing' and degree of zig-zagging were not taken into account in this experiment. Experiments were performed in triplicate and individual trials can be found in the supplementary data. A worm strain containing a *mec-4p::GFP* translational fusion was used to view the mechanosensory neurons (as the MEC-4 ion channel protein is only expressed in those six neurons). Scale bar: 50 µm.

The results of the neurite branching assay were quantified as the percentage of worms that presented neurite branching on adult day 1, 8, and 15 (Fig. 11). The results of the T-test indicate that there is a significant difference when comparing both the NGM and B_{12}+ conditions to the B_{12}- condition, but not between the NGM and the B_{12}+ conditions. The B_{12}-deficient worms were found to have earlier-onset, as well as more severe neurite branching overall. Therefore, we can conclude that vitamin B_{12} is important for the maintenance of neuronal health in these nematodes, and that B_{12} deficiency increases the rate and severity of neuronal degradation.

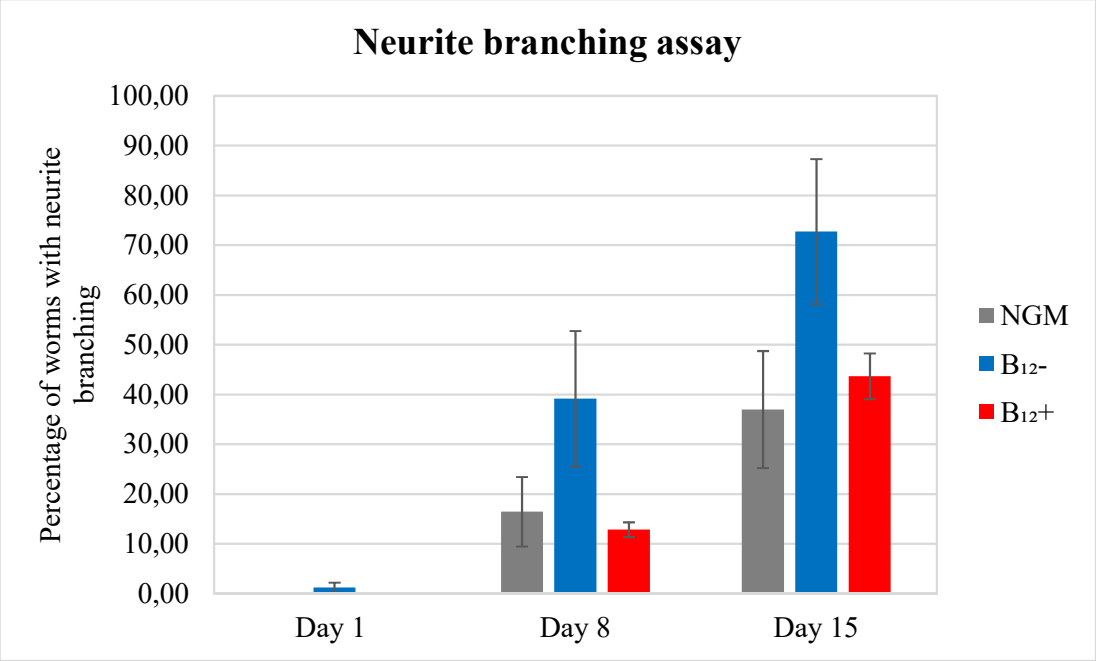


Figure 11. Neurite branching assay. Carrying out a two-tailed T-test of the data, on day 1 there was no significant difference between and of the conditions. On day 8 there was a significant difference between both the NGM and B_{12}- conditions (P-value = 0) and the B_{12}+ and the B_{12}- conditions (P-value = 0.0001), but not between the NGM and the B_{12}+ conditions (P-value = 0.48392). On day 15, the results were the same: a significant difference between NGM and B_{12}- (P-value = 0) and B_{12}+ and B_{12}- (P-value = 0), but no significant difference between NGM and B_{12}+ (P-value = 0.33204). Experiments were performed in triplicate and the raw data from each trial can be found in Fig. S7 of the supplemental data.

Using the worm as a model to understand B_{12} uptake and localisation

As B_{12} comes from the diet, understanding how it is absorbed into an organism and identifying the tissues in which B_{12} it localises to is important for understanding its function. In humans this has been previously studied, albeit not completely discerned (Kozyraki *et al.*, 2013). However, the uptake and transport mechanisms, as well as the final localisation of vitamin B_{12} in *C. elegans* is not well described. This is important as there are parasitic worms which cause vitamin B_{12} deficiency, and by studying the uptake of B_{12} in *C. elegans* we may learn more about how they compete for B_{12} in the intestine, and might discern a target against this. By synthesising a fluorescent B_{12} molecule we can observe the uptake and localisation of this molecule *in vivo*, as these nematodes are inherently transparent. Fluorescent cyanocobalamin has previously been found to localise to coelomocyte cells in *C. elegans* (Lawrence *et al.*, 2018). There are six coelomocytes in *C. elegans*, which are found in pairs in the head, midbody, and tail of the animal. While these cells are fixed in the worm's body, they are thought to have immune and scavenging functions, as they are capable of taking up and accumulating various macromolecules by endocytosis, however they are not capable of phagocytosis.

The aim of this previous experiment was to determine whether the fluorescent cobalamin analogues could be made and whether they would be taken up by a variety of organisms. Here, we wanted to expand on this by testing whether worms deficient in B_{12} altered the uptake or end point location of the fluorescent B_{12}. In other words, if cells show a preference for regular B_{12} that overrides the uptake and/or use of the fluorescent analogues. We first synthesised the fluorescent cyanocobalamin using BODIPY® TR-X as a fluorophore (Fig. 12). This particular fluorophore was used due to its excitation in the red spectrum (excitation: 588 nm, emission: 616 nm), as *C. elegans* autofluoresce in the blue/violet area of the spectrum (Martin *et al.*, 2002). This allowed us to distinguish the gut autofluorescence of the worm from the BODIPY-B_{12}.

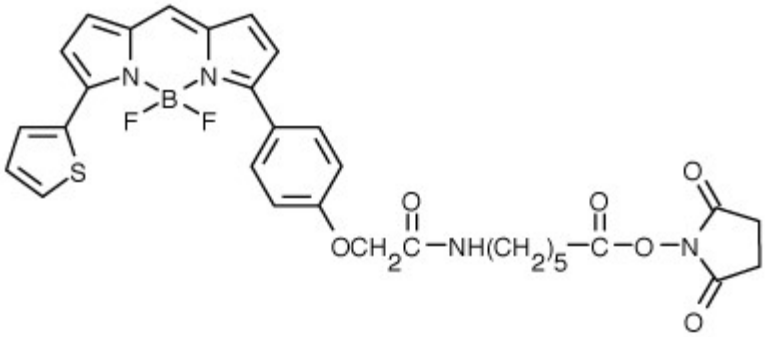


Figure 12. BODIPY® TR-X fluorescent dye molecule. The molecule has a seven-atom aminohexanoyl spacer ("X") separating the amine-reactive NHS ester (right side) from the fluorophore (left side). The NHS ester can be used to label the primary amines (R-NH_2) of molecules. The spacer separates the fluorophore from the molecule it is conjugated with, reducing the interaction between the two. (Thermofisher.com, 2016)

BODIPY-B_{12} was synthesised by linking the BODIPY® TR-X fluorophore to cyanocobalamin, as described previously (Lawrence *et al.*, 2018). The ribose-5'-hydroxyl group of cyanocobalamin was activated with 1,1'-Carbonyldiimidazole (CDI) (McEwan *et al.*, 1999; Lee and Grissom 2009). This was coupled with 1,2-Diaminoethane, which formed a primary amine at this location (Fig. 13). This allowed us to attach the BODIPY® TR-X to the cyanocobalamin at the correct point, as it is an amine-reactive dye. Once the reaction was complete, as it only had a rate of about 50%, we used several rounds of HPLC to purify it.

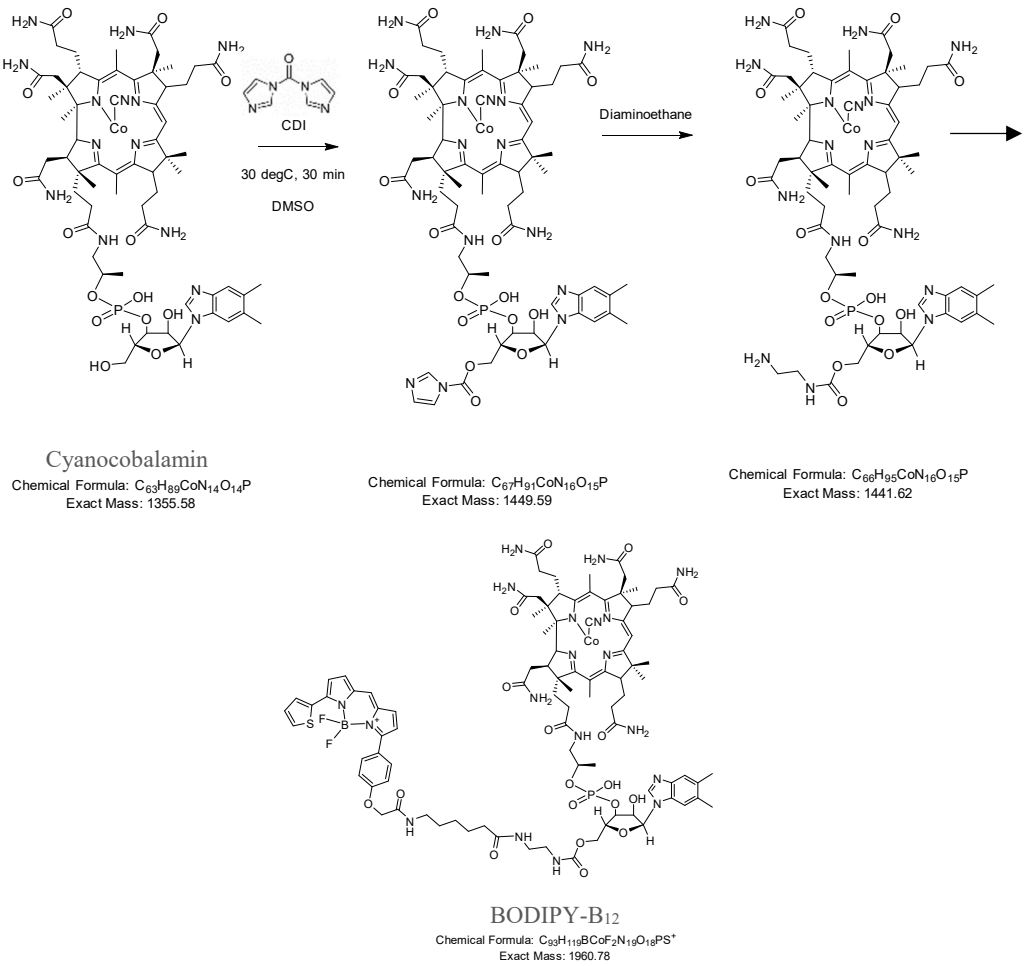


Figure 13. Diagram of the chemical synthesis of BODIPY® TR-X ribose-linked cobalamin. The BoB_{12} was synthesised as described previously. (Lawrence *et al.*, 2018)

The product was subsequently purified several times to ensure the purity of the sample: first by semi-preparative HPLC and subsequently by preparative HPLC-MS. The purification allowed us to remove large amounts of impurities, whilst the preparative HPLC-MS enabled us to separate the regular cyanocobalamin from our fluorescent BoB_{12} sample as much as possible, as well as carrying out mass spectrometry to confirm that we had synthesised the right product (Fig.14). As we were primarily testing whether BoB_{12}, in the absence of B_{12}, would localise to somewhere other than the coelomocytes, it was essential to get as pure of a sample as possible.

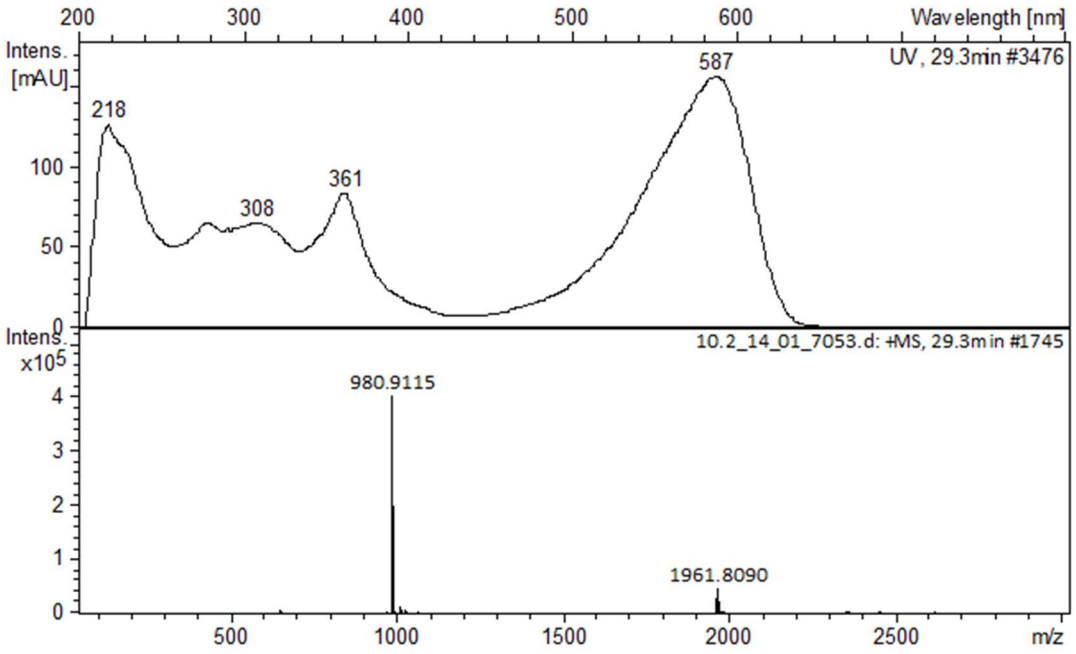


Figure 14. HPLC-MS of BODIPY® TR-X ribose linked cobalamin (BoB_{12}). Results from sample run on HPLC-MS, which was later used in our localisation and rescue experiments. The UV absorbance curve has peaks at the expected wavelengths: ~361 nm for cyanocobalamin and 588 nm for BODIPY® TR-X. The m/z also correlated with the expected results. More detailed information can be found in Fig. S5 of the supplemental data.

To compare the preference in uptake of B_{12} and BoB_{12}, we studied the uptake of the fluorescent cyanocobalamin on the B_{12}-deficient worms grown on our solid M9 media by introducing BODIPY-B_{12} into *E. coli* and then feeding it to the worms. To do this *E. coli* OP50 was transformed with a pET-BAD plasmid containing the *btuB* and *btuF* genes and used as an intermediary to transfer the BoB_{12} to the worms, as described previously (Lawrence *et al*., 2018). BtuB is an outer membrane *E. coli* protein that actively transports B_{12} analogues indiscriminately into the periplasm. The trans-periplasmic protein TonB interacts with BtuB to drive the reaction (Cadieux and Kadner, 1999). Calcium increases the affinity of cyanocobalamin for BtuB by a factor of 50 to 100 (Chimento *et al.*, 2003). Overproduction of BtuB in *E. coli* increases the amount of B_{12} uptake (Heller *et al.*, 1985). BtuF is a periplasmic protein also involved in CNCbl uptake (Cadieux *et al.*, 2002).

To induce the *E. coli* to take up as much of the fluorescent cyanocobalamin as possible, it was transformed with a pET-BAD *btuB-btuF* vector (Fig. S6 of supplemental data). This increased the amount of BtuB and BtuF transporters in each bacterium, maximising BoB_{12} internalisation.

