Genetic Analysis of Rhythmic Behavior in *C. elegans*

Jeanna M. Wheeler

A dissertation submitted in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

University of Washington

2005

Program Authorized to Offer Degree: Genome Sciences

INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

University of Washington
Graduate School

This is to certify that I have examined this copy of a doctoral dissertation by

Jeanna M. Wheeler

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Chair of the Supervisory Committee:

James H. Thomas

Reading Committee:

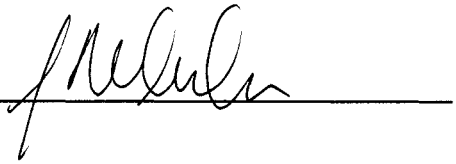
James H. Thomas

Robert Waterston

William Zagotta

Date: 11/28/05

In presenting this dissertation in partial fulfillment of the requirements for the doctoral degree at the University of Washington, I agree that the Library shall make its copies freely available for inspection. I further agree that extensive copying of the dissertation is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for copying or reproduction of this dissertation may be referred to Proquest Information and Learning, 300 North Zeeb Road, Ann Arbor, MI 48106-1346, 1-800-521-0600, to whom the author has granted "the right to reproduce and sell (a) copies of the manuscript in microform and/or (b) printed copies of the manuscript made from microform.

Signature: 

Date: 11/29/05

University of Washington

Abstract

Genetic Analysis of Rhythmic Behavior in *C. elegans*

Jeanna M. Wheeler

Chair of the Supervisory Committee:
Professor James H. Thomas
Department of Genome Sciences

How do genetic networks control the behavior of an organism? To approach this problem, I chose the model organism *C. elegans*, a simple metazoan that displays several easily observed behavioral programs such as locomotion, egg-laying, and defecation. *C. elegans* defecation is a rhythmic behavior regulated by the intestine, and consists of three muscle contractions occurring at regular intervals. My results suggest that chemosensation and external mechanosensation play no role in regulating the defecation cycle. However, conditions affecting the metabolism of the animal (e.g. starvation, poor food quality, defects in genes important for basic cellular functions) lead to alterations in the cycle period. In addition, I present evidence suggesting that the amount of food consumed is sensed by internal mechanosensors.

Several mutants had been previously isolated for their effects on the defecation rhythm, but in most cases their molecular identity was unknown. One of these mutants is

dec-2(sa89), which causes a long defecation period. My work reveals that *dec-2* encodes a novel secreted protein expressed exclusively in the hypodermis, in contrast to other Dec genes, all of which function in the intestine.

dec-2(sa89) is allelic to *osm-7(n1515)*, and both mutations cause resistance to osmotic stress. I show that adaptation to osmotic stress alters the defecation behavior of wild-type *C. elegans* to the same extent observed in *osm-7/dec-2* mutants. *C. elegans* adapts to osmotic stress by increasing glycerol production, and I find that *osm-7/dec-2* mutants have high basal levels of glycerol in the absence of osmotic stress. I have also identified several other genes with mutant phenotypes similar to that of *osm-7/dec-2*. These include *osm-11*, a gene I identified based on homology to *osm-7/dec-2*, and three collagen genes required for the formation of a substructure in the cuticle.

These results lead me to propose a model in which *osm-7* and *osm-11* are secreted from the hypodermis, interact with the cuticle, and function as negative regulators of the response to osmotic stress. This hypothesis reveals interesting parallels with osmotic stress response in yeast, and future work on these mutants should provide insight into general mechanisms of stress resistance.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to thank my advisor, Jim Thomas, for his support and guidance throughout this project. His excellence as a scientist and his devotion to a small roundworm will continue to be a source of inspiration. I would also like to thank all the members of the Thomas lab that contributed to an active and intellectually stimulating work environment. Most of all, I thank my parents for supporting me through my education and allowing me to follow my dreams, wherever they might lead me.

Chapter One:
Regulation of the ultradian defecation rhythm of *C. elegans*

Introduction

Biological rhythms are present in species from bacteria to mammals, and regulate diverse behaviors. Rhythms are divided arbitrarily by period length into ultradian (shorter than 24 hours), circadian (approximately 24 hours), and infradian (longer than 24 hours). Another criteria for categorizing biological rhythms is the number of cells required to maintain the rhythm. For instance, lamprey swimming behavior (Grillner 2003) and crustacean gut peristalsis (Selverston 2005) are examples of rhythms regulated by central pattern generators (CPGs), which are composed of small neural networks. Other biological rhythms are generated by cellular oscillators that act in a cell autonomous manner. One example of this type of rhythm is the ultradian oscillation of yeast respiration, which peaks every 40 minutes. The transcription of most yeast genes is affected by this metabolic rhythm (Klevecz *et al.* 2004), but the molecular nature of the pacemaker responsible for maintaining rhythmicity is not understood. Cellular oscillators responsible for circadian rhythms have been examined at a molecular level in diverse organisms, including *Neurospora* (Loros & Dunlap 2001), *Drosophila*, and mammals (Stanewsky 2003). Most of these oscillators are based on transcriptional feedback loops. Positive loop elements activate transcription of the negative loop elements, which in turn function to inhibit the activity of the positive elements. Gradual decay of the negative elements leads to reactivation of the cycle (Bell-Pedersen *et al.* 2005). While the molecular details of various biological clocks differ, there are a few unifying characteristics. By definition, a biological clock must be able to keep time in the absence of external cues, such as in constant darkness or temperature. In addition, biological

clocks are often temperature compensated over a physiological range of temperatures, and can be reset by an external stimulus, or *zeitgeber*, that entrains the phase of the oscillation (Schibler & Naef 2005).

C. elegans displays an ultradian behavior, the defecation rhythm, which has the qualities of a biological clock (Liu & Thomas 1994). Thus defecation behavior can serve as a model for rhythmic behaviors, and for the study of the genetic control of behavior in general. The defecation motor program is not required for survival under laboratory conditions and is easily observed using a dissecting microscope. These qualities have allowed for the use of genetic screens to identify mutants with defects in various aspects of the motor program (Thomas 1990, Iwasaki et al. 1995). The molecular nature of the cellular oscillator responsible for the defecation rhythm will be discussed in more detail in the next chapter.

C. elegans defecation is achieved by a series of muscle contractions that occur approximately every 50 seconds when abundant food is present. Figure 1 shows a wild type worm performing the defecation motor program (DMP). The first step is the simultaneous contraction of the dorsal and ventral body wall muscles, beginning at the posterior of the animal and moving anteriorly in a wave-like fashion. This posterior body wall muscle contraction (pBoc) causes mixing of the gut contents and forces them toward the anterior of the intestine. Approximately four seconds after relaxation of the pBoc, a quick sharp contraction of the anterior body wall muscles (aBoc) occurs which forces the posterior bulb of the pharynx toward the anterior end of the intestine. Immediately

following the aBoc, a set of muscles at the posterior of the animal contracts to open the anus and expel some of the gut contents (Exp).

