A size threshold governs *Caenorhabditis elegans* developmental progression

Sravanti Uppaluri and Clifford P. Brangwynne

Department of Chemical and Biological Engineering, Princeton University, Princeton, NJ 08544, USA

The growth of organisms from humans to bacteria is affected by environmental conditions. However, mechanisms governing growth and size control are not well understood, particularly in the context of changes in food availability in developing multicellular organisms. Here, we use a novel microfluidic platform to study the impact of diet on the growth and development of the nematode *Caenorhabditis elegans*. This device allows us to observe individual worms throughout larval development, quantify their growth as well as pinpoint the moulting transitions marking successive developmental stages. Under conditions of low food availability, worms grow very slowly, but do not moult until they have achieved a threshold size. The time spent in larval stages can be extended by over an order of magnitude, in agreement with a simple threshold size model. Thus, a critical worm size appears to trigger developmental progression, and may contribute to prolonged lifespan under dietary restriction.

1. Introduction

The growth and development of multicellular organisms are critically linked to their nutritional status. A wide variety of organisms exhibit developmental growth rates that depend strongly on their food intake, which can also impact the final size of the organism [1–3]. Humans provide a striking example, as average human height has increased by 5–10% over the last 150 years, owing to nutritional improvements associated with industrialization [4]. Moreover, nutritional changes are closely linked to the onset and progression of diseases, such as diabetes, cancer and obesity [5]. Paradoxically, caloric restriction is also found to increase lifespan in many organisms [6]. The effects of diet on growth and development are thus ubiquitous, but still poorly understood.

*Caenorhabditis elegans* provides a powerful model to study the effects of nutrient availability on development, due to its short lifespan, genetic tractability and molecular homology to humans. Moreover, the four distinct larval stages (L1–L4) during *C. elegans* growth provide easily detectable developmental milestones. During each larval transition, the worm undergoes a moulting process, shedding its collagen-rich outer cuticle, similar to the moulting seen in many arthropods [7]. The stereotyped and coordinated developmental changes in *C. elegans*, including cell differentiation and tissue morphogenesis [8], underscore the importance of regulating the moulting process in the face of altered food availability or other environmental changes. Consistent with this, interfering with the oscillatory expression of certain heterochronic genes uncouples moulting timing from completion of stage-specific development, causing major growth abnormalities [9,10]. Moreover, in the absence of food, L1 larvae can completely arrest development and survive for weeks without moulting or any other morphological changes [11]; L2 worms can also undergo a transition to a quiescent dauer form [12]. Despite the strong dependence of *C. elegans* growth and development on nutrition, we lack a mechanistic understanding of the cues that trigger moulting and developmental progression.

Previous work in other organisms has suggested that there is either a timer or a body size-based trigger for moulting transitions in ecdysozoan growth [13]; this is analogous to ‘Timer’ versus ‘Sizer’ models for the proliferation of individual cells [14,15]. For example, the hormone ecdysone has been shown to be a secreted signal important for the timing of larval stage transitions in the moth *Manduca*...
sextr. Ecdysone is secreted at a precisely controlled critical size threshold, which prepares the moth for metamorphosis [16]. If larvae have not reached the critical size due to low food availability, they will undergo additional growth phases and intercalary moults until they have achieved the required size for the metamorphic moult [17]. In the fruitfly D. melanogaster, the prothoracic gland assesses when critical weight for pupation has been reached; premature pupation induced in larvae with enlarged prothoracic glands results in lethality [18]. Thus, moulting transitions are essential milestones intrinsically linked to proper development.

Progress in studying this problem in C. elegans has been hampered by an inability to make high-resolution measurements of worm growth and development. The challenge is partly due to the enormous growth individual worms undergo during the course of their lifetime (L1 worms are approx. 200 µm in length and grow to over 1 mm by adulthood). Moreover, growing a large ensemble of worms on a Petri dish only yields average population-level behaviour; this misses worm-to-worm variations that can provide insights into the underlying regulatory dynamics. This could affect the interpretation of longevity studies in worms, which are typically conducted by survival assays in which worms are cultured under various growth conditions or genetic backgrounds, and the percentage of living worms is recorded over time [19]. Indeed, while these assays have provided insights into many factors involved in worm lifespan [20,21], little or no quantitative insights have been obtained about the worm’s larval development before reaching adulthood.