The *E. coli* containing the *btuBF* pET-BAD plasmid was grown in the dark as indicated in the methods section. To verify that the BODIPY-B_{12} had been taken up by the *E. coli*, the cultures were spun down and washed three times in M9 media to remove any BoB_{12} that had not been taken up. After this, the pellet was resuspended in M9 media and imaged on an Olympus IX-81 inverted fluorescence microscope at 100x magnification (Fig. 15). As seen in the image, some of the *E. coli* had internalised the fluorescent cyanocobalamin.

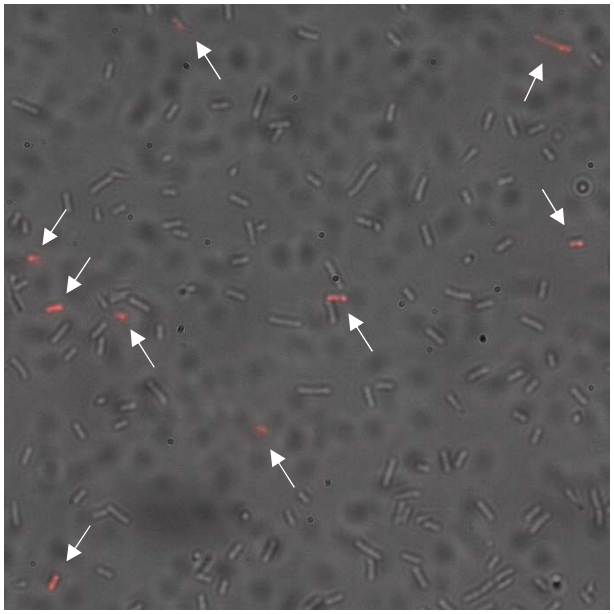


Figure 15. Image of *btuBF* pET-BAD plasmid-expressing *E. coli* containing BoB_{12}. BoB_{12} was introduced into the *E. coli* as described previously by Lawrence *et al.* (2018). Cells were imaged on an Olympus IX-81 inverted fluorescence microscope at 100x magnification using an RFP 580 filter. Brightfield images were then overlaid with the fluorescence images to confirm colocalisation of BoB_{12} and *E. coli,* verifying the uptake of BoB_{12}. The white arrows point to the OP50 *E. coli* cells which have taken up the fluorescent cyanocobalamin (approx. 6%).

Once it was confirmed that the BoB_{12} had been internalised by the BtuBF-overexpressing *E. coli*, non-B_{12}-supplemented M9 agar plates were seeded with the culture to be used in the B_{12}-localisation study. L4 stage worms were placed on to these plates and incubated at 20°C. Three days later, the now-adult progeny was imaged. All reactions and procedures involving the BoB_{12} analogue, including incubation of plates and worms, were performed in dark/low light conditions. Overlaying the brightfield images with the fluorescent ones, we concluded that the fluorescent B_{12} localised to the coelomocytes (Fig. 16).

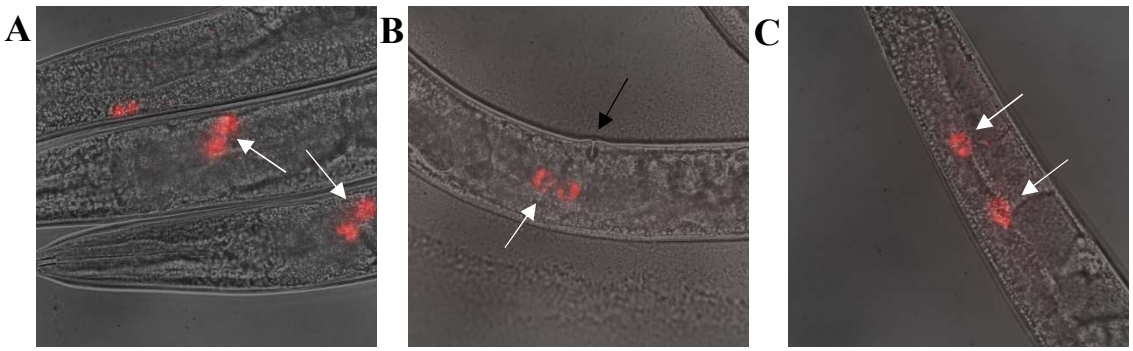


Figure 16. BoB_{12} localisation to the coelomocytes in adult *C. elegans*. Arrows point to coelomocyte cells. **A:** Head section coelomocyte pair of two *C. elegans*. **B:** Midbody coelomocytes (black arrow points to vulva for reference). **C:** Tail coelomocyte pair. Worms were imaged on an Olympus IX-81 inverted fluorescence microscope at 60x magnification using an RFP 580 filter, on 2% agarose pads with 0.06% tetramisole hydrochloride to immobilise the worms. Brightfield images were then overlaid with the fluorescence images to confirm the localisation of the BoB_{12} to the coelomocytes.

From the results obtained, we can conclude that BODIPY® TR-X ribose linked cobalamin localises to the coelomocyte cells even in B_{12}-deficient *C. elegans*. The confirmation that the worms were able to take up this cobalamin analogue supports the previously described method that this technique can be used to tag and follow macromolecules *in vivo*. In addition, it shows that worms do not necessarily have a preference for regular cyanocobalamin.

We then tried a different method to study the localisation of the BoB_{12} in worms, to see whether the state of the media had any effect in the uptake of this molecule. We attempted introduced the fluorescent cyanocobalamin into the worms by incubating them in axenic liquid media (prepared as described previously by Samuel *et al.*, 2014),

supplemented with 3.3µM of BoB_{12}. In contrast to our previous work on solid media however, we noted that the majority of the fluorescent B_{12} analogue became stuck in the nematode's mouth (Fig. 17), by which we concluded that the protocol designed by Lawrence *et al.* (2018) was more effective at introducing the fluorescent cobalamin analogue into the worm.

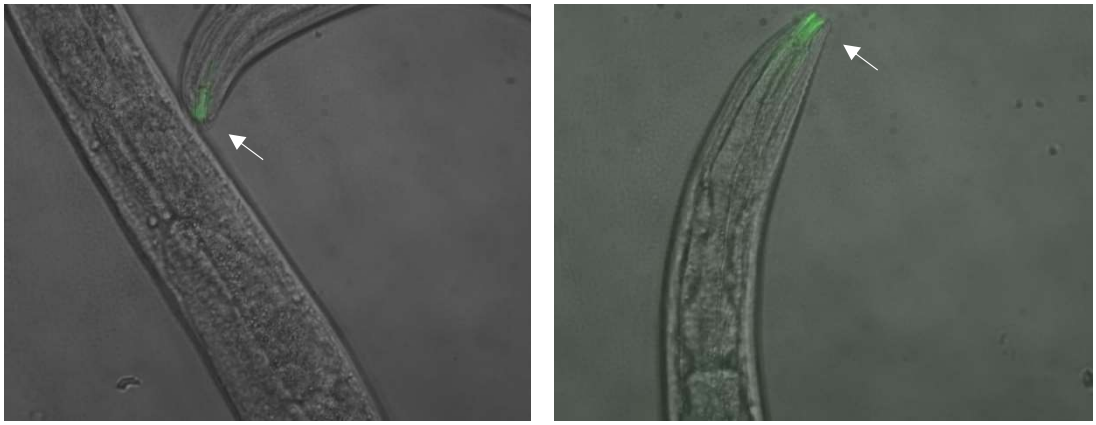


Figure 17. Adult *C. elegans* grown in liquid axenic media supplemented with BoB_{12}. Arrows point to the mouths of the nematodes, where fluorescence can be seen, indicating that the fluorescent B_{12} analogue is caught in the worm's mouth. Worms were imaged at 40x magnification.

Rescuing the B_{12}-deficient phenotype with cobalamin analogues

With the B_{12}-deficiency phenotype established and with a certain degree of confidence that the BoB_{12} can be taken up into the worm (Fig. 16), we tried rescuing the physiological phenotypes associated with B_{12} deficiency with the fluorescent B_{12} analogue to determine its ability to function as normal B_{12}. For this we compared it with cyanocobalamin, as well as pseudocobalamin. Pseudocobalamin is produced by some bacteria, especially cyanobacteria, but significantly it is not bioactive in humans. We included it in our rescue experiment as we wanted to investigate whether this molecule is biologically active in the nematodes. This would be an interesting find regarding parasitic nematode worms and their ability to utilise pseudocobalamin ingested through diet from other foods. We tried rescuing the reduced brood size phenotype of B_{12}-deficient *C. elegans* as it had consistent results across all three trials and was significantly reduced when compared to the NGM and B_{12}+ conditions.

To create the five conditions and maintain them as comparable as possible, we used the OP50 containing the pET-BAD *btuBF* plasmid in all of them, and the cobalamin analogues were all added to a final concentration of 1 µM, along with arabinose to induce their uptake. For the first condition, we used NGM plates seeded with the plasmid-containing OP50 grown in LB. The other four conditions used non-supplemented M9 agar plates. In the second condition, we seeded the plates with the OP50 grown in minimal *E. coli* M9 media, which acted as a negative control with no B_{12} present. For the final three testing conditions we grew the plasmid-expressing OP50 *E. coli* in M9 media supplemented with either cyanocobalamin, pseudocobalamin, of BODIPY® TR-X ribose-linked cyanocobalamin.

For the cobalamin analogue brood size rescue experiment, B_{12}-deficient nematodes were transferred at stage L4 from the B_{12}-deficient M9 media to the five different conditions, which tested the ability of each media to rescue the decreased brood size phenotype found in B_{12}-deficient worms.

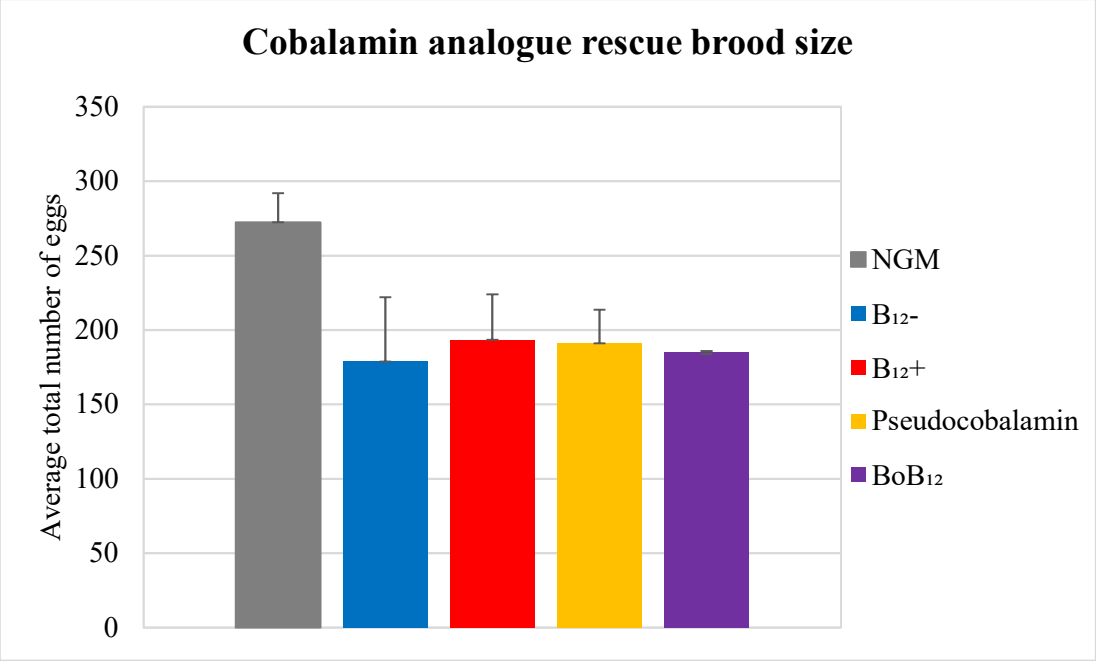


Figure 18. Reduced brood size B_{12}-deficiency phenotype rescue experiment with cobalamin analogues. Only the difference between the NGM and all other conditions is significant with a two-tailed T-test (NGM vs B_{12}- p=3,56364E-05; NGM vs B_{12}+ p=4,70571E-06; NGM vs pseudocobalamin p=1,02448E-07; NGM vs BoB_{12} p=1,98521E-08). Experiments were performed in triplicate and the individual trials can be found in Fig. S8 of the supplemental data annex.

From this we can conclude that placing the B_{12}-deficient worms at stage L4 on the different medias did rescue the decreased fertility of the B_{12}-deficient worms. We cannot say with certainty whether the pseudocobalamin and BoB_{12} analogues are bioactive in the worms and if they would rescue the B_{12}-deficiency phenotype from the results of this experiment. The NGM condition did rescue the decreased brood size characteristic of B_{12}-deficient worms, however this media is very rich in nutrients compared to the M9 media that the other conditions are based upon. We could postulate that it is a matter of the worms not being incubated for long enough on the other conditions, as it has been previously determined in our other brood size experiments that worms grown permanently on the B_{12}+ do have normal, healthy brood sizes, however additional experiments would be necessary to confirm this hypothesis.

Discussion

The goal of this project was to investigate the effects of vitamin B_{12} deficiency in *C. elegans*, and to study the localisation of this vitamin *in vivo.* In addition, the ability of pseudocobalamin and various cobalamin analogues to rescue the B_{12}-deficient phenotype was studied. The B_{12}-deficiency phenotype included delayed life cycle, shortened life span, reduced fertility, and neuronal deterioration.

Establishing a protocol for producing B_{12} deficient worms

The main goal was broken down into smaller aims, the first being to determine a protocol for producing B_{12}-deficient *C. elegans* at the University of Kent. *C. elegans* was made vitamin B_{12}-deficient by growing them on minimal M9 media with *E. coli* grown in M9 media as a food source. The ability of our media to make the worms B_{12}-deficient was confirmed with an *acdh-1*-deletion strain of *C. elegans*, which cannot survive on B_{12}-deficient media (Watson *et al.*, 2016). Our results indicated that by the fifth generation, which is when we started using the N2-strain worms for experiments, all of the *acdh-1*-deletion strain worms were dead. This result confirmed that B_{12}-deficiency can be induced in *C. elegans,* and that it can be produced in a relatively short period of time.

Determining the phenotypic effect of vitamin B_{12} deficiency in *C. elegans*

The second aim was to study the phenotype generated by vitamin B_{12} deficiency in *C. elegans*. We carried out experiments confirming previously published data on B_{12} deficiency in these nematodes (delayed life cycle, reduced fertility, and reduced lifespan), as well as studied new phenotypes associated with this condition (Bito *et al.,* 2013). It is to note that previously published results did not use worms grown on NGM as a control (Bito *et al.*, 2013), which made it difficult to elucidate which effects were a result of the worms being grown on minimal media and which were actually caused by a deficiency of vitamin B_{12}, as well as if the experiments had been carried out correctly (as there are no previous studies determining the expected results). In addition, from those studies, we could not confirm whether the B_{12}-supplemented condition was rescuing the worms to the level of those grown on standard, nutrient-

rich NGM or just partially ameliorating the symptoms of B_{12} deficiency. We wanted to confirm these results, as well as expand on them, particularly focusing on the role of B_{12} in ageing.

The first set of experiments studied the nematode's life cycle and developmental rate. The results indicate that B_{12}-deficient worms take approximately one day longer to develop from an egg to adult stage, and that supplementing the M9 media with vitamin B_{12} completely rescues this delayed lifecycle phenotype to the same levels as worms grown on nutrient-rich NGM. This is consistent with previously published findings, however our results also indicate that the B_{12}-supplemented condition rescued the worms completely, with no significant difference between it and the NGM condition (Bito *et al.*, 2013). From this, we can conclude that B_{12} is important in larval development.