The period of the defecation cycle can remain constant (~50 seconds) across the range of permissive growth temperatures (19-30°C) for *C. elegans* (Liu & Thomas 1994). The temperature insensitivity of the cycle indicates that defecation is carefully regulated and that there is an optimum length for the cycle period which the organism attempts to maintain. This idea is also supported by the observation that mutants with altered cycle timing are generally slow-growing and scrawny, presumably due to reduced nutrition (Iwasaki *et al.* 1995). However, temperature does have an effect on the defecation cycle period when worms are shifted to a temperature other than the cultivation temperature. For instance, worms cultivated at 20°C have a faster than normal cycle when shifted to 25°C and a slow cycle at 15°C (Branicky *et al.* 2001). Mutants have been isolated (*clk-1* and *dec-7*) which are defective for temperature-dependent regulation of the cycle, indicating that this aspect of *C. elegans* defecation is also under genetic control (Branicky *et al.* 2001).

Stimulation of *C. elegans* by light touch with an eyelash causes the animal to reverse its movement and back up quickly. The same stimulus can reset the phase of the defecation cycle; the next defecation event is often in phase with the time of the touch rather than in phase with the previous defecation event (Thomas 1990). This result is comparable to reset of other rhythmic behaviors by external stimuli, such as light reset of the circadian clock, and is consistent with the idea that there is a molecular clock in control of *C.*

elegans defecation. Although the neural circuit regulating the locomotion response to light touch has been well characterized (Chalfie 1993), it is currently not clear how this pathway intersects with the defecation clock.

When a wild-type worm enters an area lacking food, it ceases defecating. Upon returning to food, the next defecation event is often in phase with the previous one (Liu & Thomas 1994). This indicates the presence of a molecular clock that keeps time in the absence of the defecation motor program. In addition, when worms are moved from a thick bacterial lawn to a diluted lawn, the cycle period is lengthened (Liu & Thomas 1994). Thus, *C. elegans* is able to sense the concentration of food in the environment and adjust the defecation cycle period accordingly. The mechanism whereby information about food concentration is sensed and conveyed to the defecation clock is not understood. It has been shown that *C. elegans* regulates its locomotion in response to mechanosensory cues from bacteria (Sawin *et al.* 2000). It is possible that similar cues contribute to regulation of the defecation cycle, but other possible mechanisms for sensing of bacterial concentration include chemosensation and gut distention, and I address all of these possibilities below.

The aim of this project was to extend previous studies, in order to better understand how *C. elegans* senses food concentration and translates this into an altered defecation cycle period. I have examined the effects of various environmental conditions and several loss-of-function mutations on the regulation of defecation timing. I have also carried out a

small-scale RNAi screen for the Dec phenotype, in an effort to identify genes important for the maintainance of a normal defecation cycle period.

Materials and Methods

Strains and nematode growth conditions

Worms were cultured using standard methods (Brenner 1974). Unless otherwise noted, all strains were grown at 20°C on NGM agar plates with *E. coli* OP50 as the food source. Strains used in this study were N2 Bristol wild type, CB1112 *cat-2(e1112)*, CB1387 *daf-10(e1387)*, DA522 *eat-13(ad522)*, DA606 *eat-10(ad606)*, JT10719 *tph-1(mg280); unc-64(e246)*, and KP4 *glr-1(n2461)*. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources.

Behavioral assays

For all conditions, worms were assayed at 20°C as first-day adults. Observations were made with the plate lids on to minimize disturbances caused by air currents, and care was taken not to disturb the animals by tapping the plates. Observations were recorded using the Etho program developed by J. Thomas.

For "rinsed bacteria" and "supernatant" feeding conditions, *E. coli* were grown to exponential growth phase, then centrifuged to separate the bacteria from the growth media. NGM agar plates were flooded with the growth media supernatant, then inverted

and allowed to dry briefly before using for assays. *E. coli* removed from the supernatant were rinsed twice in M9, then concentrated and placed on NGM agar plates to create thick lawns. Plates were used within 10 minutes to minimize growth of the bacteria.

Sephadex G-200 beads (Sigma Chemicals) were suspended in S basal buffer at 30 mg/ml and autoclaved. Approximately 200μl of this mixture was transferred to an agar plate with no bacteria and allowed to dry. Animals were picked onto these plates, taking care to transfer as little food as possible, and then allowed to acclimate for 10 minutes before assaying.

Fluoresbrite YG 1.0 micron microspheres (Polysciences Inc.) were used as a food substitute. These beads are small enough to be ingested by *C. elegans* and can be observed passing through the entire digestive tract. Approximately 200μl of the bead suspension was transferred to an agar plate with no bacteria and allowed to dry. Animals were picked onto these plates individually and then assayed after 10 minutes to allow time for bacteria to clear the gut and the beads to enter.

For the "3 hour starve" assay condition, worms were raised to adulthood in the presence of an excess of food, then shifted to plates with no food. After three hours, the worms were moved back to the food source and assayed, allowing 10 minutes for the worms to recover from the transfer.

"No cholesterol" plates are standard NGM plates with no cholesterol added. This agar probably contains small amounts of phytosterols from other media components. Well-fed N2 worms were allowed to lay eggs on these plates, and the progeny were assayed for defecation phenotypes as young adults.

Diluted food plates were prepared as follows: *E. coli* OP50 were grown to exponential phase, spun down, and resuspended in M9 before being adjusted to OD_{600} 1.4. This "1x" solution was diluted to make a range of bacterial concentrations. The bacteria were spread onto NGM plates and allowed to dry for 30 minutes. A ring of 4M fructose was placed around the edges of the plates to prevent worm escape. Plates were used within 30 minutes of seeding to minimize bacterial growth. Worms were allowed to adjust to the assay environment for at least 10 minutes before any cycles were recorded. N2 controls were assayed on the same plate as mutant worms, on the same day.

RNAi experiments

Bacterial strains used for RNAi knockdown were obtained from the Ahringer library (MRC Geneservice). Growth plates used for RNAi were prepared as previously described (Fraser 2000). L4 animals were placed on dsRNA-producing bacteria and raised at 15°C for 72 hours, then transferred to fresh plates at 20°C. These animals were allowed to lay eggs for three hours and then removed from the plates. Progeny from the second set of plates were assayed 4 days later for defecation phenotypes. For each strain, six animals were assayed for four cycle periods each. The growth rate of these animals was assessed by counting the number of worms that were at the adult, L4, or L3 stage.

At the time of the assay, all control worms had reached adulthood and thus slow growth was defined as any strain that displayed a majority of animals still at L4 or L3 stages.

