Effects of early life starvation stress on adult foraging behavior in *Caenorhabditis elegans*

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ABSTRACT

The work presented in this thesis explores the effect of early-life starvation stress on adult foraging behavior in the nematode *C. elegans*. When faced with stringent environmental conditions like scarcity of food in early larval stages, *C. elegans* go into an arrested developmental stage called dauer from which they can recover when food becomes available again. We examined if the characteristic foraging behavior seen in *C. elegans* adults are in any way changed in animals experiencing dauer earlier in their lifetime. We established that post-dauer animals show reduced exploratory foraging behavior and that this behavioral plasticity in response to early life stress is seen in a wild isolate, CB4856 but not in the lab-adapted N2 strain.

ABRÉGÉ

L' étude présentée dans cette thèse explore les effets du stress de privation de nourriture chez le jeune *C. elegans* sur son comportement de recherche de nourriture à l' âge adulte. Lorsqu' il fait face à des conditions environnement difficiles telles que la rareté de la nourriture lors de premiers stades larvaires, *C. elegans* entre dans une stase de développement nommée stade dauer, dont ils peuvent en sortir lorsque la nourriture redevient disponible. Nous avons évalué dans quelle mesure les caractéristiques du comportement de recherche de nourriture observes chez *C. elegans* adulte sont affectées chez les animaux ayant traversé le stade dauer au cours des phases précoces de leur vie. Nous avons dmontr que les animaux post-dauer prsentent des comportements explorateurs réduits. Nous avons aussi montré cette plasticité comportementale, en réponse au stress précoce, observée chez le nématode sauvage isolé CB4856, est toutefois absente chez la lignée N2 adaptée aux laboratoires.

CONTRIBUTION OF AUTHORS

Kai Homer, an undergraduate student worked with me on some of the Lawn-leaving and Trail assays. Melanie Desrochers together with Dr. Michael Hendricks developed the method for calculation of the bearing angle used in Fig 4.8. Figures 2.1 and 2.3 were made by Dr. Michael Hendricks. Visou Ady translated the Abstract of this thesis into French.

Contents

List of Figures

Chapter 1

Introduction

Environmental influences in early development can have indelible effects on adult physiology and behavior. Coupled with genetic background, stressful conditions in early childhood can often lead to predisposition to chronic diseases later in life. The exact developmental mechanisms responding to early-life environmental stress is not well understood. In this work, we aimed to understand how nutritional stress during early development affects adult foraging behavior using the nematode, *Caenorhabditis elegans*, as a model organism. Long-term studies in mammalian systems are difficult owing to their long lifespan, and complex interplay between large number of genes, neuronal networks and environmental factors. The short life-cycle, genetic tractability and the anatomical simplicity of the neuronal network makes the worm an ideal model organism to study environmental programming of adult behavior. Nematodes also share conserved genetic, metabolic, and neuromodulatory pathways with humans. This project thus provides an opportunity to investigate how starvation stress affects developmental plasticity and affects adult behavioral traits.

Hypothesis and specific aims

Starvation at early development directly affects *C. elegans* development and induces dauer arrest. We hypothesized that even when dauer animals are rescued and recovered for several days with high nutrient conditions, the early-life adverse environmental conditions cause permanent

changes in their foraging behavior. We performed a number of behavioral assays to delineate behavioral differences between animals having experienced dauer arrest in their development in comparison to animals not exposed to early-life starvation stress. The specific aims of this study were:

- To explore whether early-life starvation stress can lead to permanent changes in behavior.
- To explore if such plasticity is dependant on genetic variation.
- To identify if there is a critical period for starvation stress in larval stages which results in behavioral plasticity.
- To identify locomotory patterns underlying changes in foraging behavior.

Chapter 2

Background

2.1 Effects of early life stress

A large number of epidemiological studies have linked poor maternal diet and childhood malnutrition, to development of adult-onset metabolic disorders, obesity and Type-II diabetes, particularly in developing countries and low income populations [Hales and Barker, 2001; Hales et al., 1991; Yajnik et al., 2003]. A recent study mimicking such chronic under-nutrition in developing countries, showed that rats which are under-nourished for over fifty generations exhibit metabolic abnormalities, which cannot be reversed by unrestricted diet for two generations [Hardikar et al., 2015]. Studies in humans have linked early life abuse to mental disorders in later life [Graham et al., 1999; Heim and Nemeroff, 2001]. Behavioral ecologists have also identified a number of instances where animals adapt to environmental cues at certain critical periods early in their developmental history, which permanently alter their adult phenotype and behavior [Bateson et al., 2004].

2.2 Theoretical model for environment-dependent plasticity

This study is based on the theoretical framework of the adaptive tuning hypothesis [Sih, 2011]. It proposes that in an invariant environment, plasticity is not favoured; but under fluctuating conditions, animals adapt traits tuned towards a predicted environment. This also depends on

the plasticity of the genotype, where a high plasticity genotype has high responsivity towards environmental fluctuations, which is optimal if predicted environments match with actual conditions (Fig 2.1). However, if there is a mismatch between the predicted and actual environments, these changes become mal-adapative and lead to predisposition to diseases [Hales et al., 1991; Hales and Barker, 2001]. In the 1980s, epidemiological associations between a person's place of birth and increased rates of diseases as adults living elsewhere, explained this with the "thrifty phenotype hypothesis" [Hales and Barker, 1992, 2001]. It claims that poor maternal diet and low childhood nutrition leads to adaptive physiological changes in expectation of similar environment to continue through the organism's lifetime. When this is in contrast to actual adult environment with plentiful resources, it leads to metabolic disorders like Type-II diabetes. Epidemiological studies in populations suffering famines have also shown increased susceptibility to obesity, cardiovascular diseases, microalbuminuria and mental disorders like schizophrenia, some of which are heritable [Painter et al., 2008; Song et al., 2009].

2.3 *C. elegans* as a model for behavioral plasticity

C. elegans has an invariant developmental cell lineage and a stereotyped pattern of connectivity between its 302 neurons [Sulston et al., 1983; White et al., 1986]. The N2 strain has been commonly used to develop *C. elegans* as a model organism. It is genetically tractable and previous studies have established clear correlations between activity of specific neurons to resulting behaviors. This makes *C. elegans* an ideal organism to use for comparative studies of behavioral plasticity, as animals of the same strain are genetically identical, and have same neuronal organization, differing only in past experiences.

Worms are known to exhibit experience-dependent behavior responding to mechanosensory tap stimuli [Rose et al., 2005] and to associate a particular temperature or salt concentration to presence of food, and will develop a preference towards these stimuli later on, even in the absence of food [Hedgecock and Russell, 1975; Wen et al., 1997; Ohno and Iino, 2014]. Previous work on associative learning and plasticity in *C. elegans* have shown that animals previously exposed to pathogenic bacteria learn to avoid it on future encounters [Zhang et al., 2005]. This

form of associative learning is short term and the learned behavior is lost in a timescale of hours. A recent work described long term associative aversive learning in *C. elegans*, where animals retain a memory of being exposed to pathogenic bacteria in the L1 larval stage and this behavioral plasticity is permanent over the animal's lifetime [Jin et al., 2016]. In this project, we examine a similar long-term plasticity in *C. elegans* behavior in response to early-life starvation stress.

2.4 *C. elegans* life cycle and environmental sensitivity

C. elegans have a short life cycle; in the presence of abundant food it takes only three days for newly hatched eggs to pass through four larval stages (L1 through L4) to grow into reproductively active adults. Upon facing stressful conditions like scarcity of food, high population density or restrictive temperatures in the course of its development, the L2 larvae of *C. elegans* may enter an arrested developmental stage, called dauer [Golden and Riddle, 1982, 1984; Cassada and Russell, 1975]. Animals can remain in this dauer stage, without feeding and with altered metabolic activity, for upto three months [Cassada and Russell, 1975]. Once environmental conditions improve, these animals continue their development to grow into seemingly "normal" reproductive adults (Fig. 2.2). Dauer entry clearly demonstrates how environmental conditions can drastically alter *C. elegans* development.

As a paradigm of transient starvation during development, we rescued worms, which have passed through starvation-induced dauer in the course of their development, on normal food conditions until they reached young adulthood. These animals, henceforth referred to as post-dauers, appear morphologically indistinguishable from their well-fed counterparts, animals which feed *ad libitum*. Sarah Hall and colleagues have shown that the *C. elegans* N2 strain retain a cellular memory of this altered developmental history induced by stressful environment and exhibit changes in gene expression levels, histone markers and endogenous siRNA pathways [Hall et al., 2010, 2013]. They also reported greater fecundity and longer mean lifespan in post-dauer animals [Hall et al., 2010]. These studies corroborate with our hypothesis that acute environmental stress at early development may induce permanent changes in adult life.

No work till date has looked into behavioral alterations in post-dauer animals.

2.4.1 Dauer

The decision of entering the dauer developmental arrest occurs in the late L1 stage in the *C. elegans* life cycle and is regulated by adverse environmental stimuli [Hu, 2007] like shortage of food, high temperatures and high levels of pheromone indicative of high population density [Golden and Riddle, 1982; Cassada and Russell, 1975]. To withstand such environmental stress, dauer larvae undergo morphological changes and develop a tougher cuticle [Cassada and Russell, 1975] and a plug on their mouthparts which stops feeding [Riddle et al., 1981]. This cuticle makes them resistant to surfactant treatment [Cassada and Russell, 1975] and this can be used a method to select dauer larvae in a mixed population of animals. They are also thinner and longer than well-fed L2 animals [Cassada and Russell, 1975]. Electron microscopy studies of animals in the dauer stage have also reported remodelling of the sensory cilia [Albert and Riddle, 1983; Schroeder and Flatt, 2014]. Dauer pheromone, one of the key stimuli for induction of dauer has been found to be a mix of different ascarosides [Butcher et al., 2007; Jeong et al., 2005; Srinivasan et al., 2008]. They most likely bind to G-protein receptors and regulate dauer arrest via guanylyl cyclases [Thomas et al., 1993]. Genetic studies of dauer-constitutive and dauer-defective mutants have identified DAF-12, a nuclear receptor as the final target for all dauer pathways [Hu, 2007]. A number of studies have also identified the TGFβ -like, insulin-like pathways and steroid hormone pathways to be involved in regulating the dauer arrest [Hu, 2007].

