THE GENETIC BASIS OF NATURAL VARIATION IN THE RESPONSE TO ADULT STARVATION IN *CAENORHABDITIS ELEGANS*

by

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DISSERTATION ABSTRACT

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Title: Assessment of Natural Variation in the Response to Adult Starvation in *Caenorhabditis elegans*

Caenorhabditis elegans typically feeds on rotting fruit and plant material in a fluctuating natural habitat, a boom-and-bust lifestyle. Moreover, stage specific developmental responses to low food concentration suggest that starvation-like conditions are a regular occurrence. In order to assess variation in the *C. elegans* starvation response under precisely controlled conditions and simultaneously phenotype a large number of individuals with high precision, we have developed a microfluidic device that, when combined with image scanning technology, allows for high-throughput assessment at a temporal resolution not previously feasible and applied this to a large mapping panel of fully sequenced intercross lines. Under these conditions worms exhibit a markedly reduced adult lifespan with strain-dependent variation in starvation resistance, ranging from <24 hours to ~120 hours. Genome-wide mapping of the responses of more than 7,855 individuals suggests four loci of large effects. Three of these loci are associated with single genes (*ash-2, exc-6,* and *dpy*-28) and the fourth is a ~26 KB region on Chromosome V encompassing several genes. Backcross with selection confirmed the effect of the Chromosome V locus. Overall, there is a clear genetic basis for natural variation in the response to food availability within this species.

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CHAPTER I

INTRODUCTION

Adaptation by natural selection hinges entirely on variation in fitness among individuals. Traits that have a clear and direct bearing on fitness are termed 'life history traits' and since life history traits determine an individual's survival and reproduction they are the major components of fitness (Houle, 2001; Brommer, 2007; Nussey, Wilson, & Brommer, 2007; Braendle, Heyland, & Flatt, 2011). Therefore, the study of life history evolution is about understanding adaptation, seeking to explain how evolutionary forces such as natural selection shape organisms to optimize reproduction and survival when faced with ecological challenges from the surrounding environment.

Life history theory historically views evolution as a problem of optimization (Parker & Maynard Smith, 1990; Houle, 2001; Braendle, Heyland, & Flatt, 2011). The idea being that given a set of ecological factors (such as availability of food or presence of predators) that impact an organism's ability to survive and reproduce, and given that there are constraints and trade-offs intrinsic to the individual, evolution will drive that population toward a combination of life history traits that maximizes reproductive success (Bennett & Lenski, 2007; Braendle, Heyland, & Flatt, 2011; Guillaume & Otto, 2012). To find a solution for this problem it is then necessary to define: 1) what the extrinsic, environmental factors are and how they affect survival and reproduction; and 2) how intrinsic trade-offs and other constraints limit the ways in which the traits themselves can evolve. Moreover, there must be genetic variation underlying the affected traits because natural selection can only increase or decrease the frequency of alleles. If

there is no genetic variation, there is nothing for selection to act upon. This can be a bit tricky, however. A common assumption is that phenotypic variation in a trait is directly dependent on underlying genetic variation and that population-level changes in phenotypes over time arise via natural selection acting on ancestral genotypes (Stearns, 2000; Sane, Miranda, & Agashe, 2018). This is a very logical assumption and there are clear examples of phenotypes for which this is the case. For example, multiple alleles affecting the persistence of lactase in humans have been identified. Each is associated with a distinct geographic region and period of time suggesting that there have been repeated independent origins of lactase persistence driven by natural selection during the onset of dairy farming in these regions (Itan, Jones, Ingram, Swallow, & Thomas, 2010). Therefore, the variation in phenotype of lactose tolerance/intolerance is directly tied to the genotype associated with lactase persistence.

However, such a direct relationship is not always the case. For example, one of the most commonly studied traits is height. It is easily measured and because it has a normal distribution within populations statistical analysis is relatively straightforward. A 2015 study showed that any influence of natural selection acting on height was minimal, if it exists at all, suggesting there is no significant force to push the trait in any particular direction (Stulp, Barrett, Tropf, & Mills). The genetic basis for height as evidenced by twin studies consistently has a heritability of 80-90% suggesting a strong genetic basis for heritability in this trait (Silventoinen, et al., 2003; Jelenkovic, et al., 2016). Conversely, when variation in height is examined through genome-wide mapping approaches (looking across individuals rather than within families), a large number of associated genes are identified but they collectively account for a relatively low

proportion of the variation in height (~45%) (Wood, et al, 2014; Shi, et al., 2016). So how can it be that the genetic basis of heritability for height could be so strong while the genetic basis for variation is relatively small?

One possibility is that common single nucleotide polymorphisms (SNPs) with effect sizes so small they are far below statistical significance are so numerous that they account for much of the missing variation in complex traits (Anderson, et al., 2014; Shi et al., 2016; Boyle, et al., 2017). Several studies looking for the 'missing heritability' in human complex trait studies have supported this (Yang, et al., 2010; Shi, Kichaev, & Pasaniuc, 2016). For example, Shi et. al. (2016) found low-effect common variants to account for 21% of the heritability in forearm bone mineral density and 94% of the heritability in levels of high-density lipoprotein.

Another likely contributing factor is plasticity – the ability of a single genotype to produce different phenotypes across different environments. Several studies conducted in *Arabidopsis thaliana* and maize examining the genetic basis for plasticity in traits such as flowering time point to the conclusion that plasticity itself is largely governed by myriad small-effect loci, suggesting that the phenotypic effect of the small-effect loci which appear to underlie the 'missing heritability' are, at least in part, the genetic element responsible for the phenotypic manifestation of plasticity (Buckler, et al., 2009; Fournier-Level, et. al., 2011; Li, Cheng, Spokas, Palmer, & Borevitz, 2013; Sasaki, Zhang, Atwell, Meng, & Nordborg, 2015; Gage et al., 2017). Because plasticity is a modulation of the phenotypic expression of a genotype, it can be acted upon by natural selection and affect the genetic response across environments. Moreover, if there is adaptive variation in plasticity itself (different genotypes have different levels of plasticity), then selection can

be expected to move toward an optimal reaction norm which maximizes fitness across multiple environments (Stearns, 2000; Anderson, Wagner, Rushworth, Prasad, & Mitchell-Olds, 2013). This also suggests that plasticity in a trait can act as a buffer against environmentally induced changes in other traits in order to achieve or maintain optimality of fitness (Gage et al., 2017). All of these effects can act singly or combined to create a scenario where there is more phenotypic variation than can be accounted for by a straightforward relationship with genotypic variation. However, the phenotypic effect of plasticity is a response to variation in the extrinsic variable of the environment, so studies which hold the environment rigorously constant should be able to minimize the variation due to plasticity allowing the intrinsic genetic basis to be more clearly examined and thus contribute to a better understanding of the actual components on which natural selection acts.

Another factor influencing the evolution of life history traits in addition to optimization via plasticity, is the constraint of fitness trade-offs (Stearns, 1989; Zera & Harshman, 2001; Morales-Ramos, Kelstrup, Rojas, & Emery, 2019). If there were no constraints, then fitness, i.e. survival and reproduction, would evolve to be maximal at all life stages. Logically, this would lead to "Darwinian demons" (Law, 1979), organisms that begin to reproduce as soon as they are born, produce infinite offspring, and essentially live forever. However, these organisms do not exist. Extrinsic resources are finite and intrinsic life history traits are subject to trade-offs, so natural selection is constrained to maximize fitness within the limits of extrinsic and intrinsic boundaries. Typically, trade-offs are described by negative phenotypic or genetic correlations between components of fitness within an individual such that an increase in fitness in one

trait results in a decrease in fitness via another. At the genetic level, this can be the result of a single gene that affects multiple traits (pleiotropy) or be due to selection acting on an allele that is in some way coupled to another so that they are frequently passed on together (linkage disequilibrium). This could possibly be a result of physical proximity, genes are so close to each other that recombination has not separated alleles, or there could be some functional constraint where certain combinations of alleles have been optimized such that they segregate together.

An example of antagonistic pleiotropy is the *age-1* gene in the nematode *Caenorhabditis elegans*. Hypermorphic alleles at this locus can extend adult lifespan up to 80% longer than the wild-type allele but at the expense of reduced starvation resistance such that when food is intermittent they are rapidly outcompeted by wild-type individuals (Walker, McColl, Jenkins, Harris, & Lithgow, 2000; Jenkins, McColl, & Lithgow, 2004). Linkage disequilibrium can be illustrated by a connection between alleles of the human leukocyte antigen (HLA) locus and the *HFE* hemochromatosis gene, mutations in which can lead to the excessive retention of iron in the body. Individuals with an H63D mutation at this locus, which has the potential to cause hemochromatosis, have an unusually high frequency of also carrying the HLA-A29 allele despite the fact that they are separated by ~4 MB (Cardoso et al., 2002; Pacho et al., 2004; Yassin et al., 2014; Rodriguez et al., 2015). However, individuals with this allelic combination, while having a predisposition toward development of hemochromatosis, have also been demonstrated to have a higher than average level of $CD8^+$ T lymphocytes, which play a critical role in the speed at which the immune system recognizes and responds to pathogenic antigens (Cardoso et al., 2002). This suggests this may be a trade-off between a selective

advantage for linking this combination of alleles despite the disadvantage of the tendency toward hemochromatosis (Cardoso et al., 2002).

At a more physiological level, trade-offs can be caused by antagonism in the allocation of limited resources within an individual. For example, when faced with a shortage of food, processes typically increasing growth and reproduction may generally be slowed in favor of investing energy in processes important for tissue maintenance, stress resistance, and extended survival (Templeman & Murphy, 2017). However, this type of trade-off may have a genetic basis and different genotypes may differ in how resources are allocated. Therefore, variation in trade-off strategies can drive genotypically grouped subsets of individuals in a population to optimize fitness in a differential manner and examination of this variation can be informative of how natural selection is acting on a population.

Historically it has been difficult to examine questions of how natural selection acts on the genetic basis of life history traits. Given the problem of missing heritability and the influence of plasticity, it has been difficult to robustly define what the genetic basis is when traits are complex. Moreover, the ubiquitous nature of small effect loci combined with observations that the en masse effect can be of phenotypic importance in heterogenous environments suggests that pleiotropy is pervasive as the effects of these loci are not unique to any one specific trait (Walsh & Blows, 2009; Boyle, Li, & Pritchard, 2017). But, this also supports a genetic basis for trade-offs to constrain optimization in response to natural selection. Examining the genetic basis of complex life history traits can give direct insights into the structure of pleiotropy and provides a foundation for functional analysis. Given knowledge of how extrinsic environmental

factors and intrinsic organismal factors affect survival and reproduction, an optimality framework can be used to examine adaptation by natural selection. In summary, examining variation in life history traits contributes to understanding how natural selection drives populations to optimize reproductive success and shapes the evolution of phenotypes in the process.

Dissertation Outline

The research described in this dissertation was done using the nematode *Caenorhbditis elegans* as a model system. As a model organism *Caenorhabditis elegans* is ideal for the study of the evolution of life history traits. *C. elegans* typically reproduces via hermaphrodite self-fertilization but males do occur, usually at a low frequency, and hermaphrodites are capable of outcrossing (Barriére & Félix, 2005). Because of this reproductive system, inbreeding is the norm and nearly clonal populations can exist without detrimental effects but it is also possible to use outbreeding to manipulate the levels of genetic variation in a controlled fashion. *C. elegans* also has a fully sequenced and well-characterized genome which enables the identification and characterization of genetic information (The *C. elegans* Sequencing Consortium, 1998). The time required for *C. elegans* to produce a new generation is 3-4 days and large populations can readily be maintained in a small amount of space making it feasible to maintain and study multiple cohorts simultaneously with large enough population sizes to facilitate the statistical power needed for GWA mapping.