Lifespan experiments were then carried out with FuDR at 25°C which, from experience, reduces the amount of internal hatching seen at 20°C. The first two trials were found to have a significant difference between the NGM condition and the B_{12}-deficient condition, but not between the B_{12}-supplemented and the B_{12}-deficient condition. However, both the last trial and the combined data were found to have a significant difference between all three conditions. The results from the first two trials could have been due to human error, as these were the first experiments carried out. However, the results are inconclusive, and more trials should be carried out to verify them. It was to be expected that the worms grown on the minimal media would be worse off than those grown on the nutrient-rich NGM plates, however supplementing with B_{12} seemed to partially restore this. Our experiments were carried out at 25°C compared to previously published data, which performed them at 20°C and showed that B_{12}-deficient worms had a shorter lifespan compared to the ones that had vitamin B_{12} supplemented in the media (Bito *et al.*, 2013). We note that this previously published paper does not have the standard NGM media as a control for the minimal media agar plates, which, as our results show, prevented them from painting the whole picture: that although supplementation with cyanocobalamin alleviates the detrimental symptoms of B_{12}-deficiency on lifespan, it cannot rescue them to normal levels. These results indicate that while vitamin B_{12} may play a part in longevity, general nutrient availability has a more important role in regulating lifespan.

Fecundity, another marker of nematode health, was measured via brood size assay, which determines the number of viable offspring laid by each worm. Our results show that the B_{12}-deficient worms have a significantly reduced brood size, and that the worms grown on the B_{12}-supplemented condition had no significant difference with the NGM condition. These results indicate that B_{12} is important in fecundity and that it can rescue the decreased fertility associated with B_{12}-deficiency to normal levels. Whilst this correlates with previously published data in that B_{12}-deficient worms have reduced brood sizes compared to those grown on B_{12}-supplemented M9 media, previously published data shown extremely low numbers of eggs laid (approx. 85 eggs/worms for the B_{12}-supplemented M9 condition and approx. 55 eggs/worms for the non-supplemented M9 condition), as well as no NGM control (Bito *et al.*, 2013). Having the NGM condition as a control allowed us to verify whether the reduced brood size effect was due to B_{12}-deficiency or the nutrient-poor minimal media, as well as if the experiment was carried out correctly (as the average number of eggs N2 WT *C. elegans* lay on NGM media at 20°C is thoroughly studied). The results of our NGM condition indicate that our experiment was carried out correctly, as N2 worms lay an average of 250-300 eggs at 20°C (Hodgkin *et al.*, 1991). In addition, while our B_{12}-deficient brood size was much lower than our control brood sizes (approx. 150 progeny/worm compared to 250 progeny/worm in the controls), it was still significantly higher than the published data. Thus, the previously published results clash with our findings, where the B_{12}-supplemented condition was rescued completely to the level of the NGM condition, and there are very low numbers of eggs laid per worm compared to our results. This raises the question of whether the previously published data on the effect of B_{12} deficiency on brood size is correct. More fecundity experiments should be carried out to corroborate this.

Using worms to model the role of B_{12} in neurodegeneration

Whilst *C. elegans* is used as a model for several neurodegenerative diseases, and neurite branching has been defined as a marker for neurodegeneration, the effects of B_{12} deficiency on neural health in this organism had not been studied. This study reports that B_{12}-deficiency in *C. elegans* increases neuronal ageing, and that this can be rescued by supplementing the media with cyanocobalamin.

B_{12} is known to impact on neuronal integrity and function in humans (Savage, 1995; Kocer, 2009; Chien Tu *et al.*, 2017; Irevall, 2017). To explore the possibility that this could be studied at the molecular basis in worms we examined the effect of B_{12} deficiency on neurite branching in ageing worms. In worms, neurite branching can be correlated with neuronal ageing and deterioration. To examine the role of B_{12} in neuronal health and ageing, we used CZ10175 strain *C. elegans,* which have a zdIs5 transgene containing a *mec-4p::GFP* translational fusion, which allows us to view the mechanosensory neurons (as MEC-4 is only present in these six neurons). A neurite branching assay was carried out by performing a timed egg lay and imaging the CZ10175 worms 3 days later, which was day 1 of adult stage. It is to note that most of the B_{12}-deficient worms were at L4 stage at day 3 post-timed egg-lay, however this was the most accurate was of synchronising the worms. Even though the B_{12}-deficient worms arrived at adult stage after the worms on the B_{12}-supplemented and NGM conditions, they presented neurite branching earlier on and more severely than the others. These results show us that *C. elegans* can be used as a model for the neuronal effects of vitamin B_{12} deficiency, as B_{12} has been shown to impact on neuronal integrity and function in humans (Savage, 1995; Kocer, 2009; Chien Tu *et al.*, 2017; Irevall, 2017). In addition, our results indicate that B_{12} deficiency increases the onset and severity of neuronal ageing and deterioration in these worms.

C. elegans is often used as a model for neurodegenerative diseases, of which a large part are tauopathies (e.g. Alzheimer's disease) (Li and Le, 2013; Wang *et al.*, 2018). The aggregation of filamentous tau is the second hallmark of Alzheimer's disease, where they form neurofibrillary tangles (Shirafuji *et al.*, 2018). Protein with tau-like repeats-1 (PTL-1) is the homologue of mammalian tau and MAP2 in *C. elegans* and regulates neuronal health (deviation from WT levels is detrimental to the worms) (Gordon *et al.*, 2008; Chew *et al.*, 2013; Chew *et al.*, 2014; Chew, Götz and Nicholas, 2014). Previous studies have shown that hyperphosphorylated tau aggregation causes morphological abnormalities in mechanosensory neurons in *C. elegans* (Kraemer *et al.*, 2003; Miyasaka *et al.*, 2005; Miyasaka *et al.*, 2016, Miyasaka *et al.*, 2018). Vitamin B_{12} was found to bind to tau cysteine residues, preventing tau fibrillation and further aggregation, which suggests another way that cobalamin prevents neural degeneration (Rafiee *et al.*, 2017). Alzheimer's is a tauopathy caused by protein aggregation, and B_{12} has also been found to reduce fibrillation in this specific disease (Alam *et al.*, 2017).

Cobalamin deficiency also causes accumulation of homocysteine (hyperhomocysteinemia), which has been classified as a risk factor for neurodegenerative diseases, such as Alzheimer's disease and dementia (Werder, 2010). When *C. elegans* become B_{12}-deficient, MeCbl is not available as a cofactor for methionine synthase (MS), and homocysteine cannot be converted to methionine (Fig. 19), which causes the accumulation of homocysteine in the cytosol. Homocysteine has recently been shown to increase the amount of tau protein and its phosphorylation, oligomerisation, and aggregation, all of which are associated with cell toxicity (Shirafuji *et al.*, 2018).

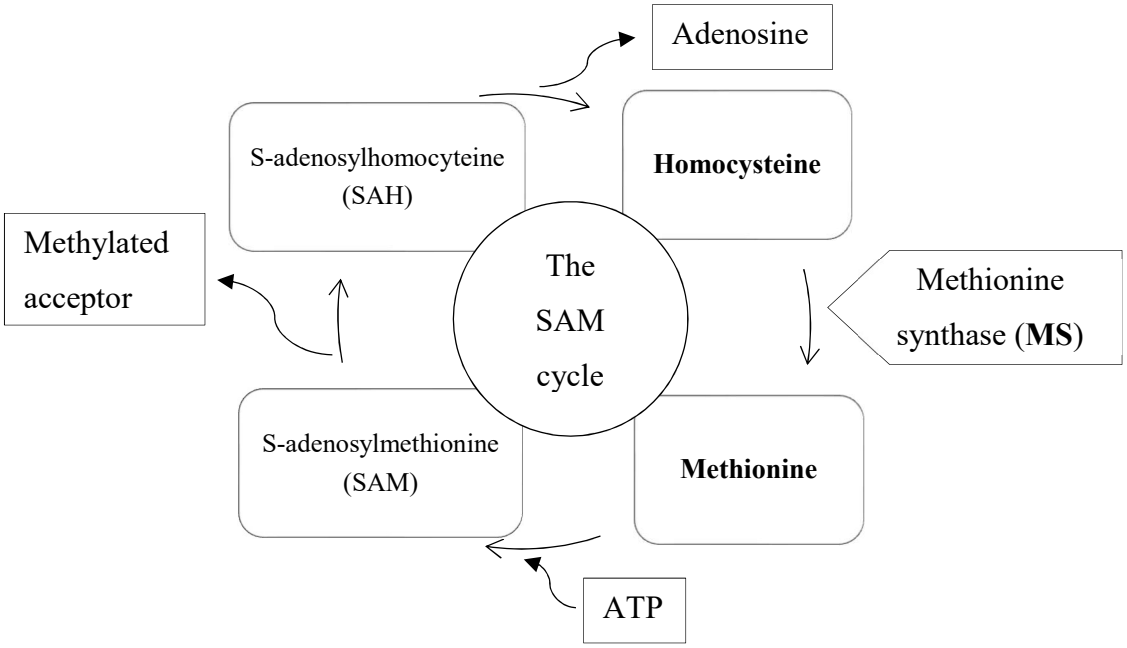


Figure 19. Simplified diagram of the SAM cycle. Methionine synthase (MS) catabolises the reaction of homocysteine to methionine in the cytosol, using MeCbl as a cofactor. B_{12}-deficiency inactivates MS, consequently causing the accumulation of homocysteine in the cell.

Fluorescent B_{12} localises to the coelomocytes in worms

Whilst B_{12} absorption and transport has been studied in humans, there is still little-to-no information for the model organism *C. elegans.* These nematodes cannot be used as a model for B_{12} absorption in humans, as the main B_{12}-binding proteins in humans, intrinsic factor (IF) and transcobalamin, have not been seen in *C. elegans.* However, the study of cobalamin transport in these worms could show us how parasitic worms

compete for the uptake of vitamin B_{12} in the small intestine, as well as corroborate a new method for tracking macromolecules *in vivo* using fluorophores. Lawrence *et al.* (2018), showed that BODIPY® TR-X ribose linked cobalamin (BoB_{12}) localised to the coelomocytes in these nematodes. There are six coelomocytes cells in *C. elegans,* which are thought to carry out immunological and scavenging functions (Fares and Grant, 2002). They are able to endocytose a wide variety of macromolecules and have high lysosome biogenesis rates, however, unlike macrophages, they are incapable of carrying out phagocytosis (Treusch *et al.*, 2004; Ewbank, 2002). These cells are 10-15 µm in diameter and have a fixed position in the worm, with two localising to the head region, two to the midbody, and two to the tail end, meaning that the material it takes up must be transported there. In addition, coelomocytes have been found to not be essential for the growth or survival of the nematodes (Fares and Greenwald, 2001).

The localisation of BoB_{12} to the coelomocytes even in B_{12}-deficient *C. elegans* may indicate that it is not being recognised as vitamin B_{12} or that the fluorophore is being cleaved off when cyanocobalamin is converted to MeCbl or AdoCbl in the worm. Alternatively, *C. elegans* only need trace amounts of vitamin B_{12} to survive. The fluorescent B_{12} may be localising to other cells in minute amounts, with the excess being endocytosed by the coelomocytes. The concentration of fluorescence in the coelomocytes, paired with the gut autofluorescence of *C. elegans*, may cause the few molecules of BoB_{12} localising to other cells to be overlooked. Alternative tests such as immunostaining could be carried out to verify the localisation of B_{12} in the worms. However, the fact that the fluorescent B_{12} was absorbed and transported in the worm corroborates the results published by Lawrence *et al.* (2018), showing that fluorophores could be used to tag macromolecules to follow them *in vivo* in *C. elegans*.

Rescue of B_{12}-deficiency phenotype with cobalamin analogues

To test whether vitamin B_{12} deficiency could be rescued, we carried out a brood size rescue experiment with a variety of cobalamin analogues. To test whether the BoB_{12} was bioactive, we included it in this experiment. Vitamin B_{12}-deficient L4-stage worms were transferred to five different conditions: NGM, B_{12}- (no B_{12}), B_{12}+ (cyanocobalamin), pseudocobalamin, and BoB_{12}. The conditions containing the analogues were supplemented by seeding B_{12}-deficient M9 media plates with *btuBF*

OP50 *E. coli* grown in culture with 1 μM of each analogue. This was done due to minute amounts of BoB_{12} and pseudocobalamin available, which did not allow us to supplement the agar with high concentrations of the molecules. Comparing the number of eggs laid in the rescue experiment with the normal brood size (where the worms were grown continuously on each condition), only the NGM condition rescued the brood size to normal levels. All other conditions (M9 media supplemented with cobalamin analogues and pseudocobalamin) did not rescue this detrimental phenotype.

As we saw that worms grown permanently on B_{12}-supplemented M9 media were rescued (fig. 5), we may infer that transferring B_{12}-deficient worms at the L4 stage does not give them enough time to be rescued by the time they develop into the adult stage and begin laying eggs. Another reason for the conditions not rescuing the B_{12}-deficiency phenotype is that supplementing the worms' food source rather the solid media has a different phenotypic effect. This experiment could be repeated by transferring the worms at an earlier stage or carrying out a timed egg-lay. Unfortunately, pseudocobalamin and BoB_{12} are scarce, and maintaining strains on media containing these molecules for extended periods of time is not viable. From this experiment, we can conclude that the availability of a rich source of nutrients can rescue the B_{12}-deficiency-induced phenotype of decreased fertility in a very short period of time, whilst supplementation with B_{12} analogues is not enough to do this.

Conclusion

We set out to study the physiological effect of vitamin B_{12} deficiency, as well as the localisation of B_{12}, in *C. elegans.* In addition, the ability of pseudocobalamin and various cobalamin analogues to rescue the B_{12}-deficient phenotype was tested.

Here, we have demonstrated the physiological relevance of B_{12} in larval development, fecundity and normal lifespan. In addition, for the first time, we demonstrate a role for B_{12} in maintaining neuronal integrity during the ageing process. These studies, combined with the fact that we can also generate fluorescent cobalamin that can be taken up and stored in the worm, means that the Tullet and Warren laboratories can move forward and use the worm as a whole organism model to further understand the uptake and action of B_{12} *in vivo*. In the future this work could benefit humans with B_{12} deficiency.

References

Alam, P., Siddiqi, M., Chaturvedi, S., Zaman, M. and Khan, R. (2017). Vitamin B_{12} offers neuronal cell protection by inhibiting Aβ-42 amyloid fibrillation. *International Journal of Biological Macromolecules*, 99, pp.477-482.

Alexander, A., Marfil, V. and Li, C. (2014). Use of Caenorhabditis elegans as a model to study Alzheimer's disease and other neurodegenerative diseases. *Frontiers in Genetics*, 5.

Allen, L. (2008). Causes of Vitamin B_{12} and Folate Deficiency. *Food and Nutrition Bulletin*, 29(2_suppl1), pp.S20-S34.

Altun, Z. and Hall, D. (2011). Hermaphrodite - Nervous System - General Description. [online] *Wormatlas.org*. Available at: http://www.wormatlas.org/hermaphrodite/nervous/Neuroframeset.html.

Andres, E. (2004). Vitamin B_{12} (cobalamin) deficiency in elderly patients. *Canadian Medical Association Journal*, 171(3), pp.251-259.

Antony, A. (2003). Vegetarianism and vitamin B-12 (cobalamin) deficiency. *The American Journal of Clinical Nutrition*, 78(1), pp.3-6.

Battaglia-Hsu, S, N Akchiche, N Noel, J-M Alberto, E Jeannesson, CE Orozco-Barrios, D Martinez-Fong, J-L Daval, and J-L Guéant. (2009). Vitamin B_{12} Deficiency Reduces Proliferation and Promotes Differentiation of Neuroblastoma Cells and up-Regulates PP2A, proNGF, and TACE. *Proc. Natl. Acad. Sci. U. S. A.* 106(51), pp.21930–35.