2.5 Theoretical model for foraging behavior

Since we are studying adaptive changes in foraging behavior, the second theoretical framework for our study is the optimal foraging theory, in particular Charnov's Marginal Value Theorem [Charnov, 1976]. Foraging is an essential behavior in all animals, where an animal must be able to integrate sensory cues from the environment and its internal state to decide when to forage

and when to feed on a patch currently available to them. The marginal value theorem proposes that the optimal time for an animal to leave its foraging ground occurs when it senses that the current patch of food is lower in resource level compared to the average level of the entire habitat (Fig 2.3). This is rationalized by the expectation that the energy spent in foraging and the associated risks will be compensated by accessing a patch with a higher resource level. This framework has been previously used to study foraging behavior in *C. elegans* in the context of genetic variation, satiety and ambient conditions [Bendesky et al., 2011; Milward et al., 2011; Calhoun et al., 2014, 2015], but has not been used to examine developmental plasticity under environmental fluctuations.

2.6 *C. elegans* locomotion and foraging

C. elegans move on solid surfaces using an undulatory sinusoidal motion and swim in liquid media. In their natural habitat, they possibly use a combination of the two to navigate complex three-dimensional environments. In the laboratory, animals are maintained on agar plates where they lie on their sides and exhibit dorso-ventral body bends to crawl on the surface. The body bends can result in three behavioral outputs (Fig2.4A): forward movement, reversals and turns resulting from varying degrees of the body bend amplitudes: a complete nose to tail touch is called an omega turn [Gray et al., 2005].

2.6.1 Navigational strategies

C. elegans exhibit chemotactic behavior towards environmental cues like volatile odors or water-soluble attractants by employing specific navigational strategies. Studies by Pierce-Shimomura *et al.* found that worms explore their environment by using a combination of bouts of reversals and turns, called “pirouettes” and forward “runs”[Pierce-Shimomura et al., 1999]. They found that the number of pirouettes is dependent on the temporal change in a chemo-attractive gradient and increases when a animal is moving down the gradient. This is similar to the biased random walk observed in bacterial klinokinesis where characteristic forward move-

ment or "runs" are interrupted by sharp turns or "tumbles" [Pierce-Shimomura et al., 1999]. The other strategy was identified more recently by Yuichi Iino *et al.* where biased head swings in the direction of a positive chemical gradient gradually steers an animal towards the gradient's peak[Iino and Yoshida, 2009]. This klinotaxis strategy is dependent on the spatial gradient of chemo-attractive stimuli[Iino and Yoshida, 2009]. *C. elegans* use a combination of these two strategies to navigate their environment and locate positive chemical cues (Fig2.4B).

2.6.2 Temporal dynamics of food search

C. elegans locomotion dynamics changes with time when placed in a plate without food from an environment with abundant food (Fig2.4B). Studies by Jesse Gray [Gray et al., 2005] showed that animals had a high frequency of turns and reversals immediately after removal from food. The speed of animals also increase indicating that they are actively sampling the environment and looking for food in the vicinity. This had been described as the local search phase of foraging behavior in *C. elegans*. If there is no food found even after 15 minutes of intense local search, they shift to a dispersal mode to locate distant sources of food. This phase is characterized by continuous long forward runs and suppression of reorientations [Gray et al., 2005].

2.6.3 Neuronal circuit involved in foraging

The *C. elegans* connectome gives us detailed information about neuronal connectivity and putative pathways regulating behavior [White et al., 1986]. Gray *et al.* used laber ablation to define a neural circuitry regulating navigation of *C. elegans*, and identified neurons affecting probability of turns and reversals when an animal is removed from food cues [Gray et al., 2005]. Sensory inputs from AWC, ASK and ASI project onto two layers of interneurons which synapse onto forward (PVC, AVB) and reverse command interneurons (AVA, AVD, AVE) [Chalfie et al., 1985; Gray et al., 2005; Zheng et al., 1999], which ultimately control locomotion of the animal. The interneurons AIB and RIB feed onto the SMD motor neurons and initiate turns whereas the AIY and RIM pathway drive reversals [Gray et al., 2005]. The *C. elegans* foraging cir-

cuit employs a larger network with overlapping set of neurons (Fig2.5). This involves neurons like AQR, PQR, URX and BAG involved in sensing O_2 and CO_2 levels, which reflects density of bacterial concentrations [Gray et al., 2004; Rogers et al., 2006; Busch and Olofsson, 2012; Milward et al., 2011] and sensory neurons are known to respond directly or indirectly to food related olfactory cues [Zaslaver et al., 2015; Chalasani et al., 2007].

2.6.4 Behavior on food

When on food, *C. elegans* display either an active roaming behavior or more a resting dwelling behavior, characterized by lower speed and frequent reorientations [Fujiwara et al., 2002; Ben Arous et al., 2009]. It has been shown that previous experience modulates such food-related locomotion, where poor food source and starvation conditions increase proportion of time spent dwelling [Shtonda and Avery, 2006; Ben Arous et al., 2009]. Neuromodulators like serotonin and the TGF-β and insulin signaling pathways are involved in its regulation. Other studies have also shown that there are changes in foraging and on-food behavior, depending on previous exposure to different quality diets or, depending on whether they are satiated or hungry [Shtonda and Avery, 2006; Gallagher et al., 2013].

2.6.5 Environmental and experience-dependant factors regulating foraging

C. elegans can evaluate a number of external cues to attenuate their foraging strategies and optimize the leaving time from a food patch. Previous studies have shown that *C. elegans* exhibit food-choices which are mostly dictated by the ease by which they can ingest the bacteria [Shtonda and Avery, 2006]. Certain strains like *E. coli* HB101 and *Commamonas sp.* were identified as preferred strains, whereas *Bacillus megaterium* is of a lower grade. They show increased chemotaxis towards good food and increased leaving behavior from less preferred ones [Shtonda and Avery, 2006]. Bacterial choice can not only affect food-seeking behavior, but can also alter behavior on food. The roaming behavior characterized by longer runs are reduced on preferred bacterial sources and is regulated by the AIY interneuron [Shtonda and

Avery, 2006]. *C. elegans* also avoid pathogenic bacterial strains like *Pseudomonas aeruginosa* PA14 and *Serratia marscescens* via serotonin modulation in ADF neurons [Zhang et al., 2005] .

Ambient oxygen and carbon dioxide levels are also known to affect foraging. Higher concentration of bacteria typically found on the edges of bacterial lawns can change local O_2/CO_2 concentrations. Gaseous concentrations thus act as as an indirect measure for food availability for *C. elegans* and high oxygen levels have been found to promote food-leaving [Milward et al., 2011]. Variations in the *gcy-35* gene among different *C. elegans* isolates can alter neural circuits involved in sensing oxygen and carbon dioxide [Gray et al., 2004; Cheung et al., 2005; Rogers et al., 2006]. Recent work also found that the size of a bacterial patch can affect search dynamics on leaving the lawn [Calhoun et al., 2015, 2014]. Animals from smaller patches have a reduced local search behavior. Interestingly, it is not the absolute size of the patch which regulate this behavior but the frequency of lawn border encounters. In a smaller patch, an animal encounters the border more frequently, which is known to have a different local environment with respect to nutrient concentration and gaseous stimuli. Two neurons ASK and ASI modulate this experience-dependent short-term plasticity in local search [Calhoun et al., 2015; Hendricks, 2015].

C. elegans also show an increased food-leaving activity with depleting patches, confirming with the marginal value theorem [Milward et al., 2011]. Milward *et al.* reported that as density of bacteria decreases in a food patch over a long periods of time, animals have a higher tendency to leave the lawn [Milward et al., 2011]. Their speed increases as food concentration depletes and reversals at lawn boundaries are suppressed [Milward et al., 2011]. This mechanism involves a large neural circuitry including neurons involved in oxygen sensation like AQR, PQR, URX and neurons like ASER, involved in gustatory plasticity.

2.6.6 Natural variation and the genetic basis of foraging

In the wild, *C. elegans* are found in fruit compost heaps and humus [Hodgkin and Doniach, 1997], where they feed on resident bacteria [Frézal and Félix, 2015; Felix and Duveau, 2012].

Depending on environmental fluctuations, such resources can be largely patchy and thus foraging for better resources is of enormous importance. The lab reference strain, N2 was maintained for several years in agar plates with a single patch of *E. coli* as its food source, before it was frozen. *E. coli* is not known to be a food source for wild *C. elegans*, but was chosen for its easy availability in laboratories [Frézal and Félix, 2015]. Owing to the organism's short generation time, the N2 animals cycled for many generations, adapted to lab conditions and developed a number of mutations (notably in the *npr-1* gene), which significantly altered its gene expression profile and behavior [Bendesky et al., 2011; de Bono and Bargmann, 1998; Gloria-Soria and Azevedo, 2008; Milward et al., 2011].

Polymorphisms in the *npr-1* gene led to strikingly different foraging patterns in different wild isolates of *C. elegans*. Strains with low NPR-1 activity, like the Hawaiian CB4856, aggregate on food, whereas the common laboratory wild-type strain N2, which has a high activity *npr-1* allele, display solitary feeding behavior. Natural variation in another gene, *tyra-3*, encoding a tyramine-activated G protein-coupled receptor also affects exploration decisions in wild-type *C. elegans*, and *npr-1* was found to be epistatic to it [Bendesky et al., 2011].

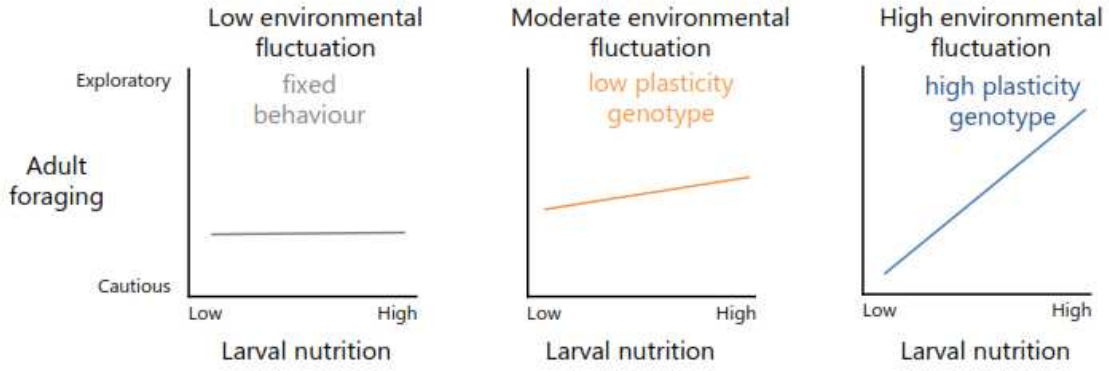


Figure 2.1: Adaptive tuning hypothesis. This theoretical model predicts that traits tuned towards gaining environment specific advantage should be plastic in a variable environment and fixed in a static environment.

