Adult *C. elegans* Exhibit Physiological Abnormalities When Early Gut Development is Partially Compromised

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ABSTRACT

The nematode *Caenorhabditis elegans* displays developmental robustness, such that nearly all embryos show normal development across a range of conditions. We are investigating properties of adults derived from embryos that are partially compromised for a very early step in the specification of the intestine (gut) primordium. As the gut provides essential nutritional functions to the animal, we hypothesize that is there are any abnormalities remaining in these adults, they might be detectable through indirect measurements of characteristics that depend on normal metabolism and physiology. Here, we examine adults derived from strains in which early embryonic gut development has been partially compromised and quantify three physiological properties: resistance to oxidative stress by exposure to hydrogen peroxide, mean rate of pharyngeal pumping, and average life span. Our results show that when specification is mildly compromised, oxidative stress resistance increases by 35%, pharyngeal pumping decreases by 11%, and life span decreases by 20%. In a more severely compromised strain, oxidative stress resistance decreases by 11%, pharyngeal pumping decreases by 27%, and life span decreases by 23%. The results show that function of the intestine, and general metabolic health, are indeed affected when gut specification is compromised. We propose that in *C. elegans*, proper function of the adult gut requires robust early progenitor specification, and that there are limits to the ability of later gut development to compensate for early perturbations in specification.

Keywords: *C. elegans*, gut specification, differentiation, adult defects, longevity, oxidative stress, pharyngeal pumping, metabolism

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Kollan Doan is a second-year Cell, Molecular, and Developmental Biology major. His research investigates the relationship between gene regulatory networks and gut specification in the model system, the nematode *Caenorhabditis elegans* under the guidance of Dr. Morris Maduro. He is a member of CNAS Science Ambassadors and Delta SIFY in which he works with outreach to inspire underprivileged students in pursuing STEM careers. He plans to apply to medical school within the next few years.

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Morris F. Maduro is a Professor in the Department of Biology and a past recipient of a Distinguished Teaching Award at the University of California, Riverside. His work focuses on the ways in which genes direct the development of animals, using the nematode *C. elegans* as a model system. Undergraduates are regularly involved in research in his laboratory.

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INTRODUCTION

The nematode *Caenorhabditis elegans* is an ideal model system for genetic studies due to its short life span and simple anatomy. Embryonic development takes approximately 12 hours at 25°C, whereupon the embryos hatch as first-stage larvae (L1). Approximately 26 hours after hatching and three larval molts (L1, L2, and L3), the animal enters the fourth-stage larvae (L4) whose prominent feature is the developing vulva. After 10 hours and a final molt, the animal develops to a young adult. The young adult matures to a full adult capable of egg-laying after eight hours. The adult consists of approximately 1000 cells, ~300 of which are neurons (Sulston et al. 1983). The *C. elegans* intestine has been a useful model for organ development and function. The fully developed intestine is a simple tube consisting of 20 cells at the end of development with a total of 34 nuclei at adulthood. The gene network that controls specification of the gut progenitor, a single embryonic cell called E, is well understood (Maduro 2006). The E cell is specified through the activation of several genes in simple gene network. The maternal factor SKN-1 activates the zygotic *med-1,2* GATA factor genes whose products contribute, along with input from maternal factors POP-1 and PAL-1, to the activation of the *end-1,3* genes to specify the E fate (Maduro et al. 2001, 2007). Downstream of specification, the GATA factors ELT-2 with contribution from ELT-7 maintain the intestinal fate, through initial activation of their genes by END-1,3 and autoregulation of *elt-2,7* to maintain their expression (McGhee et al. 2009; Fukushige et al. 1998). The simplest interpretation of this model is that gut development follows two phases: an early specification phase that gives the gut progenitor cells their identity, followed by a differentiation phase that maintains the intestinal fate.