Results and Discussion

Role of chemosensation in the response to food

C. elegans is sensitive to the concentration of food in the environment, as is indicated by the ability of worms to rapidly adjust the length of the defecation cycle in response to changes in food concentration. Under standard laboratory conditions, the *E. coli* food source is present in excess. *C. elegans* is essentially immersed in the bacterial lawn, and feeds almost constantly at maximal speed. Liu and Thomas (1994) observed that the average cycle period of wild-type worms begins to lengthen at food concentrations 10 times more dilute than the standard lawn, and continues to lengthen gradually as food concentration is decreased. Most animals cease defecating at dilutions of 100-fold or more. An obvious candidate for the sensation of food concentration is the chemosensory system of *C. elegans*. The worm genome contains an astonishing number of putative chemoreceptors (Bargmann 1998), and behavioral assays reveal that *C. elegans* responds to a large number of chemical stimuli (Bargmann *et al.* 1993). However, preliminary results indicated that mutants which are defective in chemosensation display wild-type defecation behavior (Thomas 1990). This information led me to the hypothesis that chemosensation is not required for regulation of the cycle in response to food concentration. To address this, I carried out the following two experiments.

First, I asked whether a growing bacterial culture might produce some soluble factor that is sensed by *C. elegans*. The concentration of this soluble factor could vary based on environmental bacterial concentration, and thus provide a signal for modulation of the defecation rate. As shown in Table 1, I found that worms in the presence of bacterial growth media (with the bacteria removed) almost never defecated, and when they did, the cycle period was very long. Conversely, a bacterial culture that had been rinsed to remove any chemicals present in the growth media caused worms to behave identically to those assayed under normal feeding conditions. These results are consistent with the conclusion that there is no soluble factor secreted by bacteria that contributes to the regulation of defecation timing. However, it remains possible that the signal is a labile compound (thus missing from the growth media plates) which is rapidly produced by the bacteria (thus present even in the rinsed bacteria).

Previous results indicated that chemosensation is not required for a wild-type defecation cycle period when food is abundant. To extend these results, I asked whether mutants with defects in chemosensation might have an impaired ability to sense decreases in food concentration. *daf-10* mutants have defects in all chemosensory cilia and exhibit abnormal chemotaxis (Perkins *et al.* 1986). Figure 2A shows that *daf-10* mutants regulate their defecation cycle period normally in response to decreased food stimulus. Thus, I find no evidence that chemosensory pathways are involved in the response of *C. elegans* to lowered food concentration.

Role of mechanosensation in the response to food

Sawin *et al.* (2000) reported that *C. elegans* alters its locomotion behavior in response to a bacterial lawn, and that this "basal slowing response" is mediated by dopaminergic mechanosensory neurons. Worms crawling on a matrix of Sephadex beads exhibit locomotion behavior similar to that of worms crawling on a bacterial lawn. I tested the defecation behavior of *C. elegans* under similar assay conditions, and observed that the worms ceased defecating (Table 1). Thus, this type of mechanosensory stimulus is not sufficient to maintain wild-type defecation behavior, and different pathways must regulate locomotion and defecation in response to food concentration. This result is also consistent with my finding (see below) that dopaminergic signaling is not required for a wild-type defecation cycle period.

I also tested the possibility that mechanosensory inputs to the defecation clock may be internal (e.g. in the pharynx or gut) rather than external. I assayed the defecation behavior of *C. elegans* in the presence of a food substitute, non-digestible beads that are small enough to be ingested. I found that the worms maintained a normal defecation cycle period while ingesting these beads, despite the absence of significant amounts of bacteria (Table 1). This suggests that *C. elegans* senses the amount of bacteria actually consumed, rather than the amount of bacteria present in the environment, when determining its rate of defecation. It also suggests that quantity is more important than quality, at least at the time of consumption, since the defecation cycle does not immediately respond to ingestion of non-digestible material. The conclusion that there are internal mechanosensory inputs to the defecation clock is also supported by the

observation that constipated mutants, which exhibit distention of the gut lumen, have slightly shorter than normal defecation cycle periods (Elaine Round, personal communication).

Role of starvation state in the response to food

It has been reported that a period of starvation affects several behaviors in *C. elegans*, including thermotaxis (Mori 1999) and chemotaxis (Colbert & Bargmann 1997). The locomotion of worms in response to a lawn of food is also altered by the starvation state of the animals (Sawin *et al.* 2000). Worms normally slow down upon entering a bacterial lawn, but this response is exaggerated if the animals have been starved prior to the assay. This is known as the "enhanced slowing response". Table 1 shows that the defecation cycle period of *C. elegans* is not altered upon return to food after 30 minutes of starvation, but is significantly lengthened after 3 hours of starvation. This is in contrast to the results of locomotion assays, where 30 minutes of starvation was sufficient to induce a behavioral change (Sawin *et al.* 2000). This result supports the conclusion that food is sensed by at least two distinct mechanisms, and that locomotion and defecation are regulated by separate pathways. However, it is also possible that the enhanced slowing response and the defecation cycle are regulated by starvation via the same pathway, but require a different threshold of stimulus to produce a behavioral response (see below).

C. elegans cannot synthesize sterols *de novo*, and thus cholesterol must be supplied in the growth media. Worms that are cultivated in the complete absence of cholesterol have

lowered brood size and slow growth rate, and their progeny do not develop to adulthood (Merris *et al.* 2003). To test whether lack of a single nutrient could affect the defecation cycle, even in the presence of excess bacteria, I measured the cycle period of *C. elegans* raised on plates with no added cholesterol. I observed that cholesterol deprivation causes a severe lengthening of the cycle period even in the first generation of animals exposed to this treatment (Table 1). Thus, nutrient availability provides an important input to the defecation clock, rather than the simple presence or absence of bacteria. It should also be noted that several mutants that lack enzymes required for fatty acid metabolism, and thus have altered lipid content, have also been shown to have long defecation cycle periods (Kniazeva *et al.* 2003).

Another way to assess the effects of starvation on the defecation cycle is to use mutants that have defective pharyngeal pumping. These eating-defective (Eat) mutants are presumed to be slightly starved due to a decreased capacity to ingest bacteria. It was previously reported that two Eat mutants, *eat-2* and *unc-89*, have slightly longer than normal defecation cycle periods (Thomas 1990). However, these strains display several other pleiotropies, and it is not clear whether their defecation defect is caused specifically by the Eat phenotype. Thus, I wanted to test for defecation defects in mutants that have a strong Eat phenotype with no obvious pleiotropies. *eat-10(ad606)* and *eat-13(ad522)* meet these qualifications (Avery 1993). Table 2 shows that the average cycle period of the *eat-10* and *eat-13* mutant strains is wild-type, and Figure 2B shows that *eat-10* worms are also capable of altering their defecation cycle in response to lowered food concentration. One possible explanation for these results is that although *eat-10* and *eat-*

13 cannot ingest bacteria as well as the wild type, they are still able to eat enough to avoid triggering the starvation response. It is also possible that these strains are starved, but that chronic starvation of these mutants has a different effect than that of acute starvation of the wild type. These observations suggest that a defect in pumping alone is not sufficient to cause alteration of the defecation cycle period. However, it remains unclear exactly why *eat-2* and *unc-89* display a long cycle phenotype.