The project described in Chapter II of this dissertation investigates the genetic basis of starvation response, a life history trait in *C. elegans*, and discusses the evolution

of two diverging strategies for optimizing reproduction during periods of starvation. There is ample evidence that starvation is a regular occurrence in wild populations of *C. elegans* and the resulting lack of nutrition has a direct impact on both reproduction and survival making the study of starvation response both ecologically and evolutionarily relevant as a life history trait (Barrière & Félix, 2014; Frézal & Félix, 2015). Previous work has shown that *C. elegans* has two very different strategies for reproducing when starved, one in which adult hermaphrodites sacrifice themselves to matriphagy and one where they effectively put reproductive maturation on hold (Angelo & Van Gilst, 2009; Seidel & Kimble, 2011; Burnaevskiy et al., 2018). However, studying starvation response in *C. elegans* has historically been complicated for two main reasons: 1) individuals try to flee in search of food and end up dying from causes other than starvation so assays have been limited to relatively small sample sizes and 2) because *C. elegans* are bacteriovores it is very difficult to maintain a starvation environment free of potentially edible contaminants without introducing complicating effects of handling and intervention during the course of the experiment. To address these issues, I created a novel microfluidic approach which has several advantages over traditional starvation assays. First, I created a microfluidic device which houses fifty worms per device in a contained space such that they are individually separated from each other and cannot flee. This device has constant perfusion of a sterile solution so any potential environmental contaminants are immediately removed without any handling or intervention and it is physically structured so that worms move in the same sigmoidal pattern as they would in their natural environment, i.e. even though this is a liquid based environment, worms are crawling rather than swimming. Second, one entire device is ~20 mm x 70 mm so fifty

individual worms are housed in an area of 1400 mm^2. Traditionally, the equivalent experimental setup would require fifty petri plates with a 35 mm diameter. When laid out side-by-side this amounts to ~61,250 mm^2, a ~44-fold difference in the amount of space required. Third, previous assays required manual observation of worm lifespans resulting in limitations on the temporal resolution of the data. I coupled the microfluidic device with an automated imaging system. In this system, the microfluidic devices are placed on flatbed computer scanners (Epson V700, model B11B178011) allowing simultaneous imaging of 600 individuals per scanner (12 microfluidic devices per scanner). The main limitation in this system is the time required for the scanner to complete a scan at the chosen resolution, and in this experiment that time limit was 4 minutes per microfluidic device. This results in a temporal resolution of 50 individuals being assayed every 48 minutes, a much finer scale than previously possible with manual assays. Moreover, this resolution scales with the number of scanners. Using two scanners at a time, 7,855 individuals were assayed for this study.

The *C. elegans* lines used in this project represent a unique genetic resource that allowed genome-wide mapping of the genetic basis of starvation response to be done with a degree of precision not available otherwise. The *Caenorhabditis elegans* Multiparental Experimental Evolution (CeMEE) mapping panel was derived in such a way that linkage disequilibrium is greatly reduced (Noble et al., 2017). This allows for the phenotypic effects of individual loci to manifest without confounding effects from linked loci. In addition, because *C. elegans* primarily reproduces through self-fertilization, each individual line in the CeMEE panel is essentially a population of homozygous clones. Therefore, genetic variation is spread between lines and not within

lines. When assayed at the scale previously mentioned, this allows for a much clearer picture of the relationship between a given genotype and the starvation response phenotype.

Lastly, I used a novel alternative mapping approach to verify the results of the GWA mapping. Backcrossing with selection is a method to isolate genes with large effects, originally proposed by Sewall Wright in 1952 and traditionally used for 'improvement' in breeding programs or to examine the additive effects of a quantitative trait locus (Wright, 1952; Hill, 1998; Luo, WU, & Kearsey, 2002; Hospital, 2005). The idea is to introgress a characteristic from a donor parent into the genetic background of a recurrent parent thereby isolating loci of relatively large effect (Wright, 1952; Hospital, 2001; Luo, WU, & Kearsey, 2002). As generations progress, offspring are selected for the characteristic of interest and backcrossed to the recurrent parent. When selection is applied, the proportion of the donor genome reduces by half at each generation except on the chromosome with the locus of interest where the rate of decrease is slower (Hill, 1997; Hospital, 2001; Luo, WU, & Kearsey, 2002; Hospital, 2005). Typically, this results in a signature of linkage drag where the loci of interest are selected for but so is the linked surrounding region, therefore using this scheme to precisely identify the genetic basis of a quantitative trait is not feasible. However, in this study, backcross with selection is not being used to identify loci of interest, rather it is applied as a technique to verify loci previously identified through GWA mapping. In addition, as previously discussed, the CeMEE panel lines used in this study have markedly reduced linkage disequilibrium so the effect of linkage drag should be correspondingly reduced. To our

knowledge this is the first instance of this technique being applied to *C. elegans* and thus represents a novel use of backcross with selection.

In Chapter III, I summarize the work from Chapter II and discuss how natural selection is likely acting on genetic variation for starvation response in *C. elegans* to produce two diverging life history traits.

CHAPTER II

HIGH THROUGHPUT ASSESSMENT OF NATURAL VARIATION IN THE RESISTANCE TO STARVATION STRESS IN *C. ELEGANS* USING MICROFLUIDICS

Introduction

In a natural ecological setting organismal behavior is governed by their energetic state, which in turn directly impacts how that organism invests its energy when the availability of resources in the environment is uncertain. These behaviors typically manifest as trade-offs between investing energy in somatic maintenance or allocating resources toward reproduction, which may lead to delays or shifts in reproduction over the course of an individual's life under conditions of reduced resource availability. For example, multiple bird species change how they invest in broods when resources are scarce, partitioning resources toward somatic development and delaying or foregoing reproduction for the season (Covas, Doutrelant, & du Plessis, 2004; Shaw & Levin, 2012; Mourocq et al., 2016). Similarly, some arachnid species address the challenge of limited resources in a manner completely counter to this via increased investment in reproduction to the point of facultative matriphagy (Stearns, 1989; Evans, Wallis, & Elgar, 1995; Kim, Roland, & Horel, 2000; Tizo-Pedroso & Del-Claro, 2005; Salomon, Aflalo, Coll, & Lubin, 2015). Other species have evolved to physically and temporally separate somatic growth and maintenance from reproduction, particularly in migratory birds and fish that leave their natal homes for more resource-rich environments before returning back to their breeding grounds and investing the acquired energy resources directly into

reproduction (Stabell, 1984; Weber, Ens, & Houston, 1998; Bradbury et al., 2014; Moore et al., 2014). In its most extreme form, for instance in salmonids, the transition from somatic energy acquisition to reproductive investment is total, resulting in one-time, semelparous reproduction and death (Briggs, 1953; Kindsvater, Braun, Otto, & Reynolds, 2016).

Physiological effects of resource limitation can also more directly and immediately affect how individuals partition energy toward reproduction when nutrition is limited. For example, many mammalian species can delay implantation of fertilized eggs such that fetal development is postponed until nutrition is adequate, or for other species menstrual cycles become irregular and spontaneous abortion rates increase, and for unicellular organisms, nutritional state controls whether an individual develops to a reproductive state at all (Sandell, 1990; Bulik, et al., 1999; Trites & Donnelly, 2003; Kempes, Dutkiewicz, & Follows, 2011). The fact that such a broad diversity of species have evolved many independent mechanisms to partition resources in the face of nutrient limitation suggests that the trade-off between somatic and reproductive investment is common to most organisms. What is missing is a thorough understanding of the genetic and functional basis of the underlying systems that are involved in structuring that tradeoff.

As a model organism *Caenorhabditis elegans* has been widely used in laboratory settings for nearly 50 years (Brenner, 1974). In the past approximately 15 years, the natural ecology of *C. elegans* has begun to be investigated (Félix & Braendle, 2010; Frézal & Félix, 2015). Breeding populations are typically found in decomposing vegetation in a continuum of settings ranging from human-affiliated (orchard and urban

garden) to wild (forest and riverbank) (Félix and Braendle, 2010; Barriére and Félix, 2014). Data collected from the same site over several periods suggests a boom-and-bust lifestyle. Genotypes have been observed to expand then shrink then expand, or go extinct followed by repopulation then disappear again (Félix and Braendle, 2010; Richaud, Zhang, Lee, Lee, & Félix, 2018). Given that *C. elegans* feeds on the bacteria associated with rotting plant material, which is subject to seasonal cycles in natural settings, it is likely that starvation conditions are a regular occurrence. Developmentally, *C. elegans* has unique stage specific responses to low food concentration which suggests that starvation-like conditions are naturally a regular occurrence in its environment and starvation a historically selective evolutionary pressure. If no food is present upon hatching, individuals arrest in the first larval stage (L1) only exiting that stage when sufficient food can be found (Johnson, Mitchell, Kline, Kemal, & Foy, 1984; Baugh, 2013; Roux, Langhans, Huynh, & Kenyon, 2016). If food is lacking early in the third (L3) or early in the fourth (L4) larval stage, development will halt in the respective stage (Schindler, Baugh, & Sherwood, 2014). If food runs low between the first and second larval stage (L2), *C. elegans* develops through the alternative dauer morph (Klass & Hirsh, 1976; Fielenbach & Antebi, 2008; Zhou, Pincus, & Slack, 2011; Roux, Langhans, Huynh, & Kenyon, 2016).

In addition to starvation induced larval arrest and induction of the dauer morph, a developmental response to starvation occurs in some individuals at the transition from the last larval stage (L4) to reproductive adult, termed adult reproductive diapause (ARD) (Angelo & Van Gilst, 2009; Seidel & Kimble, 2011; Burnaevskiy et al., 2018). If starvation occurs during this time, most individuals will continue to develop into

reproductive adults and die via facultative matricide (aka "bagging") because egg-laying is inhibited by starvation (Seidel & Kimble, 2011). However, in those worms that do not bag, the intestine and somatic gonad atrophy, dead embryos appear in the uterus, and the germline becomes reduced while other tissues continue to show signs of aging (Angelo and Van Gilst, 2009; Seidel and Kimble, 2011; Burnaevskiy et al., 2018). When food is reintroduced, these individuals exit ARD, growth resumes, germline stem cells repopulate, atrophy of the intestine and somatic gonad reverse, and animals become again capable of producing progeny, living a normal adult lifespan.

There is extensive knowledge regarding *C. elegans* molecular and cellular biology along with its development and behavior. But, nearly all this information has come from a single, lab-domesticated genotype, largely ignoring the genetic diversity and natural variation present within the species and the related variety in phenotypes (Sterken, Snoek, Kammenga, & Andersen, 2015). Because the primary mode of reproduction is self-fertilization and the rate of outcrossing extremely low, populations sampled on the scale of <10 m often contain mostly individuals with identical or nearly identical genotypes (Barriére & Félix, 2005; Félix & Braendle, 2010). Even in samples taken from actively reproducing populations where mixed genotypes were found, very few are heterozygous and when examined repeatedly over a few years no lasting recombination was observed (Barriére & Félix, 2007; Rockman & Kruglyak, 2009; Andersen et al., 2012; Richaud, Zhang, Lee, Lee, & Félix, 2018). Recent simulations suggest these small-scale population dynamics are the result of demes founded by 3-10 individuals, suggesting the population structure is more appropriately depicted as 'coexisting and competing homozygous clones' (Richaud, Zhang, Lee, Lee, & Félix, 2018). Further, low levels of outcrossing

lead to coadaptation of genomic loci. This results in selection acting against heterozygotes and/or recombinant genotypes and in fact outbreeding depression is frequent in *C. elegans* (Barriére & Félix, 2007; Dolgin, Charlesworth, Baird, & Cutter, 2007). So, while the use of a single genotype has facilitated many discoveries, the genetic diversity of *C. elegans* represents an interesting and largely untapped resource. Moreover, this natural population structure suggests that life history traits have the potential to be adapting via natural selection in parallel, sharing the same set of extrinsic factors while evolving under diverging internal constraints. Given the known differences in adult *C. elegans* responses to food availability, this is very likely the case in the context of starvation response.

Since the primary behavior of *C. elegans* when food becomes scarce is to leave the area, this typically results in individuals in a plate-based environment climbing up the walls of the plate where they desiccate and die, complicating phenotypic analysis of starvation response in these environments. Liquid-based assays can accommodate larger numbers without the loss to 'walling', but this leads to changes in behavior and physiology within the worms (Pierce-Shimomura et al., 2008). Moreover, both approaches are subject to the buildup of metabolic byproducts, which lead to the progressive alteration of the environment over time (Kaplan et al., 2011). This can be minimized by transferring individuals to fresh plates and/or conditions, but the physical handling and manipulation required introduces the possibility of stress and injury. The amount of labor required by these assays also limits the throughput and temporal resolution, such that most assays performed with larger samples are conducted with population cohorts and lack individual longitudinal information. Here, we develop a

microfluidics approach that allows individual responses to be assayed at high temporal resolution using an automated scanner-based image acquisition system (based on Banse, Blue, Robinson, Jarrett, & Phillips, 2019). We are able to standardize the environment by using constant perfusion to flush metabolic byproducts and use an artificial dirt (pillared) structure (Lockery et al., 2008) so as to allow worms to display plate-like behavior and physiology.

We coupled the scale, resolution, and throughput of microfluidics with whole-genome data for association mapping of adult starvation response in *C. elegans* using 72 recombinant inbred lines (RILs) from the *C. elegans* multiparental experimental evolution (CeMEE) panel (Noble et al., 2017). These lines were created by hybridization of 16 wild isolates, followed by 140 generations of mixed selfing and outcrossing then a further 50 generations selfing, all under standard laboratory conditions (Figure 1). The CeMEE panel captures 22% of the known polymorphisms segregating in wild populations and greater than 95% of the genome harbors nucleotide diversity. Moreover, intrachromosomal linkage decays to near background levels by 0.5 cM on average and interchromosomal disequilibrium is weak across chromosomes (Noble et al., 2017). In contrast, as mentioned above, natural populations have high linkage disequilibrium and the average SNP diversity is ~0.3% (though in hypervariable regions it can reach >16%) (Cutter, 2006; Thompson et al., 2015). The low diversity and high linkage of natural populations can complicate standard GWAS approaches because correlated SNPs covering long genomic regions make it difficult to pinpoint loci relevant to the trait being studied and multiple correction testing becomes overly conservative (Graustein, Gaspar, Walters, & Palopoli, 2002; Rockman, Skrovanek, & Kruglyak, 2010; Andersen et al.,

2012). Using the CeMEE panel lines, coupled with verification via a backcross with selection approach, we are able to examine the individual effects of natural genetic variants on adult starvation resistance at a finer resolution than possible in the presence of high linkage disequilibrium. We find that the genetic basis of adult lifespan under microfluidic starvation conditions is polygenic with additive contributions from at least four loci, one which appears to be also pleiotropic for the ability to produce offspring after extended starvation.