Bito, T., Matsunaga, Y., Yabuta, Y., Kawano, T. and Watanabe, F. (2013). Vitamin B_{12} deficiency in Caenorhabditis elegans results in loss of fertility, extended life cycle, and reduced lifespan. *FEBS Open Bio*, 3(1), pp.112-117.

Bito, T., Yabuta, Y., Ichiyanagi, T., Kawano, T. and Watanabe, F. (2014). A dodecylamine derivative of cyanocobalamin potently inhibits the activities of cobalamin-dependent methylmalonyl-CoA mutase and methionine synthase of Caenorhabditis elegans. *FEBS Open Bio*, 4(1), pp.722-729.

Bito, T. and Watanabe, F. (2016). Biochemistry, function, and deficiency of vitamin B_{12} in Caenorhabditis elegans. *Experimental Biology and Medicine*, 241(15), pp.1663-1668.

Bito, T., Misaki, T., Yabuta, Y., Ishikawa, T., Kawano, T. and Watanabe, F. (2017). Vitamin B_{12} deficiency results in severe oxidative stress, leading to memory retention impairment in Caenorhabditis elegans. *Redox Biology*, 11, pp.21-29.

Bonsdorff, B. and Gordin, R. (2009). Castle's Test (with Vitamin B_{12} and Normal Gastric Juice) in the Ileum in Patients with Genuine and Patients with Tapeworm Pernicious Anaemia. *Acta Medica Scandinavica*, 208(1-6), pp.193-197.

Cadieux, N. and Kadner, R. (1999). Site-directed disulfide bonding reveals an interaction site between energy-coupling protein TonB and BtuB, the outer membrane cobalamin transporter. *Proceedings of the National Academy of Sciences*, 96(19), pp.10673-10678.

Cadieux, N., Bradbeer, C., Reeger-Schneider, E., Koster, W., Mohanty, A., Wiener, M. and Kadner, R. (2002). Identification of the Periplasmic Cobalamin-Binding Protein BtuF of Escherichia coli. *Journal of Bacteriology*, 184(3), pp.706-717.

Chew, Y., Fan, X., Gotz, J. and Nicholas, H. (2013). PTL-1 regulates neuronal integrity and lifespan in *C. elegans*. *Journal of Cell Science*, 126(9), pp.2079-2091

Chew, Y., Fan, X., Götz, J. and Nicholas, H. (2014). Regulation of age-related structural integrity in neurons by protein with tau-like repeats (PTL-1) is cell autonomous. *Scientific Reports*, 4(1).

Chew, Y., Götz, J. and Nicholas, H. (2014). Neuronal protein with tau-like repeats (PTL-1) regulates intestinal SKN-1 nuclear accumulation in response to oxidative stress. *Aging Cell*, 14(1), pp.148-151.

Chien Tu, M., Ping Lo, C., Feng Huang, C., Hsuan Hsu, Y. and Lan Wang, T. (2017). Neurological presentations and therapeutic responses to cobalamin deficiency. *Neuropsychiatry*, 07(03).

Chimento, D., Mohanty, A., Kadner, R. and Wiener, M. (2003). Substrate-induced transmembrane signaling in the cobalamin transporter BtuB. *Nature Structural & Molecular Biology*, 10(5), pp.394-401.

Commons.wikimedia.org. (2018). *File:Cyanocobalamin.svg - Wikimedia Commons*. [online] Available at: https://commons.wikimedia.org/w/index.php?curid=5197720.

Daphna K Dror, Lindsay H Allen. (2008). Effect of vitamin B_{12} deficiency on neurodevelopment in infants: current knowledge and possible mechanisms. *Nutrition Reviews*, 66(5), pp.250–255.

De Rosa, A., Rossi, F., Lieto, M., Bruno, R., De Renzo, A., Palma, V., Quarantelli, M. and De Michele, G. (2012). Subacute combined degeneration of the spinal cord in a vegan. *Clinical Neurology and Neurosurgery*, 114(7), pp.1000-1002.

Edirimanne, V., Woo, C., Siow, Y., Pierce, G., Xie, J. and O, K. (2007). Homocysteine stimulates NADPH oxidase-mediated superoxide production leading to endothelial dysfunction in rats. *Canadian Journal of Physiology and Pharmacology*, 85(12), pp.1236-1247.

Fang, H., Kang, J. and Zhang, D. (2017). Microbial production of vitamin B_{12}: a review and future perspectives. *Microbial Cell Factories*, 16(1).

Fenech, M. (2001). The role of folic acid and Vitamin B_{12} in genomic stability of human cells. *Mutation Research. Fundamental and Molecular Mechanisms of Mutagenesis,* 475(1-2), pp.57-67.

Fielenbach, N. and Antebi, A. (2008). *C. elegans* dauer formation and the molecular basis of plasticity. *Genes and Development*, 22(16), pp.2149–2165.

Gems, D. and Riddle, D. (2000). Defining Wild-Type Life Span in Caenorhabditis elegans. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 55(5), pp.B215-B219.

Gherasim, C., Lofgren, M. and Banerjee, R. (2013). Navigating the B_{12} Road: Assimilation, Delivery, and Disorders of Cobalamin. *Journal of Biological Chemistry*, 288(19), pp.13186-13193.

Gordon, T., Hegedus, J. and Tam, S. (2004). Adaptive and maladaptive motor axonal sprouting in aging and motoneuron disease. *Neurological Research*, 26(2), pp.174-185.

Gordon, P., Hingula, L., Krasny, M., Swienckowski, J., Pokrywka, N. and Raley-Susman, K. (2008). The invertebrate microtubule-associated protein PTL-1 functions in mechanosensation and development in Caenorhabditis elegans. *Development Genes and Evolution*, 218(10), pp.541-551.

Green, R. (1995). Metabolite assays in cobalamin and folate deficiency. *Baillière's Clinical Haematology*, 8(3), pp.533-566.

Greer, JP, Arber, DA, Glader, B, List, AF, Means, RT, Paraskevas, F & Rodgers, GM. (2014). *Wintrobe's clinical hematology: Thirteenth edition.* Philadelphia, PA: Wolters Kluwer/Lippincott Williams & Wilkins. ISBN 978-1-4511-7268-3. Chapter 36: Megaloblastic anemias: disorders of impaired DNA synthesis by Ralph Carmel**.**

Hashimoto, M. and Masliah, E. (2003). Cycles of aberrant synaptic sprouting and neurodegeneration in Alzheimer's and dementia with Lewy bodies. *Neurochemical Research*, 28(11), pp.1743-1756.

Heller, K., Mann, B. and Kadner, R. (1985). Cloning and Expression of the Gene for the Vitamin B_{12} Receptor Protein in the Outer Membrane of Escherichia coli. *Journal of Bacteriology*, 161(3), pp. 896-903.

Herrmann, W., Schorr, H., Bodis, M., Knapp, J., Muller, A., Stein, G. and Geisel, J. (2000). Role of homocysteine, cystathionine and methylmalonic acid measurement for diagnosis of vitamin deficiency in high-aged subjects. *European Journal of Clinical Investigation*, 30(12), pp.1083-1089.

Herrmann, W. and Obeid, R. (2011). Cobalamin Deficiency. *Subcellular Biochemistry*, pp.301-322.

Herrmann, W. and Obeid, R. (2011). Homocysteine: a biomarker in neurodegenerative diseases. *Clinical Chemistry and Laboratory Medicine*, 49(3).

Hodgkin, J. and Barnes, T. (1991). More is Not Better: Brood Size and Population Growth in a Self-Fertilizing Nematode. *Proceedings of the Royal Society B: Biological Sciences,* 246(1315), pp.19-24.

Irevall, T., Axelsson, I. and Naumburg, E. (2016). B_{12} deficiency is common in infants and is accompanied by serious neurological symptoms. *Acta Paediatrica*, 106(1), pp.101-104.

Kocer, B., Engur, S., Ak, F. and Yılmaz, M. (2009). Serum vitamin B_{12}, folate, and homocysteine levels and their association with clinical and electrophysiological parameters in multiple sclerosis. *Journal of Clinical Neuroscience*, 16(3), pp.399-403.

Kozyraki, R. and Cases, O. (2013). Vitamin B_{12} absorption: Mammalian physiology and acquired and inherited disorders. *Biochimie*, 95(5), pp.1002-1007.

Kraemer, B., Zhang, B., Leverenz, J., Thomas, J., Trojanowski, J. and Schellenberg, G. (2003). Neurodegeneration and defective neurotransmission in a Caenorhabditis elegans model of tauopathy. *Proceedings of the National Academy of Sciences*, 100(17), pp.9980-9985.

Kumar, M. and Kozlowski, P. (2017). Electronic and structural properties of Cob(I)alamin: Ramifications for B 12 -dependent processes. *Coordination Chemistry Reviews*, 333, pp.71-81.

la Marca, G., Malvagia, S., Pasquini, E., Innocenti, M., Donati, M. and Zammarchi, E. (2007). Rapid 2nd-Tier Test for Measurement of 3-OH-Propionic and Methylmalonic Acids on Dried Blood Spots: Reducing the False-Positive Rate for Propionylcarnitine during Expanded Newborn Screening by Liquid Chromatography-Tandem Mass Spectrometry. *Clinical Chemistry*, 53(7), pp.1364-1369.

Labuschagne, C. and Brenkman, A. (2013). Current methods in quantifying ROS and oxidative damage in Caenorhabditis elegans and other model organism of aging. *Ageing Research Reviews*, 12(4), pp.918-930.

Lawrence, A., Nemoto-Smith, E., Deery, E., Baker, J., Schroeder, S., Brown, D., Tullet, J., Howard, M., Brown, I., Smith, A., Boshoff, H., Barry, C. and

Warren, M. (2018). Construction of Fluorescent Analogs to Follow the Uptake and Distribution of Cobalamin (Vitamin B_{12}) in Bacteria, Worms, and Plants. *Cell Chemical Biology.*

Lee, M, and CB Grissom. 2009. Design, Synthesis, and Characterization of Fluorescent Cobalamin Analogues with High Quantum Efficiencies. *Org. Lett.* 11 (12): 2499–2502.

Li, J. and Le, W. (2013). Modeling neurodegenerative diseases in Caenorhabditis elegans. *Experimental Neurology*, 250, pp.94-103.

Lian Chew, Y., Fan, X., Götz, J. and Nicholas, H. (2013). Aging in the nervous system of Caenorhabditis elegans. *Communicative & Integrative Biology*, 6(5), p.e25288.

Lian Chew, Y., Fan, X., Götz, J. and Nicholas, H. (2013). Aging in the nervous system of Caenorhabditis elegans. *Communicative & Integrative Biology*, 6(5), p.e25288.

MacNeil, L., Watson, E., Arda, H., Zhu, L. and Walhout, A. (2013). Diet-Induced Developmental Acceleration Independent of TOR and Insulin in *C. elegans*. *Cell*, 153(1), pp.240-252.

Martens, H. Barg, M. Warren, D. Jah, J. (2002). Microbial production of vitamin B_{12}. *Applied Microbiology and Biotechnology*, 58(3), pp.275-285.

Martin, K., Hart, C., Schulenberg, B., Jones, L. and Patton, W. (2002). Simultaneous red/green dual fluorescence detection on electroblots using BODIPY TR-X succinimidyl ester and ELF 39 phosphate. *PROTEOMICS*, 2(5), pp.499-512.

Masliah, E., Mallory, M., DeTeresa, R., Alford, M. and Hansen, L. (1993). Differing patterns of aberrant neuronal sprouting in Alzheimer's disease with and without Lewy bodies. *Brain Research*, 617(2), pp.258-266.

McEwan, J., Veitch, H. and Russell-Jones, G. (1999). Synthesis and Biological Activity of Ribose-5‘-Carbamate Derivatives of Vitamin B_{12}. *Bioconjugate Chemistry*, 10(6), pp.1131-1136.

Miller, A., Korem, M., Almog, R. and Galboiz, Y. (2005). Vitamin B_{12}, demyelination, remyelination and repair in multiple sclerosis. *Journal of the Neurological Sciences*, 233(1-2), pp.93-97.

Miyasaka, T., Ding, Z., Gengyo-Ando, K., Oue, M., Yamaguchi, H., Mitani, S. and Ihara, Y. (2005). Progressive neurodegeneration in *C. elegans* model of tauopathy. *Neurobiology of Disease*, 20(2), pp.372-383.

Miyasaka, T., Xie, C., Yoshimura, S., Shinzaki, Y., Yoshina, S., Kage-Nakadai, E., Mitani, S. and Ihara, Y. (2016). Curcumin improves tau-induced neuronal dysfunction of nematodes. *Neurobiology of Aging*, 39, pp.69-81.

Miyasaka, T., Shinzaki, Y., Yoshimura, S., Yoshina, S., Kage-Nakadai, E., Mitani, S. and Ihara, Y. (2018). Imbalanced Expression of Tau and Tubulin Induces Neuronal Dysfunction in *C. elegans* Models of Tauopathy. *Frontiers in Neuroscience*, 12.

Morita, S., Miwa, H., Kihira, T. and Kondo, T. (2003). Cerebellar ataxia and leukoencephalopathy associated with cobalamin deficiency. *Journal of the Neurological Sciences*, 216(1), pp.183-184.

Na, H., Ponomarova, O., Giese, G. and Walhout, A. (2018). *C. elegans* MRP-5 Exports Vitamin B_{12} from Mother to Offspring to Support Embryonic Development. *Cell Reports*, 22(12), pp.3126-3133.

Naik, S., Mahalle, N. and Bhide, V. (2018). Identification of vitamin B_{12} deficiency in vegetarian Indians. *British Journal of Nutrition*, 119(6), pp.629-635.

Nemoto-Smith, E. (2017). *Synthesis of Cobalamin Analogues Using Enzymatic and Chemical Modification Methods, and Subsequent Identification of Cobalamin Localisation in a Variety of Organisms*. PhD. The University of Kent.

Obeid, R. and Herrmann, W. (2006). Mechanisms of homocysteine neurotoxicity in neurodegenerative diseases with special reference to dementia. *FEBS Letters*, 580(13), pp.2994-3005.

Obeid, R., Fedosov, S. and Nexo, E. (2015). Cobalamin coenzyme forms are not likely to be superior to cyano- and hydroxyl-cobalamin in prevention or treatment of

cobalamin deficiency. *Molecular Nutrition & Food Research*, 59(7), pp.1364-1372.

Otzen, C., Bardl, B., Jacobsen, I., Nett, M. and Brock, M. (2014). Candida albicans Utilizes a Modified β-Oxidation Pathway for the Degradation of Toxic Propionyl-CoA. *Journal of Biological Chemistry*, 289(12), pp.8151-8169.

Palmer, A., Kamynina, E., Field, M. and Stover, P. (2017). Folate rescues vitamin B_{12} depletion-induced inhibition of nuclear thymidylate biosynthesis and genome instability. *Proceedings of the National Academy of Sciences*, 114(20), pp.E4095-E4102.

Pan, C., Peng, C., Chen, C. and McIntire, S. (2011). Genetic analysis of age-dependent defects of the Caenorhabditis elegans touch receptor neurons. *Proceedings of the National Academy of Sciences*, 108(22), pp.9274-9279.

Pawlak, R. (2015). Is Vitamin B_{12} Deficiency a Risk Factor for Cardiovascular Disease in Vegetarians?. *American Journal of Preventive Medicine*, 48(6), pp.e11-e26.

Rafiee, S., Asadollahi, K., Riazi, G., Ahmadian, S. and Saboury, A. (2017). Vitamin B_{12} Inhibits Tau Fibrillization via Binding to Cysteine Residues of Tau. *ACS Chemical Neuroscience*, 8(12), pp.2676-2682.