Microfluidic technology has been leveraged for various applications in nematode biology—from immobilization for imaging to laser ablation in neuronal and ageing studies [22–25]. Worm growth has also been studied using an array of chambers in which L4 worms were individually loaded to monitor development to adulthood [26], or using a device developed to observe collections of 30–40 adults worms [27]. A device with multiple observation chambers for adult worms was exploited to study chemical effects on worm behaviour [28] and oil-in-water emulsions were used to encapsulate individual worm embryos [29,30] or L1s [31] which could develop to adulthood. While encapsulation allows the study of even early larval stages which are otherwise challenging, proper feeding and the physiological effect of surfactant required for droplet production limit the applicability of this method [32].

Here, we introduce a novel microfluidic platform, the ‘growthChip’, to study C. elegans development from the earliest larval stage to adulthood, at a high spatio-temporal resolution within a well-controlled environment. This technology allows us to track individual worms through larval development, and determine their growth rates and moulting transitions as a function of food (bacteria) availability. We use this system to show that C. elegans moulting transitions are controlled by a size threshold: worms do not moult and progress to the next developmental stage until reaching the appropriate size. This size threshold imposes a developmental delay under low food availability, which could have implications for lifespan extension under dietary restriction.

2. Material and methods

Full methods are given in the electronic supplementary material, methods.

(a) Microfluidics

Standard multilayer photolithography is used to fabricate the growthChip consisting of three successive layers; 10, 35 and 50 µm in height for optimal trapping and worm growth.

(b) Worm culture

Wild-type N2 and smu-3(ok1430) strain worms were obtained from the Caenorhabditis Genetics Center (CGC). Well-fed gravid adult worms that were grown on NGM plates were bleached to extract embryos using a standard protocol [33]. For ‘off-chip’ experiments, 200 synchronized L1s were grown in S-Medium supplemented with the desired concentration of bacteria. Worms were imaged and resuspended in fresh growth medium every 24 h. Time to L1–L2 moult was recorded as the first day a worm was observed either with a shedding cuticle or with developing germ cells—a characteristic of the L2 stage, and a 1-day error was assumed.

(c) Device design and operation

Flow fields were simulated with COMSOL Multiphysics software using a stationary solution. We base device optimization on fluidic resistance R dependent on the channel dimensions [34]. Worms were grown on-chip in S-Medium supplemented with OP50 E. coli bacteria. Images were acquired for each growth chamber using an ASI motorized stage equipped with a linear encoder at 10 min intervals and a 5x objective on an inverted Zeiss 200 M microscope.

(d) Image processing and data analysis

Growth curves were extracted from timelapse movies using a custom image processing routine (MATLAB, MathWorks). The worm is approximated as a cylinder to measure body volume as in [35].

(e) Particle image velocimetry

One micrometre green fluorescent polystyrene beads suspended in the S-Medium supplemented with OP50 E. coli were used to visualize and quantify flow fields using PIVLab [36].

3. Results

(a) Development of a custom worm growthChip

Attempting to observe individual C. elegans worm throughout their development presents major challenges. Observation chambers must confine worms while allowing for delivery of nutrients (bacteria) and removal of waste. However, this is difficult since the worm can squeeze through spaces narrower than its own body diameter which changes over 10-fold during development. The growthChip technology that we have developed precisely captures single C. elegans embryos in successive cup-shaped traps that each lead to isolated ‘growth chambers’; a similar strategy was used in [37]. A wide inlet facilitates embryo entry and bacterial flow, while a parallel array of much narrower outlet channels act as a filter; this allows waste and fluid to be flushed out while retaining even the smallest larvae and provides an enclosed culture chamber. The height of the growth chamber is increased to a total of 90 µm using a third layer, preventing space constraints from affecting growth. A motorized stage facilitates the simultaneous imaging of dozens of growth chambers on a single device, at high temporal resolution. A schematic of the growthChip and its operation is shown in figure 1a. To isolate single embryos, the volumetric flow
rates were optimized based on fluidic resistance [34], causing
an embryo to preferentially enter a trap if the trap is not already
filled (figure 1d). Newly hatched *C. elegans* larvae can easily
swim through channels more than or equal to 15 μm. There-
fore to reduce flow resistance through the growth chamber,
while preventing escape of larvae, the outlet comprises
multiple small outlet channels (10×10 μm).