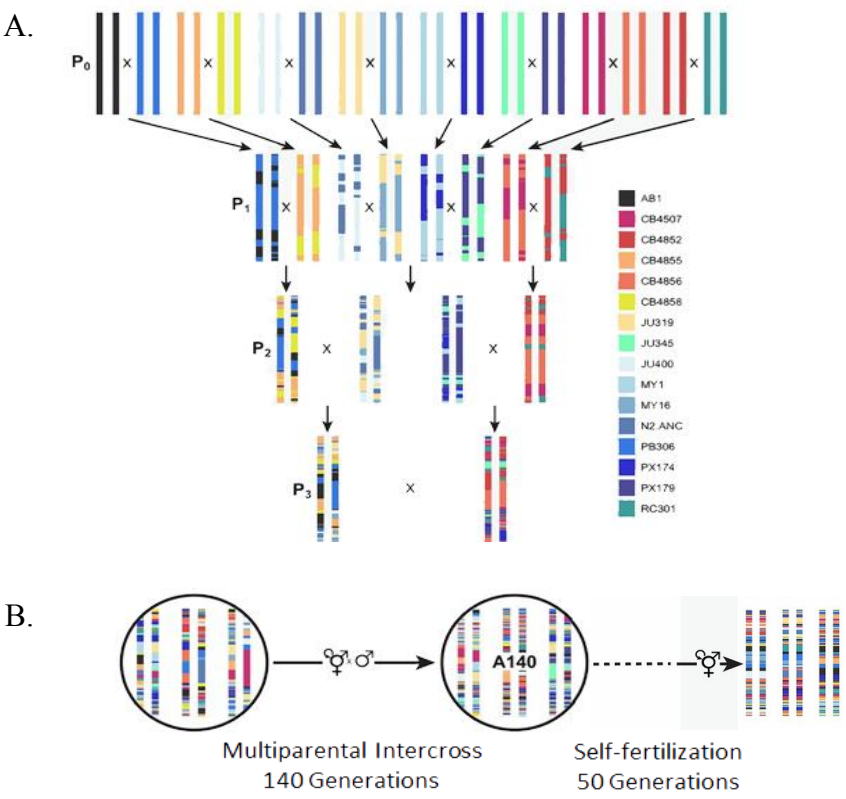


Figure 1: Derivation scheme for the C. elegans Multiparental Experimental Evolution (CeMEE) panel. The CeMEE panel captures 22% of the known polymorphisms segregating in wild populations and greater than 95% of the genome harbors nucleotide diversity. Intrachromosomal linkage decays to near background levels by 0.5 cM on average and interchromosomal disequilibrium is weak (r^2 0.99, 0.95 quantiles = 0.538, 0.051 within chromosomes vs. 0.037, and 0.022) across chromosomes. Adapted from Noble et al., 2017.

Materials And Methods

Mapping Lines

All *C. elegans* strains were maintained with standard culturing conditions on NGM lite agar with *E. coli* OP50 as a food source and maintained at 20°C (Brenner, 1964). The strains used for genetic mapping are recombinant inbred lines (RILs) from the *C. elegans* Multiparental Experimental Evolution (CeMEE) panel (Noble et al., 2017). The strains used here are listed in Supplemental Table I.

Phenotyping of starvation response using microfluidics

The Starvation Arena (Figure 2) was designed with Vectorworks Fundamentals (Vectorworks, Inc.). Using standard soft lithography methods (Whitesides, Ostuni, Takayama, Jiang, & Ingber, 2001), single layer devices were fabricated with polydimethylsiloxane (PDMS) and bonded to a glass microscopy slide by air plasma exposure (see Banse, Blue, Robinson, Jarrett, & Phillips, 2019). A biopsy punch was used to open inlet and outlet channels. Embryos were harvested via bleaching and immediately placed onto OP50 seeded plates at a density of ~5000 individuals/100 mm petrie plate. When populations reached the L3 larval stage, additional OP50 was added in excess so individuals could feed ad libitum. Populations were monitored until ~3 early adults were visible. At this time the majority of individuals in the population were in either very late L4 larval stage or extremely early adulthood. Hermaphrodites were immediately picked into a prepared microfluidic chip (see section below) at a density of 1 individual per arena (50 arenas per chip), and image acquisition initiated. All starvation experiments were performed in S Basal lacking cholesterol (5.85g NaCl, 1g K_2HPO_4, 6g KH_2PO_4, H_2O to 1 liter) sterilized by autoclaving.

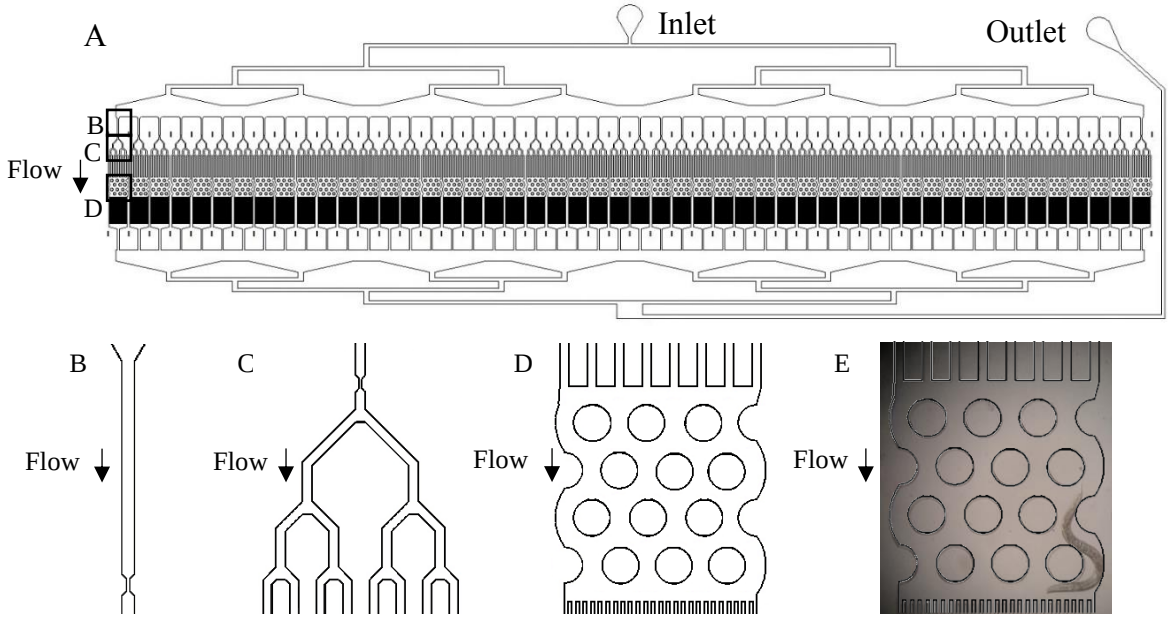


Figure 2: Microfluidic chip design. A) The device is designed to have a single array of 50 pillared arenas. Flow through the device enters at the inlet hole, perfuses through the device, and exits at the outlet hole. B) Movement of worms into the arenas is facilitated by a 0.52 mm upstream holding channel (1 per arena) with a 0.015 mm constriction point. This enables pre-loading of 50 animals followed by a brief increase in pressure sufficient to move the animals into the arenas. C) Each arena has a bifurcated section preceding the pillared portion to keep animals in the arenas. D) The arena is ~1.2 mm x ~0.9 mm with twelve 0.2 mm diameter pillars spaced 0.1 mm apart. E) An individual arena housing an adult *C. elegans* hermaphrodite.

Experimental Protocol (Microfluidic Device Setup)

Four hours before use, microfluidic starvation arenas (aka chips) were first filled with an 1% w/v solution of Pluronic F-127 dissolved in S-basal made w/o cholesterol then allowed to sit for ~40 minutes. This procedure lowers adhesion to the PDMS surface (Wu, 2009). Care was taken to ensure that no air was allowed into the chips from this point forward. The chips were then flushed with S-basal lacking cholesterol and 50 worms picked into the inlet port of each device. Tubing was connected to the chips and additional S-basal flushed through under manually controlled pressure until all worms were aligned properly in the array formation at the constriction point (Figure 2). Then,

pressure was manually increased to a level sufficient to cause the expansion of the constriction point such that worms pass through and into the pillared portion of the arena. Lifespan assays of *C. elegans* using the same constriction point in a similar device but done in the presence of food result in a normal lifespan suggesting the passage of worms through the constriction point in this manner does not result in injury (P.C. Phillips, personal communication).

Once worms had moved into the arenas, chips were placed on flatbed document scanners (Epson V700, model B11B178011) and connected to a mechanically pressurized fluid system with inflow air pressure set at 3 psi (Figure 3). To ensure that pressure stayed consistent throughout the course of a given experiment, an intermediate air tank was used as a buffer. In this system the pressure source coming in to the intermediate tank was ~80 psi and subject to potential transient increases or decreases as the initiating compressor turned on and off. In order to buffer against these potential fluctuations and reduce the pressure to an appropriate level, a regulator was placed on the outflow side of the intermediate air tank such that the flow of fluid into the chips was ~ 3 psi (Figure 3).

The air-line exiting the buffer tank was bifurcated into 4 tubing lines, each pressurizing a sealed 1 L bottle of S-basal made without cholesterol as previously described. The bottle caps had been modified with 4 ports: 1 terminal inlet port for air tubing and 3 exit ports for liquid supply lines. Each liquid supply line extended from the basal surface of the 1 L bottle to an inlet port of a microfluidic chip placed on a scanner (Figure 3). Once all chips were connected and fluid flow visually verified by monitoring

the outflow through the arena device exit ports, image acquisition was initiated. Fluid flow was continuous throughout the course of any given experiment.

Image Analysis and Survival Estimation

Scans of an individual microfluidic chip were initiated every 4 minutes in a sequential manner resulting in a rate of 1 image every 48 minutes for each of 12 chips per scanner. Images were collected for a period of 5 days using *C. elegans* Lifespan Machinery software (Stroustrup, et al. 2013; for additional technical details see Banse, Blue, Robinson, Jarrett, & Phillips, 2019). Image processing was performed using ImageJ 1.50i (Schneider, Rasband, & Eliceiri, 2012). TIFF images were downloaded as a stack (one stack per chip) with brightness and contrast adjusted as necessary. Image stacks were manually scanned and worms examined individually. An individual was determined dead when movement was no longer visible for a minimum period of 4 frames (3 hours and 12 minutes). Individuals still alive at the conclusion of the experiment were censored and included in this manner. Analysis of Kaplan-Meier survivorship curves with log rank test for statistical differences was performed using JMP Statistical Software (SAS Institute Inc., JMP Pro 13, 2017). Possible differences in the shape of survivorship curves were assessed using the fit to a Gompertz mortality model using the R package flexsurv (Jackson, 2016).

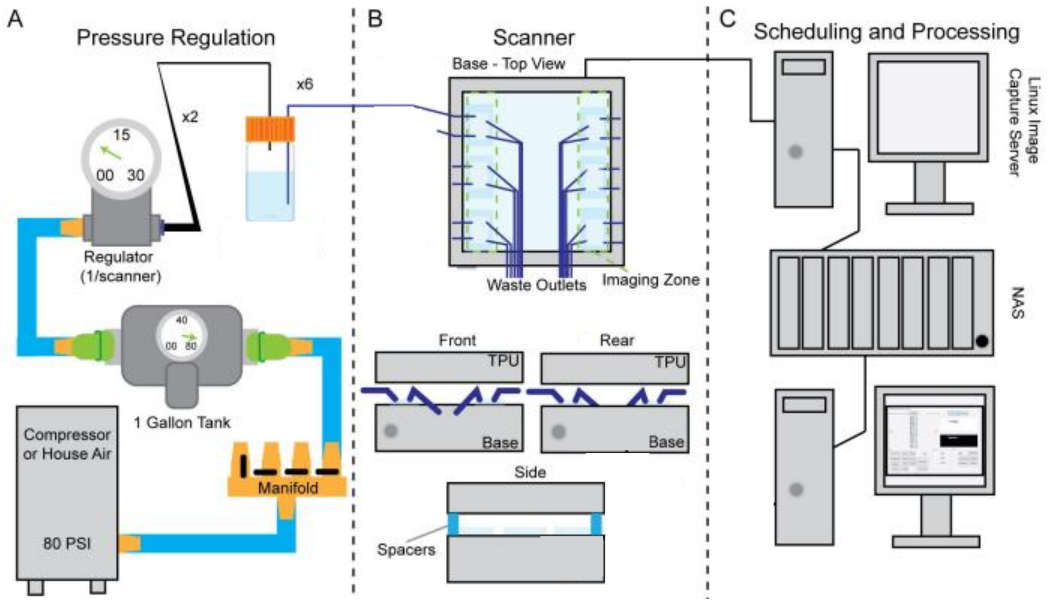


Figure 3: Pressure regulation and image capture schematic. A) Pressurized air was split through a manifold into a 1-gallon cushion tank. Air exiting the cushion tank was regulated with a microregulator set to 3-4 psi. This air was then passed through bifurcated tubing (Rain Bird ¼" blank tubing and ¼" barbed couplers) and into four 1-liter bottles. Each bottle lid had four 1.5 mm OD stainless steel stems passing through. One stem connected the pressurized air and the other three were connected internally to ~50 mm of tubing extending to the bottom of the bottle, and externally to 1.5 mm ID tubing extending to the inlet ports of the microfluidic chips. B) Microfluidic chips bonded to 50 mm x 75 mm glass slides were arranged on scanner with 3 slides per side (2 chips per slide), ~15 mm from the scanner edge. C) Image capture was done at timed intervals using a Linux version of the *C. elegans* Lifespan Machine software (Stroustrup et al., 2013) with images stored on a local network storage device. Image processing was performed using ImageJ 1.50i (Schneider, Rasband, & Eliceiri, 2012). Adapted from Banse, Blue, Robinson, Jarrett, & Phillips, 2019.