Role of neurotransmitters in the response to food

Mutations in the gene encoding choline acetyltransferase (*cha-1*) cause a lack of acetylcholine and severe defects in synaptic transmission. Partial loss-of-function *cha-1* mutants have defects in locomotion, egg-laying, and defecation (Rand 1989, Bany *et al.* 2003, Thomas 1990). However, other mutations causing defects in synaptic transmission do not have a large effect on defecation behavior (Thomas 1990). In particular, mutations in *unc-17*, which encodes the transporter responsible for loading acetylcholine into synaptic vesicles, cause phenotypes very similar to those of *cha-1* mutants with the exception of defecation behavior (Nguyen *et al.* 1995, Thomas 1990). It remains unclear why *cha-1* is the only mutant in its class that also has a long defecation cycle.

Sawin *et al.* (2000) reported that mutants with defects in serotonin and dopamine metabolism exhibited altered locomotion behavior in response to food. *cat-2* encodes tyrosine hydroxylase, an enzyme required for dopamine synthesis (Lints and Emmons 1999). *tph-1* encodes tryptophan hydroxylase and is required for serotonin synthesis (Sze *et al.* 2000). I examined the defecation behavior of these mutants, to determine whether

dopamine or serotonin signaling is required for a wild-type defecation cycle period. I also included the mutant *glr-1*, which encodes a glutamate receptor (Maricq *et al.* 1995), in order to examine the effect of a defect in glutamate signaling. As shown in Table 2, none of these mutants have a very large effect on the defecation cycle period. *tph-1* mutants have a slightly longer than normal cycle period, indicating that serotonin signaling may provide input to the defecation clock. This is interesting because serotonin mediates the enhanced slowing response, which is the locomotory response that starved worms exhibit upon return to food. Thus, starvation may lead to a serotonin-mediated signal that affects both locomotion and defecation. In contrast, I have found that external mechanical stimulation, which is sufficient to elicit the basal slowing response mediated by dopamine, does not affect cycle period and neither does elimination of dopamine signaling.

Correlation between slow growth and long defecation cycle period

All previously identified mutations causing altered cycle period have also been characterized as slow-growing (Iwasaki *et al.* 1995). In general, defecation-defective (Dec) strains take an additional day to reach adulthood as compared to the wild type. This fact has been exploited for genetic mapping by myself and others, and here I also use the slow-growth phenotype (Gro) to conduct a candidate RNAi screen. Large-scale RNAi screens have been carried out in *C. elegans* and have identified several hundred genes that produce a Gro phenotype when their expression is reduced (Simmer *et al.* 2003, Kamath *et al.* 2003, Maeda *et al.* 2001). Two genes known to cause a Dec phenotype (*dec-2* and *flr-1*) were annotated as Gro and Clr (clear) in large-scale screens.

I chose 18 clones from the Ahringer RNAi library that had been annotated as giving a Gro phenotype. Preference was given to clones annotated as only Gro or Gro Clr, to avoid ones with additional phenotypes that might complicate the analysis. As shown in Table 3, the molecular identities of the corresponding genes are diverse. I hypothesized that genes involved in basic metabolic functions, such as structural ribosomal components and tRNA synthetases, would be Gro but not Dec, and that genes interacting with the defecation clock, giving a Gro Dec phenotype, would be rare. I found that out of 18 clones, eight did not give a Gro phenotype (suggesting that the RNAi was not working). Of these eight non-Gro clones, seven had normal defecation timing. Among the ten clones that did cause slow growth, I observed the following defecation phenotypes: one short cycle period (37 sec.), one normal cycle period (57 sec.), and nine long cycle periods (< 70 sec.). Based on a Fisher's Exact Test of the data, the correlation between slow-growth and abnormal defecation cycle period is highly significant ($p=0.0008$).

These data show that the Dec phenotype is much more common than previously thought. Furthermore, I propose that these data indicate that the metabolic rate of the animal provides input to the defecation clock. Similar to the effects of starvation on *C. elegans*, a defect in translation or another essential process would cause a shortage of important metabolites. This deficiency could theoretically be sensed by the defecation clock through the same pathways that sense starvation state. Although it remains possible that a long defecation cycle is in some cases the cause of slow growth, I propose that the reverse is most often true. Thus, many defects cause slow growth of the animal, and

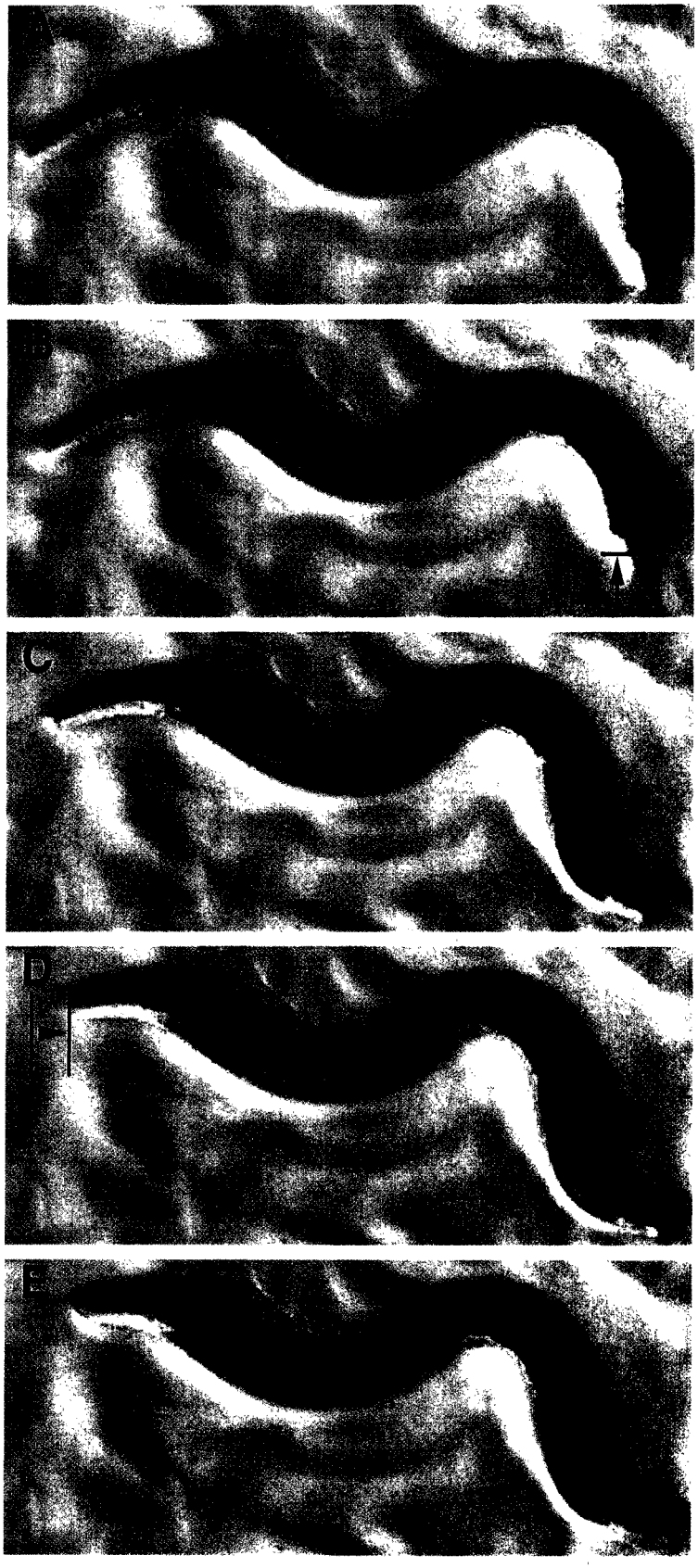
subsequently the defecation cycle is altered in response. A long defecation cycle may allow food to remain in the intestine longer, thereby allowing the animal to extract more nutrients from the food.