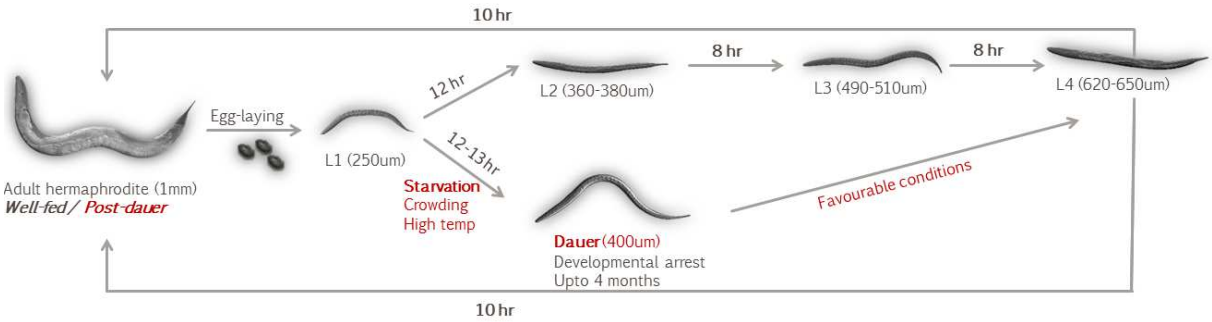


Figure 2.2: *C. elegans* life cycle. *C. elegans* passes through four larval stages to become a reproductively active adult. Starvation, crowding or high temperature at late L1 stages can induce animals into dauer.

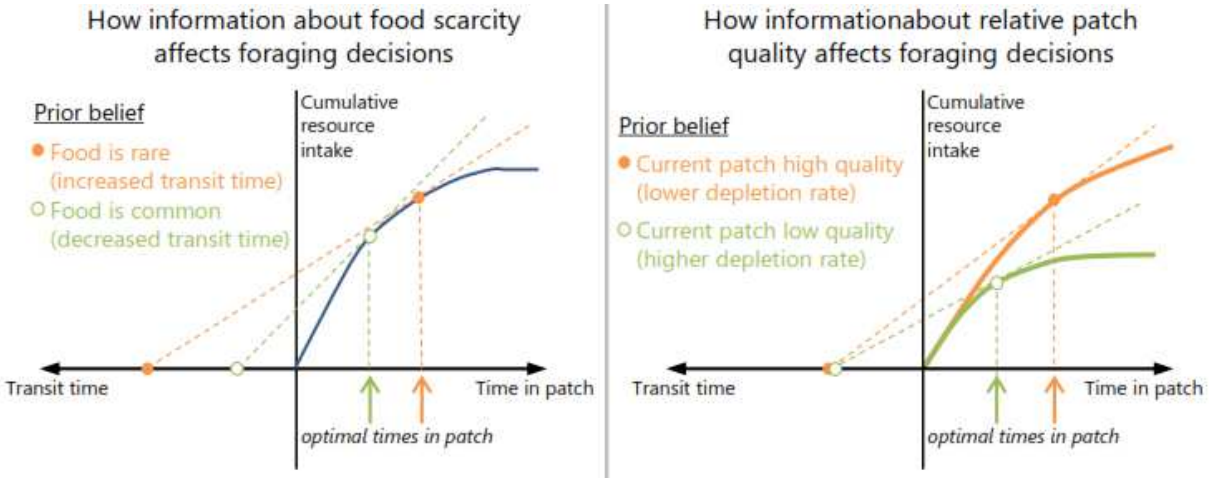
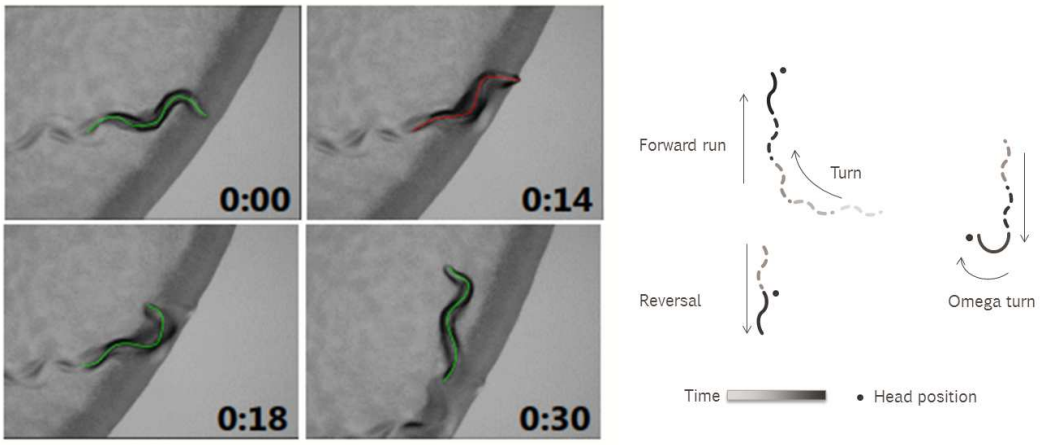
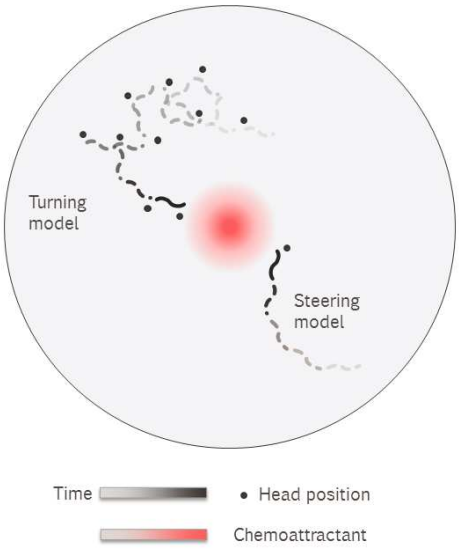


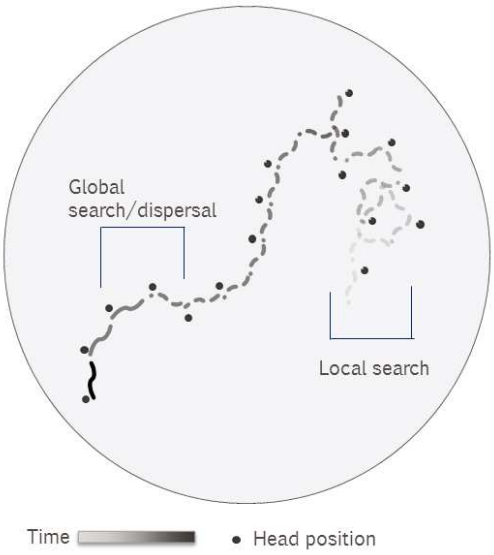
Figure 2.3: Theoretical framework of foraging. The Marginal Value Theorem determines the optimal time for leaving a food patch, depending on the animal's idea about spatial distribution of resources in a patchy environment.



(A) *C. elegans* locomotion



(B) *C. elegans* navigational strategies



(C) Temporal dynamics of food search

Figure 2.4: *C. elegans* locomotion and foraging. A) Panel at time 0:00 minutes shows forward run, followed by border encounter and reversal at 0:14 minutes, a turn at 0:18 minutes and continuation of forward run after reorientation at 0:30 minutes. B) The turning model involves a biased random walk strategy with high frequency of turns and reversals. The steering model is a klinotaxis behavior with biased head swings towards a preferred stimuli resulting in gradual steering. C) On removal from food, the first 15 minutes are characterized by large number of reorientations- the local search phase followed by longer runs and suppressed reversals- the dispersal phase.

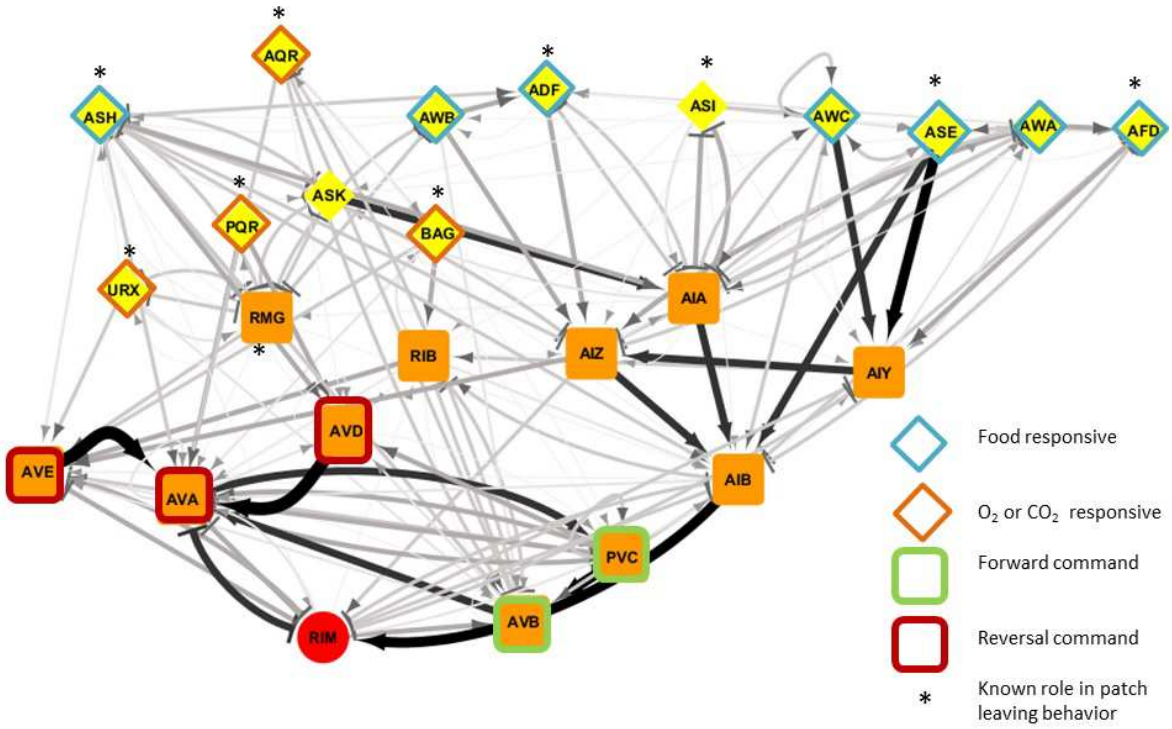


Figure 2.5: Neurons involved in foraging. Network representing all sensory neurons that are known to be food-responsive or involved in patch-leaving and aggregation behaviors. Downstream neurons include interneurons which connect to the forward and reverse command neurons.