Genome Wide Association Test

The CeMEE strains used here contained 352,665 SNPs spread across 72 recombinant inbred lines (Noble et al., 2017, Supplemental Table 1). Using Plink v.1.9, SNPs with more than 10% missing calls across the lines were filtered from the analysis (www.cog-genomics.org/plink/1.9/; Chang et al., 2015). Further, individual lines were required to have genotypes at ≥ 95% of sites to be retained. GWAS analysis of survival

time in the absence of food was conducted on the trimmed data using an additive linear regression in Plink v.1.9, using the following options: --assoc qt-means --adjust gc. Plink binary files were converted to the rrBLUP format. GWA mapping was performed in rrBLUP using the above genotype data and the quantitative trait of time-until-death for each individual in the microfluidic starvation environment (Endelman, 2011). A kinship matrix was calculated with the default A.mat function from within the GWAS function. Finally, SNPs with a minor allele frequency of less than 5% were excluded. After applying these filters, a total of 248,374 SNPs and 7,855 individuals remained.

Backcross with Selection

In order to independently test the mapping of potential QTL influencing starvation response, a backcross with selection approach was implemented using two lines CeMEE lines that were found to display highly divergent starvation response phenotypes: A6140L110 as the 'short-lived' parent and GA450L40 as the 'long-lived' parent. Since males arise from non-disjunction of the X chromosome in meiosis and occur at a very low frequency in *C. elegans*, male-enriched lines were created by passaging an equal number of males to hermaphrodites (~20 each) every generation until a sufficient number of males was consistently present (~5 generations and ~20% male frequency). After this, 25 day 1 adult hermaphrodites were selected from each parent line (n = 50), picked to an isolated plate (1 line per plate), and allowed to self-fertilize for 24 hours in order to deplete self-sperm. Once this period had passed, hermaphrodites were transferred to a new plate and an equal number of males from the opposing parent line were added. After ~48 hours, eggs (F1 offspring) were harvested through treatment with sodium-hypochlorite (Stiernagle, 2006) and immediately placed on OP50 seeded plates.

To allow for the segregation of any potential recessive alleles, all odd-numbered generations were allowed to develop without interference and self-fertilize, with eggs harvested as previously described.

Even-numbered generations, assumed to be ¼ homozygous for loci of interest, were allowed to develop to late L4/early adulthood and picked into microfluidic chips in the same manner as previously described under the Phenotyping of Starvation Response Using Microfluidics section but with one critical difference: pressure was not increased to a level sufficient to open the constriction point and move individuals into the arenas. Worms were kept upstream of the constriction point so surviving individuals could be recovered as follows: 1) chips were placed on scanners with image acquisition initiated as previously described, 2) images were monitored such that chips could be removed from scanners when approximately 20% of individuals remained alive or 24 hours had passed, whichever came first, 3) after removal, tubing was connected in reverse so the direction of fluid flow was reversed and the remaining individuals could be manually flushed out through the inlet ports and picked onto OP50 seeded plates.

Survivors were allowed to recover/self-fertilize on OP50 seeded plates for a period of 24 hours. After this, they were picked to new plates and males from the 'short-lived' parent line were added in a 1:1 ratio. Eggs were harvested via bleaching after ~48 hours and the resulting odd-numbered generation was handled as previously described. This cycle was repeated up to and through the F11 generation.

Following thirteen generations of the above procedure (five generations of actual backcrossing), hermaphrodites were subjected to selection one final time and twenty survivors picked to individual plates as founders for near-isogenic lines (NILs, n = 20).

Each of these lines was maintained in isolation and self-fertilized for 10 generations before being phenotyped again.

Genomic Sequencing of NILs

Nematodes were maintained under standard lab conditions as described (Brenner, 1974). For preparation of genomic DNA, ~10 - 20,000 synchronized L1 staged worms in M9 media were processed with the Zymo genomic DNA Kit following Proteinase K digestion for 3-4 hours at 55° C. Genomic DNA concentration was quantified with a Qubit fluorimetric reader using the HS kit. Genomic DNA was processed for Illumina sequencing with the Nextera DNA Library kit per manufactures instructions. Individual samples were multiplexed, combined in equal molar ratios and sequenced on a Hi-Seq 4000 with 100bp of Paired end reads. (University of Oregon Sequencing Facility, Eugene, OR).

Results

Variation in Adult Lifespan of CeMEE Panel Lines under Microfluidic Starvation Conditions

We assayed 72 CeMEE panel lines for starvation response using our novel microfluidic approach (Figure 4). Each CeMEE line represents a unique combination of the genetic variants segregating within the derived ancestral population (Figure 1). Median survival times vary by up to six-fold (range from 14.1 hours to 86.5 hours, Supplemental Table I). Partitioning variation within and between lines using analysis of variation of the survival time of each of the 7855 individuals in the experiment reveals that genotype accounts for 37% of the total variation observed in the experiment

(Restricted Maximum Likelihood, upper 95% CI = 276.98, lower 95% CI = 127.68, SE = 38.09, $p < 0.0001$, JMP Pro13, 2017). Overall, there appears to be substantial variation in both median survival time and in the shape of the mortality trajectories of each line.

In this system, late L4/very early adults are removed from a plate-based environment with abundant food and placed directly into a microfluidic environment in the absence of food. The typical response of *C. elegans* individuals when food becomes scarce is to seek out a new location where food is present (Shtonda & Avery, 2006; Kang & Avery, 2009). In this experimental setup individuals can attempt to flee as normal but ultimately are retained within the pillared arena until death. The measurements presented here capture the variety of starvation responses in our CeMEE panel lines. The survivorship curves presented in Figure 4 appear to be dividable into two categories

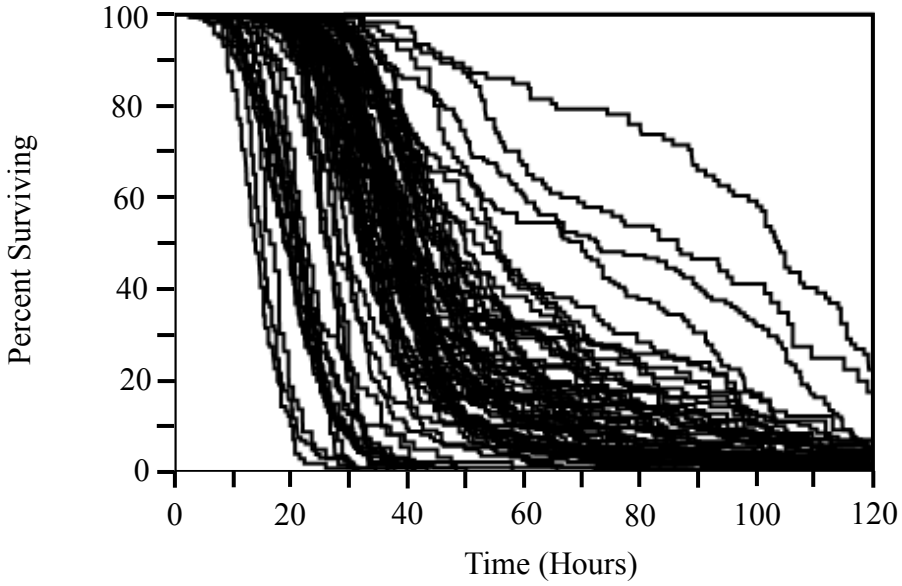


Figure 4: CeMEE panel adult starvation survival curves. Survival curves of 72 CeMEE panel lines. Each curve represents one CeMEE line with technical replicates ≥ 2; replicate assays initiated on different days. At time 0, animals are early adults and within 1 hour of placement into the microfluidic starvation environment. Analysis of Kaplan-Meier lifespan curves with log rank test for statistical differences was performed using JMP Statistical Software (SAS Institute Inc., JMP Pro 13, 2017)

based on the curve shapes. The first are curves with a sigmoidal shape. In this group, individuals tend to die off quite rapidly and these comprise lines with lower mean and median survival lengths (Supplemental Table 1). The second group are curves that have a more consistently gradual decrease in shape. The individuals in this group die off slowly and make up the lines with higher mean and median survival times. This is suggestive of two different sources of mortality in response to starvation and is consistent with previous observations of adult *C. elegans* where, when confronted with a loss of food, some hermaphrodites respond by sacrificing themselves to matriphagy and others enter a state of reproductive diapause (Angelo & Van Gilst, 2009; Seidel & Kimble, 2011; Burnaevskiy et al., 2018).

Genetic Variation and Median Survival

To identify genetic variation contributing to differences in the time-until-death phenotype under microfluidic starvation conditions, we used a GWA mapping approach. For this approach we treated the time to death of each individual as a quantitative trait (n = 7855). Plink and rrBLUP identified a similar set of SNPs as significant contributors to

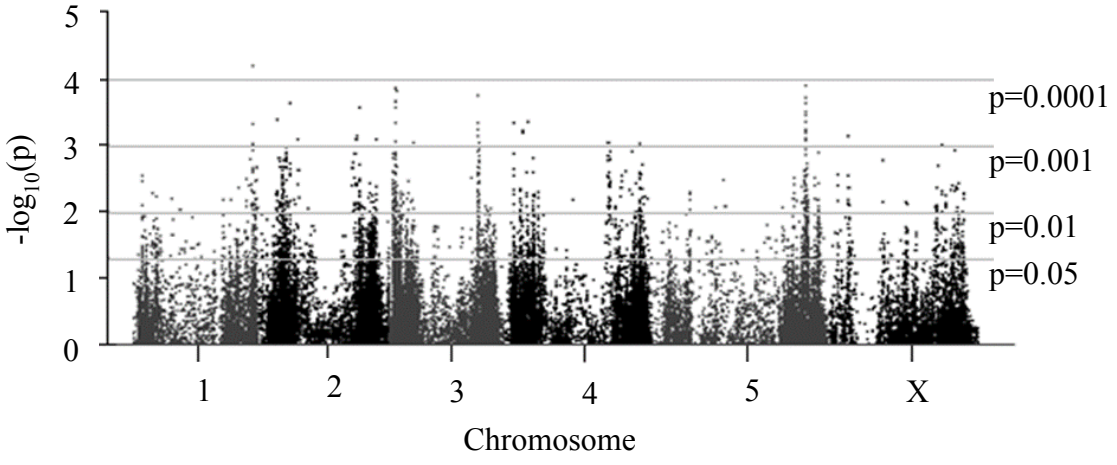


Figure 5: Genome-Wide Association Manhattan plot (rrBLUP). Distribution of variants identified through genome-wide mapping with rrBLUP.

this phenotype but with variance in the absolute levels of significance across the genome (Supplemental Figure 1, Supplemental Table 2). Because of the similarity, only the rrBLUP results will be presented here (Figure 5, Table 1).

Potential causative genetic variation underlying each QTL was identified by first extracting all SNPs from each dataset where $-\log_{10}(p) \geq 3.50$, sufficient to select the majority of SNPs with $R^2 > 0.10$ (SNPs below this value do not show a clear direction of influence on median survival time, see Supplemental Figure 2). This resulted in a total of 40 focal SNPs, however several of these were adjacent and redundant so this number was further collapsed as described in Table 1 down to a total of 15 loci. We then searched for genes within appropriate regions on either side of each SNP (see Table 1 for description of search regions). Next, we plotted median survival times against genotype to examine directionality of influence (Figure 6). Eight SNPs identified in our GWA mapping were either heavily influenced by a single outlier or the genotype/phenotype correlation did not have a clearly identifiable direction and so were not given any further consideration (Supplemental Figure 2). Four of the SNPs on chromosome V are in close proximity and largely redundant (n = 57, 55, 56, and 54), discussed in further detail below.