In this work, we are interested in the extent to which the differentiation phase is self-correcting for mild defects in specification. In many systems, when early embryonic development is partially compromised, organ development undergoes canalization to result in an otherwise normal tissue (Waddington 1942). It is thought that canalization normally buffers stochastic differences among embryos that result from changes in the environment and variations

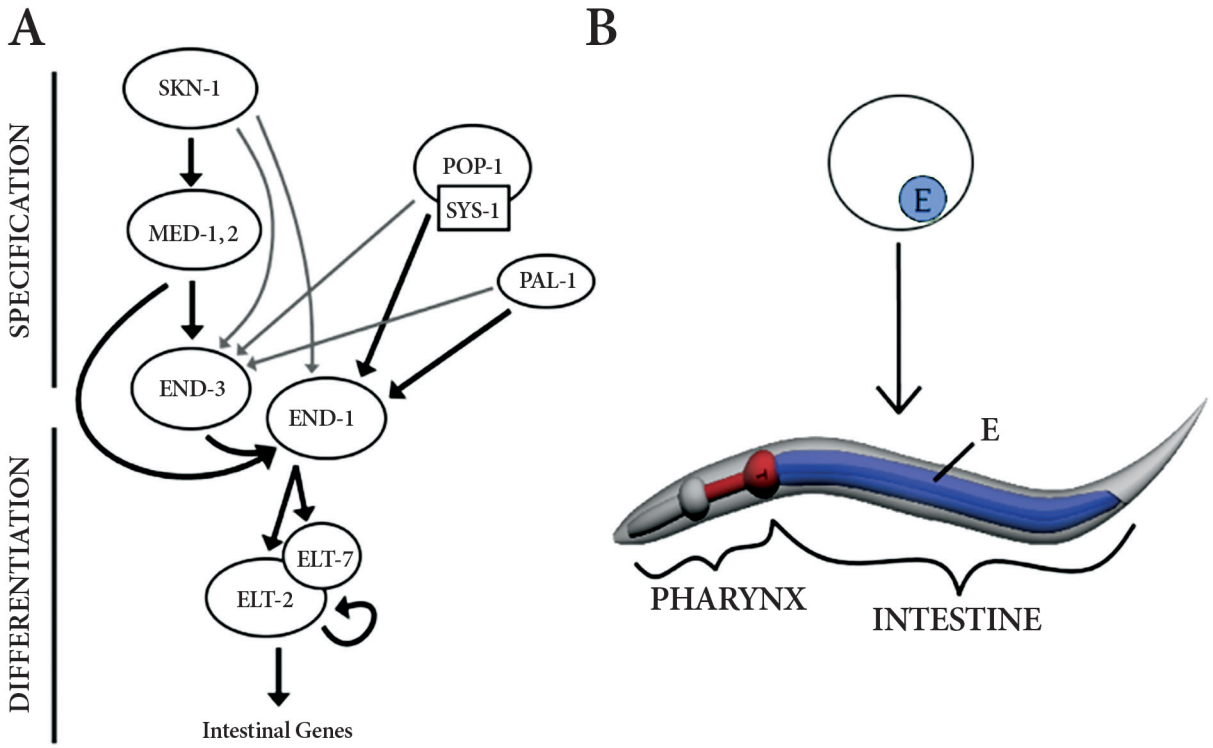

Figure 1: *A)* C. elegans *gene regulatory network for the specification of E showing required convergent upstream inputs of SKN-1, POP-1/SYS-1, and PAL-1 (Maduro 2008). B) Progenitor cell E produces the entire intestine (colored blue).*

Table 1: *Summary of strains used in experiments. Data from Maduro et al. 2007, 2015.*

Strain	Description	% of Embryos Making Any Amount of Gut Multicultural
MS1810	*end-1*(-), *end-3*(-) double mutant carrying wild-type *end-1*(+) and *end-3*(+) transgene, used as a control	100% (n>200)
MS1809	*end-1*(-), *end-3*(-) double mutant carrying *end-1*(+) and *end-3*(+) transgene, lacking binding sites for the MED proteins	75% (n=459)
MS404	*med-1*(-), *end-3*(-) double mutant	42% (n=251)

in gene expression. Unlike most other systems, the *C. elegans* embryo is "mosaic": if an embryonic cell is unable to adopt a specified lineage or is missing entirely, the normal descendants will not be replaced (Sulston et al. 1983). When the gene network that specifies gut in *C. elegans* is partially compromised, not all E lineage cells adopt a gut fate in all embryos (Maduro et al. 2015). The worms that do create a functional gut and survive into adulthood exhibit a variety of visible defects that suggest metabolic function is impaired, but this has not yet been tested directly (Maduro et al. 2015).