Chapter 3

Materials and Methods

3.1 Maintenance of animals

All *C. elegans* strains were maintained at 22°C using standard methods on Nematode Growth Medium (NGM) plates seeded with the *E. coli* HB101 strain as the food source. Young-adult hermaphrodites were used for all experiments. All strains were obtained from the Caenorhabditis Genetics Centre (CGC). The N2 strain isolated from mushroom compost near Bristol, United Kingdom was identified in the 1960s and maintained by different labs till it was deposited at the CGC in 1993. The CGC maintains frozen stocks of strains to limit rapid adaptation to current environments. The CB4856 strain was isolated from a pineapple field in Hawaii in 1972. The current stock at the CGC was thawed in 2014 from stock frozen in 1995.

Generation of post-dauer, post-L1 diapause and post-starvation animals

Animals raised on abundant food throughout their lifetime were termed as "well-fed" through this manuscript. Plates left for about 4-5 days led to depletion of food and animals starved at early larval stages were induced into the developmental arrest phase, dauer. Dauer larvae have a thicker cuticle structure and are resistant to surfactant treatment [Cassada and Russell, 1975; Popham and Webster, 1979; Swanson and Riddle, 1981]. These starvation-induced dauers were selected by exposing them to 1% sodium dodecyl sulphate (SDS) for 30 minutes. Animals were washed and placed back on an abundant food source after the SDS selection till early adulthood

and were termed as “post-dauer” animals.

C. elegans go into another developmental arrest stage, called L1 diapause if they are starved at early L1 larval stages, for example in situations where eggs hatch in an environment with no food [Baugh, 2013; Rechavi et al., 2014]. We wanted to check if animals experiencing the L1 diapause can also exhibit changes in adult behavior. Eggs were isolated by washing a plate with large number of gravid adults and eggs with a solution with one part of sodium hydroxide to two parts sodium hypochlorite. When these eggs were plated on a NGM plate with no bacterial food source, the newly hatched L1 larvae go into the L1 diapause stage [Baugh, 2013; Rechavi et al., 2014]. These animals arrested in the L1 diapause stage were manually picked and placed back on food till young adulthood and termed as “post-L1 diapause” animals.

By plating eggs isolated using the sodium hypochlorite solution on a bacteria seeded plate the age of a population could be synced. As this synced population exhausted food over a period of 4-5 days, a fraction of them go into the dauer stage. We wanted to identify if dauer is essential for the behavioral plasticity or if early life starvation is sufficient. We selected animals which were starved for the same period of time in the same developmental timescale, but did not go into dauer. Larval worms without the dauer morphology were manually picked and put back on food till young adulthood and termed as “post-starvation’ animals. The same plate was then treated with SDS to isolate dauers to obtain the “post-dauer” set.

3.2 Lawn-leaving assay

A lawn-leaving assay, adapted from a recent publication [Bendesky et al., 2011] was conducted to measure explorative behavior. We seeded 6cm NGM plates with 10μl of a fresh overnight culture of *E.coli* HB101, diluted in LB to OD_{600}=0.2, 24 hours before the assay. The lawn thickness was considerably lower than the density of bacteria on animal maintenance plates. Ten young-adult animals were picked from a maintenance plate and allowed to condition on the thinner lawn for 30 minutes before commencement of a 60 minutes assay period. The assay used by Bendesky *et al.* involved a separate conditioning plate with thin lawn conditions, from which animals were picked up on to the assay plate after allowing 30 minutes of acclimatization.

We omitted this step of having a separate conditioning plate since animals often reacted to mechanical stimuli during transfer, and elicited an initial escape response, which could have be misinterpreted as explorative behavior during the assay period. During the 60 minute assay period, behavior was monitored manually and by recording videos at 2 frames per second(fps) using a Dinolite USB microscope camera. Number of animals exploring outside the lawn were recorded at 2-minute intervals. A typical bacterial food patch on an agar plate has varying levels of bacterial concentration, with the highest level at the edges [Calhoun et al., 2015]. When *C. elegans* feed on such a patch, they normally reverse on encountering the lawn boundary and turn away in order to remain on food. Favouring exploration over exploitation of the existing food patch would therefore require suppression of these reversals to exit from the food patch. To quantify the number of times animals actively decided to forage, we quantified the number of lawn-leaving events.

3.3 Trail assay

To overcome the shortcomings of being able to record only a limited number of hour-long videos in a given day, we shifted to a more high-throughput assay method, called the "Trail assay" henceforth. We seeded 9cm NGM plates with 10μl of fresh overnight culture of *E.coli* HB101, diluted in water to OD_{600} = 0.1, 20-24 hours before the assay. We placed a single young-adult animal on each plate for an assay period of 1 hour. Each worm was removed after the assay period and the plates incubated overnight in a 37°C humidified incubator. Worms defecate at a very frequent rate of once every 50 seconds [Croll and Smith, 1978; Liu and Thomas, 1994]. The defecation leaves a trail of bacteria which grows in the incubator, allowing a convenient way of observing trails left by the foraging animal in combination with the sinusoidal tracks left on the soft agar surface. The trails were traced out and a grid with the smallest square measuring 35 sq.mm was used to quantify the area explored by the animal.

3.4 Grid assay

To examine if the difference in foraging activity was a result of locomotory defects in the post-dauer animals, we performed an assay to quantify locomotion when on food. We seeded 6cm NGM agar plates with 600μl of fresh overnight *E.coli* HB101 culture, such that the bacterial lawn covered the entire surface area of the plate. A single animal was transferred to the center of the plate and allowed to move around for 16 hours. The worm was removed after the assay and its tracks on the bacterial lawn were traced and quantified with a grid, similar to the previous assay.

3.5 Dot Matrix assay

To induce leaving behavior, we reduced the size of bacterial lawns by seeding a 4x8 grid of 2μl *E. coli* HB101 culture, diluted to the same density as the trail assay performed before. We predicted that the small size of the lawn and presence of other patches outside more accurately replicates a patchy resource environment and further induces the animal to leave the initial food patch. A single animal was placed upon a central food patch and we then quantified the number of patches explored once it exited the initial patch. To further quantify the distance it traversed, we scored its exploratory behavior. The initial patch was assigned a score of 1, with its first neighbor as 2, second neighbor as 3 and so on. The summation of the scores of the patches explored by the animal was taken as a measure of its exploratory foraging activity.

3.6 Microfluidic devices

Microfluidic devices allow for more careful observation of animals in an environment less likely to be affected by environmental fluctuations like temperature and humidity. The devices allow for laminar flow of liquids under precise spatio-temporal control. We used devices designed by Dirk Albrecht [Albrecht and Bargmann, 2011], where a 2sq.cm behavioral arena made of poly-dimethylsiloxane (PDMS) is sealed to a hydrophobic glass surface. They consist of an array

of microposts, 200μm in diameter, 70μm deep and spaced 300μm apart, which allow normal sinusoidal motion by *C. elegans* seen on solid surfaces, while being exposed to liquid stimuli. Designs were generated using AutoCAD (Autodesk) and SU-8 photoresists were commercially ordered. Silicon masters were fabricated using soft lithography in collaboration with Xinyu Liu's lab in the Mechanical Engineering Department, McGill University. PDMS devices were made by mixing Sylgard 184 A and B (Dow Corning) in a 1:10 ratio by weight. We used devices either irreversibly bonded by plasma activation or reversibly sealed by clipping together glass pieces treated with (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (Sigma). Devices were degassed prior to use using a vacuum dessicator and 2.5 % Pluronic (Sigma) solution was washed through device to avoid bubbles during flow of liquid stimuli.

We used the pulse chip design [Albrecht and Bargmann, 2011] where different liquid stimuli can flow through the entire device in temporal pulses. We used alternating pulses of NGM buffer (without peptone) and *E. coli* HB101 culture grown overnight in NGM media at 28°C without shaking. We used a combination of temporal switches of one 3 minute pulse starting with buffer, followed by three 1 minute switches and three 30 second switches, recording for a total time of 15 minutes. About 20 animals were used for each device and the assay replicated over multiple trials. Videos were recorded using a Dinolite USB microscope camera at 30 fps in a custom-made chamber with diffused lighting from a LED panel.

Analysis

Videos were decimated using the VirtualDub software and background subtracted in Fiji. Individual animals were recognized by defining a threshold of pixels for each worm. The automated tracking option in the TrackMate plugin (Fiji) was used to get (x,y) coordinates of each animal over the length of the assay period. The coordinate values were used to draw direction vectors sampled over every alternate time frame (lag). The angle between two consecutive vectors was defined as the bearing angle and calculated as the cosine of the two directional vectors. The raw data was analysed on JMP 11.2 (SAS).

3.7 Data Analysis

Our plate assays were generally reliable and there was always a consistent difference between well-fed and post-dauer foraging in the CB4856 strain. However, temperature and humidity variation in the lab often increased or decreased exploration of both conditions. These effects were independent from our developmental stress conditions (data not shown). However, to account for this, we always used data where different conditions were tested on the same day under same conditions. We did not have any prior expectation of the distribution of our data, so we chose non-parametric methods for analysing statistical significance. The Wilcoxon Rank Sum Test was used when comparing between two strains or conditions. All statistical analysis were performed and graphs made with JMP11.2 software (SAS). Videos were formatted using the VirtualDub1.10.4 (Avery Lee) software and analysed using plugins in Fiji [Schindelin et al., 2012] .