The most suggestive locus is on the left arm of chromosome III (BP 1,107,965). The alternate genotype is present in 46 of the CeMEE lines and represents an average drop in median survival time of ~11.5 hours. This locus accounts for 18.1% of the variation between lines. As the genotype file used for this analysis was aligned under an earlier version of the reference genome (WS245), verification of the genotypes was done in order to provide the most up-to-date position information. In this case, verification of the genotype shows a matching T → A substitution reported in the current version of the

C. elegans reference genome (WS269) at position 1,107,975, within an intron of the gene *exc-6* (www.wormbase.org).

On the right arm of chromosome III is the next locus, at position 10,768,218. This locus accounts for 17.8% of variation and reflects an average decrease in median survival time of ~11.1 hours. Thirty of the CeMEE lines have the alternate genotype. However, verification of the genotype showed the nearest matching site is a T → C substitution at base 10,768,084 in an intron of the gene *dpy-28*. This substitution partitions appropriately with the lines but in the opposite direction than expected, i.e. lines that were initially identified as having the alternate genotype match the reference at this locus and vice versa. In our dataset this locus partitioned equally with a SNP at III: 10,770,501 so these loci are more likely to be representative of an effect coming from the ~2 KB region spanning from one SNP to the other rather than either individual variant, thus making the above discrepancy not surprising (Table 1).

A third locus of interest is on the right arm of chromosome II (BP 11,986,204). This locus represents a ~10.7 hour decrease in median survival time. Twenty-five of our tested lines have the alternate genotype and verification matches a G → A substitution in an intron of the gene *ash-2* at position 11,986,223. This locus accounts for 15.6% of the variation in median survival time between lines.

A region on the right arm of chromosome V (BP 18,271,385) is the last locus of interest. It accounts for 11% of variation and reflects an average decrease in median survival of ~10 hours. Fifty-five of our tested lines have the alternate genotype, a known missense mutation in the gene *Y37H2C.4* located at position 18,271,413 in the most current version of the *C. elegans* reference genome (WS269). However, this locus represents the focal

point of a ~26 KB region where multiple genetic variants are acting together to affect median survival across the CeMEE lines. Three other loci identified as significant in the GWA mapping surround this SNP and have only slightly different membership across the CeMEE panel lines (n = 57 and n = 56 and 54, respectively; Table 1, Supplemental Table 3). Due to the difference in membership they have somewhat lower p-values and were initially examined as separate contributory sources. However, on closer examination it became apparent that the only time a significant increase in median survival was seen was when the first three variants match the reference genotype (Figure 7). The fourth variant, V:18,293,029, does not appear to contribute any additional effect (see Supplemental Figure 3 for side-by-side comparison of 3- and 4-locus combinatorial genotype effect sizes) and so it is not included in further analysis. Of our 72 lines, 53 have the alternate (-) genotype at all three loci and 5 have an intermediate combination (+/- combinations). There does not appear to be any significant difference between these lines. However, the remaining 14 lines have the reference genotype (+) at all 3 loci and this group displays the ~10 hour increase in median survival time. This is not to say that any individual SNP exerts a greater influence or that a specific combination is necessarily meaningful, more intermediate data points are needed, this is only to suggest that it is variation in this region as a whole influencing the survival phenotype.

Chromosome	SNP	log10(p)	# of lines	R^2	Genes	Region
I	14359698	4.23	8	0.11	*Y105E8A.3*	Single
II	3704344	3.66	6	0.05	*srx-107*	Single
II	**11986204**	**3.60**	**25**	**0.16**	***ash-2***	**Single**
III	922537	3.90	60	0.12	*pef-1*	Single
III	924366	3.88	61	0.11	*pef-1*	Single
III	974499	3.60	62	0.10	*R06B10.2*	108 BP
III	998200	3.71	63	0.11	*trp-2, R06B10.7*	8.2 KB
III	1010961	3.68	62	0.09	*Y34F4.2, Y3F4.6*	←1.5 KB
III	**1107965**	**3.85**	**46**	**0.18**	***exc-6***	**Single**
III	**10768218**	**3.78**	**30**	**0.18**	***dpy-28***	**2 KB→**
V	18266401	3.67	57	0.11	*R10E8.6, fbxa-213*	4 KB/26 KB
V	**18271385**	**3.94**	**55**	**0.11**	***Y37H2C.4***	**Single/26 KB**
V	18277648	3.75	56	0.12	*Y37H2C.1, Y37H2C.5*	2.7 KB/26 KB
V	18293029	3.54	54	0.12	*Y51A2A.12*	Single/26 KB
V	18312716	3.75	24	0.06	*fbxa-117*	Single

Table 1: SNPs identified through GWA mapping as significant contributors to median survival length in the microfluidic starvation environment. SNPs with -$\log_{10}$(p) above 3.5 were selected. The cutoff of 3.5 was chosen because it was sufficient to capture the majority of significant SNPs with R^2 values above 0.10. In a few instances there were multiple adjacent SNPs of equal significance, in these cases the SNP located in the middle was chosen as a representative SNP and the size of the associated region is listed in the Region column. See results section for detailed information on the Chromsome V region. In two instances there were two adjacent SNPs with equal significance values, for these there is an arrow indicating the direction of the region associated with the representative SNP. Associated genes were identified by searching within the specified region or within 1 KB on either side for single SNPs. After examination of the per line median survival times plotted by genotype (Figure 6), the four SNPs in bold have clear directionality and were considered the loci of most significance (also see Supplemental Figure 2).

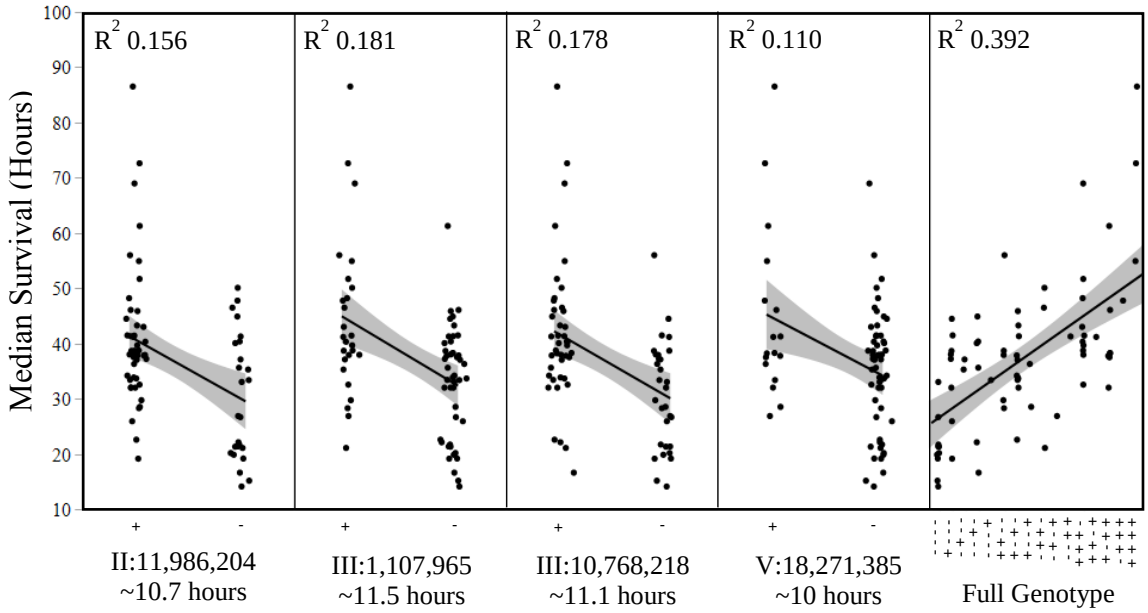


Figure 6: Effect sizes of SNPs contributing to differences in median lifespan under microfluidic starvation conditions. Genotypes denoted as '+' are the N2 reference allele. All alleles are biallelic.

Because linkage in our tested lines is low, the genetic components underlying the variation seen here are likely to be influencing the trait in a mostly additive fashion. If this is true, then the expectation is that for any line with a genotype containing a combination of two or more advantageous alleles, the increase in lifespan over those without the alleles should equal the sum of the increase for those with the respective single allele:

$$A_1B_1 \ldots X_1 = (\overline{A}_1 - \overline{A}_0) + (\overline{B}_1 - \overline{B}_0) + \cdots + (\overline{X}_1 - \overline{X}_0)$$

This appears to be the case. Figure 6 shows the best fit line and the median survival time values for all genotype combinations in this dataset. While the overall increase predicted by the best fit line is approximately 30 hours, the observed increase across the mean

values for the two extremes is 42.3 hours which nearly matches the strictly additive model prediction (10.7 + 11.5 + 11.1 + 10 = 43.3). However, more data points for the double and triple genotype combinations are needed to confirm this with certainty.

Backcross with Selection

Because of the crossing strategy used to create the CeMEE mapping panel, each line represents a mosaic of variants segregating in natural populations. But, due to the multiple generations of inbreeding by selfing after the initial crossing, the variants are largely fixed within lines, with each line largely representing a static mosaic of fixed variants spread throughout the genomes (Figure 1). With this in mind we chose to use a

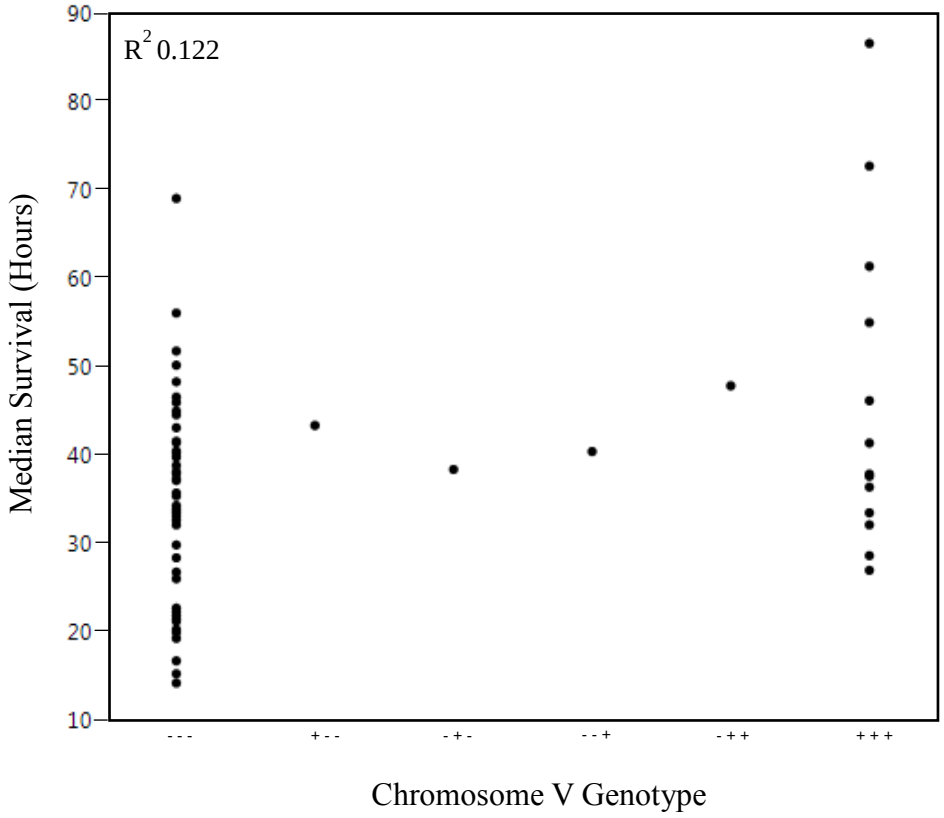


Figure 7: Effect size of combinatorial genotypes at Chromosome V locus. Median survival time values plotted against the combinatorial genotypes for the significant loci identified through GWA mapping within the ~26 KB region on the right arm of Chromosome V. Alleles denoted as '+' match the N2 reference.

backcross with selection strategy to isolate the variants contributing to starvation response. We chose one line which displayed the slow-dying/long-lived phenotype and another which displayed the fast-dying/short-lived phenotype (Figure 4, Supplemental Table 1) to be the founding parents then continually selected for the long-lived phenotype while repeatedly crossing into the short-lived genome as described in the methods section.

The near-isogenic lines derived from the backcross with selection segregated into two phenotypic groups matching the respective parent phenotype in a ratio of 11:6 long to short, with an average difference of 10 hours in median lifespan (Figure 8). Comparison of the genomic sequences from 8 NILs (4 long-lived and 4 short-lived) plus the parent lines shows a region on the right arm of chromosome V, starting around base 18,200,000 and continuing to the end of the chromosome containing variants common to only the long-lived NILs and long-lived parent (Figure 9).

Within this region there is fine-scale variation where the long-lived NILs possess variants matching only the short-lived parent and/or novel mutations in between small blocks of the long-lived genotype (Supplemental Figure 4). As such, recombination has occurred with retention of the presumably necessary variants from the long-lived parent.