Samuel, T., Sinclair, J., Pinter, K. and Hamza, I. (2014). Culturing Caenorhabditis elegans in Axenic Liquid Media and Creation of Transgenic Worms by Microparticle Bombardment. *Journal of Visualized Experiments*, (90), e51796.

Savage, D. and Lindenbaum, J. (1995). Neurological complications of acquired cobalamin deficiency: clinical aspects. *Baillière's Clinical Haematology*, 8(3), pp.657-678.

Scalabrino, G., Veber, D. and Tredici, G. (2014). Relationships between cobalamin, epidermal growth factor, and normal prions in the myelin maintenance of central nervous system. *The International Journal of Biochemistry & Cell Biology*, 55, pp.232-241.

Shirafuji, N., Hamano, T., Yen, S., Kanaan, N., Yoshida, H., Hayashi, K., Ikawa, M., Yamamura, O., Kuriyama, M. and Nakamoto, Y. (2018). Homocysteine

Increases Tau Phosphorylation, Truncation and Oligomerization. *International Journal of Molecular Sciences*, 19(3), p.891.

Shyambabu, C., Sinha, S., Taly, A., Vijayan, J. and Kovoor, J. (2008). Serum vitamin B_{12} deficiency and hyperhomocystinemia: A reversible cause of acute chorea, cerebellar ataxia in an adult with cerebral ischemia. *Journal of the Neurological Sciences*, 273(1-2), pp.152-154.

Solomon, L. (2015). Functional cobalamin (vitamin B_{12}) deficiency: role of advanced age and disorders associated with increased oxidative stress. *European Journal of Clinical Nutrition*, 69(6), pp.687-692.

Suzuki, H., Kerr, R., Bianchi, L., Frøkjær-Jensen, C., Slone, D., Xue, J., Gerstbrein, B., Driscoll, M. and Schafer, W. (2003). In Vivo Imaging of *C. elegans* Mechanosensory Neurons Demonstrates a Specific Role for the MEC-4 Channel in the Process of Gentle Touch Sensation. *Neuron*, 39(6), pp.1005-1017.

Tank, E., Rodgers, K. and Kenyon, C. (2011). Spontaneous Age-Related Neurite Branching in Caenorhabditis elegans. *Journal of Neuroscience*, 31(25), pp.9279-9288.

Taskesen, M., Yaramis, A., Pirinccioglu, A. and Ekici, F. (2012). Cranial magnetic resonance imaging findings of nutritional Vitamin B_{12} deficiency in 15 hypotonic infants. *European Journal of Paediatric Neurology*, 16(3), pp.266-270.

Thermofisher.com. (2016). BODIPY™ TR-X NHS Ester (Succinimidyl Ester). [online] Available at: https://www.thermofisher.com/content/dam/LifeTech/Documents/chemstructures/images/2016.jpg.

Toth, M., Melentijevic, I., Shah, L., Bhatia, A., Lu, K., Talwar, A., Naji, H., Ibanez-Ventoso, C., Ghose, P., Jevince, A., Xue, J., Herndon, L., Bhanot, G., Rongo, C., Hall, D. and Driscoll, M. (2012). Neurite Sprouting and Synapse Deterioration in the Aging Caenorhabditis elegans Nervous System. *Journal of Neuroscience*, 32(26), pp.8778-8790.

Wang, C., Saar, V., Leung, K., Chen, L. and Wong, G. (2018). Human amyloid β peptide and tau co-expression impairs behavior and causes specific gene

expression changes in Caenorhabditis elegans. *Neurobiology of Disease*, 109, pp.88-101.

Watanabe, F., Katsura, H., Takenaka, S., Fujita, T., Abe, K., Tamura, Y., Nakatsuka, T. and Nakano, Y. (1999). Pseudovitamin B_{12} is the Predominant Cobamide of an Algal Health Food, Spirulina Tablets. *Journal of Agricultural and Food Chemistry*, 47(11), pp.4736-4741.

Watanabe, F., Tanioka, Y., Miyamoto, E., Fujita, T., Takenaka, H. and Nakano, Y. (2007). Purification and Characterization of Corrinoid-Compounds from the Dried Powder of an Edible Cyanobacterium, Nostoc commune (Ishikurage). *Journal of Nutritional Science and Vitaminology*, 53(2), pp.183-186.

Watanabe, F., Yabuta, Y., Tanioka, Y. and Bito, T. (2013). Biologically Active Vitamin B_{12} Compounds in Foods for Preventing Deficiency among Vegetarians and Elderly Subjects. *Journal of Agricultural and Food Chemistry*, 61(28), pp.6769-6775.

Watson, E., MacNeil, L., Ritter, A., Yilmaz, L., Rosebrock, A., Caudy, A. and Walhout, A. (2014). Interspecies Systems Biology Uncovers Metabolites Affecting *C. elegans* Gene Expression and Life History Traits. *Cell*, 156(4), pp.759-770.

Watson, E. and Walhout, A. (2014). Caenorhabditis elegans metabolic gene regulatory networks govern the cellular economy. *Trends in Endocrinology & Metabolism*, 25(10), pp.502-508.

Watson, E., Olin-Sandoval, V., Hoy, M., Li, C., Louisse, T., Yao, V., Mori, A., Holdorf, A., Troyanskaya, O., Ralser, M. and Walhout, A. (2016). Metabolic network rewiring of propionate flux compensates vitamin B_{12} deficiency in *C. elegans*. *eLife*, 5.

Werder, S. (2010). Cobalamin deficiency, hyperhomocysteinemia, and dementia. *Neuropsychiatric Disease and Treatment*, p.159.

White, J., Southgate, E., Thomson, J. and Brenner, S. (1986). The Structure of the Nervous System of the Nematode Caenorhabditis elegans. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 314(1165), pp.1-340.

Wiseman, M. (1992). The COMA Report: Dietary Reference Values for Food Energy and Nutrients for the United Kingdom. *British Food Journal*, 94(3), pp.7-9.

Yen, C. and Curran, S. (2016). Gene-diet interactions and aging in *C. elegans*. *Experimental Gerontology*, 86, pp.106-112.

Zhang, B., Xiao, R., Ronan, E., He, Y., Hsu, A., Liu, J. and Xu, X. (2015). Environmental Temperature Differentially Modulates *C. elegans* Longevity through a Thermosensitive TRP Channel. *Cell Reports*, 11(9), pp.1414-1424.

Supplemental data

Red: mutation, yellow: flanking sequence, blue: external primers, green: internal primers.

Δacdh-1(ok1489) genomic secuence

>ok1489 *ok1489* with 250 bp flanks

ttgtgacgcaaggtgtatttgaaaatggggtaagttaattcttattacttaaattttaatgttatgga
aaaggggtttatatttacactgtttattatgaaaacctaaaaaaagtataataagaaggaagattgat
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ctgatctaattgtacatgttctagagttataaatgattatcataaaatagaagctcatcatcggagct
ttatataatgagcgatattatttttttcaaatatttcatatcaaaaagtatgtcttagaatctgaac
cgttgtgaaatgaatattctgaaaaaaattcaattgaattcaaatgtttcaaggttgcaacatcaaca
tcagctcaatgtgtaaaatggctcggtggtgttggattcacaaaagaat

WT genomic sequence

>ok1489 wild type, with 250 bp flanks

ttgtgacgcaaggtgtatttgaaaatggggtaagttaattcttattacttaaattttaatgttatgga
aaaggggtttatatttacactgtttattatgaaaacctaaaaaaagtataataagaaggaagattgat
aaatactttataactttgtaaggtaacgtttcagtaaaatcaatttcatggaaatatccaaaaacttt
tgcattaaacacttaacttatttaactaattatgctataaaaatgagaaagttttagattgacttttc
tagttcaaaacgattgtaagcagatttatttatagacctgttaagaaacgaaaaaatcttccagctaa
tgggtgttcatgttcctgaagaatacggaggctctggatcctcttttttcaatgcaatgattgtcatt
gaagagttggcaaaaactgatccatctgtatctgcaatggttggaattcacaacaccctccctgtatc
aatgattatagattatggaacagaAGAACAAAAACTAAAATACTTGCCTCGTCTCTGTTCTGATAGTC
TTGCATCCTTCTGTATCTCAGAATCAGGTGCTGGATCCGATGCATTTGCATTGAAAACTATTGCAAAA
CGAGATGGAGATCACTTTTTGATCTCTGGAACTAAAATGTGGATTACTAATTCAGGAGAGGCACAGGT
TTTTGTGGTATTTGCAAATGCAGATCCTAGCCAAAAATACAAAGGTATCACATGTTTTATTGTGGAAA
GATCAGCGGATGGTCTTACTGTAGATAAGGAGGTATGTTCAGTTTTTAACTTTAGAGCAAAAATGAAA
AAAAAATTTACAGGAAGACAAACTTGGAATCCGAGCTTCATCCACTTGTCAAGTTCATTTTGACAATG
TTCGTGTCCACAAATCAGCAATTCTGGGAGAGTATGGAAAAGGTATGAGATTCTAGTAAAATCAATCA
ATAACTTGCATGTTTCCAGGCTACAAATACGCAATTGAATGCCTAAATGCAGGTAGAATTGCCATTGG

AGCACAAATGATTGGACTGGCTCAGGGTTGTTTCGATCAAACAATTCCATATCTTCAACAGAGAGAAC
AGTTCGGTCAGAgacttattgattttcaggttatttttgaaattgggtttgaatttttgatacacaaa
tgttcagggtttacaacatcagattgcacaagctagaactgaaattgaggccgcacgtctactagtgt
ataatgctgcccgtatgaaagaatatggaataccatatgtacgagaagctgcaatggcgaaactgttt
gcatcacaggtaatttgataaattgtagaaacaccacaggcacagagctcgtgctctgatctaattgt
acatgttctagagttataaatgattatcataaaatagaagctcatcatcggagctttatataatgagc
gatattatttttttcaaatatttcatatcaaaaagtatgtcttagaatctgaaccgttgtgaaatga
atattctgaaaaaaattcaattgaattcaaatgtttcaaggttgcaacatcaaacatcagctcaatgt
gtaaaatggctcggtggtgttggattcacaaaagaat

5’ external primer:	gaatacggaggctctggatcctc
3’ external primer:	gttgatgttgcaaccttgaaac
5’ internal primer:	CTCAGAATCAGGTGCTGGATCCG
3’ internal primer:	GATCGAAACAACCCTGAGCCAG

Figure S1. VC1011 (*acdh-1*-deletion) strain genotyping using single-worm PCR. Figure shows the *ok1489* gene transcripts of the VC1011 and the wildtype strains, as well as the primers designed for the PCR. The expected PCR products for the N2 strain were 1190 bp with the external primers and 500 bp with the internal primers. For the VC1011 strain the expected products were 590 bp with the internal primers and no product with the internal primers.

A.

	Day 1			Day 2			Day 3			Day 4		
	Size (µm)			Size (µm)			Size (µm)			Size (µm)		
Worm	**B_{12}-**	**B_{12}+**	**NGM**	**B_{12}-**	**B_{12}+**	**NGM**	**B_{12}-**	**B_{12}+**	**NGM**	**B_{12}-**	**B_{12}+**	**NGM**
1	322,7	367,74	353,37	590,24	719,65	823,68	914,76	1103,63	969,05	1057,21	1019,36	1198,94
2	415,7	345,83	445,81	537,95	688,64	776,47	1000,76	1130,78	1070,03	1104,98	1010,53	1146,6
3	432,98	353,21	346,38	532,88	624,09	691,66	920,94	1107,01	1037,49	1183,25	1064,5	1133,89
4	297,19	358,91	419,77	460,22	666,19	815,07	843,03	1005,49	1001,12	1083,01	1183,19	1006,7
5	288,68	358,55	382,92	503,97	797,59	740,65	879,98	1182,31	1140,61	1111,51	1162,81	1110,69
6	312,61	426,44	357,76	544,72	697,89	660,93	935,6	1117,07	822,5	1073,15	1074,68	959,08
7	469,61	344,54	388,78	647	728,95	787,44	923,23	1104,93	982,6	1012,87	982,48	1002,11
8	310,92	322,47	425,89	520,18	561,6	578,6	862,22	1054,82	958,8	1093,69	1089,12	1001,86
9	341,39	404,24	316,1	501,41	720,04	591,43	840,15	1327,97	1028,16	1017,09	992,77	1209,83
10	303,08	426,69	380,48	548,23	648,67	615,65	922	1125,61	959,27	1027,58	1174,41	962,83
11	288,09			538,05	678,25	741,29	887,66	1149,87	1014,2	1141,61	1239,1	1100,62
12				501,43	636,01	789,6	850,86	1037,94	881,49	1041	1312,59	1135,77
13				494,68	642,16	788,46	922,66	1014,91	922,62	1134,76	1121,43	1121,91
14				488,47	684,29	630,44		1101,04	941,59	1069,72	1160,66	1165,31
15					704,9	735,97		968,1	941,76	1117,9	1109,07	1337,41
16						577,49		1018,77		1088,78	1191,98	1082,35
17						626,84		1078,78		1026,4	1188,57	1124,48
18								890,47		1111,44	1243,86	1140,89
19								1121,84		1019,11	1329,48	1068,25
20								1044,64		1120,49		1159,92
21										1153,82		1122,11
22										1189,45		1102,76
23										1163,95		1014,96
24										1116,43		1317,33
25										1159,72		1142,5
26										1125,43		
27										1165,01		
28										1152,19		
Average	343,90455	370,862	381,726	529,245	679,928	704,21588	900,29615	1084,299	978,086	1102,1982	1139,5047	1114,764
STDEV	64,364645	35,896116	40,127359	46,611575	54,849596	87,560549	45,492668	89,513329	76,91953	53,145043	101,92958	94,155571
T test	0,248037531			1,46597E-08			1,2062E-08			0,155299025		
		0,531544491			0,349712659			0,000663841			0,41517379	
		0,121311294			1,7988E-07			0,003063307			0,55959152	

B.