Chapter 4

Results

4.1 Animals experiencing early life starvation-induced dauer show adaptive tuning of foraging behavior in a *C. elegans* wild isolate

To explore whether early-life starvation stress has any effect on adult behavior, we looked for behavioral differences between animals grown in abundance of food, termed "well-fed" and animals rescued from starvation-induced dauer, termed "post-dauers". In the laboratory, foraging by *C. elegans* involves leaving a bacterial lawn on an agar plate.

We conducted our initial Lawn-leaving assays using the lab reference strain, N2, and a wild-isolate sourced from pineapple fields in Hawaii, CB4856, referred as HW henceforth. Assays were repeated over multiple days and data pooled together (n=80 for N2; n=120 for HW). N2 animals had a high percentage of animals remaining on the lawn and there was no significant difference between well-fed and post-dauer animals (Fig4.1). Well-fed animals of the HW CB4856 strain showed a significantly lower number of animals remaining on the lawn, in comparison to N2 animals and their post-dauer counterparts (Fig4.1). On an average, nine out of ten animals of the post-dauer animals chose to remain on the food patch, instead of the characteristic high lawn-leaving behavior shown by the well-fed animals.

It is possible that in a population of worms, some may be more exploratory than the others.

Observing behavior of individual animals is a way to get around this potential caveat of our previous assay method. We used the Trail Assay and measured the area covered by a single animal as it foraged outside the bacterial lawn on which it was placed. This was quantified by measuring number of squares of a grid through which the animal's tracks passed. We confirmed that the N2 strain did not exhibit any plasticity in behavior in response to passage through dauer, and there was no significant difference ($p>0.05$) between number of squares entered by post-dauers and well-fed animals (Fig4.2) (n=50). In the HW CB4856 strain, the number of squares explored by post-dauers (n=40) were significantly fewer than well-fed animals (n=53; $p<0.001$).

To induce animals to have more lawn-leaving behavior, we used the Dot Matrix Assay where a single animal was placed on a grid of tiny food patches (Fig4.3). HW well-fed (n=19) animals had higher foraging rates and explored more number of patches than post-dauers (n=14) ($p<0.05$). The distance travelled from the original patch was quantified with as a summation of scores associated with individual patches, with patches further away having higher scores. The well-fed animals travelled further and had a higher score than post-dauers ($p<0.001$). This assay proved to be somewhat time-consuming too, as the grids needed to be made with precision. Only the HW strain was tested using this assay, and it corroborated the findings from the other assays.

4.2 The role of genetic background in long-term plasticity of foraging behavior

C. elegans isolates have a range of exploratory foraging activity and lawn-leaving propensity [Bendesky et al., 2011]. The HW CB4856 strain is known to have markedly higher baseline levels of lawn-leaving behavior [Bendesky et al., 2011] compared to the N2 strain. Our lawn-leaving rates in these two strains matched with previous literature [Bendesky et al., 2011], where the HW animals had significantly higher leaving behavior than N2 animals (Fig:4.1). Interestingly, we found that the adaptive plasticity in foraging behavior occurs in the wild-isolate HW,

but not in the N2 strain. In both the lawn-leaving assay and the trail assay, N2 post-dauers had similar behavior to its well-fed counterparts (Fig:4.1, 4.2).

4.3 Post-dauer animals do not show any difference while on food

The reduced foraging behavior shown by the post-dauer animals may indicate a defective locomotory system, rather than a choice to be more cautious in their approach to exploration. To explore this possibility, we used the Grid Assay and quantified the area explored on a plate completely covered with food. No significant difference was found between the movement by the post-dauer and the well-fed animals on food, for both HW (n=20) and N2(n=16) strains (Fig 4.4) ($p>0.05$). Further analysis using single worm tracking did not reflect a reduced speed for the post-dauer animals (preliminary data not shown). This supports our "thrifty phenotype" hypothesis and show that post-dauer animals do retain a memory of the early-life stringent conditions and choose to exploit an available food source rather than exploring.

4.4 Necessity and sufficiency of going through dauer for adaptive tuning of adult foraging

Our hypothesis was that starvation in early development acts as an environmental stressor which alters adult foraging behavior. In its natural environment, *C. elegans* often face starvation conditions and most animals observed in the wild are found to exist in the dauer stage [Frézal and Félix, 2015]. Thus the adaptive effects of experiencing starvation-induced dauer should be ecologically relevant. Going into the dauer development arrest gives a clear indication that stringent environmental conditions have affected the animal's developmental pathway.

When starved at early adulthood, only a sub-population of animals go into dauer and can winter out stressful conditions. To differentiate between whether transient starvation without going through dauer is sufficient, or whether the experience of dauer is necessary, we performed

Trail assays (refer Materials and methods) with animals which were starved equally but did not go in the dauer stage (post-starvation). These animals were starved for the same time period and in the same developmental time window as post-dauer animals. We found that the experience of dauer is indeed necessary to show a lower exploratory phenotype (Fig 4.5). Exploration by post-starvation HW animals was not significantly different from well-fed ones ($p<0.001$). We found the Trail Assay to be the most efficient method of observing foraging behavior for a large number of animals, and used it to test out further conditions.

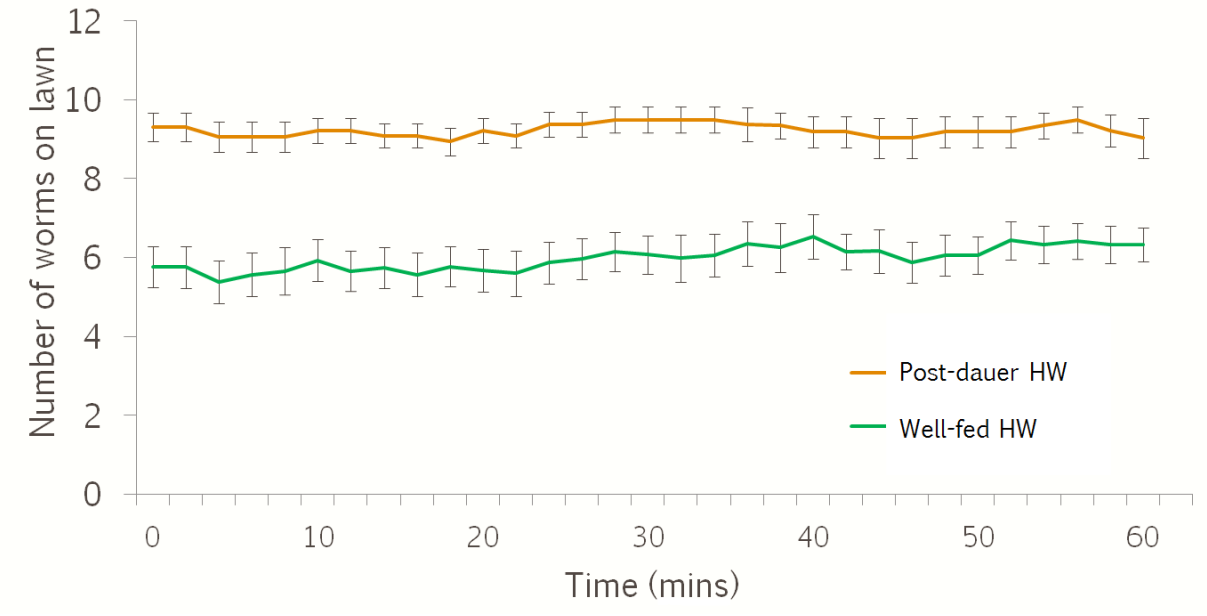
C. elegans also exhibit another arrest stage called L1 diapause, which occurs when eggs hatch on a environment with no food [Baugh, 2013; Rechavi et al., 2014]. It has been argued that the L1 diapause is more like a starvation induced quiescence, and not a true diapause like the dauer stage, where the arrest occurs at a specific stage in the life cycle, is initiated by specific environmental cues, and, metabolism is altered in preparation for stress [Baugh, 2013]. Animals in the L1 arrest phase are stress resistant and are used to maintain frozen stocks. Recent work has showed that post L1-diapause worms have impaired feeding behavior, changes in brood size, higher incidence of males in the progeny, and, increased tolerance to heat stress which could be transmitted over generations [Jobson et al., 2015]. Since this arrest phase is triggered by early-life starvation, we wanted to examine if this could also lead to the kind of behavioral plasticity we observed in the post-dauer animals. We performed Trail Assays on young adult animals which had experienced L1 diapause (n=15), and found no difference in their foraging strategy compared to well-fed animals (Fig 4.6) ($p>0.05$).

4.5 Determining differences in underlying locomotory patterns associated with foraging

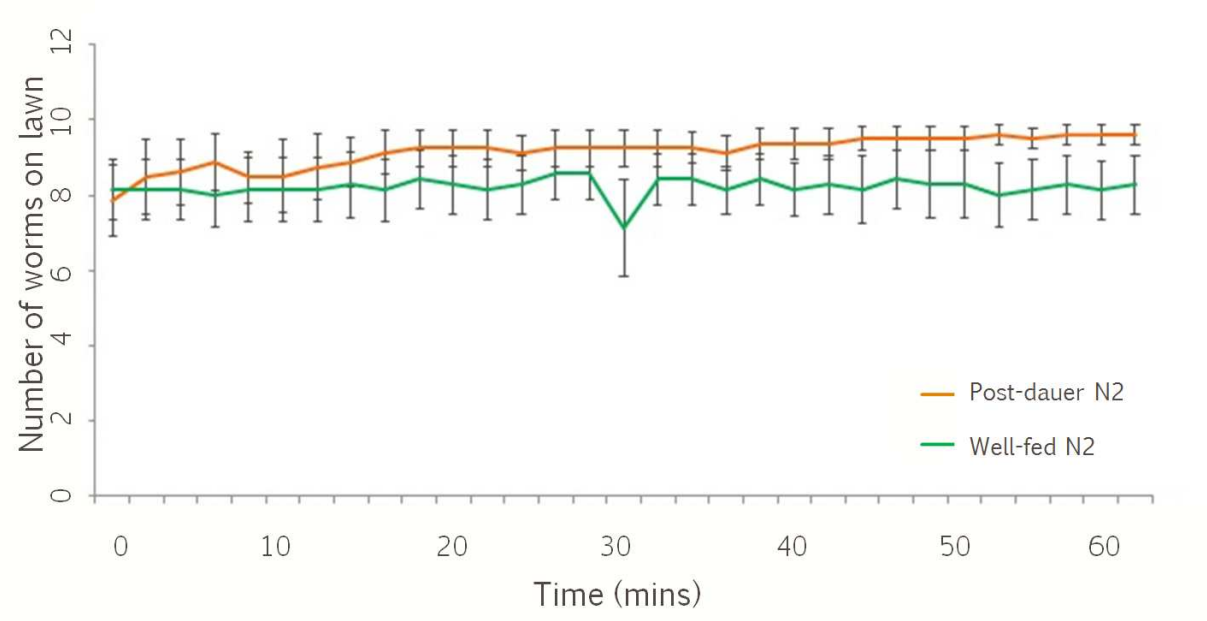
We established that post-dauer HW animals are less exploratory than well-fed animals. Next we aimed to understand the underlying locomotory dynamics involved in this reduced lawn-leaving behavior. We quantified the number of leaving events in our lawn-leaving assays. This gives us a clear readout of decisions taken in favour of exploration when animals encounter a lawn

boundary. We found that HW post-dauers had significantly lesser number of leaving events, compared to their well-fed counterparts (Fig 4.7B). They also returned less once they left the lawn. Consistent with our previous results, there was no difference between N2 well-fed and post-dauer animals.