Discussion

Organisms respond in a multitude of ways when faced with resource limitation. It has been well-established in *C. elegans* that dietary restriction and food deprivation can significantly extend overall lifespan (see Uno & Nishida, 2016 for a comprehensive review). In early larval stages (L3 and prior) individuals can arrest or adopt alternate development strategies to cope, allowing them to stay in these states for an extended amount of time until food becomes sufficient and development resumes as normal (Klass & Hirsh, 1976; Johnson, Mitchell, Kline, Kemal, & Foy, 1984; Fielenbach & Antebi, 2008; Zhou, Pincus, & Slack, 2011; Baugh, 2013; Schindler, Baugh, & Sherwood, 2014; Roux, Langhans, Huynh, & Kenyon, 2016). When food is reduced or lost during later days of adulthood (day 2 and after) individuals also typically have an increase in lifespan over their well-fed counterparts (Kaberlein et al., 2006). However, the picture is not quite

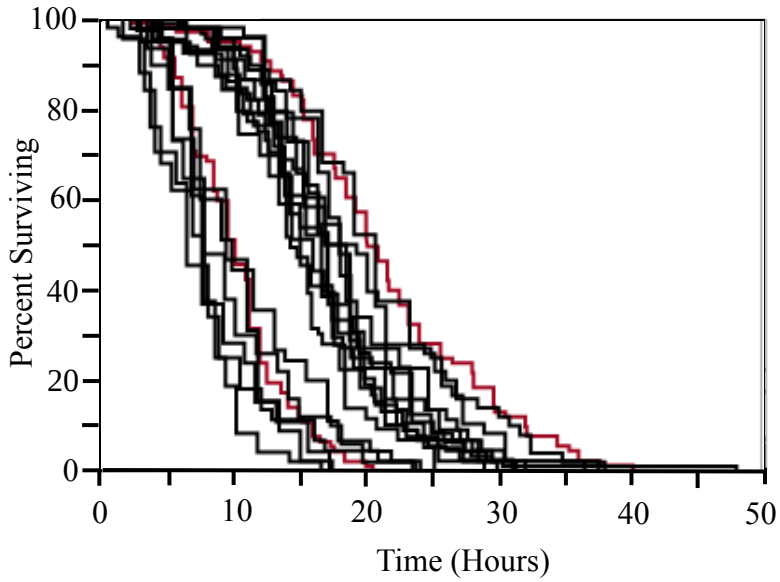


Figure 8: Survival curves of adults from the near-isogenic lines in the microfluidic starvation environment. Adult hermaphrodite survival was measured in the same manner as previously described. The near-isogenic lines segregated into a ratio of 11:6 (long:short) with an average mean difference of ~10 hours. Black lines are near-isogenic lines, red lines are the CeMEE parental lines (A6140L110 and GA450L40).

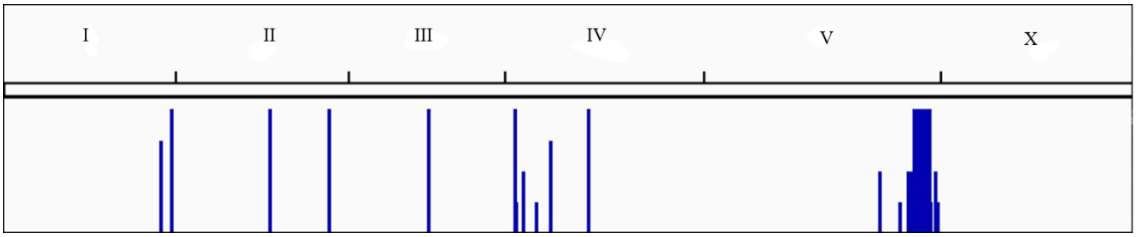


Figure 9: Chromosomal regions from long-lived parent retained by long-lived NILs. Dark blue regions represent the portions of each chromosome shared between the long-lived NILs and the long-lived parent exclusively. The height of the dark blue bar reflects the number of long-lived NILs (1-4) matching the parent sequence. The block on Chromosome V starts around base 18,200,000 and extends to the end of the chromosome.

as clear during the last larval stage (L4) and very early adulthood. This is the time during which *C. elegans* directs much of its energy into reproductive processes under normal circumstances and it has been shown that loss of food during this period can lead to very different outcomes (Angelo & Van Gilst, 2009; Seidel & Kimble, 2011; Burnaevskiy et al., 2018). Based on the expression patterns of genes involved in vulval development, the L4 larval stage can be roughly divided into three substages; early (hours 0-4), middle (hours 4-8), and late (hours 8-9) (Mok, Sternberg, & Inoue, 2015). While the effect of food removal on development through these substages is not entirely clear, it is evident that when food is removed during late L4 and/or the transition into adulthood, as was done in this study, individuals either undergo facultative vivipary (where eggs are fertilized but not laid, thereby hatching within the body of the parent hermaphrodite, also referred to as 'bagging'), or enter a state of adult reproductive diapause (Angelo & Van Gilst, 2009; Seidel & Kimble, 2011; Burnaevskiy et al., 2018). The two different shapes of curves seen in Figure 4 likely reflect this – the 'rapid' death of the sigmoid shape reflects lines where the majority of individuals bagged and diapause lines are those with the comparatively 'slow' deaths of the gradually decreasing slope. Our GWA mapping

identified four loci with clearly additive effects underlying the variation in starvation response. Two of the loci are individual SNPs within introns of the genes *ash-2* and *exc-6*, the other two are representative of larger haplotype blocks: 1) a ~2 KB region associated with the gene *dpy-28* and 2) ~26 KB region encompassing several genes on Chromosome V (Table 1).

Consistent with the lifespan curves, animals recovered from the microfluidic devices during the backcross with selection had either bagged or displayed the very poor morphological appearance with reduced intestines and somatic gonads, similar to the description of animals in adult diapause described in Angelo & Van Gilst (2009), when first placed on recovery plates. Moreover, as in the Angelo & Van Gilst (2009) study, this poor morphological appearance gave way to a fully rejuvenated appearance by recovery day two in the individuals who produced offspring. While this seems to suggest that individuals can enter a state of adult reproductive diapause in the microfluidic starvation environment there is one striking difference between the individuals in this study and other previous studies where individuals have been reported to remain in the diapause state for up to at least 3 weeks, i.e. 504 hours (Burnaevskiy, et al., 2018). In the microfluidic starvation environment, the maximum lifespan observed was ~140 hours. There are several, not mutually exclusive, possibilities for this difference: 1) the previous studies were done on unseeded agar plates typically using antibiotics to suppress the growth of bacteria whereas our microfluidic setup uses constant perfusion of sterile fluid to create an environment devoid of bacteria and other substances and so there may be an effect of the plate environment, 2) when on plates, animals are in contact with each other and presumably take chemical cues from one another which may influence the diapause

state, in our microfluidic chip animals are separated and the constant perfusion means that any chemical forms of communication are washed away, 3) data on adult reproductive diapause has been collected primarily, if not exclusively, from animals with the N2 genetic background in which case adult diapause as reported may be unique to that background, and 4) adult reproductive diapause may be a complex trait involving the cohesive function of multiple linked loci which have become uncoupled in the CeMEE panel (Figure 1).

While the backcross with selection was intended to be a mapping approach parallel to GWA, the selection approach imposes a slightly different phenotypic criterion than was measured for GWA. In the GWA approach, adult individuals were placed into the starvation environment and monitored without further intervention until all were dead. In the backcross with selection, adult individuals were placed into the starvation environment and monitored until ~20% of the population remained or 24 hours had passed, whichever came first. Surviving individuals were then removed and the fraction which produced offspring are the genotypes that progressed to the next backcross. Therefore, when survival lengths were measured for the backcross with selection, the individuals had been selected to 1) have an increased length of survival and 2) be able to

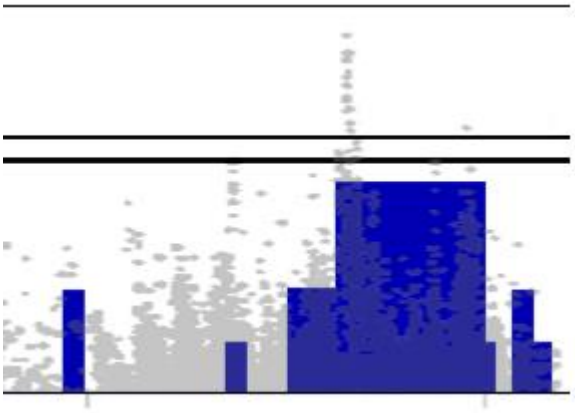


Figure 10: GWA mapping peaks from Chromosome V match the retained region in the long-lived NILs. An overlay of the GWA Manhattan plot shows the same region of significance as the region retained in the NILs.

produce offspring when food was reintroduced after ~80% of the population had succumbed to starvation. However, an overlay of the region retained in the long-lived NILs and the significant chromosome V region from the GWA shows that the regions match (Figure 10). It is likely that total length of survival and ability to produce offspring post-starvation are related but different traits, therefore it is not necessarily surprising that variants from 3 out of the 4 loci significant in the GWA approach were not retained in the long-lived genotypes resulting from the backcross with selection.

The retained region spans ~2.7 MB, beginning around position V:18,200,000 while the GWA locus has the approximate genomic position of V:18,264,009-18,293,029, a length of ~26 KB. This is the locus contributing an average 10-hour increase in median lifespan among the CeMEE panel lines and the same average difference is seen between the short- and long-lived NILs. If linkage and epistatic interactions between this locus and other genomic regions are truly diminished (as expected for lines from the CeMEE panel), then the phenotype resulting from introgression of this locus into a different genetic background would be expected to have only the additive effect. Given that the average change in median survival is the same in the NIL lines as it is for lines in the CeMEE panel, then this 1) reaffirms the reduction/loss of linkage disequilibrium in the CeMEE panel and 2) confirms this effect as additive (Wright, 1952; Hill, 1998; Luo, WU, & Kearsey, 2002; Hospital, 2005**)**. Moreover, this chromosome (and more specifically this region) has the only sign of linkage drag. None of the other chromosomes have retained significant portions of the long-lived parent genome (Figure 9**)**. A comparison across the two groups (short- vs long-lived NILs) gives a Cohen's D of 0.81, suggesting this is a locus of large effect and

supporting the identification of this locus as a significant contributor to median survival in the GWA mapping approach. Moreover, it is likely that variants within this locus have a directed effect on post-starvation reproductive ability. This appears to be an almost textbook example of the repeated selection and backcross scheme first proposed by Wright in 1952 (and expanded to include *inter se* mating as was done here by Hill in 1998) for isolating QTL of large effect.

The three loci identified in the GWA mapping which are absent from the backcross results have interesting physiological potential as candidates for influencing overall lifespan through starvation response. The gene *ash-2*, associated with the locus at II:11,986,204, is part of a histone methyltransferase complex known to be involved in regulating adult lifespan through its effects on H3K4 methylation in the *C. elegans* germline (Greer et al., 2010; Greer et al., 2011; Zuryn et al., 2014; Robert et al., 2014). Given that this locus already has a known role in affecting lifespan through reproduction, further investigation of variation in this gene under the context of starvation response is warranted. The gene *exc-6*, associated with the III:1,107,965 locus, is expressed throughout the pharynx, rectal gland cells, and the reproductive system in both larval and adult individuals and localizes to filamentous actin (Shaye & Greenwald, 2016; Hegsted, Wright, Votra, & Pruyne, 2016). However, it does not appear to have any previously reported role in affecting lifespan or starvation response, but *exc-6* mutants do exhibit defects in ovulation, suggesting this locus does have a significant role in reproduction and may influence survival in a starvation environment by restricting ovulation (Hegsted, Wright, Votra, & Pruyne, 2016). It would be interesting to examine the relationship

between variation in *exc-6* and the bagging/ARD decision as presumably reduced ovulation would push energy investment in the direction of somatic maintenance.

The third locus, at III:10,768,218, is associated with the gene *dpy-28*. This gene is involved in meiotic sister chromatid segregation, mitotic sister chromatid segregation, negative regulation of reciprocal meiotic recombination, and localizes to the intracellular organelle (Hernandez, et al., 2018). Interestingly, mutants of *dpy-28* have been reported to be unable to enter the dauer arrest state via an effect on the transcription factor DAF-16/FOXO, a widely recognized stress response pathway with dramatic effects on lifespan and starvation response in *C. elegans* (Weinkove, Halstead, Gems, & Divecha, 2006; Dumas et al., 2013; Uno & Nishida, 2016). The genetic basis for this role is suggested to be mediated by a role of *dpy-28* in dosage compensation, such that *dpy-28* mutants may have elevated expression of X-linked genes that typically promote dauer bypass. This raises the possibility that variants at this locus may promote the bagging phenotype by making it difficult or preventing individuals from entering into an arrested state in response to starvation cues. Of course, any of these putative effects would need to be confirmed via genetic transformation, and it is formally possible that some of these SNPs could influence the regulation of genes that are not directly adjacent to them (Pastinen, 2010; 1000 Genomes Project Consortium, 2012).