The simulation in figure 1b illustrates streamlines at the
trap and the connectivity between each chamber. A suspen-
sion of embryos flows through the device until all traps are
filled (figure 1c). Applied pressure is manually increased
causing the inlet channel to expand and pushes the embryo
through to the growth chamber (shown in figure 1e), where
it can complete embryogenesis. Once loading is complete,
the device is flushed for 18–24 h with M9 buffer for L1
synchronization before switching to liquid culture containing
bacteria. The loading process does not affect embryo viability
and the number of larvae that escape the chamber is very
small (approx. 3%).

Using particle image velocimetry (PIV) [36], we found
that the flow fields and relative flow rates into the growth
chamber and bypass channel match the design criteria
(figure 1d). Furthermore, the ratio of flow rates $\frac{Q_{\text{bypass}}}{Q_{\text{inlet}}}$ < 1 remain constant even after 24 h of bacterial flow,
ensuring that delivery of nutrients is consistent throughout
the experiment (see the electronic supplementary material,
methods for details). A constant flow rate of 50 μl h⁻¹
during the entire experiment minimizes accumulation of
bacteria in the device, ensures consistent delivery of food to
the worm and replenishes the entire volume of the chamber
within approximately 5 s.

Individual worm growth is monitored by imaging at
desired time intervals using a microscope equipped with a
programmable motorized stage to move from one growth
chamber to the next. Figure 2 illustrates the volume change
of a single worm from the L1 through to the L4 stage in the
growthChip extracted from a timelapse movie. A straight-
forward image processing algorithm allows us to assume a
cylindrical shape for the worm; the lateral area and
'backbone' length of the worm (figure 2b) are used to obtain
the worm volume as described in Material and methods sec-
tion. Volumetric growth obtained at a time resolution of
10 min is shown in figure 2c.

### (b) growthChip reveals distinct growth rates
for each larval stage

Typical growth curves for *C. elegans* development found in
the literature are sigmoidal in shape with exponential con-
tinuous growth through larval stages followed by growth
'saturation' once adulthood is reached [38]. These measure-
ments are derived from population averages. Such data
have two major drawbacks: (i) sampling time limits the tem-
poral resolution of measurements and (ii) features of interest
at the individual level are averaged out. High temporal resol-
ution imaging of individual worm development in the

---

**Figure 1.** growthChip design allows monitoring of individual worms through development. (a) Schematic of growthChip. Bacterial suspension flows through the PDMS chip using a syringe pump and is kept well mixed with a stir plate. A motorized stage is used to image each growth chamber. (b) Illustration of growthChip geometry showing growth chambers are arranged in series. Isolated embryos enter through the traps. (c) Single embryos (black and red arrowheads) are successively trapped for subsequent entry into the chamber (see (e)). (d) COMSOL simulation (methods) showing close up of boxed area in (b), illustrating flow streamlines that match experiment; experimentally determined PIV flow fields shown in right (e). A slight increase in pressure after traps are filled expands the entry slightly (blue arrow) allowing embryos to squeeze through the entry. Scale bar 100 μm.

---
growthChip reveals discontinuous growth dynamics. Worms grow at four distinct rates, with growth curves exhibiting a ‘piecewise linear’ behaviour (figure 2c). Closer examination of the images reveals that the four regimes correspond to each of the four larval stages, and that growth rate transitions correspond to moult events (figure 2d). Small dips in volume between each growth regime appear to arise from the volume due to loss of the old cuticle. These dips enable us to determine the precise timing of the moult.

(c) Size threshold for moult

Monitoring the growth dynamics of individual worms reveals significant variability in worm to worm growth rate as shown in figure 3. This variability could be used to help elucidate the mechanism underlying the decision to moult. One possibility is that worms contain an internal clock which establishes the duration of each larval stage. Such a ‘Timer’ model simply predicts that the duration of each developmental stage, $\tau_i$, is a constant: $\tau_i = \tau_T$. Here $i$ corresponds to each of the four larval stages ($i = 1, \ldots, 4$). Alternatively, moults may occur only at a critical size, suggesting a mechanism for measuring body size.

This ‘Sizer’ model predicts that moult occurs at a constant size $V_s$ such that $V_i = V_0 + \sum_{i=1}^{4} V_i \tau_i$, where $V_i$ is the volumetric growth rate, and $V_0$ is the initial volume of the newly hatched worm. In the L1 stage, we see that larvae that exhibit faster growth tend to moult earlier (figure 4a). Moreover, across all larval stages the growth rate is also negatively correlated with duration in the stage, as shown in figure 4b. These data are inconsistent with the Timer model, but instead could suggest that the moult transitions occur once the worm grows to a specific size (see lines figure 4a).