Our lawn-leaving data suggested that even when post-dauer HWs move outside the lawn, they explore a region close to the bacterial lawn and do not cover a large area. We hypothesized that when off food, these animals may have increased number of turns and reversals, characteristic of the local-search phase in worm foraging and thus would show differences in the microfluidic setup of the pulse chip where we can expose them to temporal pulses of food versus buffer. We observed both well-fed (n=20) and post-dauer (n=20) HW animals show larger bearing angles corresponding to turns when in the buffer stream indicative of the local search behavior. Contrary to our expectations, we did not observe any obvious difference between the post-dauer and well-fed conditions (Fig 4.8).

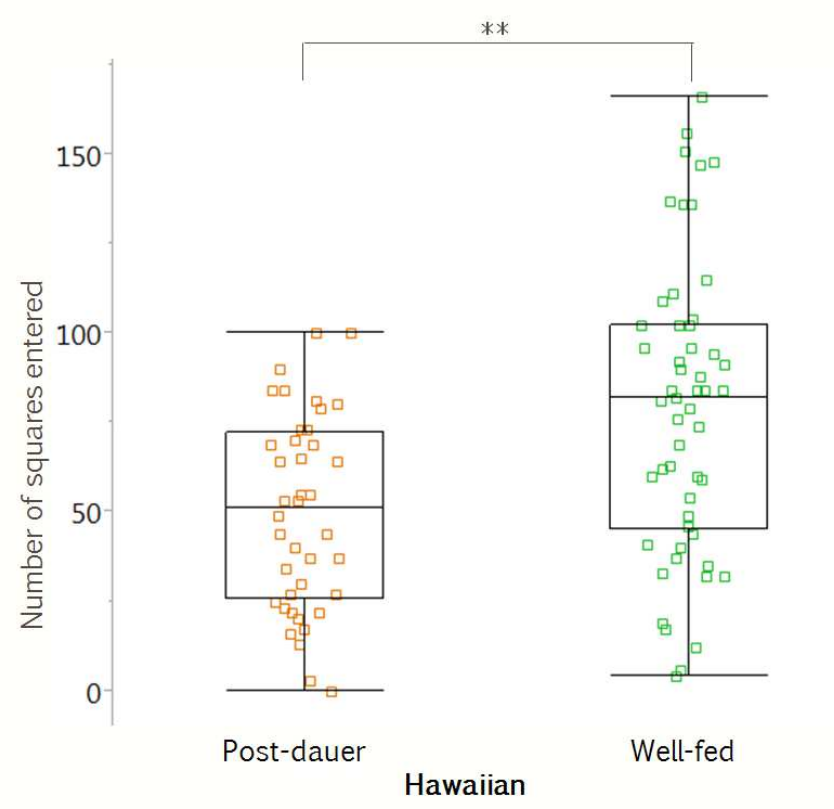


(A) Lawn-leaving assay- HW

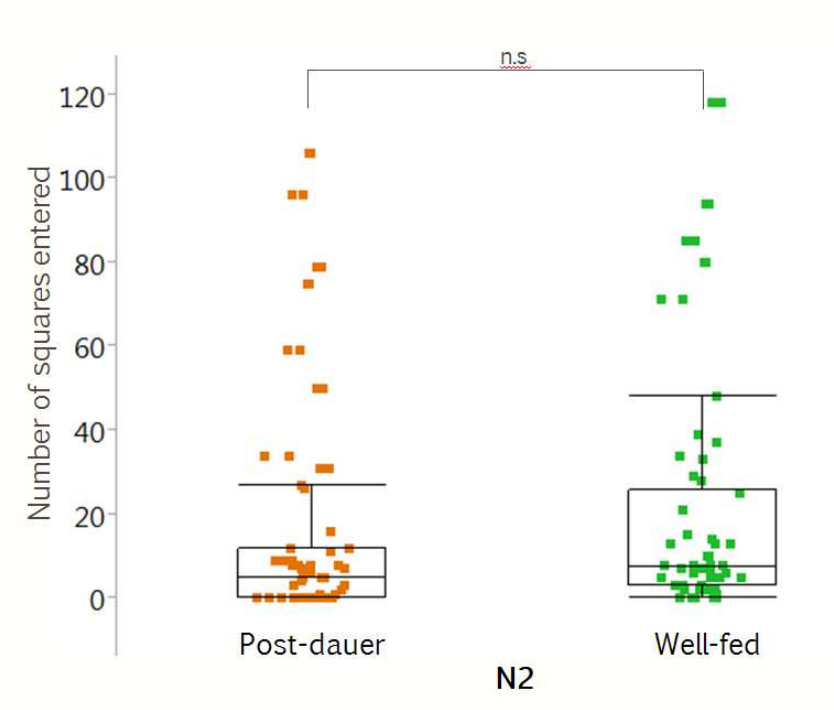


(B) Lawn leaving assay-N2

Figure 4.1: Post-dauer animals show adaptive tuning of behavior. A) Lawn-leaving assays quantified number of animals off the lawn at 2-minute intervals for a 1 hour assay period. i) A larger population of the well-fed HW animals remained outside the lawn at every time interval compared to its post-dauer counterparts. ii) No significant difference was observed between the post dauer and well-fed N2 animals. Error bars, s.e.m.

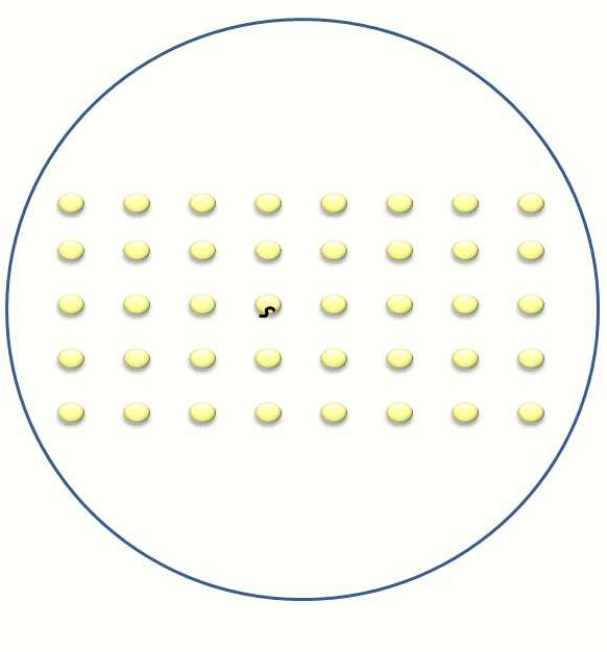


(A)

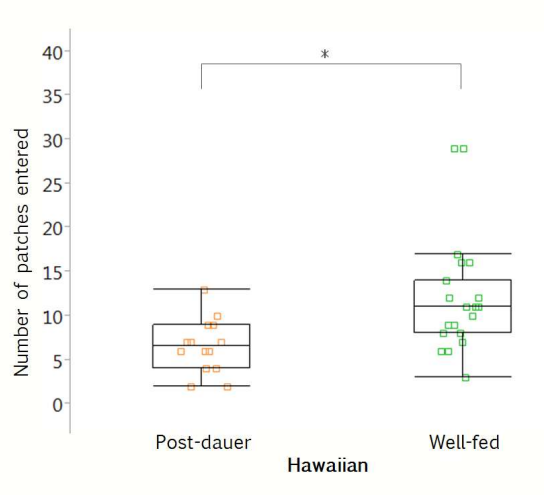


(B)

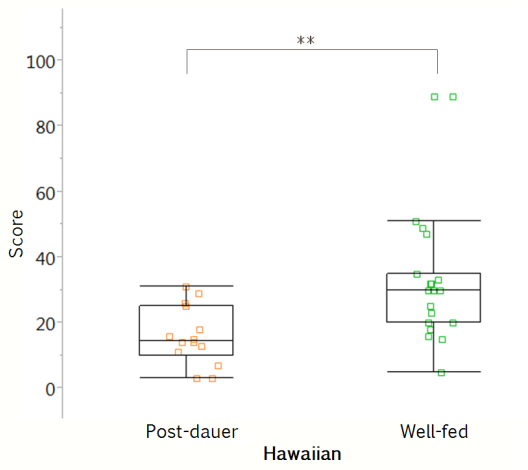
Figure 4.2: Post-dauer animals show adaptive tuning of behavior. The trail assay quantified area explored outside a bacterial lawn for a 1 hour assay period. A) HW post-dauer animals showed significantly lower foraging behavior than the well-fed animals. $p<0.001$ by Wilcoxon test. B) The N2 animals did not show a significant difference between the two sets. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s., not significant.