Conclusions

I have extended current knowledge about what types of stimuli provide input to the molecular clock in control of the *C. elegans* defecation rhythm. I find that chemosensation, while an obvious candidate for responses to food, is dispensable for normal regulation of the defecation cycle. I also find that although external mechanosensory input is not sufficient to maintain a wild-type defecation cycle in the absence of food, internal mechanical stimulation is sufficient. Both total starvation and lack of cholesterol cause a lengthening of the cycle period. This response may be partially mediated by serotonin, since serotonin is known to mediate other responses to food. In addition, defects in serotonin signaling have an effect on cycle period of similar magnitude to that caused by starvation. Finally, I have presented data showing that loss of function in many genes can cause alterations in the defecation cycle period, and speculate that slow growth and defecation phenotypes are causally linked.

The regulation of defecation timing appears to be complex, and is most likely controlled by a web of several interacting genetic pathways rather than by a single linear pathway. Some of these pathways must converge on the IP_3 receptor in the intestine, but it is not known whether there might be IP_3R-independent regulation of the cycle period. I

propose the following model for regulation of the defecation cycle in response to food. The response of *C. elegans* to lowered food concentration is fast, occurring within one or a few cycle periods. This fast response is most likely regulated directly by food intake, as measured by internal mechanosensors in the pharynx or gut. In addition to this initial measure of food quantity, *C. elegans* must also have a method for measuring the quality of food consumed. When food is abundant but poor quality (as in low cholesterol conditions), the cycle period lengthens over the course of several hours or more. This slow response of the defecation cycle period is also triggered by starvation, serotonin defeciency, and by downregulation of genes important for general cellular metabolism. There are probably many metabolic pathways that provide input to the defecation clock. Based on current evidence, defective translaton or shortages of ATP, amino acids, sterols, or fatty acids can cause lengthening of the defecation cycle period. Additional support for this model could be obtained by observing the response of *C. elegans* to media deficient in a range of different nutrients, or by obtaining a time course of the response to lowered food quality by continuous observation of individual worms.


C
D
E

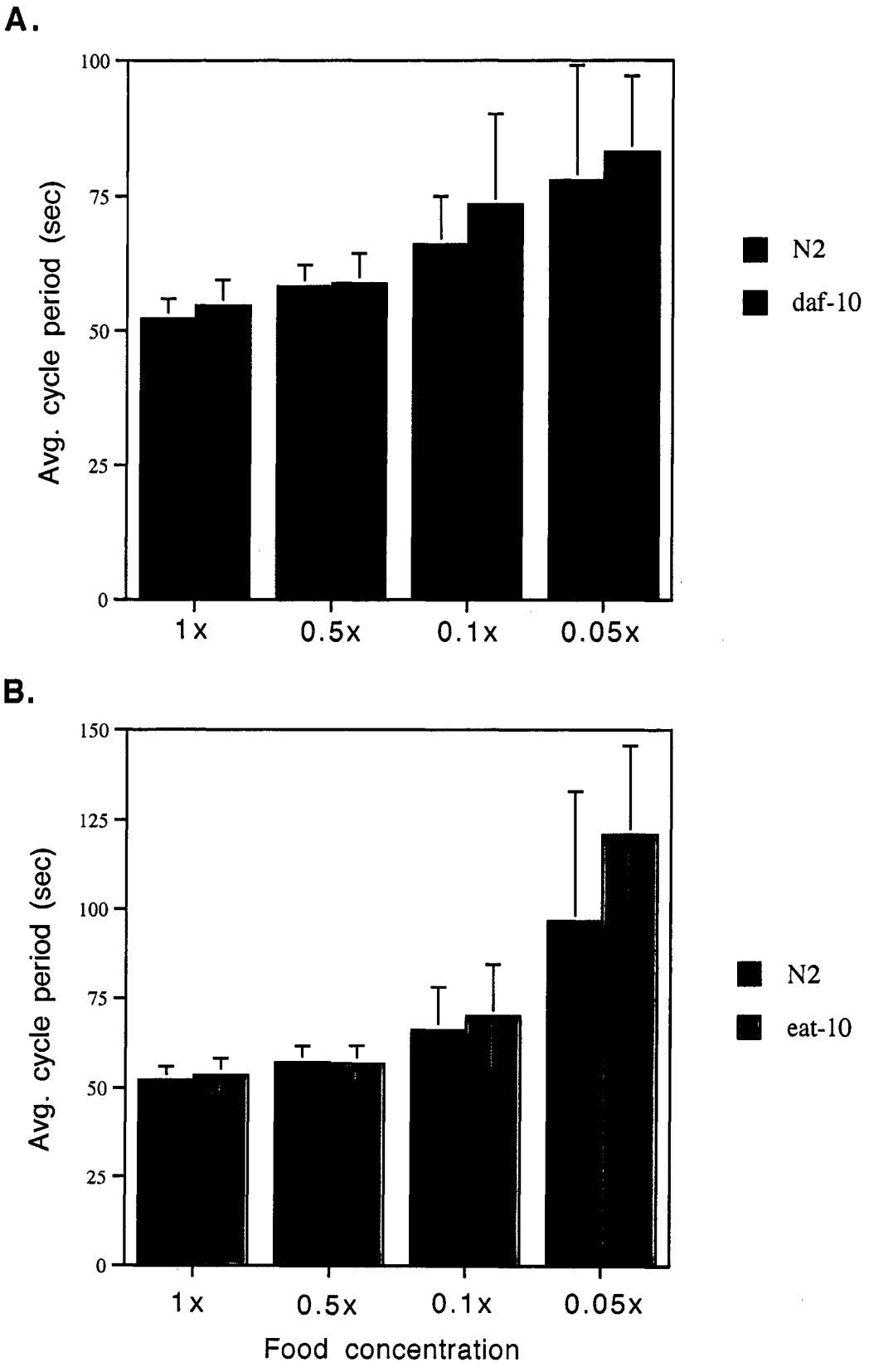

A.
100
75
50
25
0
Avg. cycle period (sec)
1x
0.5x
0.1x
0.05x
N2
daf-10
B.
150
125
100
75
50
25
0
Avg. cycle period (sec)
1x
0.5x
0.1x
0.05x
Food concentration
N2
eat-10