	Day 1			Day 2			Day 3			Day 4		
	Size (µm)			Size (µm)			Size (µm)			Size (µm)		
Worm	**B_{12}-**	**B_{12}+**	**NGM**	**B_{12}-**	**B_{12}+**	**NGM**	**B_{12}-**	**B_{12}+**	**NGM**	**B_{12}-**	**B_{12}+**	**NGM**
1	293,64	362,72	367,74	473,01	737,41	786,38	887,26	1125,73	999,52	1134,04	1198,54	1350,06
2	277,56	307,33	384,4	484,34	740,34	749,26	897,34	1114,83	1061,49	1114,71	1166,43	1144,45
3	279,11	337,55	369,47	474,95	776,09	723,51	903,27	1088,14	1015,55	1052,05	1052,88	1305,8
4	284,92	327,52	349,27	551,06	800,04	715,14	883,57	1032,79	1037,88	1208,76	1283,98	1187,11
5	300,92	317,49	390,52	488,84	722,85	744,69	929,3	1152,91	1191,38	1031,44	1096,7	1166,56
6	305,47	326,33	359,48	504,14	727,14	751,74	898,8	1093,51	1107,32	1010,59	1100,16	1163,93
7	267,11	331,46	397,36	516,55	728,63	756,97	935,35	1080,8	1127,75	1097,25	1032,26	1112,99
8	294,45	317,57	394,96	533,45	789,05	755,27	909,32	1007,5	1050,05	1065,11	1023,96	1185,92
9	301	337,47	393,23	580,23	649,14	733,91	991,95	1100,96	1035,26	965,15	1130,31	1097,45
10	289,81	337,51	393,15	509,99	673,19	736,79	871	1019,89	1062,8	1113,8	1099,57	1184,49
11	281,78	448,43	406,82	450,61	719,9	718,23	912,48	1100,38	1248,22	1035,79	1089,09	1088,9
12	280,22	336,06	389,21	527,4	885,65	745,06	879,34	1088,77	1015,93	1220,82	1119,56	1085,46
13	333,16	320,18	388,48	532,18	757,23	800,3	939,17	1096,57	1142,85	987,06	1136,44	1005,91
14	294,98	364,98	390,14	539,23	756,84	789,38	959,48	1033,05	1158,89	1082,44	1107,2	1161,51
15	273,14	333,49	395,44	474,76	727,7	789,09	887,57	1108,83	1018,15	1021,98	1169,01	1178,13
16	268,96	326,71	389,43	462,05	656,45	759,48	858,93	1093,09	1185,61	1050,38	1105,24	1255,73
17	273,51	334,01	449,27	484,8	734,32	786,2	891,35	1043,46	1097,88	1113,03	1068,48	1214,7
18	262,65	317,87	423,57	591,23	724,42	720,82	921,83	1127,92	1213,56	963,51	1120,34	1166,3
19	280,47	343,97	427,66	490,98	715,61	819,18	893,7	1092,86	1038,19	1105,59	1185,34	1049,51
20	281,09	349,45	449,96	520,82	689,37	640,37	846,48	1009,33	1089,7	1045,53	1103,09	1149,83
21	292,43	338,26	406,81	422,23	742,15	664,43	969,59	1135,56	1155,46	1054,42	1338,12	1127,43
22	275,97	312,09	407,49	476,56	776,84	766,27	860,08	1244,45	1172,48	1101,75	1095,46	1144,36
23	293,8	355,3	405,42	508,55	781,32	766,15	885,74	1205,14	1077,76	1168,98	1274,01	1214,19
24	288,3	319,12	381,49	539,71	620,22	767,58	829,41	999,76	1080	1048,49	1109,02	1078,13
25	302,85	348,77	415,62	496,78	725,88	713,75	906,28	1023,02	1066,36	1041,45	1220,31	1141,98
26	283,27	355,2	365,91	501,94	625,16	790,92	907,06	1028,06	1093,73	1107,46	1227,81	1136,12
27		365,94	401,47	505,57	798,11	735,48	872,34	1082,98	1044,51	1058,13	1244,84	1186,62
28		314,29	400,83	544,8	638,78	783,37	854,51	1066,56	1035,14	1093,5	1260,48	1291,44
29		347,1	399,2	530,3	784,42	682,01	833,91	1103,61	1031,17	1043,17	1145,13	1139,12
30		363,2	444,08	640,7	821,26	672,91	895,68	974,49	1038,21	1007,13	1306,83	1258,84
31		354,43	379,43	473,94	686,09	771,51	826,72	1052,9	1034,83	1001,5	1174,13	1244,24
32			392,68	499,36	664,25	775,18	858,56	1026,99	1006,36	1041,09	1261,37	1190,03
33			408,19	516,03	701,49	792,33	840,39	1058,1	1022,73	1094,1	1161,35	1185,4
34			398,49	536,2	702,67	728,45	855,33	1082,54	1006,58	1081,91	1166,76	1043,6
35			392,16	499,32	737,55	751,46	887,52	1093,28	1020,28	993,75	1120,66	1153,63
36			393,21	459,56	700,3	746,49	885,43	1202,37	1081,63	1054,92	1300,85	1102,99
37			388,16	526,01	693,85	701,93	876,27	1036,68	1137,19	1069,02	1156,17	1209,3
38			425,27	502,71	670,91	763,97	885,14	1338,21	1041,9	1105,31	1173,04	1106,3
39			458,81	485,29	769,42	768,16	924,16	1083,64	1033,95	1110,85	1163,9	1245,49
40			424,47	480,97		753,89	845,72	1104,75	1008,32	1155,92	1313,49	1151,92
41			418,06	538,48		661,58	817,68	1231,24	1021,45	1003,38	1256,29	1138,93
42			410,23	511,62		732,89	857,46	986,42	1021,8	1030,45	1185,67	1012,99
43			399,46	519,16		703,46	870,62	1104,95	1017,45	1007,2	1153,25	1042,97
44			416,36	517,92		715,85	949,51	1104,02	1064,95	1136,12	1176,67	1103,9
45			393,54			670,5	810,71	1024,25	981,03	1052,66	1004,75	1146,39
46			371,05				916,26	1082,93	1041,93	1060,14	1212,61	1194,72
47			403,49				844,07	1093,34	1077,68	988,58	1195,6	1142,53
48			401,63				807,31	1142,61	1015,86	1069,66	1144,68	1110,74
49			420,79				860,68	1064,56	1020,81	997,48	1066,53	1104,99
50			429,56				935,41	1014,36	1113,03	1018,67	1113,19	1019,37
51							946,71	1210,78	1036,34	1040,33	1170,76	1184,87
52							887,11	1085,58	1026,66	1024,5	1131,36	1161,19
53							825,31	1094,73	1203,78	1044,25	1133,93	1102,15
54							899,44	1139,28	1056,24	1017,53	1092,03	1077,75
55							909,77	1008,16	1100,35	1032,64	1136,05	1112,96
56							876,25	1072,03	1020,7	1046,97	1245,95	1092,63
57							919,32	1097,6	1024,31	989,95	1023,23	1129,3
58							873,62	1023,86	1068,59	1096,67	1213,52	1143,32
59							891,55	1053,41	1166,4	1088,78	1370,99	1115,34
60							821,11	1086,63	1055,5	1119,93	1160,64	1108,31
Average	286,945	340,38065	401,2584	509,64386	726,97538	742,27311	885,28717	1086,307	1070,3572	1061,9628	1163,1668	1147,5203
STDEV	14,820915	26,174336	22,83627	38,447041	56,347101	40,957569	40,497839	58,926459	61,594711	54,984906	80,878479	69,264238

T test	2,37406E-06			7,2377E-13			6,2366E-14			0,001807228		
		2,17947E-06			0,558287719			0,842261384			0,072608939	
		2,28249E-10			4,71069E-17			8,56284E-08			2,70882E-06	

C.

	Day 1			Day 2			Day 3			Day 4		
	Size (µm)			Size (µm)			Size (µm)			Size (µm)		
Worm	**B_{12}-**	**B_{12}+**	**NGM**	**B_{12}-**	**B_{12}+**	**NGM**	**B_{12}-**	**B_{12}+**	**NGM**	**B_{12}-**	**B_{12}+**	**NGM**
1	273,48	322,86	388,14	448,1	620,63	580,992	768,31	904,17	1101,39	986,43	1066,3	1191,1
2	347,13	340,98	362,58	416,94	676,66	787,95	844,99	1064,97	1070,54	1040,68	1042,85	990,94
3	359,35	540,59	402,78	535,23	632,25	735,02	754,13	982,67	1098,77	1011,31	1036,26	1127,06
4	302,23	360,21	333,13	505,54	617,81	749,09	799,67	1017,97	1089,62	1035,86	1044,45	1118,05
5	253,28	316,14	310,03	556,61	701,38	718,92	732,78	1012,62	1127,2	1035,56	1192,94	1329,26
6	353,43	350,63	388,3	633,35	628,18	801,64	771,31	1015,95	1076,51	986,24	1137,43	1132,46
7	352,57	322,73	348,2	523,53	584,17	686,13	780,51	1042,16	1161,62	1044,74	1182,26	1171,34
8	317,22	314,93	393,73	537,76	705,08	757,94	686,85	822,82	1196,28	1096,21	1074,75	1072,49
9	290,96	307,91	444,15	560,07	670,82	738,18	737,4	966,47	1062,64	1036,66	1201,95	1154,52
10	306,6	315,54	376,63	510,98	679,11	687,72	737,3	1039,63	1074,17	1034,29	1136,75	1111,63
11	280,95	309,43	312,42	494,26	820,16	686,81	733,57	925,23	982,88	1014,49	1150,2	1116,33
12	310,63	320,42	390,41	512,78	712	765,95	741,66	1007,09	912,43	1041,64	1007,78	1248,16
13	308,94	311,02	396,21	455,07	656,93	752,07	931,94	1005,12	1039,71	1056,12	1162,61	1193,5
14	329,35	318,53	343,43	534,36	650,85	738,24	774,18	959,23	1081,15	1022,96	1140,9	1208,47
15	372,92	310,88	323,22	558,41	662,84	710,26	959,21	1132,4	1109,35	1048,79	1093,09	1185,46
16	339,7	362,85	371,73	461,86	607,11	674,06	793,13	1038,74	998,12	1033,78	1117,91	1068,53
17	319,14	371,55	356,3	485,94	611,98	609,88	808,47	968,8	1105,15	996,4	1100,6	1191,08
18	308,47	330,42	388,08	528,02	681,1	755,83	766,76	1018,77	1191,47	1076,6	1028,33	1237,73
19	360,58	333,49	407	522,66	611,39	702,46	816,83	926,67	1021,08	1046,19	1036,48	1178,94
20	323,2	311,92	320,72	502,46	666,72	744,25	959,31	1018,68	1096,87	1027,07	1041,8	1071,97
21	364,27	337,61	387,72	480,44	637,71	665,06	750,99	921,64	1119,52	965,7	1098,76	1186,02
22	335,09	369,65	325,44	519,08	743,41	700,12	754,53	1011,18	1083,33	1055,56	1052,37	1050,77
23	306,19	335,18	324,09	424,36	668,06	675,18	705,76	951,34	997,76	1040,73	1118,16	1195,99
24	312,03	330,91	397,74	440,14	595,49	662,12	876,78	1072,34	1085,59	1190,62	1104,97	1023,82
25	322,11	308,06	358,58	475,39	645,25	563,77	692,08	954,69	1061,05	1020,39	1233,78	1087,62
26	301,34	361,43	387,52	444,1	605,49	840,1	754,3	917,49	1006,8	1021,18	1127,43	1162,02
27	296,45	324,04	454,58	446,93	679,65	587,7	674,25	929,1	1107,95	1092,94	1088,35	1158,2
28	292,2	310,76	448,77	472,47	608,09	572,13	928,76	932,86	1125,33	986,29	1070,82	1295,52
29	300,83	330,05	312,7	470,31	674,33	674,22	658,27	935,1	1075,39	983,94	1119,88	1092,3
30	331,23	306,68	404,46	520,41	612,15	590,82	828,31	981,13	1081,12	1033,31	1124,61	1094,25
31	304,19	325,32	448,28	458,13	672,56	750,81	834,54	954,75	1071,66	1083,55	1161,28	1261,24
32	301,32	358,86	396,88	469,49	713,02	725,72	866,53	937,74	1161,8	984,67	1108,59	1150,36
33	359,23	344,75	387,96	559,01	716,18	851,15	863,22	947,76	1142,2	962,78	1175,1	1170,96
34	318,26	330,86	310,64	475,96	670,48	692,62	714,34	945,3	1191,28	979,43	1170,99	1204,56
35	293,71	334,74	322,17	477,07	686,34	751,1	771,63	959,24	1075,23	1038,07	1099,59	1085,04
36	297,91	317,68	454,57	516,13	709,17	631,17	815,69	977,93	1236,77	1011,91	1091,98	1155,47
37	312,02	338,51	322,97	448,89	619,61	667,38	969,4	1025,49	1132,03	1034,66	1151,36	1034,85
38	332,4	349,12	384,77	490,36	610,33	702,3	899,5	1011,8	1154,35	1032,1	1176,89	1147,81
39	298,37	311,12	392,37	495,88	645,23	773,03	872,74	1003,55	1070,75	1103,82	1079,46	1103,34
40	289,54	328,96	310,01	484,07	638,97	719,24	785,45	996,21	1102,34	1052	1166,44	1192,7
41	367,46	361,6	319,72	552,34	645,26	691,56	756,29	943,22	1126,13	1071,39	1009,71	1284,66
42	368,07	327,3	415,61	538,74	676,49	804,68	738,38	1041,34	1187,15	978,59	1083,83	1397,26
43	320,41	335,13	349,47	482,43	672,15	810,7	808,25	1003,41	1148,99	1024,86	1227,69	1408,28
44	338,43	365,78	420,12	414,67	702,14	680,49	860,33	966,48	1156,35	1081,86	1174,99	1286,86
45	317,44	332,12	353,85	467,34	602,59	802,55	787,65	916,25	1021,51	1197,13	1114,67	1341,35
46	286,52	364,01	453,36		737,46	761,2	823,67	991,13	1170,14	982,09	1104,64	1187,95
47	316,7	353,63	410,02		586,64	722,03			1034,63	1056,07	1127,96	1262,17
48	328,59	322,5	447,39		603,31	693,89			1079,61	1041,18	1144,24	1351,8
49		329,98	410,83		673,45	686,93			1172,36	1012,61	1132,89	1229,43
50		351,88	387,77		705,37	798,62			1092,97	1006,02	1087,33	1247,01
51		330,01	398,24		709,31	746,9			1101,34	1034,94	1131,09	1237,27
52		336,29	354,64		581,06	638,03			1048,45	1024,32	1125,05	1332,65
53		312,26	411,32		710,07	587,83			1173,46	1057,86	1122,28	1265,52
54		348,36	395,33		655,83	698,54			1161,24	1007,22	1152,47	1321,8
55		355,07	324,92		686,32	750,97			1065,55	1103,5	1045,18	1348,76
56			397,6		595,04	805,24			1079,94	1024,91	1090,14	1382,57
57			366,51		749,98	802,32			1015,5	978,4	1149,93	1251,44
58			417,2		645,17	705,1			1027		1121,28	1093,03
59			367,17		646,91	741,17			1000,4		1176,55	1144,32
60			338,91		662,26	675,47			1097,24		1137,2	1298,59
Average	319,2175	337,33164	377,1565	495,73489	659,925	713,05587	797,60761	986,22	1093,986	1035,5898	1116,2595	1193,743
STDEV	27,234596	33,497864	42,328508	44,864032	47,561966	67,71545	77,152664	63,954628	61,891689	45,888454	51,804302	98,933556
T test	0,157315742			4,53319E-08			1,89742E-10			0,000192867		
		0,325490237			0,02193598			0,000774796			0,045154722	
		0,001207811			8,33692E-11			1,42968E-12			1,84437E-07	

Figure S2. Developmental assay (raw data). The numbers indicate the size of each worm (in µm), measured as described in the 'Methods' section, as well as the average size of the worms from each condition per day. A standard two-tailed T-test was used to analyse the data and determine the significant difference between the conditions. **A.** Trial 1. **B.** Trial 2. **C.** Trial 3.

A.

% N2 NGM		
# days	Dead	Censored
1	0	0
2	0	0
4	0	3
8	1	1
10	8	0
12	16	0
15	48	1
17	20	0
18	0	0
20	2	0

% N2 NGM	
Time (days)	% Survival
1	100
2	100
4	100
8	98,969072
10	90,634835
12	73,966359
15	23,960933
17	2,178267
18	2,178267
20	0

% N2 B_{12}+		
# days	Dead	Censored
1	0	0
2	0	0
4	0	1
8	12	21
10	15	2
12	23	0
15	18	0
17	7	0
18	1	0

% N2 B_{12}+	
Time (days)	% Survival
1	100
2	100
4	100
8	87,878788
10	67,906336
12	36,031933
15	11,086749
17	1,385844
18	0,000000

% N2 B_{12}-		
# days	Dead	Censored
1	0	0
2	0	0
4	0	1
8	7	0
10	30	0
12	32	0
15	30	0

% N2 B_{12}-	
Time (days)	% Survival
1	100
2	100
4	100
8	92,929293
10	62,626263
12	30,30303
15	0

Mean/Median Lifespan

Name	No. of subjects	Restricted mean			Age in days at % mortality					
		Days	Std. error	95% C.I.	25%	50%	75%	90%	100%	95% Median C.I.
N2 NGM	100	14.56	0.24	14.09 ~ 15.02	12	15	17	18	20	- ~ -
N2 B12+	100	12.43	0.31	11.83 ~ 13.04	10	12	15	17	18	- ~ -
N2 B12-	100	12.02	0.23	11.58 ~ 12.46	10	12	15	-	-	- ~ -

Test Result Log-Rank Test

Condition	Statistics		
	Chi^2	P-value	Bonferroni P-value
N2 NGM *v.s.* N2 B12+	22.32	2.3e-06	4.6e-06
N2 NGM *v.s.* N2 B12-	51.65	0.0e+00	0.0e+00
N2 B12+ *v.s.* N2 NGM	22.32	2.3e-06	4.6e-06
N2 B12+ *v.s.* N2 B12-	2.26	0.1327	0.2654
N2 B12- *v.s.* N2 NGM	51.65	0.0e+00	0.0e+00
N2 B12- *v.s.* N2 B12+	2.26	0.1327	0.2654

B.