(A) Dot matrix assay setup



(B) Number of patches explored



(C) Scored distance travelled from initial patch

Figure 4.3: Dot matrix assay. A) A single animal was placed in the center of a 4x8 matrix of tiny bacterial lawns. B) HW post-dauer animals explored significantly lesser number of patches. $p<0.05$ by Wilcoxon test. C) Score was calculated as the summation of scores associated with individual patches, with higher scores associated with lawns farther away from initial lawn. Post animals when exploring visited neighbouring lawns, and had a significantly lower score ($p<0.01$) by Wilcoxon test. * $p<0.05$, ** $p<0.01$.

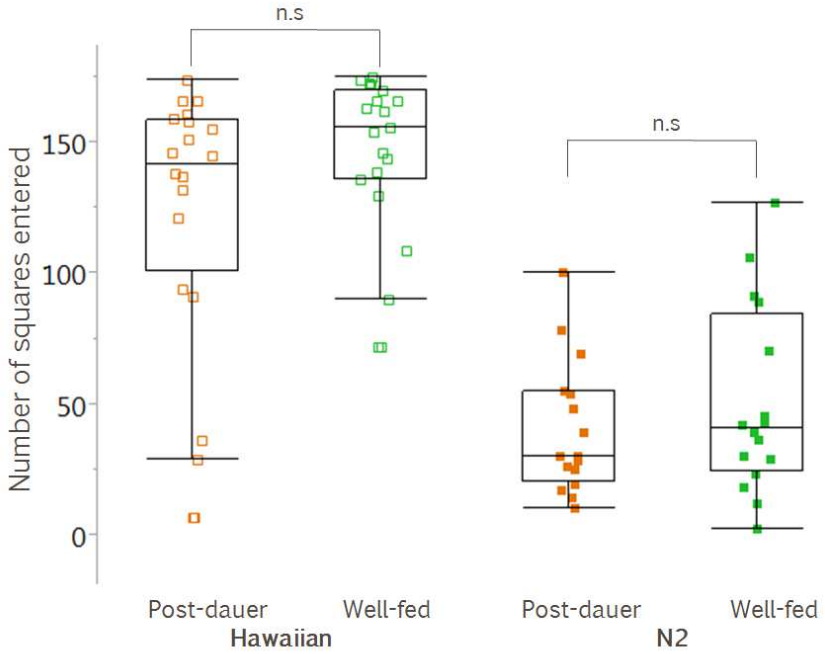


Figure 4.4: Movement on food. Quantification of the area explored on a plate completely covered with food showed no significant difference between post dauer and well-fed animals, of both N2 and HW strains. $p>0.05$ by Wilcoxon test. n.s., not significant

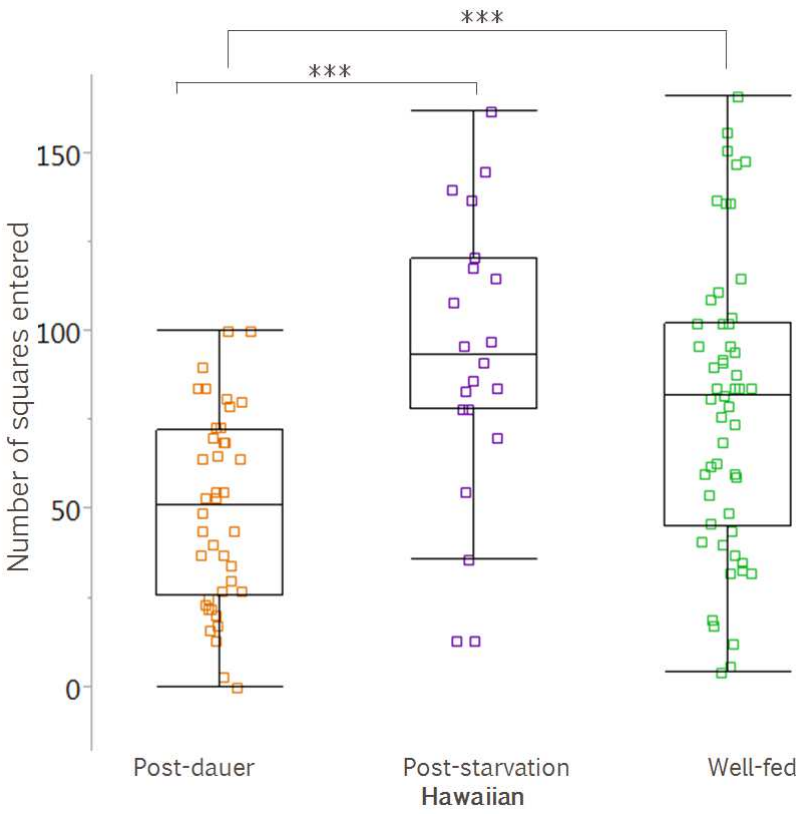


Figure 4.5: Necessity of dauer. Trail assay with worms starved equally but not going through dauer (Post-starvation) showed that Post-starvation animals forage similar to well-fed animals. Both Post-starvation and Well-fed animals show significantly higher foraging behavior than post-dauers in the HW strain. $p<0.001$ by Wilcoxon test. *** $p<0.001$

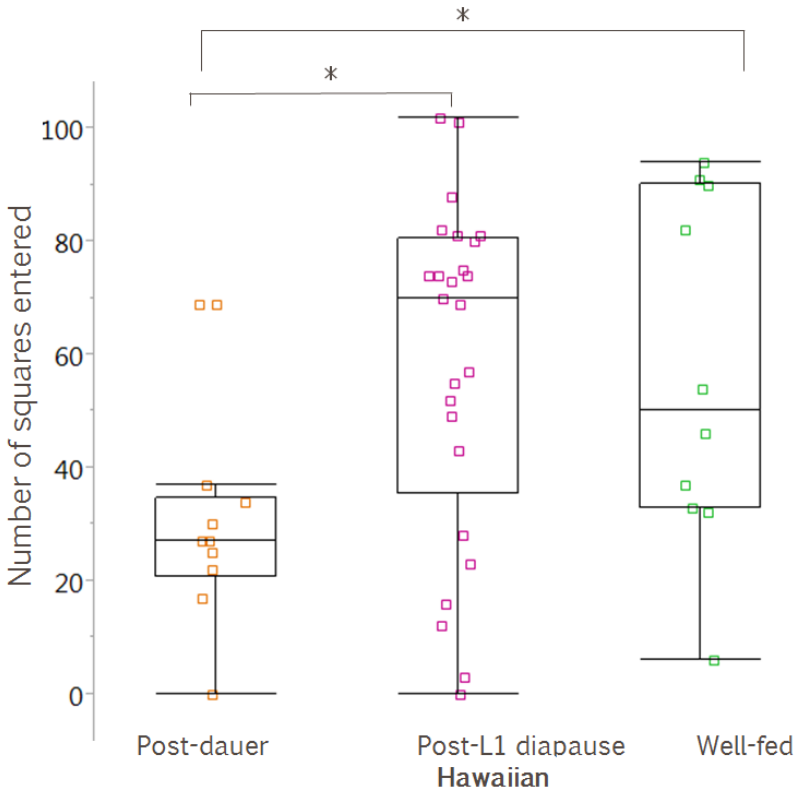
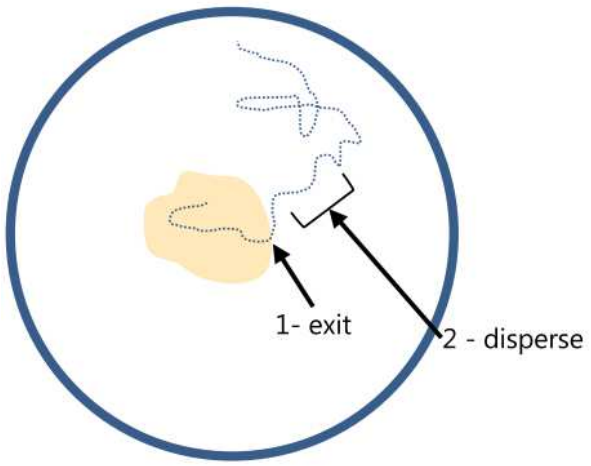
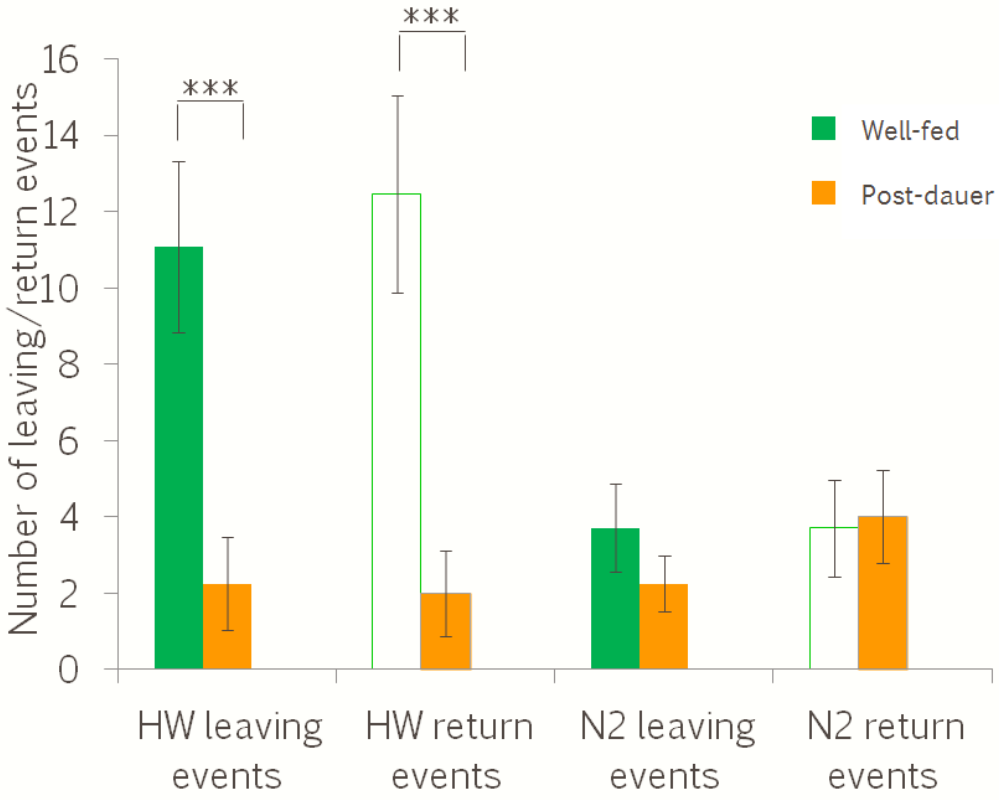