In this study, we determined the degree to which survival of partial gut differentiation manifested a change in the physiological state of animals compared to controls, focusing on three quantifiable behaviors with implications in metabolism. The first of these was resistance to oxidative stress. Endogenous or exogenous reactive oxygen species (ROS) can damage various components of the cell. These radicals, resulting from oxygen consumption during metabolism, can impair function by oxidizing macromolecules (Golden et al. 2002). *C. elegans* can be subjected to oxidative stress by exposure of compounds to assess for the ability to withstand free radicals. Hence, we evaluated survival time of animals in liquid buffer containing 3 mM hydrogen peroxide. The second behavior measured was pharyngeal pumping. To consume nutrients, usually bacteria or single-celled fungi, *C. elegans* larvae and adults pump food into the intestine using the pharynx, an organ that is functionally equivalent to the human esophagus. The rate of pharyngeal pumping is regulated depending on the presence of food, and function of the pharynx muscles themselves depends on the overall health of the animal (Croll 1978; Horvitz et al. 1982; Chiang et al. 2006). We measured the rate of pharyngeal pumping on agar plates in the presence and absence of food (bacteria) by direct microscopic observation. The third behavior measured is life span. The ability of *C. elegans* to survive under laboratory conditions is directly related to their overall health (Golden et al. 2002). To measure life span, we maintained animals on agar plates in the presence of food, eliminating animals that ceased movement and became unresponsive to touch.

METHODOLOGY

Nematode Strains and Animal Handling. Three strains were used, consisting of a control (MS1810) and two strains that exhibit compromised gut specification: MS1809 and MS404. Shown in Table 1, MS1809 has mild defects in gut specification such that its embryos make some amount of gut approximately 75% of the time. Strain MS404 has more severe defects in gut specification and whose embryos make gut approximately 42% of the time. Strains were age-synchronized by rinsing and bleaching of gravid adult worms. Synchronized embryos were collected via centrifugation then pipetted onto petri dishes consisting of Nematode Growth Medium (NGM) and seeded with OP50, an *E. coli* bacterial strain that is standard for growing *C. elegans* (Brenner 1974). Embryos were left to hatch and raised at 23°C for approximately three days until late L4/early adulthood was reached. Age-synchronized adult worms were randomly picked and allocated for use in all experiments. Assays for oxidative stress resistance, pharyngeal pumping rate, and life span were performed in triplicate according to previously established procedures, summarized below (Kenyon et al. 1993; Larsen 1993; Chiang et al. 2006).

Oxidative Stress Resistance. To measure resistance to oxidative stress, commercially available 3% Hydrogen Peroxide (H_2O_2) was diluted down from approximately 882 mM to 3 mM in M9 buffer. Fifty microliters of 3 mM H_2O_2 were pipetted into six wells each per strain of a 96-well microtiter plate. Four worms at late L4/early adulthood

were allocated per well across six wells for a total of 24 worms per strain. Observations lasted over a period of eight hours beginning with t = 0 hours and recording the number of worms alive after each hour. The assay was repeated three times and the number of worms alive each hour was summed to determine the average number of worms alive each hour per strain. Student's t-test was used to determine significance in differences between control and compromised strains.

Pharyngeal Pumping. To determine pharyngeal pumping rates, 70 worms per strain, synchronized at late L4/early adulthood stage, were transferred to new plates. Half were transferred to plates seeded with OP50 as food with the other half transferred to unseeded plates with no food. Observations began 30 minutes after transferring worms to plates to allow for acclimation. The pharyngeal pumping rate was defined as the number of contractions in the pharyngeal terminal bulb in a time of one minute. An ANOVA test was use to analyze the differences in pharyngeal pumping rate among and between the control strain and compromised strains.

Life Span. Animals grown under the conditions described above were selected for longevity assay as follows. Fifty age-synchronized worms at late L4/early adulthood from each strain were transferred to new, seeded plates every day for three to four days until egg-laying ceased. The worms were then transferred every two to three days to new plates until all worms were deceased, ascertained by cessation of movement and lack of response to gentle touch. Observations were taken daily, beginning with day one of adulthood, and the number of worms that were still alive on each subsequent day was recorded. Worms that died prematurely due to hatching of embryos inside the mothers, or who escaped the agar surface, were censored from the results. Student's t-test was used to determine significance between control and compromised strains.

RESULTS

Resistance to Oxidative Stress. We evaluated survival of adults in the presence of 3 mM hydrogen peroxide (H_2O_2). Mean survival time was calculated from the survival of individual animals over an eight-hour time period.