Table 1. Effect of various feeding conditions on the defecation behavior of *C. elegans*. Average cycle period is the unweighted average of all measured cycle periods. S.D. = standard deviation. *No defecation events were observed. † $p < 0.0001$

Feeding condition	Avg. cycle period ± S.D.	Minutes observed (# worms)
Rinsed bacteria	52.5 ± 3.0	20.6 (5)
Supernatant	193.1 ± 34.9 †	39.5 (3)
Sephadex (large beads)	*	15.4 (3)
Food substitute (small beads)	51.1 ± 11.8	21.6 (4)
30 minute starve	52.8 ± 5.4	24.7 (5)
3 hour starve	64.6 ± 3.8 †	25.1 (5)
No cholesterol	93.6 ± 10.1 †	21.1 (3)

Table 2. Defecation behavior of several mutant strains. All assays performed at 20°C using adult hermaphrodites. Average cycle period is the unweighted average of all measured cycle periods. S.D. = standard deviation † $p < 0.0001$

Genotype	Phenotype	Avg. cycle period ± S.D.	Total cycle periods
N2	Wild type	52.2 ± 3.7	64
eat-10	Defective pumping	53.6 ± 4.7	25
eat-13	Defective pumping	51.8 ± 9.2	46
cat-2	Dopamine deficient	50.2 ± 4.2	72
tph-1	Serotonin deficient	60.4 ± 8.2 †	55
glr-1	Defective glutamate signaling	55.0 ± 4.7	55

Table 3. RNAi screen for genes affecting growth rate and defecation. Average cycle period is the unweighted average of all measured cycle periods. Blank vector negative control is HT115 bacterial strain containing the L4440 vector with no insert. *flr-1* and *dec-2* were included as positive controls, since mutations in these genes were previously known to cause a Dec phenotype. A "+" indicates wild-type growth rate, with all individuals reaching adulthood by the time of the assay. Slow-growth ("-") indicates the presence of individuals at larval stages.

Gene	Identity	Avg. cycle period (sec)	Growth rate
F02D10.5	Sodium channel (*flr-1*)	31	-
K04E7.2	H+/peptide transporter (*opt-2*)	37	-
C37A2.7	Ribosomal protein	40	+
F29G6.3	Novel protein	41	+
B0361.5	Phosphatidylserine carboxylase	42	+
Blank vector		44	+
M142.6	Ubiquitin ligase	45	+
Y48A6B.3	Ribosomal protein	48	+
F08B12.2	Ubiquitin ligase (*prx-12*)	49	+
ZC395.3	Cation transporter	52	+
C25A1.6	rRNA processing	57	-
C34E10.4	tRNA synthetase (*wrs-2*)	71	+
C53B7.4	ATP synthase (*asg-1*)	79	-
Y54G11A.10	Cell junction protein (*lin-7*)	93	-
F14F4.3	CFTR homologue (*mrp-5*)	94	-
F56D1.3	Ribosomal protein	94	-
T05D4.4	Novel protein (*dec-2*)	95	-
W03B1.4	tRNA synthetase (*srs-1*)	97	-
F43C1.3	Zinc finger protein	99	-
B0261.4	Ribosomal protein	99	-
F54C9.6	Mitochondrial ATPase	110	-

Chapter Two:

Molecular identity and tissue expression of *dec-2*

Introduction

Genetic screens have identified many mutations that alter the defecation behavior of *C. elegans*. Thomas (1990) screened for a constipated (Con) phenotype and described several classes of mutants. While most of these Con mutants are defective for one or more of the muscle contractions of the defecation motor program (the Pbo, Abo, Exp, and Aex classes), there is one class that affects only the timing of the motor program (Dec). Worms carrying a mutation from this class exhibit an abnormal defecation cycle period but execute the motor program normally. This result revealed that the defecation clock is genetically distinct from the motor program, and led to a second screen by Iwasaki *et al.* (1995) that specifically identified Dec mutants. This screen identified an additional eleven genes that produce a long (Dec-L) or short (Dec-s) cycle period when mutated. Table 4 gives a list of all the known Dec genes.

One gene, originally named *dec-4*, was subsequently shown to encode the inositol triphosphate (IP_3) receptor of *C. elegans* and was renamed *itr-1*. The IP_3 receptor is a calcium channel that resides in the endoplasmic reticulum, and releases calcium stores from the ER into the cytoplasm (Berridge 1993). Dal Santo *et al.* (1999) found that *itr-1* loss-of-function causes a long cycle, overexpression causes a short cycle, and an *itr-1* null mutation eliminates the cycle entirely. This suggests that the IP_3 receptor is the molecular timekeeper for the defecation clock. In addition, Dal Santo *et al.* presented evidence that ITR-1 is required in the intestine for a normal defecation cycle period, and that calcium oscillations occur in the intestinal cells. A calcium spike in the posterior

intestinal cell immediately precedes each pBoc event. These results indicate that the defecation cycle is under non-neuronal control, and that the clock resides in the intestinal cells. It has been shown in other systems that the IP_3 receptor is capable of creating intracellular calcium oscillations, and that these oscillations can be propagated intercellularly as calcium waves (Deitmer *et al.* 1998). In *C. elegans*, a calcium wave propagates through the intestinal cells following the posterior calcium spike, and the *inx-16* innexin gap junction protein is required for normal wave propagation (Maureen Peters and Kouichi Iwasaki, personal communication).

It is not clear whether other Dec genes interact directly with *itr-1* or how they might affect calcium signaling in the intestine. *unc-43* encodes the calcium/calmodulin-dependent kinase type II, and its expression is required in the intestine for normal defecation behavior (Liz Newton, personal communication). Thus it is possible that UNC-43 directly modifies ITR-1 to affect cycle period. *goa-1(lf)* and *egl-30(gf)* are mutations in G proteins that function upstream of *egl-8*/phospholipase C (Miller *et al.* 1999) and thus may alter the defecation cycle period via changes in IP_3 levels. The three Flr genes (*flr-1*, *flr-3*, and *flr-4*) were originally isolated for their fluoride resistance, and their function is required in the intestine for normal defecation cycle period (Take-Uchi *et al.* 1998). *flr-1* encodes a sodium channel, which could conceivably affect intestinal calcium signaling by altering intracellular ion levels, but its function is not well understood.

The identification of the above Dec genes provides hints about how the defecation clock might work, but *itr-1* remains the only gene for which a clear function has been proposed. Identification of additional genes may further our understanding of the clock mechanism and reveal connections between the other Dec genes. I chose to work on *dec-2* because among the uncloned Dec genes, it has the phenotype most similar to *itr-1*. *dec-2(sa89)* animals are scrawny, slow-growing, and have a very long average cycle period.