Figure 4.6: Post L1-diapause animals do not show reduced foraging. Trail assay with animals previously arrested in L1-diapause (Post-L1 diapause) did not show significant difference with well-fed HW animals. Both Well-fed and Post-diapause animals showed significantly higher area explored compared to post-dauer animals. $p<0.05$ by Wilcoxon test. * $p<0.05$



(A) Diagrammatic representation of lawn boundary decision



(B) Lawn leaving events

Figure 4.7: A) A decision to exit when encountering food boundary favours foraging exploration. Otherwise an animal can also decide to reverse and stay on the lawn. B) HW well-fed animals have a significantly higher number of patch-leaving and return events compared to post-dauer animals. N2 animals did not show a significant difference between the two sets. ($p<0.0001$, Wilcoxon test)

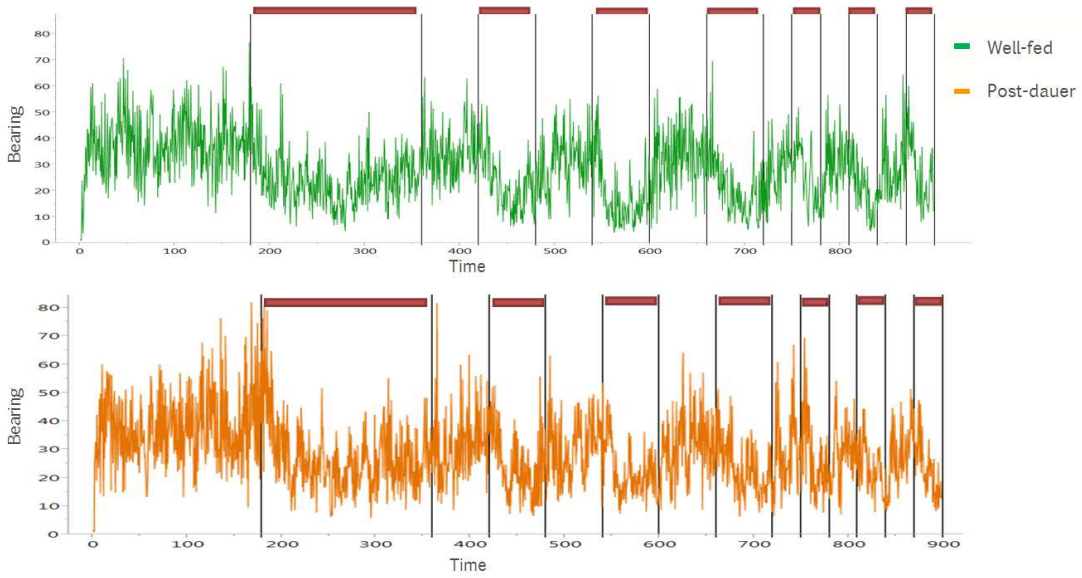


Figure 4.8: Change in bearing angle. Mean bearing angle was measured for 15 minutes with alternating temporal pulses of bacterial culture (food) and buffer. Red bars indicate stimuli i.e. food presentation. Time is measured in seconds.

Chapter 5

Discussion

5.1 Long-term behavioral plasticity in response to early-life starvation in the wild isolate CB4856

Our results showed that the experience of starvation-induced dauer can permamnently affect foraging behavior in the *C. elegans* wild isolate HW CB4856. These observations are consistent with our theoretical frameworks, where animals experiencing starvation can predict their environment to be low in resource availability (Fig2.1), and hence adapt to be more cautious in their approach, and would prefer exploiting a food patch at hand, rather than being more exploratory (Fig2.3). The domesticated N2 strain did not exhibit environmental dependence on adult foraging behavior (Fig4.1, Fig4.2). Both the well-fed and post-dauer N2 animals preferred to remain on the lawn for most part of the assay period. This lack of plasticity may be explained by the domestication of N2 under laboratory conditions, where they do not expect a patchy environment. Alternatively, the low baseline foraging rate of N2 animals may be making it difficult to detect possibly even lower foraging rates by the post-dauer animals.

Polymorphisms in a number of genes, notably *npr-1*, incurred as a result of domestication in lab conditions, has markedly changed foraging patterns and aggregation behaviors on food in N2 animals [Bendesky et al., 2011; de Bono and Bargmann, 1998; Milward et al., 2011]. The high activity *npr-1* allele is known to be involved in the lower baseline lawn-leaving behavior in the N2 strain [Bendesky et al., 2011]. It would be interesting to explore if *npr-1* is involved

in regulating this plasticity in foraging behavior. This can be answered by looking at behavior of: 1) N2 with Hawaiian *npr-1*, 2) Hawaiian with the N2 gain-of-function *npr-1* mutation, 3) Recombinant inbred lines with a combination of N2 and HW haplotypes [Bahrami and Zhang, 2013], and 4) LSJ1, a N2-like strain, but without the *npr-1* polymorphism.

Population genomic studies in a number of *C. elegans* wild isolates identified strains which are more N2-like or HW-like [Rockman and Kruglyak, 2009]. We selected three such strains with the HW *npr-1* allele- MY1, JU258 and CB4853, but different baseline lawn-leaving rates and conducted Trail assays with them [Bendesky et al., 2011]. Our preliminary results show that there is a marked decrease in foraging rates in post-dauers of these strains, similar to our HW phenotype (data not shown). This indicates that the deficit of post-dauer behavioral change in the N2 animals may be attributed to its acquired genetic polymorphisms due to adaptation to the lab environment.

5.2 Behavioral plasticity is dependant on going through dauer

We set out to explore effects of early-life starvation stress, but found that the behavioral plasticity we observed were dependant on the experience of dauer. Classical imprinting seen in new-born geese [Lorenz, 1935] or in homing behavior in salmon [Nevitt et al., 1994] show that early life experiences can be crucial in affecting adult behavior. We tried to define a critical period for early-life starvation to affect adult foraging behavior. Starving *C. elegans* till they enter a arrested developmental phase ensures that all animals experienced starvation stress in the same developmental time window. Recent work has shown that post-L1 diapause animals have slower growth, reduced fecundity and abnormal feeding behavior [Jobson et al., 2015]. On the contrary, previous studies on post-dauers have reported higher brood size and increased fecundity [Hall et al., 2010]. This seems to suggest that animals passing through these two arrest phases adapt very different survival strategies later in life. The permanent change in foraging behavior that we observed were seen only in animals which experienced dauer, and not in response to starvation stress in other larval stages.

Dauer entry involves detection of a number of sensory cues and can be induced by starva-

tion, high temperature and high levels of dauer-pheromone present in high population densities [Golden and Riddle, 1982; Hung and Zhen, 2014]. It is possible that post-dauers may differ depending on the cause of dauer entry. It will be interesting to test if adult foraging behavior gets affected only on experiencing starvation-induced dauer or whether all post-dauers show the phenotype. Exploratory/dispersal behavior may also be affected by previous experience of crowding. We plan to induce crowding-dependent dauer by exposing animals to high concentrations of dauer pheromone, in the presence of ample food and check if these post-dauers show our observed behavioral changes. However, dauer being a stage induced under stressful environmental conditions, it may be possible that all post-dauers adapt a "cautious" phenotype as adults.

5.3 Understanding underlying locomotory patterns associated with reduced foraging in post-dauers

Our assays identified that post-dauer HWs have lower lawn-leaving behavior than well-fed animals and also explore less area outside a food patch. One can speculate that this decision making may be taking place at the lawn boundary where animals either choose to continue a forward run and favour exploration or do a reversal or turn to go back into the bacterial lawn. Single worm tracking can help in identifying decisions at the lawn border more carefully.

The other behavioral difference one can expect is in a longer time scale of minutes, where *C. elegans* employ local or global search strategies. We used microfluidic devices to explore this. Varying humidity and temperature in the current lab conditions led to variability in plate conditions for some of our plate assays. *C. elegans* are sensitive to both these factors and show isothermal tracking and preference towards specific humidity conditions when placed in different conditions from their maintainence conditions [Luo et al., 2006; Russell et al., 2014]. The use of microfluidic devices can overcome this problem, but also creates an environment which can be very different from the mechanical stimuli an animal experiences on an agar plate. When animals were exposed to alternating stimuli of bacterial culture and buffer in a

microfluidic pulse chip, we did not find any difference between the post-dauers and the well-fed worms. Different groups have used different analysis and computational strategies to quantify *C. elegans* navigation [Gray et al., 2005; Calhoun et al., 2015; Yemini and Brown, 2014]. We are still working on our analysis methods to better quantify individual turns and reversals instead of an overall change in bearing angles. But it is unlikely that our current analysis methods could not detect overall changes in number of changes in direction, which is characteristic of a local search mode. The temporal change in stimuli presentation used in the pulse chip is not a close representation of our lawn-leaving assays. Another microfluidic design - the striped stimulus pattern chip [Albrecht and Bargmann, 2011] can offer a closer replication of our plate assays. Alternating stripes of bacterial culture and buffer can be used in this device resembling a more realistic patchy foraging environment. The time spent on each of these stripes and quantification of border crossing events can support data from our lawn-leaving assays.

Chapter 6

Conclusion

This project used the nematode *C. elegans* to explore long-term plasticity in foraging behavior, in response to a starvation-induced developmental arrest stage, dauer. Environmental stressors in childhood have been linked to development of debilitating diseases in later life in humans. It is difficult to find causal relationships to such complex disorders in mammalian systems, and hence simpler model organisms like *C. elegans* are ideal for studying the effect of environment on neural circuits regulating complex behavior. Various forms of short-term plasticity have been observed in worms, and epigenetic traits in response to environmental stress have been found to be heritable. However, permanent alteration of adult behavior in response to larval nutritional stress has not been reported before. In this thesis, we showed that post-dauer animals of the HW CB4856 strain show long-term plasticity in their foraging behavior and have reduced exploratory activity. Interestingly, we also established that this plasticity is absent in the lab reference strain, N2 and thus depends on the genetic background of the animal. Since worms share conserved genetic, metabolic and developmental with mammals, this project will add to our understanding of developmental plasticity in response to environmental stress.

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