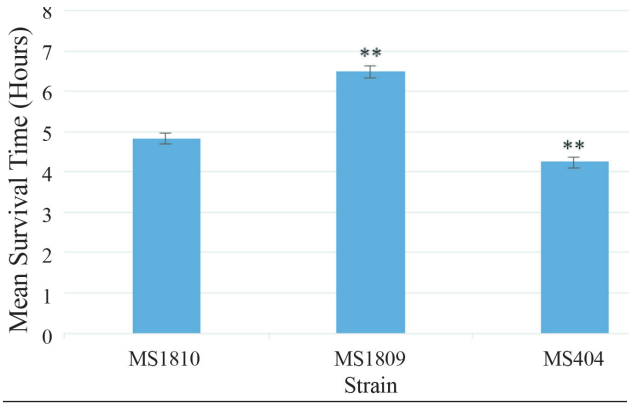

Figure 2: *The mean surviva:l time of adults in 3 mM hydrogen peroxide. **Values were significant between wild-type and compromised strains.*

The mean survival time of control MS1810 animals was 4.8 ± 0.13 hours. We found that the mean survival time of MS1809 was higher, 6.5 ± 0.15 hours. Student's t-test determined that this increase was significant ($P < 0.0001$). While MS1809 experienced increased survival in H_2O_2, MS404, a highly gut compromised strain, showed a decrease in mean survival time of 4.25 ± 0.14 hours compared to the control and mildly compromised strain. Statistical analysis showed that the susceptibility of MS404 to oxidative stress was significant ($P = 0.0033$).

Pharyngeal Pumping Rate. We measured the rate of pharyngeal pumping of our strains in the presence and absence of bacteria as described in Figure 3. In the

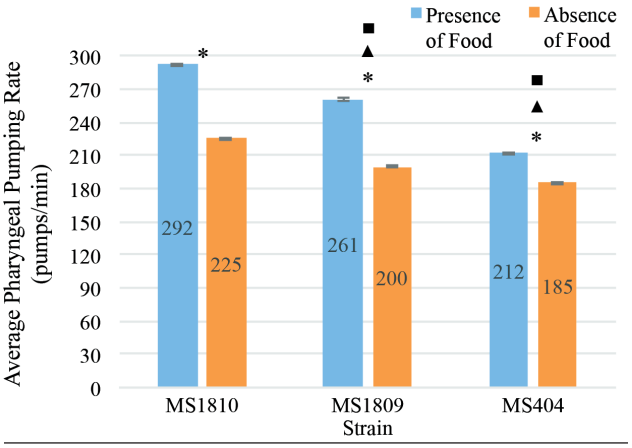

Figure 3: *The average pharyngeal pumping rate of MS1810, MS1809 and MS404 in the presence and absence of food (mean ± SEM). *Values were significant between groups in the presence and in the absence of food. ▲Values were significant when compared to wild-type in the presence of food. ■Values were significant when compared to wild-type in the absence of food.*

presence of food, the pharyngeal pumping rates across the three strains MS1810 (control), MS1809, and MS404 were measured to be 292 ± 1.5, 262 ± 1.7, and 212 ± 1.2 pumps/minute, respectively. In the absence of food, the pharyngeal pumping rates were 225 ± 1.3, 200 ± 1.1, and 185 ± 1.2 pumps/minute respectively. The decreases seen across all three strains were consistent, confirming that as the gut specification became more compromised, surviving adults showed significantly slower pharyngeal pumping rates ($P < 0.0001$).

Life Span. Using the control strain MS1810 as a baseline, we determined that both strains exhibiting embryonic gut defects experienced decreases in adult life span. The average life spans of MS1810, MS109, and MS404 were 10.7 ± 0.88, 8.5 ± 0.63, and 8.2 ± 0.52 days, respectively, as shown in Figure 4. We determined that the average life span of MS1809 adults was 20% lower ($P = 0.0515$) and that of MS404 was 23% lower ($P = 0.0178$) than the control.