% N2 NGM		
# days	Dead	Censored
1	0	0
3	0	0
4	0	3
5	0	25
6	0	4
7	1	0
8	2	0
10	11	2
11	3	0
12	2	1
13	3	0
16	6	0
17	11	0
19	25	0

% N2 NGM	
Time (days)	% Survival
1	100
3	100
4	100
5	100
6	100
7	98,507463
8	95,522388
10	79,104478
11	74,451273
12	71,349137
13	66,592528
16	57,079309
17	39,638409
19	0,000000

% N2 B_{12}+		
# days	Dead	Censored
1	0	0
3	0	5
4	0	2
5	0	4
6	0	4
7	0	0
8	6	21
10	10	0
11	6	1
12	10	0
13	13	0
16	19	0
17	5	0
19	3	0

% N2 B_{12}+	
Time (days)	% Survival
1	100
3	100
4	100
5	100
6	100
7	100
8	93,617021
10	79,644332
11	71,260718
12	57,008574
13	38,480788
16	11,401715
17	4,275643
19	0

% N2 B_{12}-		
# days	Dead	Censored
1	0	0
3	0	0
4	0	7
5	0	5
6	0	1
7	8	1
8	7	7
10	15	0
11	6	0
12	5	0
13	10	0
16	23	0
17	6	0

% N2 B_{12}-	
Time (days)	% Survival
1	100
3	100
4	100
5	100
6	100
7	90,909091
8	82,853855
10	63,733735
11	56,085686
12	49,712313
13	36,965566
16	7,648048
17	0

Condition	Statistics			
	P-value at 25%	P-value at 50%	P-value at 75%	P-value at 90%
N2 NGM *v.s.* **N2 B12+**	0.5987	1.3e-05	0.0008	0.8844
N2 NGM *v.s.* **N2 B12-**	0.0101	3.7e-05	1.2e-08	0.0273
N2 B12+ *v.s.* **N2 NGM**	0.5987	1.3e-05	0.0008	0.8844
N2 B12+ *v.s.* **N2 B12-**	0.0118	0.2126	0.0297	0.0297
N2 B12- *v.s.* **N2 NGM**	0.0101	3.7e-05	1.2e-08	0.0273
N2 B12- *v.s.* **N2 B12+**	0.0118	0.2126	0.0297	0.0297

Condition	Statistics		
	Chi^2	P-value	Bonferroni P-value
N2 NGM *v.s.* **N2 B12+**	24.00	9.6e-07	1.9e-06
N2 NGM *v.s.* **N2 B12-**	38.74	0.0e+00	0.0e+00
N2 B12+ *v.s.* **N2 NGM**	24.00	9.6e-07	1.9e-06
N2 B12+ *v.s.* **N2 B12-**	2.37	0.1233	0.2466
N2 B12- *v.s.* **N2 NGM**	38.74	0.0e+00	0.0e+00
N2 B12- *v.s.* **N2 B12+**	2.37	0.1233	0.2466

C.

% N2 NGM			% N2 NGM	
# days	Dead	Censored	Time (days)	% Survival
1	0	0	1	100
2	0	0	2	100
3	0	0	3	100
4	0	0	4	100
5	0	0	5	100
6	0	0	6	100
7	0	0	7	100
9	1	2	9	98,823529
10	0	3	10	98,823529
11	7	0	11	90,067014
13	18	0	13	67,550261
14	24	0	14	37,527923
15	0	0	15	37,527923
16	12	0	16	22,516754
18	8	0	18	12,509308
19	7	0	19	3,752792
20	3	0	20	0

% N2 B12+			% N2 B12+	
# days	Dead	Censored	Time (days)	% Survival
1	0	0	1	100
2	0	0	2	100
3	0	0	3	100
4	0	0	4	100
5	0	0	5	100
6	0	0	6	100
7	3	3	7	97
9	16	5	9	80,489362
10	17	4	10	61,745264
11	18	0	11	40,371903
13	21	1	13	15,436316
14	11	0	14	1,28636
15	0	0	15	1,28636
16	1	0	16	0

% N2 B12-			% N2 B12-	
# days	Dead	Censored	Time (days)	% Survival
1	0	0	1	100
2	0	0	2	100
3	0	0	3	100
4	0	0	4	100
5	0	0	5	100
6	0	2	6	100
7	16	0	7	83,673469
9	42	0	9	40,816327
10	16	0	10	24,489796
11	11	0	11	13,265306
13	9	0	13	4,081633
14	3	0	14	1,020408
15	0	0	15	1,020408
16	1	0	16	0

Condition	Statistics			
	P-value at 25%	P-value at 50%	P-value at 75%	P-value at 90%
N2 NGM *v.s.* **N2 B12+**	4.5e-11	1.7e-09	4.6e-05	0.4153
N2 NGM *v.s.* **N2 B12-**	3.5e-13	4.4e-13	3.4e-11	0.0002
N2 B12+ *v.s.* **N2 NGM**	4.5e-11	1.7e-09	4.6e-05	0.4153
N2 B12+ *v.s.* **N2 B12-**	2.0e-08	1.0e-07	2.9e-06	0.0003
N2 B12- *v.s.* **N2 NGM**	3.5e-13	4.4e-13	3.4e-11	0.0002
N2 B12- *v.s.* **N2 B12+**	2.0e-08	1.0e-07	2.9e-06	0.0003

Condition	Statistics		
	Chi^2	P-value	Bonferroni P-value
N2 NGM *v.s.* **N2 B12+**	71.85	0.0e+00	0.0e+00
N2 NGM *v.s.* **N2 B12-**	130.77	0.0e+00	0.0e+00
N2 B12+ *v.s.* **N2 NGM**	71.85	0.0e+00	0.0e+00
N2 B12+ *v.s.* **N2 B12-**	30.09	4.1e-08	8.2e-08
N2 B12- *v.s.* **N2 NGM**	130.77	0.0e+00	0.0e+00
N2 B12- *v.s.* **N2 B12+**	30.09	4.1e-08	8.2e-08

Figure S3. Lifespan assay. The tables show the raw data from each trial (number of dead and censored worms per day, where each trial starts with 100 L4-stage worms), as well as the equivalent percent survival rate for each condition. The two tables underneath show the statistics for each trial, from which we used the P-value, calculated with a T-test, to determine the significant difference between the conditions. **A.** Trial 1. **B.** Trial 2. **C.** Trial 3.

A.

N2 B_{12}-							
	Day						
Worm	1	2	3	4	5	6	Total
1	22	80	72	14	5	2	195
2							
3	19	79	42	28	5	1	174
4	20	85	47	30	5	1	188
5							
6	16	73	45	28	19	5	186
7	17	84	43	0	1	0	145
8	19	79	38	4	4	1	145
9	15	82	58	15	4	0	174
10							
Average	18,285714	80,285714	49,285714	17	6,1428571	1,4285714	172,42857
Standard deviation	2,4299716	3,9880775	11,799919	12,124356	5,843189	1,7182494	20,189578

N2 B_{12}+							
	Day						
Worm	1	2	3	4	5	6	Total
1	114	137	39	9	0		299
2							
3	46	97	31	5	16	6	201
4							
5	62	156	44	3	0	4	269
6	69	145	58	23	2	2	299
7							
8							
9	39	131	56	35	14	4	279
10	39	113	50	24	18	6	250
Average	61,5	129,83333	46,333333	16,5	10	4,4	266,16667
Standard deviation	28,514908	21,581628	10,366613	12,739702	8,3666003	1,6733201	36,977921

B.

N2 NGM							
	Day						
Worm	1	2	3	4	5	6	Total
1	83	149	60	1	0	0	293
2	35	126	81	1	1	0	244
3	32	128	68	8	6	1	243
4	42	150	102	1	0	0	295
5	63	135	53	2	0	0	253
6	44	86	22	2	0	0	154
7	61	85	18	2	0	0	166
8							
9							
10	42	124	83	22	2	0	273
Average	50,25	122,875	60,875	4,875	1,125	0,125	240,125
Standard deviation	17,285419	25,062422	29,396003	7,2984832	2,1001701	0,3535534	53,43738

N2 B_{12}-							
	Day						
Worm	1	2	3	4	5	6	Total
1	18	78	43	11	0	0	150
2	14	68	36	2	0	0	120
3	12	83	67	15	7	0	184
4	18	66	52	9	1	0	146
5	36	97	15	1	0	0	149
6							
7	26	49	14	9	3	0	101
8	39	56	15	3	0	0	113
9							
10	20	36	45	23	12	0	136
Average	22,875	66,625	35,875	9,125	2,875	0	137,375
Standard deviation	9,9633256	19,580876	19,68638	7,4149945	4,421942	0	26,087422

N2 B_{12}+							
	Day						
Worm	1	2	3	4	5	6	Total
1	34	121	62	0	0	0	217
2	39	135	61	18	0	0	253
3	31	133	40	0	1	0	205
4	40	112	46	12	1	0	211
5	64	120	3	0	0	0	187
6	52	123	24	2	5	0	206
7							
8	59	108	10	13	7	2	199
9	42	116	12	0	0	0	170
10	51	150	25	2	0	0	228
Average	45,777778	124,22222	31,444444	5,2222222	1,5555556	0,2222222	208,44444
Standard deviation	11,311253	13,074572	21,898123	7,0671383	2,6034166	0,6666667	23,696577

C.

N2 NGM							
	Day						
Worm	1	2	3	4	5	6	Total
1	43	114	93	29	11	0	290
2	37	119	150	28	8	1	343
3							
4	50	142	126	9	0	0	327
5	44	117	99	0	0	0	260
6	43	121	115	0	0	0	279
7	36	102	72	35	2	0	247
8	35	122	118	20	0	0	295
9	51	108	130	1	0	0	290
10							
Average	42,375	118,125	112,875	15,25	2,625	0,125	291,375
Standard deviation	6,0930288	11,80118	24,298369	14,498768	4,3732139	0,3535534	31,753234

N2 B_{12}-							
	Day						
Worm	1	2	3	4	5	6	Total
1	26	115	52	0	0	0	193
2	33	100	28	2	0	0	163
3	75	90	13	4	0	0	182
4	72	124	29	0	0	0	225
5	47	107	42	3	0	0	199
6	21	61	43	2	2	0	129
7	25	90	38	3	3	0	159
8	35	86	36	4	7	1	169
9	23	85	45	1	0	0	154
10	29	71	41	4	0	0	145
Average	38,6	92,9	36,7	2,3	1,2	0,1	171,8
Standard deviation	19,844955	19,232207	10,995454	1,5670212	2,2997584	0,3162278	28,361946

N2 B_{12}+							
	Day						
Worm	1	2	3	4	5	6	Total
1	38	136	78	2	0	0	254
2	40	134	77	11	2	0	264
3	29	109	71	35	2	0	246
4	43	173	83	5	0	0	304
5	58	105	68	26	12	3	272
6	41	174	69	2	0	0	286
7	28	172	80	1	0	0	281
8	49	200	93	27	0	0	369
9	70	145	63	25	0	0	303
10							
Average	44	149,77778	75,777778	14,888889	1,7777778	0,3333333	286,55556
Standard deviation	13,416408	32,155007	9,1210989	13,298914	3,929942	1	36,790322

Figure S4. Brood size assay. The tables show the raw data for each trial of the brood size experiments. As described in the 'Methods' section, ten worms were used per condition per trial, and their progeny was counted for each day. The worm numbers highlighted in orange indicate worms that were censored due to unnatural deaths (e.g. internal hatching), or that escaped. **A.** Trial 1. **B.** Trial 2. **C.** Trial 3.

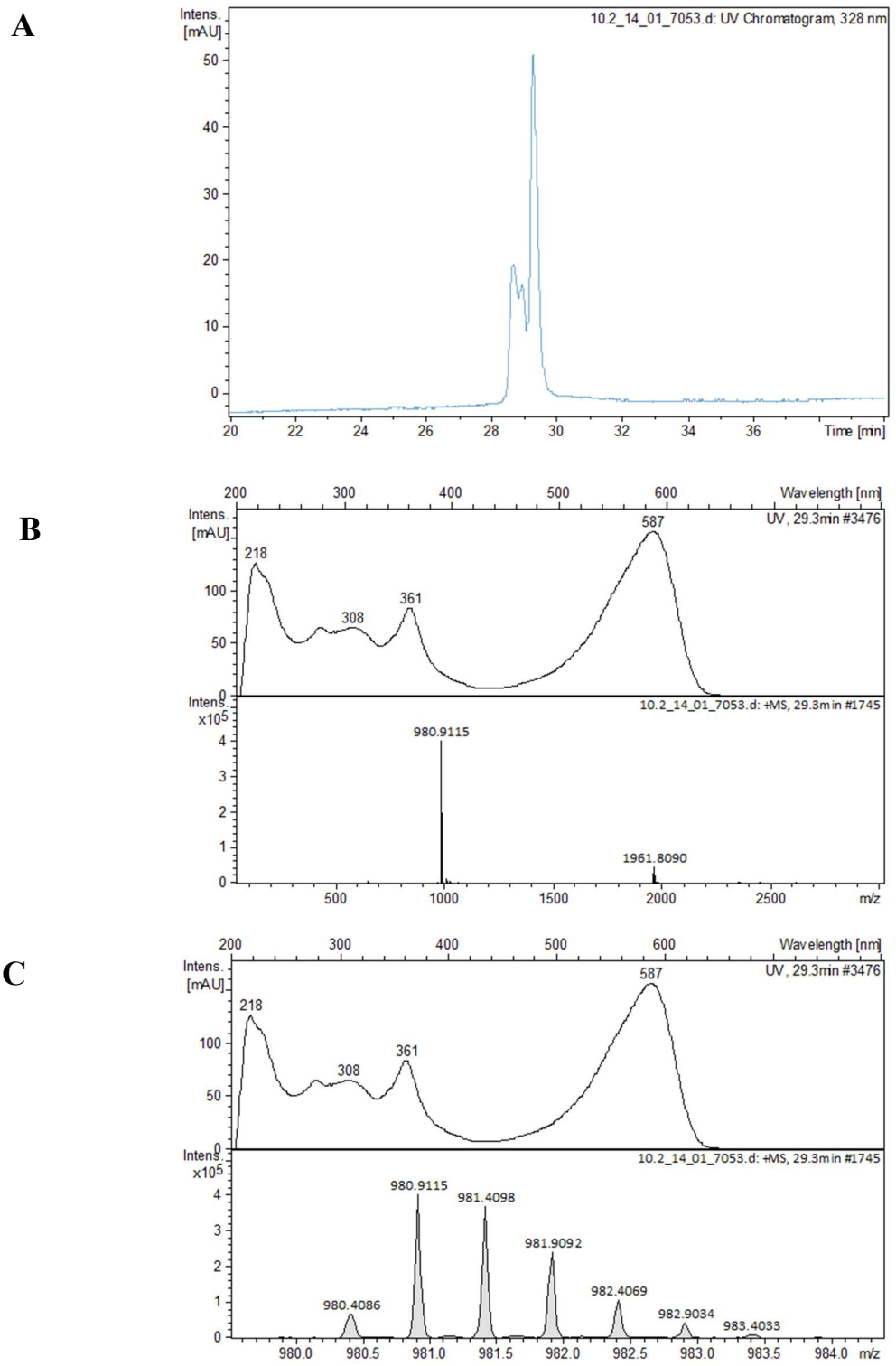

Figure S5. HPLC-MS of BODIPY® TR-X ribose linked cobalamin (BoB_{12}). **A.** Absorbance at 328 nm of sample from HPLC later used on HPLC-MS. **B.** Sample run on HPLC-MS. The UV absorbance curve has peaks at the expected wavelengths: ~361 nm for cyanocobalamin and 588 nm for BODIPY® TR-X. **C.** Detailed view of the 980.9 m/z peak. The m/z also correlated with the expected results.