To more rigorously test the size threshold model, we used different concentrations of bacterial food to vary the worm growth rate. We found that $V_i$ increases at each larval stage (figure 5a) and that $V_i$ shows a strong dependence on food availability. This can be strikingly seen in the plot of $V_i$ versus bacterial concentration, where non-zero growth was observed ($\dot{V}_i > 0$) over two orders of bacterial concentration (figure 5b).

At the lowest concentrations ($4.8 \times 10^7$–$20 \times 10^9$ cells ml$^{-1}$), the growth rate was very small, and we did not observe the L1–L2 moult even after 48 h. The dependence of L1 growth rate on nutritional availability is well described by a function of the form $\dot{V}_i(C) = \dot{V}_i^{sat}(1 + e^{-i(C-C_s)/C'})$, providing a phenomenological equation that can be used to further test the Sizer model (figure 5b, see the electronic supplementary material, methods for details). We note that the plateau in figure 5b corresponds to a saturating growth rate, $\dot{V}_i^{sat}$, pointing to a biological limit on how fast the worm can grow.

In response to starvation, L1 worms can arrest development (‘L1 arrest’) without morphological changes but with increased stress resistance [11]. Thus, our diet-restricted L1 worms that did not moult within 48 h may have simply arrested development. However, these worms exhibited a small, but distinctly non-zero, growth rate. In light of the Sizer model, this suggests that they may simply have not yet achieved the critical size. To test this, we grew synchronized L1 worms off-chip (see Material and methods), in liquid medium, at the lowest concentrations of bacteria (concentration range $4.8 \times 20 \times 10^9$ cells ml$^{-1}$). If there is a critical size, moult timing can be predicted as a function of concentration by the Sizer model $\tau_i = (V_i - V_0)/\dot{V}_i(C)$.
where $\hat{V}_1(C)$ is given by the empirically determined function illustrated in figure 5b. Here, $V_1$ is taken as the mean volume at which worms were observed to cross the L1–L2 transition within 48 h on-chip. The prediction of the Sizer model for the moult time $\tau_1$ is shown in figure 6a.

Consistent with this model, we observed that dietary restriction delayed the moult transition but did not prevent it. The transition to the L2 stage was marked by shedding of the cuticle and the appearance of germ cells (figure 6a inset)—a morphological characteristic specific to...
the L2 larvae. These worms continued to develop and can eventually reach adulthood. The Sizer model correctly predicts that worms cross the first moult only when they grow to a critical size of roughly $10^5$ μm³ (figure 6b). This is remarkable, since under severe dietary restriction the time to achieve this size is extended over an order of magnitude, from approximately 20 h at high bacterial concentrations to more than 250 h at the lowest concentrations (figure 6c).

Given the fact that the L1–L2 moult required more than 10 days to achieve at the lowest food concentration, testing the validity of the Sizer model for later developmental stages is experimentally challenging. However, for worms at high bacterial concentration, we were able to vary growth rate by a factor of approximately 2. This allows us to determine whether moult occurs at a constant size for each larval stage. Consistent with a size threshold, despite significant differences in growth rate, we find that worm size is roughly constant at each mouling transition (figure 6c). These data suggest an internal mechanism for sensing body size, and triggering developmental transitions at all C. elegans larval stages.

(d) Genetic mutations can change the size threshold

To test whether the threshold is absolute, or can be biologically tuned, we conducted similar experiments with sma-3 mutant worms. SMA-3 is involved in TGFβ signal transduction—a pathway essential for body size regulation. This mutant is known to exhibit reduced post-embryonic growth resulting in a smaller body size [39]. We measured L1 growth rate as a function of food concentration in the sma-3 mutant and found the same sigmoidal functional form in the dependence of L1 growth rate on food availability in sma-3 worms as in wild-type N2 worms (figure 5b). However, the sma-3 mutant grows more slowly and ultimately reaches a smaller size than wild-type N2 worms. Despite significant differences in growth rate at different food concentrations, sma-3 worms also cross the L1–L2 moult at the same size. However, the size at moult is shifted from approximately $1 \times 10^5$ μm³ for N2 worms down to approximately $4.5 \times 10^4$ μm³ for sma-3 worms (figure 6b). As for N2 worms, the Sizer model correctly predicts the time to reach the L1–L2 moult, again pointing to a size threshold to trigger the moult (electronic supplementary material, figure S2). These data thus provide further support for the Sizer model, but reveal that the critical size for moult can vary in worms with different genetic backgrounds.