Overall, then, we identified four loci with significant effects contributing to variation in survival time in a microfluidic starvation environment through a GWA mapping approach. One of the four loci, a locus on the right arm of chromosome V, was confirmed in a parallel backcross with selection approach. The absence of selection on any of the other three loci suggest that they were either lost by chance during the

backcross with selection or have different effect sizes under the slightly different criteria – the ability to reproduce post-extended starvation (Hill, 1998; Hospital, 2001; Hospital, 2005). Taken together this suggests that the absolute adult lifespan under starvation conditions may be partially independent of the ability to recover and reproduce after surviving extended starvation and the locus identified in both approaches may act in a pleiotropic fashion to influence both traits. When faced with a loss of food during the period of time during which *C. elegans* hermaphrodites normally balance a physiological investment of energy into maturation of the germline with investment into somatic maintenance, individuals have to make a choice of where to partition their energetic resources. Some individuals are genetically predisposed to sacrifice their soma and undergo facultative matricide while others can maintain their soma and effectively put germline maturation on pause, suggesting that the natural variation segregating in populations of *C. elegans* may be evolving two different starvation response strategies.

CHAPTER III

CONCLUSION

The evolution of life history traits within a population reflects the manner in which natural selection is acting upon that population. Life history traits themselves are adaptive strategies that optimize fitness in their related ecological context. In order to understand the relationship between these traits and natural selection it is necessary to have information about two things: 1) the extrinsic ecological factors that impact fitness and 2) the intrinsic organismal trade-offs that impose constraints.

In this work I have shown that for the nematode *Caenorhabditis elegans,* one such ecological factor is starvation. Two lines of evidence support this; first is the various alternative developmental pathways characteristic of *C. elegans* that only manifest when food is in short supply, and second is repeated sampling data showing boom-and-bust type cyclical patterns in the persistence of *C. elegans* genotypes at the same sites over time (Félix & Braendle, 2010; Richaud, Zhang, Lee, Lee, & Félix, 2018). Together these strongly suggest that extended periods of starvation are a regular part of *C. elegans* natural environment.

Intrinsically, the two different starvation response strategies seen in *C. elegans* represent a trade-off. In one scenario, early adult hermaphrodites respond to the loss of food by sacrificing their own soma in favor of producing offspring which will hatch internally and consume the parent (facultative matricide) resulting in a radically shortened lifespan (Angelo & Van Gilst, 2009; Seidel & Kimble, 2011; Burnaevskiy et al., 2018). If there is no food available in the external environment this will provide

enough of a resource for the offspring to reach one of the early life stages where starvation-dependent alternative development pathways manifest and they are better equipped to disperse in search of food. While this is one way to maximize fitness in the context of starvation by ensuring the production of offspring, it also results in a reduced level of total fecundity when compared to a normal environment. In other words, these individuals produce offspring but far fewer than they would if food were available. In the other scenario, starving early adult hermaphrodites do not develop a mature germline. Instead, they go into a state of reproductive diapause (Angelo & Van Gilst, 2009; Seidel & Kimble, 2011; Burnaevskiy et al., 2018). This allows the individual hermaphrodite to wait out the period of starvation. Once sufficient food is encountered, the hermaphrodite recovers and goes on to produce offspring as normal. In this case the number of offspring eventually produced is not reduced but there is no guarantee that sufficient food will ever be encountered, and the amount of time taken to produce offspring is lengthened (Angelo & Van Gilst, 2009; Seidel & Kimble, 2011; Burnaevskiy et al., 2018).

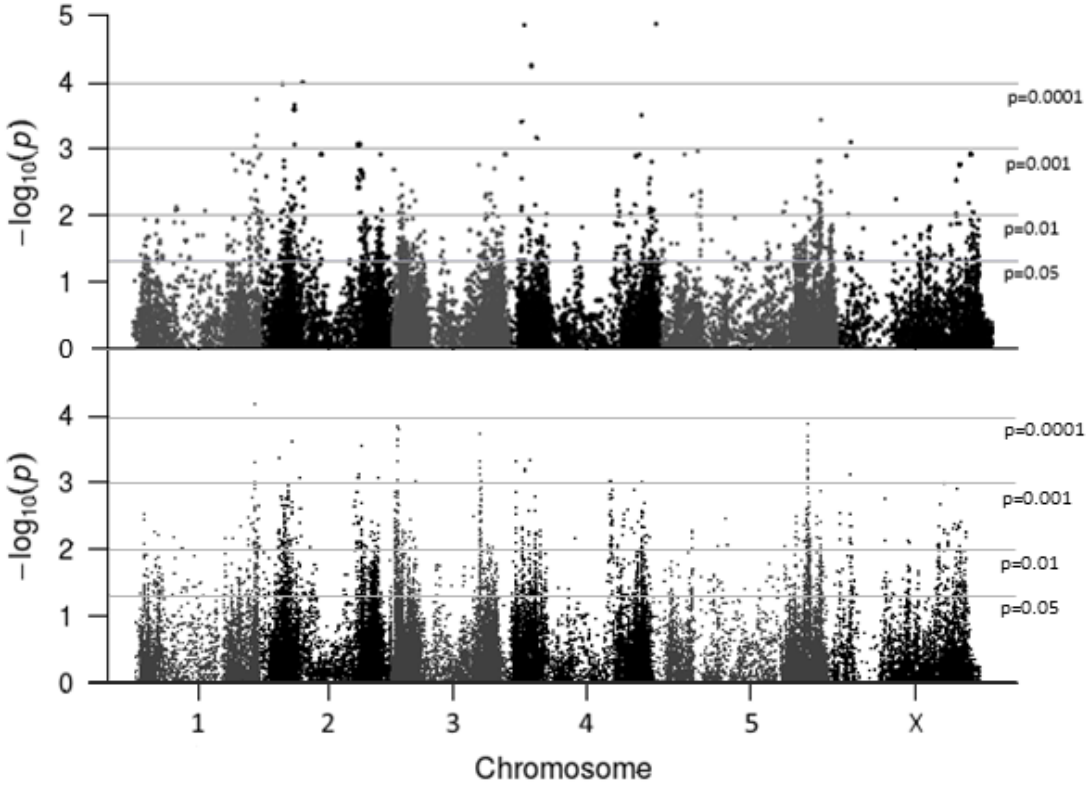
In this study I have also demonstrated that there is a likely genetic basis to the difference in these life history traits. Two different response shapes can be seen in the initial analysis of starvation survival among the mapping lines (Figure 4). The first is a sigmoidal shape common to all lines on the left-hand side, i.e. common to all lines with shorter lifespans under starvation conditions. The second is a more gradual decline common to the right-hand side, the lines with longer lifespans. While not tested directly here, these observations are consistent with predictions from observations of two different starvation response strategies. A genome-wide association (GWA) mapping of the variation in median lifespan under starvation conditions identified four loci that act in

an additive fashion to contribute to the lifespan of starved adult hermaphrodites and one of these loci was retained when a backcross with selection strategy was used to select for the ability to produce offspring post-starvation. The selection strategy used inherently selected against individuals that would undergo facultative matricide and for those who exhibit characteristics consistent with the adult reproductive diapause strategy. This suggests that a genetic basis for these life history traits exists and that there is correlated genetic variation.

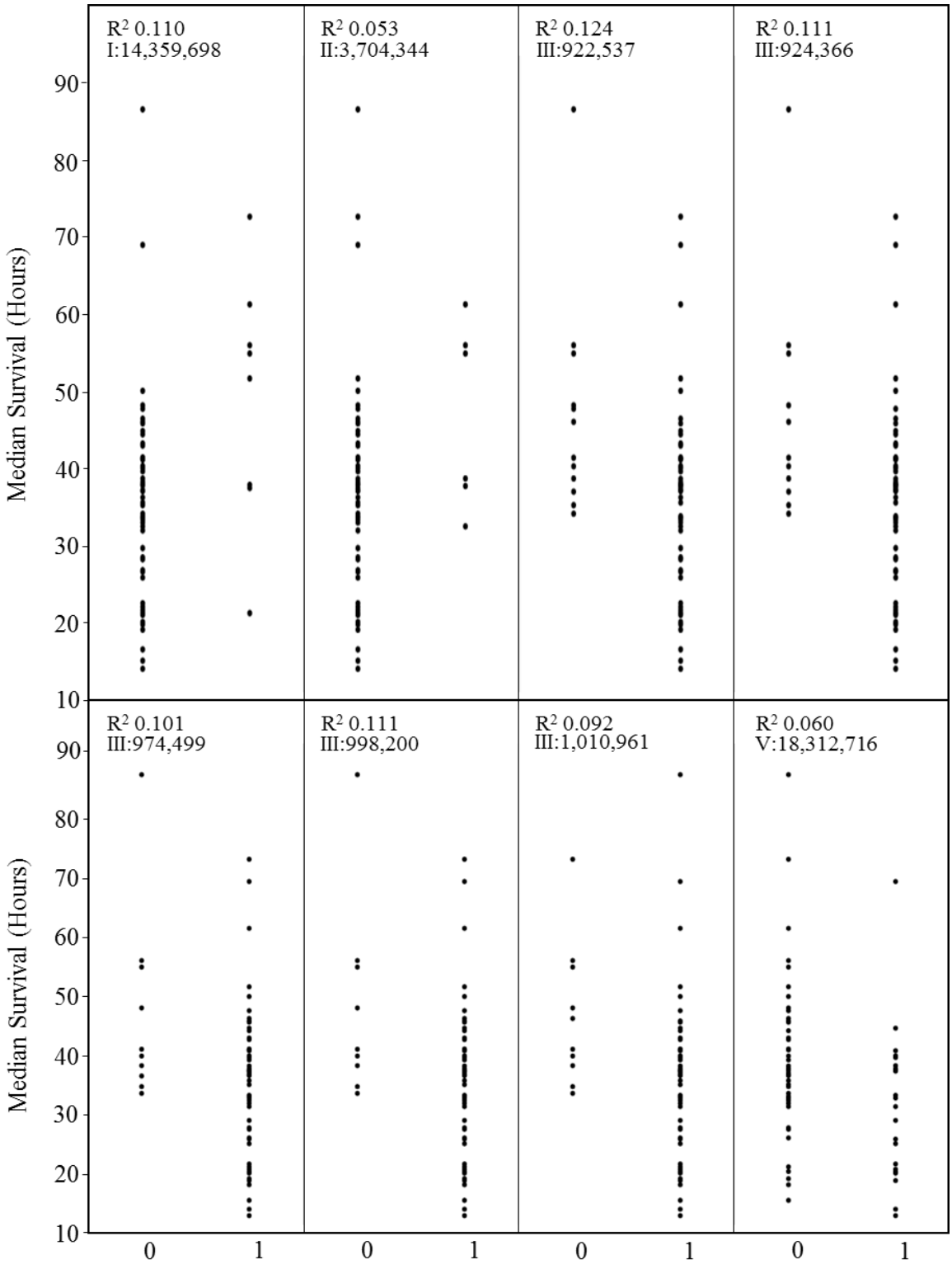
The work described in this dissertation lays the foundation of a system utilizing *C. elegans* that can be further capitalized on in several ways. First, the genetic loci identified could be functionally investigated, e.g. CRISPR could be used to swap alleles singly or in combination and the phenotypic effects characterized in a high-throughput manner using microfluidics thus providing information about the genetic mechanism underlying the trade-off between investing in somatic maintenance versus reproduction. Second, experimental evolution could be used to test specific hypotheses regarding the evolution of different starvation responses. For example, the potential advantages of the timing in the different reproduction strategies could be tested by evolving competing lines under different temporally regulated starvation schemes. Third, this system could be used in a similar fashion as presented here to investigate the genetic basis of variation in other traits. In summary, natural selection is likely acting on variation for starvation response in *C. elegans* to produce two diverging life history traits and the system presented here can be further utilized to explore adaptation to starvation in greater detail or be adapted to explore other traits.