Materials and Methods

C. elegans strains

Strains used in this study were N2 Bristol wild type, CB156 *unc-25(e156)*, CB246 *unc-64(e246)*, GE2175 *unc-32(e189) tDf6/qC1 dpy-19(e1259) glp-1(q339); him-3(e1147)*, GE2204 *unc-32(e189) tDf10/qC1 dpy-19(e1259) glp-1(q339); him-3(e1147)*, JT89 *dec-2(sa89)*, JT7358 *dpy-18(e364) dec-2(sa89)*, JT11053 *unc-25(e156) unc-64(e246)*, JT11445 *dec-2(sa89); saEx687*, JT11446 *dec-2(sa89); saEx688*, and MT1642 *lin-15(n765ts)*.

Mapping and transgenic rescue

A total of 1086 slow-growing progeny were picked from *unc-25(e156) unc-64(e246)/dec-2(sa89)* heterozygotes: 1077 segregated neither Unc marker, 9 segregated the *unc-25* shrinker phenotype, and none segregated the *unc-64* paralyzed phenotype. *tDf6/dec-2* and *tDf10/dec-2* heterozygotes were constructed as follows. *dpy-18 dec-2*/+ + heterozygous males were mated with *Df/qC1* hermaphrodites. Many F_1 animals were

scored for their defecation cycle period, and those segregating 1/4 dead eggs and 1/4 Dpy progeny were inferred to be *Df/dec-2*. (The Dpy phenotype of *dpy-18* is easily distinguished from that of the qC1 balancer chromosome.)

The cosmids used for transgenic rescue of *dec-2* were obtained from Alan Coulson at the Sanger Center (Cambridge, UK). The structure of all cosmids was confirmed by restriction mapping. Cosmids were injected into the gonads of young adult hermaphrodites at a concentration of 20 ng/μl, along with 10 ng/μl of *myo-2::gfp* marker DNA and 100 ng/μl Bluescript filler DNA. *dec-2/unc-64* heterozygotes were used for injections, since scrawny *dec-2* homozygotes proved difficult to manipulate and also tended to produce very unstable transgenic lines. Transgenic animals were identified by GFP fluorescence in the pharynx. Transgenic lines were maintained as heterozygotes until the F_3 generation and then the phenotype of GFP-expressing *dec-2* homozygotes was examined.

RNAi experiments

Genomic fragments were amplified from T05D4 using GenePairs primer sequences (Research Genetics), cloned into pCR TOPO vector (Invitrogen), then moved into the L4440 vector and transformed into the HT115 bacterial strain. Growth plates used for RNAi were prepared as previously described (Fraser *et al.* 2000). L4 animals were placed on dsRNA-producing bacteria and raised at 15°C for 72 hours, then transferred to fresh plates at 20°C. Progeny from the second set of plates were assayed 4 days later for the Dec phenotype.

Sequencing of cDNAs and genomic DNA

The exon structure of T05D4.4 was confirmed by sequencing of cDNA clones (yk723f1 and yk727g8) provided by Yuji Kohara (National Institute of Genetics, Mishima, Japan). Genomic DNA was prepared from *dec-2* worms using the DNeasy tissue kit (Qiagen). Sequencing of the *sa89* mutation was performed using bulk PCR product generated from genomic DNA and BigDye Terminator reagents (Applied Biosystems). The full length of all *dec-2* exons was sequenced, and the *sa89* mutation was identified in multiple independent PCR reactions.

Identification and alignment of T05D4.4 homologues

Proteins containing the OSR domain were identified using the NCBI BLAST server, and a multiple alignment was created using ClustalW. The WormBase gene predictions for K02F3.7 and ZK507.4 were corrected by J. Thomas using *tblastn* searches and manual inspection of the protein sequences. Phylogenetic tree was constructed using the MacVector 7.2 software package (Accelrys, Inc.).

GFP expression

A PCR fragment containing 5kb of *dec-2* genomic sequence was amplified and inserted into BamHI and SphI sites in the Fire vector p95.67. This construct contains approximately 3kb of *dec-2* promoter sequence, the complete first exon and first intron of *dec-2*, and part of the second exon fused in frame to GFP. The expression construct was injected into *lin-15(n765ts)* young adult hermaphrodites at 100 ng/μl, along with *lin-*

15(+) marker DNA at 60 ng/μl. Animals used for injections were raised at 15°C, then shifted to 25°C after injection to allow expression of the *lin-15* Muv phenotype and selection of wild-type transgenic animals.

Results and Discussion

Molecular identification of *dec-2*

Figure 3 shows the genomic region containing *dec-2*. *dec-2* had previously been mapped to the right arm of chromosome III, to the right of *unc-25* (Iwasaki et al. 1995). In order to refine the genetic map location of *dec-2*, I constructed an *unc-25 unc-64* double mutant. The Unc phenotype of these two mutations is easily distinguishable, and thus they make good markers for recombination mapping. *unc-25* animals move relatively well, but display a shrinker phenotype (simultaneous contraction of all body wall muscles) when touched on the nose. *unc-64* animals are almost completely paralyzed and display little spontaneous movement. I picked approximately 1000 progeny of *unc-25 unc-64/dec-2* heterozygotes, and identified 9 recombinants. Of these 9 recombination events, all were between *dec-2* and *unc-25*. Thus, I was unable to position *dec-2* with respect to *unc-64*, but this data suggests that *dec-2* is located close to *unc-64* on the extreme right arm of chromosome III.

In a second attempt to position *dec-2* with respect to *unc-64*, I used two deficiency strains containing deletions on chromosome III. *tDf6* is a large deletion that uncovers *dpy-18, unc-25, unc-64*, and many other markers on chromosome III. *tDf10* is a small deletion

located to the right of *unc-64*. Table 5 shows the results of this deficiency mapping. Both *tDf6/dec-2* and *tDf10/dec-2* animals displayed a Dec phenotype, indicating that *dec-2* is located in the region of overlap between these two deficiencies, to the right of *unc-64*.

There are sixteen overlapping cosmid clones between *unc-64* and the telomere of chromosome III. I attempted to purify all sixteen cosmids in this region, but thirteen were unstable and difficult to purify in the quantities required for transgenic rescue experiments. From the remaining three cosmids, I obtained the following results: F56A8 gave four transgenic lines, none of which were rescued for the Dec phenotype; T03E6 did not produce any transgenic lines; T05D4 gave three transgenic lines, two of which were fully rescued for the Dec phenotype. T05D4 is predicted to contain five genes, and thus I made constructs for RNAi knockdown of these five transcripts. Worms exposed to four of the five RNAi bacterial strains had wild-type defecation cycle periods. One RNAi strain, corresponding to T05D4.4, gave an average cycle period of 95 seconds. These worms were also slow-growing, scrawny, and indistinguishable from *dec-2(sa89)* animals in appearance. I sequenced T05D4.4 in the *dec-2(sa89)* strain and found a nonsense mutation in the sixth exon (Figure 4A). Taken together these three pieces of information (cosmid rescue, RNAi phenotype, and mutant gene sequence) indicate that *dec-2* encodes the protein T05D4.4.