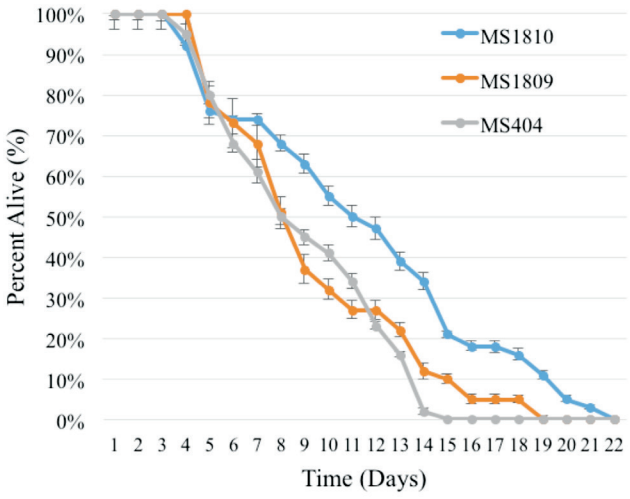

Figure 4: *Adult life span of the control strain to gut compromised strains. Differences between groups were significant (P = 0.0237).*

DISCUSSION

We found that partial gut differentiation generally led to physiological defects with more severe abnormalities exhibited from a more compromised strain, with one exception described below. Our data shows that *C. elegans* animals are unable to compensate for early gut specification defects, resulting in mild phenotypic abnormalities in the adult. This suggests that the developing intestine does not compensate for the consequences of partial specification, even though a relatively normal morphology gut is produced (Maduro et al. 2015). It was reported that the hypomorphic specification adults exhibit a slight increase in the number of gut nuclei, an increase in approximately five extra nuclei on average. These extra nuclei do not appear to be the direct cause of the physiological defects, as gain-of-function mutants in *cdc-25.1* that result in an even greater number of nuclei have not been observed to have other defects in gut function (Kostić et al. 2002).

In the mildly compromised strain MS1809, we observed an increase in resistance to oxidative stress, while in the more severely compromised strain MS404, we observed a decrease. This result was unexpected for two reasons. First, the other two behaviors measured, pharyngeal pumping and life span, both decreased in the compromised strains. Second, resistance to oxidative stress has generally been observed to correlate with an increased life span, not a decreased one (Kenyon 2010). We measured oxidative stress in MS1814, a strain with similar genotype to MS1809 but constructed in parallel, and found the same results as with MS1809 (data not shown), suggesting that the increased oxidative stress resistance in MS1809 is not the result of a spurious background mutation. Rather, the results suggest that a mild perturbation of gut specification results in the activation of a pathway that increases resistance to oxidative stress, but a stronger compromise blocks this pathway. A further experiment to determine whether pathways involved in oxidative stress resistance are indeed activated in MS1809 but not MS404 could be used to test this hypothesis of degree in gut specification.

The decreases in pharyngeal pumping and life span, and the changes in resistance to oxidative stress, collectively suggest that metabolic function is altered in the specification-compromised strains. Consistent with this, unpublished results from our laboratory show that the MS1809 and MS404 strains exhibit increases in lipid storage in the intestine. Excess lipid storage is analogous to an increase in fat cells in mammals, suggesting that our *C. elegans* strains have altered metabolism in favor of energy storage. We interpret our collective results to mean that partially-compromised gut specification results in a change to an alternate metabolic state that affects metabolism, causing changes in a variety of physiological properties such as resistance to oxidative stress and overall health. This metabolic state is similar, but not identical, to effects observed

under dietary restriction—when normal animals are subjected to limited caloric intake, they normally exhibit increases in life span and resistance to oxidative stress (Yu 1996). The results also indicate that the effects of partial gut specification on differentiation are complex, likely representing changes in a variety of pathways that cause diverse physiological abnormalities. Further experiments will be directed at identifying the major regulatory pathways that have changed as a result of compromised specification.

CONCLUSION

Through characterization of abnormalities in animals that survive partial gut specification, we were able to detect multiple phenotypic defects that suggest that proper metabolism in adults requires proper early progenitor specification. Gut function plays a fundamental role in pathogenesis of various metabolic diseases, including obesity and diabetes, which suggests that at least some of the time, these diseases may result from mild defects in early embryonic development. Our work suggests that *C. elegans* provides a platform for understanding the connection between early-acting gene networks for gut specification and the consequences on development and adult intestine function.

ACKNOWLEDGMENTS

I would like to thank Dr. Morris Maduro for his guidance, mentorship, and continued support throughout my project. I would also like to extend my thanks to Gina Broitman-Maduro and Hailey Choi for their invaluable input and insight.

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