APPENDIX

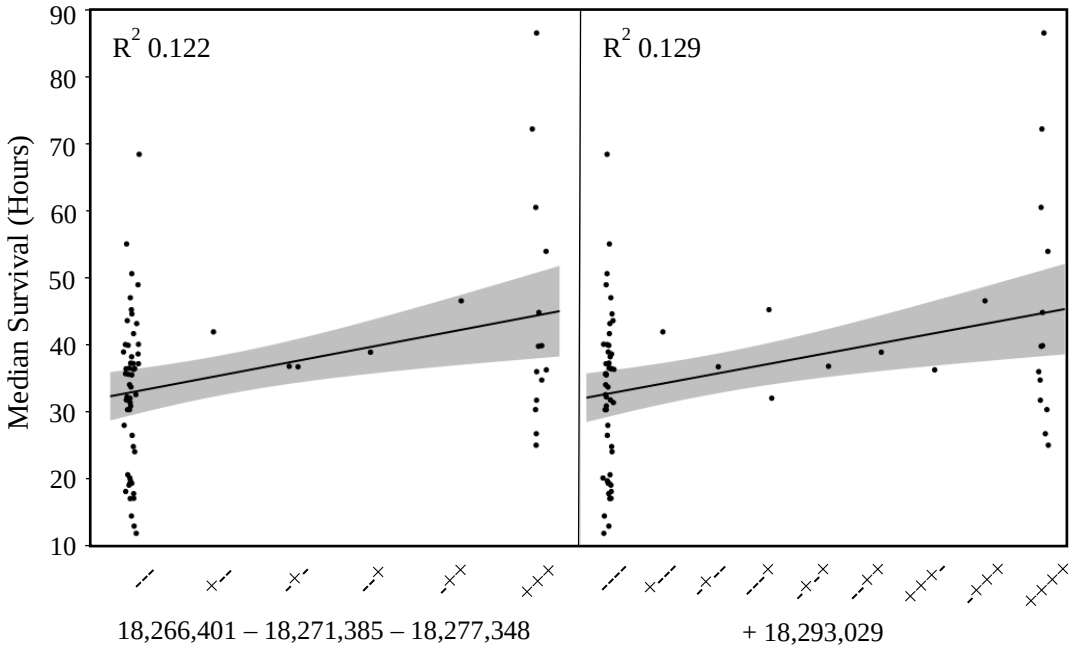
SUPPORTING INFORMATION FOR CHAPTER II



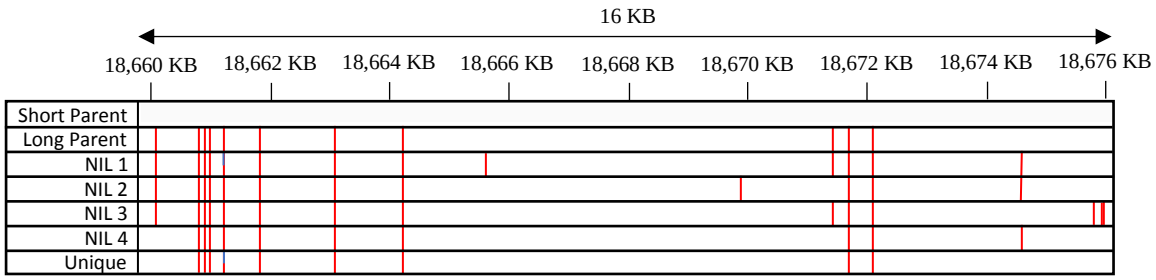
Supplemental Figure 1: Plink vs. rrBLUP Manhattan Plots. The top Manhattan plot shows results from GWA mapping of median adult survival time under starvation conditions performed in Plink v1.9, the bottom Manhattan plot shows the reported results from rrBLUP. Overall, the distribution of significant SNPs within each chromosome is the same.



Supplemental Figure 2: Effect size plot of loci not considered. Loci identified as significant through GWA mapping with rrBLUP but either heavily affected by an outlier or direction of influence on trait was unclear. Genotypes denoted as '0' are the N2 reference allele.



Supplemental Figure 3: Combinatorial genotypes at Chromosome V locus. The effect of the four-SNP genotype is not significantly different from the three-SNP genotype, therefore the SNP at V:18,293,029 was not considered a significant contributor to variation in the starvation response phenotype. Alleles denoted as '+' match the N2 reference.



Supplemental Figure 4: Fine-scale variation in NIL lines at the Chromosome V locus. Detail of the variation present among NIL lines at the Chromosome V locus. Short regions matching the long-lived parent are punctuated by recombined regions. There also appears to be a few novel SNPs. Red lines represent SNPs deviating from N2 reference genome.

CeMEE Line	Number of Individuals	Number Censored	Mean Survival (Hours)	Standard Error	Median Survival (Hours)	Lower 95%	Upper 95%
A6140L10	99	0	37.27	1.93	28.54	27.8	32.02
A6140L101	105	0	54.46	2.75	41.44	38.27	45.84
A6140L106	147	1	39.14	1.34	34.195	32.17	36.82
A6140L107	139	4	48.38	1.72	41.48	40.1	43.88
A6140L110	143	0	37.59	1.20	33.68	32.07	35.65
A6140L113	142	5	42.20	1.15	38.67	37.07	40.27
A6140L120	137	8	44.82	1.63	40.37	38.77	43.18
A6140L123	142	5	41.31	1.18	38.03	37.75	39.35
A6140L124	97	1	39.23	1.57	36.3	31.8	39
A6140L125	154	1	39.02	0.99	37.93	34.78	40.33
A6140L126	144	6	40.07	1.03	37.85	36.03	39.23
A6140L133	197	3	37.64	1.18	33.43	32.63	35.73
A6140L134	140	5	35.36	1.13	32.02	30.9	34.07
A6140L135	98	0	41.61	1.37	38.23	35.83	40.77
A6140L137	146	2	31.99	1.07	29.75	28.95	30.55
A6140L142	85	8	22.18	0.88	22.13	18.98	22.98
A6140L153	139	7	21.65	0.50	21.725	20.4	22.8
A6140L155	85	2	23.98	0.70	22.6	21	24.05
A6140L156	92	0	20.89	0.66	20.18	19.33	21.73
A6140L157	146	1	14.81	0.42	14.12	13.32	15
A6140L158	85	13	27.06	0.82	25.93	24.7	28.33
A6140L168	135	5	19.84	0.63	19.185	18.07	20.9
A6140L176	89	4	20.36	0.64	19.87	17.78	20.98
A6140L18	93	0	22.15	0.64	21.37	19.77	22.65
A6140L181	94	4	21.21	0.49	21.33	20.38	22.78
A6140L182	79	10	45.24	1.98	40.33	38.05	47.53
A6140L183	95	7	48.87	2.15	41.285	36.35	48.62
A6140L184	95	3	47.42	3.03	33.39	30.38	38.38
A6140L185	91	6	39.64	1.78	33.87	32.27	37.07
A6140L188	93	5	35.69	1.30	32.02	30.47	34.47
A6140L19	97	0	15.44	0.42	15.17	14	15.97
A6140L190	90	7	54.97	2.56	44.9	41.7	55.03
A6140L200	87	6	52.71	2.58	44.48	40.73	46.88
A6140L203	89	8	57.53	2.97	47.77	41.77	48.57
A6140L205	90	7	52.73	3.14	37.52	33.48	45.48
A6140L211	87	6	42.66	2.05	37.78	36.12	40.18
A6140L23	100	3	61.37	2.67	51.7	45.84	62.78
A6140L26	92	5	49.20	2.79	37.05	34.45	39.45
A6140L28	143	3	44.39	1.45	39.68	37.28	41.5
A6140L36	193	0	41.81	1.04	38.7	36.15	41.1

A6140L38	94	4	59.15	2.62	54.93	48.98	60.98
A6140L48	138	2	38.91	1.69	32.03	30.42	36.02
A6140L49	138	5	44.91	2.20	35.63	32.43	38.83
A6140L50	140	5	42.38	1.54	37.13	35.35	38.73
A6140L52	88	2	37.27	1.87	32.56	31.17	34.37
A6140L56	96	0	21.41	0.70	21.1	19.37	22.7
A6140L60	88	0	19.67	0.63	19.155	17.28	21.03
A6140L80	103	0	17.31	0.43	16.63	15.83	18.17
A6140L82	137	9	49.87	2.08	41.2	38.23	46.23
CA350L13	143	9	71.20	2.19	68.98	62.02	73.72
GA150L35	195	3	46.65	1.08	43.02	41.55	44.49
GA150L51	94	0	52.09	1.93	46.085	41.57	48.9
GA150L52	144	0	34.75	0.80	33.05	32.03	35.05
GA150L53	102	1	49.66	1.61	45.88	43	51
GA150L57	98	2	45.75	1.58	41.33	39.73	42.13
GA150L58	90	8	62.14	2.13	56	52	59.22
GA150L59	67	32	82.72	3.03	86.53	66.53	101.48
GA150L60	93	6	49.10	1.98	43.28	41.22	44.88
GA150L61	95	4	51.19	1.70	48.22	45.02	51.42
GA250L51	104	2	41.14	1.39	38.75	36.75	40.75
GA250L52	98	0	27.04	0.71	26.68	25.08	27.48
GA250L61	93	0	31.30	1.08	28.3	26.7	30.7
GA450L31	95	0	27.44	0.64	26.88	25.37	27.77
GA450L36	148	0	39.37	0.84	37.97	36.47	38.7
GA450L37	200	2	48.19	1.69	40.08	38.28	41.9
GA450L38	182	9	55.94	1.62	50.1	45.75	53.97
GA450L39	165	18	72.72	2.36	61.29	53.83	79.35
GA450L40	135	7	72.17	2.82	72.64	54.55	89.2
GA450L41	150	0	41.58	1.59	35.3	33.83	37.5
GT150L23	188	5	54.33	1.81	46.49	39.98	51.98
GT250L19	151	0	40.10	1.08	37.23	36.5	40.17
GT250L34	196	0	39.50	0.76	38.3	37.3	40.45
Combined	8642	296	42.48	0.26	36.33	36.02	36.78

Supplemental Table 1: Summary statistics for CeMEE panel lines. CeMEE lines phenotyped in microfluidic starvation environment.

Chromosome	SNP	PLINK -log10(p)	rrBLUP -log10(p)
IV	16875066	4.87	0.00
IV	1489316	4.86	0.00
IV	2318178	4.26	3.26
IV	2329101	4.23	3.21
II	4636338	3.99	3.11
II	4671053	3.99	3.11
II	2299897	3.97	3.41
I	14359698	3.74	4.23
II	3704344	3.66	3.66
II	3648925	3.58	0.00
II	3654708	3.58	0.00
II	3658943	3.58	0.00
II	3663176	3.58	0.00
II	3667170	3.58	0.00
II	3675301	3.58	0.00
II	3689965	3.58	0.00
II	3699906	3.58	0.00
IV	15185237	3.50	2.94

Supplemental Table 2: PLINK GWA mapping results. List of SNPs with -log10(p) > 3.50 identified by GWA mapping with PLINK, rrBLUP significance values included for comparison. GWAS analysis of survival time in the absence of food was conducted on the trimmed data using an additive linear regression in Plink v.1.9, using the following options: --assoc qt-means --adjust gc (www.cog-genomics.org/plink/1.9/; Chang et al., 2015). The genomic inflation lambda value (based on median chisq) was estimated to equal 55.3583.

	V:18266401	V:18271385	V:18277648	V:18293029
A6140L10	+	+	+	+
A6140L101	-	-	-	-
A6140L106	-	-	-	-
A6140L107	-	-	-	-
A6140L110	-	-	-	+
A6140L113	-	-	-	-
A6140L120	-	-	-	-
A6140L123	-	-	-	-
A6140L124	+	+	+	+
A6140L125	-	-	-	-
A6140L126	-	-	-	-
A6140L133	-	-	-	-
A6140L134	-	-	-	-
A6140L135	-	+	-	-
A6140L137	-	-	-	-
A6140L142	-	-	-	-
A6140L153	-	-	-	-
A6140L155	-	-	-	-
A6140L156	-	-	-	-
A6140L157	-	-	-	-
A6140L158	-	-	-	-
A6140L168	-	-	-	-
A6140L176	-	-	-	-
A6140L18	-	-	-	-
A6140L181	-	-	-	-
A6140L182	-	-	+	+
A6140L183	+	+	+	+
A6140L184	+	+	+	+
A6140L185	-	-	-	-
A6140L188	-	-	-	-
A6140L19	-	-	-	-
A6140L190	-	-	-	-
A6140L200	-	-	-	-
A6140L203	-	+	+	+
A6140L205	+	+	+	+
A6140L211	+	+	+	-
A6140L23	-	-	-	-
A6140L26	-	-	-	-
A6140L28	-	-	-	-
A6140L36	-	-	-	-

A6140L38	+	+	+	+
A6140L48	+	+	+	+
A6140L49	-	-	-	-
A6140L50	-	-	-	-
A6140L52	-	-	-	-
A6140L56	-	-	-	-
A6140L60	-	-	-	-
A6140L80	-	-	-	-
A6140L82	+	+	+	+
CA350L13	-	-	-	-
GA150L35	-	-	-	-
GA150L51	+	+	+	+
GA150L52	-	-	-	-
GA150L53	-	-	-	-
GA150L57	-	-	-	-
GA150L58	-	-	-	-
GA150L59	+	+	+	+
GA150L60	+	-	-	-
GA150L61	-	-	-	-
GA250L51	-	-	-	-
GA250L52	-	-	-	-
GA250L61	-	-	-	-
GA450L31	+	+	+	+
GA450L36	-	-	-	-
GA450L37	-	-	-	-
GA450L38	-	-	-	-
GA450L39	+	+	+	+
GA450L40	+	+	+	+
GA450L41	-	-	-	-
GT250L19	-	-	-	+
GT250L34	-	-	-	-
GT150L23	-	+	-	+
Total Alt	57	55	56	54

Supplemental Table 3: Genotypes of CeMEE panel lines at Chromosome V loci. N2 reference allele denoted as '+', alternate alleles denoted as '-'. All alleles are biallelic. Total Alt = total number of lines with alternate allele.

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