4. Discussion

Caenorhabditis elegans is a widely used model organism and we anticipate that the growthChip will find widespread use among C. elegans researchers. The growthChip enables high temporal resolution imaging of individual worms through development, which provides access to data otherwise hidden in population averages; this may be particularly useful for exploring sources of noise in growth and size control. The device could easily be adapted for RNAi screens through bacterial feeding together with developmental studies. Moreover, the ability to monitor stage-specific effects of environmental parameters on growth, such as diet (concentration and species of bacteria), temperature or chemical perturbations, and genetic backgrounds (as demonstrated here) will benefit researchers in various fields.

Using the growthChip microfluidic platform, we found that worms grow in a piecewise linear fashion through larval development. We identify a nonlinear dependence of the growth rates on bacterial concentration. Slower growing worms crossed moult transitions later. The L1 stage was extended from 24 h at high food concentrations to over 250 h under slow-growing diet-restricted conditions. The simple ‘Sizer’ model, which predicts moulting transitions occur at a critical worm size, quantitatively accounts for these data.

How organelles, cells, tissues and organisms measure size is a longstanding question that has recently received increasing attention [14,40–43]. In C. elegans, several factors may be involved in size assessment before a moult is initiated. One interesting possibility is that the properties of the collagen-rich cuticle, which encases the growing cells and tissues of the worm, give rise to an upper worm size limit, providing a mechanosensory cue for moult. Consistent with this, at each moult the cuticular sheath is about the same length.
regardless of growth temperature [44]. In *M. sexta*, tracheal size limits oxygen intake due to its salutary growth, thus contributing to the requirement for moulting [16]. In other insects, particularly in hemiptera, expanding or stretching the abdomen during growth triggers stretch receptors once a critical size has been achieved [45,46]. In line with this, stretch receptors have been identified in *C. elegans* that mediate normal locomotion [47]. Why and how worm volume dips during intermoult periods (figure 2) may help elucidate why and how worm volume stretch receptors have been identified in a critical size has been achieved [45,46]. In line with this, insects, particularly in hemiptera, expanding or stretching contribute to the requirement for moulting [16]. In other size limits oxygen intake due to its saltational growth, thus extension in adulthood. Interestingly, we were not able however, we note that we have not directly tested for lifespan phenomenon of increased lifespan under dietary restriction; development is slowed in larval stages under low food availability. Consistent with this, the moulting and genetic development programmes can be decoupled [50].

Importantly, the data presented in our manuscript do not rule out the possibility of intrinsic genetic timers. Gene expression in *C. elegans* is known to oscillate according to an intrinsic developmental timer, dictated by heterochronic genes [9,10,49]. It is conceivable that the size-dependent moulting decisions and the expressions of heterochronic gene represent separate but linked developmental programmes. Consistent with this, the moulting and genetic development programmes can be decoupled [50].

Our data point to a physical mechanism by which development is slowed in larval stages under low food availability. The requirement for achieving a critical size for developmental progression probably contributes to the well-known phenomenon of increased lifespan under dietary restriction; however, we note that we have not directly tested for lifespan extension in adulthood. Interestingly, we were not able to identify a minimum concentration of bacteria at which L1 worms ceased growth. Presumably, basal metabolic rates require a minimum food intake to sustain metabolic activity; only above this minimal level can the worm devote nutrient resources to the synthesis of new material required for increasing body mass. However, our data suggest that as food supply decreases, such that food intake becomes only marginally larger than the basal metabolic requirement, the worm growth rate approaches zero. Under these conditions, our data and model suggest that worms will spend an increasingly longer time in each developmental stage, before achieving the critical size. We are thus led to an intriguing question: if the developmental lifetime can be extended from 1 day to over 10 days, can it also be extended to 100 or 1000 days, or even longer?

Data accessibility. Data supporting this article have been uploaded as part of the electronic supplementary material.

Authors’ contributions. S.U. and C.P.B. designed the research and wrote the manuscript. S.U. carried out experiments and analysis.

Competing interests. We declare we have no competing interest.

Funding. This work was supported by the NIH Director’s New Innovator Award (1DP2GM105437-01), and the Searle Scholars Program (12-S99P-217).

Acknowledgements. We thank Stephanie Weber, Nilesh Vaidya and other members of the Brangwynne lab for helpful discussions. We thank Saurabh Vywahare at the Princeton Microfluidics Facility. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

References