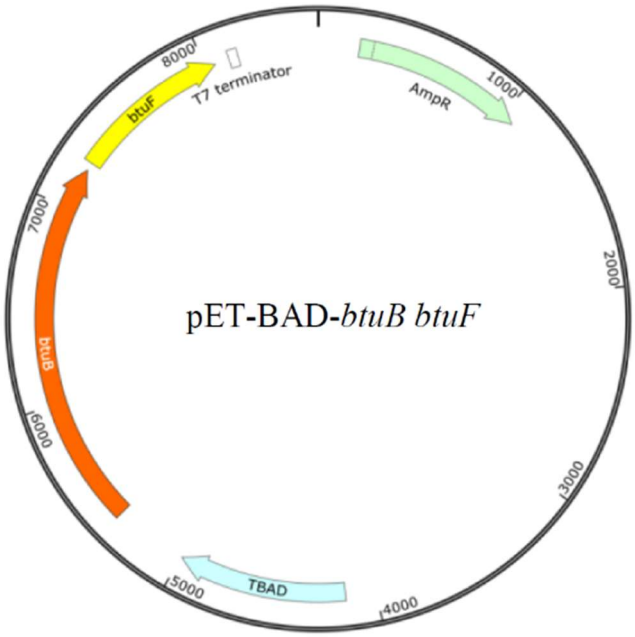

Figure S6. pET-BAD plasmid containing the *E. coli btuB* and *btuF* genes. The TBAD promoter (blue) allows induction of the *btuBF* genes (orange and yellow) by L-arabinose. AmpR (green) encodes ampicillin resistance. (Nemoto-Smith, 2017)

A.

	Day 1			Day 8			Day 15		
	B_{12}-	B_{12}+	NGM	B_{12}-	B_{12}+	NGM	B_{12}-	B_{12}+	NGM
Number of worms assayed	51	53	62	36	26	41	51	37	67
Number of worms with neurite branching	1	0	0	8	3	3	34	15	18
Percent worms with branching	1,96	0,00	0,00	22,22	11,54	7,32	66,67	40,54	26,87

B.

	Day 1			Day 8			Day 15		
	B_{12}-	B_{12}+	NGM	B_{12}-	B_{12}+	NGM	B_{12}-	B_{12}+	NGM
Number of worms assayed	75		92	59		81	37		88
Number of worms with neurite branching	1		0	29		17	24		37
Percent worms with branching	1,33		0,00	49,15		20,99	64,86		42,05

C.

	Day 1			Day 8			Day 15		
	B_{12}-	B_{12}+	NGM	B_{12}-	B_{12}+	NGM	B_{12}-	B_{12}+	NGM
Number of worms assayed	41	35	35	43	44	48	33	34	18
Number of worms with neurite branching	0	0	0	17	6	8	30	16	9
Percent worms with branching	0,00	0,00	0,00	39,53	13,64	16,67	90,91	47,06	50,00

Figure S7. Neurite branching assay. The tables show the raw data for each trial, which indicate the percentage of worms with neurite branching per day for each condition. **A.** Trial 1. **B.** Trial 2. **C.** Trial 3.

A.

NGM								
	Day							
Worm	1	2	3	4	5	6	7	Total
1	52	176	36	0	0	0	0	264
2	48	178	89	0	0	0	0	315
3	73	168	70	0	0	0	0	311
4	61	173	46	0	0	0	0	280
5	58	196	34	0	0	0	0	288
6	78	119	35	2	8	2	0	244
7	43	190	54	2	1	0	0	290
Average	59	171,42857	52	0,5714286	1,2857143	0,2857143	0	284,57143
Standard deviation	12,832251	25,085568	20,824665	0,9759001	2,9840848	0,7559289	0	25,01904

B_{12}-								
	Day							
Worm	1	2	3	4	5	6	7	Total
1	37	106	34	0	0	0	0	177
2	30	114	56	0	0	2	0	202
3	45	113	58	8	3	0	0	227
4	41	103	26	3	0	0	0	173
5	32	97	53	2	0	0	0	184
6	36	112	68	6	3	0	0	225
7	49	104	49	2	3	1	0	208
Average	38,571429	107	49,142857	3	1,2857143	0,4285714	0	199,42857
Standard deviation	6,8521807	6,2716292	14,496305	3	1,6035675	0,7867958	0	22,112268

B_{12}+								
	Day							
Worm	1	2	3	4	5	6	7	Total
1								
2	33	101	18	5	1	0	0	158
3	62	173	29	0	0	0	0	264
4	83	124	7	2	0	0	0	216
5	65	122	15	0	0	0	0	202
6	73	120	9	0	0	0	0	202
7	37	129	38	3	0	0	0	207
Average	58,833333	128,16667	19,333333	1,6666667	0,1666667	0	0	208,16667
Standard deviation	19,883829	23,961775	12,011106	2,0655911	0,4082483	0	0	33,978915

Pseudocobalamin								
	Day							
Worm	1	2	3	4	5	6	7	Total
1	41	74	22	2	5	1	0	145
2	35	99	13	3	5	3	0	158
3	43	91	71	4	1	2	0	212
4	64	83	9	2	0	1	0	159
5	31	87	50	1	2	1	0	172
6	59	101	26	3	1	2	0	192
7	60	86	39	0	3	8	0	196
Average	47,571429	88,714286	32,857143	2,1428571	2,4285714	2,5714286	0	176,28571
Standard deviation	13,239551	9,3222724	22,041087	1,3451854	1,9880596	2,5071327	0	24,294816

BoB_{12}								
	Day							
Worm	1	2	3	4	5	6	7	Total
1	46	114	14	6	7			187
2	46	118	46	0	0	0	0	210
3	57	70	34	0	1	0	0	162
4	40	115	14	10	2			181
5	47	117	28	7	0	0	0	199
6	45	109	33	4				191
7	46	124	12	10	8			200
Average	46,714286	109,57143	25,857143	5,2857143	3	0	0	190
Standard deviation	5,0896721	18,026436	12,915476	4,1918288	3,5777088	0	0	15,577762

T test NGM vs B12-	2,2149E-05
T test NGM vs B12+	0,001348685
T test NGM vs Pseudocobalamin	2,87861E-06
T test NGM vs BoB12	6,77261E-06
T test B12- vs B12+	0,603558443
T test B12- vs Pseudocobalamin	0,087198654
T test B12- vs BoB12	0,376576324
T test B12+ vs Pseudocobalamin	0,087866342
T test B12+ vs BoB12	0,268384278
T test Pseudocobalamin vs BoB12	0,236630877

B.

NGM								
	Day							
Worm	1	2	3	4	5	6	7	Total
1	34	210	38	0	0	1	0	283
2	38	200	25	1	1	2	0	267
3	53	222	51	11	1	0	0	338
4	29	156	65	9	1	2	1	263
5	42	136	42	16	0	0	0	236
6	37	226	14	2	3	4	0	286
7	35	144	44	1	1	0	0	225
8	41	212	6	10				269
9	39	109	25	11	2	5	0	191
10	36	189	9	1				235
Average	38,4	180,4	31,9	6,2	1,125	1,75	0,125	259,3
Standard deviation	6,328068	41,004607	19,31292	5,7888782	0,9910312	1,908627	0,3535534	40,290749

B_{12}-								
	Day							
Worm	1	2	3	4	5	6	7	Total
1								
2	8	50	32	28	7	2	0	127
3	13	63	47	34	10	4	1	172
4	35	52	61	29				177
5	31	84	44	5				164
6	27	34	46	50	23	5	1	186
7	25	36	45	40	35	7	0	188
8	10	33	42	22	16	10	2	135
9	24	47	46	14	4	4	0	139
10	23	61	46	20				150
Average	21,777778	51,111111	45,444444	26,888889	15,833333	5,3333333	0,6666667	159,77778
Standard deviation	9,4177728	16,541194	7,418071	13,596364	11,583034	2,8047579	0,8164966	22,813252

B_{12}+								
	Day							
Worm	1	2	3	4	5	6	7	Total
1	19	40	17	12	3	3	0	94
2	14	42	29	17	14	4	0	120
3	18	53	31	15	5	4	0	126
4	15	67	61	55	60	3	0	261
5	11	57	53	35	26	9	1	192
6	10	45	40	37	27	24	4	187
7	8	54	37	14	17	12		142
8	10	40	36	21	22	29	2	160
9	12	37	34	18	9	9	0	119
10	26	44	37	15	2	2	0	126
Average	14,3	47,9	37,5	23,9	18,5	9,9	0,7777778	152,7
Standard deviation	5,4375239	9,5038004	12,240643	13,947919	17,225627	9,4098058	1,3944334	49,09639

Pseudocobalamin								
	Day							
Worm	1	2	3	4	5	6	7	Total
1	12	101	62	34	8			217
2	41	67	42	42	33	7	0	232
3	20	54	36	40	38	25	2	215
4	13	68	41	47	24	15	1	209
5	9	51	61	56	25	3	1	206
6	27	80	62	30				199
7								
8	23	16	48	15	15	8	3	128
9	19	39	21	16	6			101
10	10	48	41	28	29	9	3	168
Average	19,333333	58,222222	46	34,222222	22,25	11,166667	1,6666667	186,11111
Standard deviation	10,185774	24,503968	13,838353	13,645309	11,585089	7,8081154	1,2110601	44,621868

BoB_{12}									
	Day								
Worm	1	2	3	4	5	6	7	8	Total
1	15	25	32	41	7	3	0	0	123
2	12	52	30	17	7	0			118
3	11	36	49	28	52	41	17	2	236
4									
5	12	43	28	30	31	14	7	1	166
6	12	62	69	41	9	10	0	0	203
7	13	41	53	25	10	5	1	0	148
8	11	56	24	17	11	6	1	0	126
9	10	56	43	21	8	7	2	0	147
10	16	58	55	40	14	8	3	0	194
Average	12,444444	47,666667	42,555556	28,888889	16,555556	10,444444	3,875	0,375	86,117647
Standard deviation	1,9436506	12,175796	15,125402	9,8671734	15,240662	12,135806	5,7678295	0,7440238	88,199973

T test NGM vs B12-	8,38922E-06
T test NGM vs B12+	5,42099E-05
T test NGM vs Pseudocobalamin	0,001752583
T test NGM vs BoB12	7,63283E-05
T test B12- vs B12+	0,688910092
T test B12- vs Pseudocobalamin	0,14110907
T test B12- vs BoB12	0,872526936
T test B12+ vs Pseudocobalamin	0,13861813
T test B12+ vs BoB12	0,646927563
T test Pseudocobalamin vs BoB12	0,255870293

C.

NGM								
	Day							
Worm	1	2	3	4	5	6	7	Total
1	41	176	103	10	5	0	0	335
2	47	192	52	11	9	4	0	315
3	43	141	19	1	0	0	0	204
4	61	197	48	0	0	0	0	306
5	35	169	65	2	3	0	0	274
6	29	176	47	3	2	0	0	257
7	28	183	46	2	0	0	0	259
8	61	173	51	9	2	0	2	298
9	26	181	78	3	0	0	0	288
10	27	178	65	0	0	0	0	270
Average	39,8	176,6	57,4	4,1	2,1	0,4	0,2	280,6
Standard deviation	13,314987	15,108497	22,336816	4,2282121	2,9608557	1,2649111	0,6324555	36,8064

B_{12}-								
	Day							
Worm	1	2	3	4	5	6	7	Total
1	11	58	68	62	23	5	2	229
2	20	82	83	43	14	0	0	242
3	33	69	65	34	19	7	5	232
4	15	67	69	19	27	1		198
5	33	77	63	40	22	10	0	245
6	19	84	65	41	16	0	0	225
7								
8	0	1	2	1	0	0	0	4
9	21	81	52	20	14	1	0	189
10								
Average	19	74	66,428571	37	19,285714	3,4285714	1,4	195,5
Standard deviation	10,941402	27,294361	24,318937	18,677718	8,219098	3,8544964	1,9148542	79,873114

B_{12}+								
	Day							
Worm	1	2	3	4	5	6	7	Total
1	30	65	64	49	8	2	1	219
2	32	63	88	44	12	6	2	247
3	23	69	67	43	15	4	1	222
4	10	77	132	57	9	7	1	293
5	23	60	88	37	7	8	5	228
6	38	70	82	55	21	8	5	279
7								
8								
9	32	68	93	56	9	8	2	268
10	8	106	66	45	8	5	3	241
Average	24,5	72,25	85	48,25	11,125	6	2,5	249,625
Standard deviation	10,77033	14,557767	22,123033	7,2259453	4,7640769	2,2038927	1,6903085	27,598848

Pseudocobalamin								
	Day							
Worm	1	2	3	4	5	6	7	Total
1	40	70	61	36	10	3	5	225
2	18	58	58	30	7	4	0	175
3	16	39	59	42	15			171
4	25	75	49	31	25	9	0	214
5	28	25	38	41	15	1	1	149
6	31	74	84	36	14	12	9	260
7	30	60	64	45	19	14	8	240
8	41	72	48	49	29	10	2	251
9	21	55	46	36	18	6	0	182
10	23	60	52	33	12	4	0	184
Average	27,3	58,8	55,9	37,9	16,4	7	2,7777778	205,1
Standard deviation	8,4859361	16,143798	12,644278	6,1904945	6,6699992	4,4440972	3,6324158	38,018855

BoB_{12}								
	Day							
Worm	1	2	3	4	5	6	7	Total
1	30	60	21	23	3	9		146
2	21	58	70	32	17	5	2	205
3	30	56	42	36	19	7	3	193
4	14	57	34	21	21	9	8	164
5	15	74	59	73	56	26	10	313
6	23	56	47	24	15	8	10	183
7	10	62	64	27	12	9	4	188
8	21	60	58	23	10	5	2	179
9	35	69	62	36	16	13	1	232
10	14	40	56	38	15	13	6	182
Average	21,3	59,2	51,3	33,3	18,4	10,4	5,1111111	198,5
Standard deviation	8,2737873	8,941787	15,180763	15,290157	14,143707	6,1318839	3,5158372	46,253528

T test NGM vs B12-	0,0204131
T test NGM vs B12+	0,0583228
T test NGM vs Pseudocobalamin	0,0002705
T test NGM vs BoB12	0,000391
T test B12- vs B12+	0,1048341
T test B12- vs Pseudocobalamin	0,761186
T test B12- vs BoB12	0,9265947
T test B12+ vs Pseudocobalamin	0,0110403
T test B12+ vs BoB12	0,010837
T test Pseudocobalamin vs BoB12	0,7315942

Figure S8. Cobalamin analogue brood size rescue assay. The tables show the raw data for each trial. The experiments were carried out as described in the 'Methods' section, with ten worms used per condition per trial. The small tables show the P-values (from a two-tailed T-test) for each pair of conditions, with the ones in red indicating those which are significantly different to one another. **A.** Trial 1. **B.** Trial 2. **C.** Trial 3.