***dec-2* encodes a novel protein**

Figure 4A shows the exon structure of T05D4.4, as predicted by WormBase and confirmed by sequencing of two cDNAs. T05D4.4 is a novel protein with no significant similarity to proteins outside of nematodes or to any previously identified functional domains. However, it does show similarity to several genes in *C. elegans* and *C. briggsae*. Figure 5A shows a partial alignment of DEC-2 with some of its homologues, and figure 5B shows the phylogenetic relationship between these genes. The greatest similarity is near the N-terminus of the protein, in a region I have named the OSR domain (based on a phenotype that will be discussed in the following chapter). The fact that this family is small and the domain is not highly conserved may explain why it was not previously noticed by search algorithms. Furthermore, the available gene predictions for K02F3.7 and ZK507.4 were incorrect, decreasing the apparent similarity (J. Thomas, personal communication). Each of the *C. elegans* family members is more closely related to its *C. briggsae* homologue than it is to any other *C. elegans* protein. However, the degree of similarity within these pairs (~75% identical) is lower than the average for orthologous gene pairs in these two species (Stein *et al.* 2003), indicating that this family may be evolving more rapidly than average for *C. elegans* proteins.

Although the function of the OSR domain is not known, there are two properties of the sequence that provide some information. First, the presence of twelve highly conserved cysteine residues, which are often important for protein stability, suggests that T05D4.4 and its homologues may have an extracellular function. All family members also contain

a signal sequence, which indicates targeting to a vesicular pathway and is also suggestive of an extracellular function.

dec-2 is expressed in the hypodermis

Other genes known to affect the defecation rhythm, such as *itr-1*, *flr-1*, and *unc-43*, have been shown to be required in the intestine for wild-type defecation behavior (Dal Santo *et al.* 1999, Take-Uchi *et al.* 1998, and Allyson McCormick, pers. comm.). Since the defecation clock resides in the intestine, I decided to determine the expression pattern of *dec-2*. If *dec-2* is co-expressed with other Dec genes in the intestine, it may affect the defecation rhythm by direct interaction with other clock components. On the other hand, if *dec-2* is not expressed in the intestine, this would support an indirect effect of *dec-2* on defecation behavior.

I observed that GFP fused to the second exon of *dec-2* and under the control of the *dec-2* upstream regulatory region was expressed exclusively in the hypodermis (Figure 6). Four out of four transgenic lines showed strong expression in the hypodermis, and expression was not observed in any other tissue. Thus, DEC-2 is not required in the intestine and cannot interact directly with ITR-1 to affect defecation rhythm. However, since DEC-2 appears to be a secreted protein, this result does not reveal the site of action of the protein. DEC-2 function may not be required in the hypodermal cells themselves, but rather at an extracellular location such as the cuticle or pseudocoelomic space.

Conclusions

I have determined the molecular identity of *dec-2*, and find that it encodes a novel secreted protein with no homologues outside of nematodes. The protein sequence of *dec-2* contains no homology to any previously identified functional domains, and thus does not provide any clues to the molecular function of *dec-2*. However, I have identified a novel family of proteins related to *dec-2* that contain a previously unidentified protein domain, which I have named the OSR domain. I have determined that DEC-2 does not function in the intestine, and thus does not interact directly with ITR-1. This leads me to the conclusion that *dec-2* is not a component of the molecular clock that controls the defecation rhythm. Rather, *dec-2* is likely to affect defecation indirectly, perhaps by impairing the general metabolism of the animal. This would be consistent with my results showing that RNAi knockdown of many basic metabolism genes can cause a long defecation cycle, in many cases as long as or longer than that caused by elimination of *dec-2* function. Thus, the Dec-L phenotype is not specific enough for identification of clock components, and candidate genes would ideally be evaluated on alternative criteria in the future to ensure identification of relevant genes.

Table 4. Mutations known to cause the Dec phenotype

Gene	Molecular identity	Map location	# of alleles	Cycle length(sec.)	Other phenotypes
cha-1	Choline acetyltransferase		11	>100	Unc, Egl-c, scrawny
egl-30	$G_q\alpha$ subunit		4 (gf)	100	Hyper, Egl-c, Gro, scrawny
flr-1	DEG/ENaC sodium channel		6	20	Resistant to fluoride
flr-3	Protein kinase		3	30	Resistant to fluoride
flr-4	Protein kinase		3	25	Resistant to fluoride
goa-1	$G_o\alpha$ subunit		8	100	Hyper, Egl-c, Gro, scrawny
itr-1	IP_3 receptor		10	90	Sterile (null), Gro, scrawny
unc-43	CaM kinase II		20	50	Unc, Egl-c, pBoc echo
dec-1		LGX 17.7	1	100	Gro, cycle age-dependent
dec-2		LGIII 21.6	2	90	Gro, scrawny
dec-7		LGIII 0.6	2	30	
dec-9		LGIV 4.7	1	40	
dec-10		LGX 2.0	1	35	
dec-11		LGIV 24.1	1	65	Gro, scrawny
dec-12		LGI –10.7	1	75	Gro, scrawny

Table 5. Results of deficiency mapping. Genotype was determined by examining the progeny of each animal for dead eggs and Dpy phenotypes. No data was obtained for some animals that produced no or few progeny, making genotype assignment impossible. Results for *tDf6* and *tDf10* are pooled since the results were the same for progeny from either mating. Approximately equal numbers of animals were tested from each mating.

Genotype	Avg. cycle period (sec.)	# of animals
Df / qC1	55	7
Df / +	57	2
qC1 / +	56	6
dpy-18 dec-2 / *Df*	106	5
dpy-18 dec-2 / *qC1*	55	8
Unknown (no progeny)	n.d.	15
		Total = 43

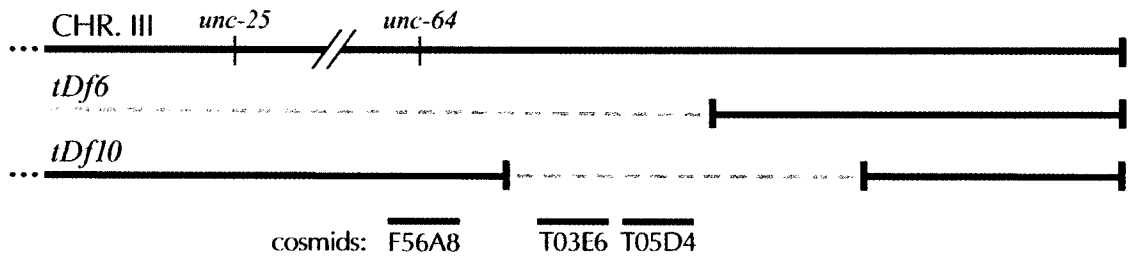


Figure 3. Genomic region of *dec-2*. The right arm of chromosome III is shown, from *unc-25* to the telomere. Regions uncovered by the tDf6 and tDf10 deletions are indicated by dashed lines. The positions of cosmids used for transgenic rescue experiments are indicated, but cosmids are not drawn to scale.

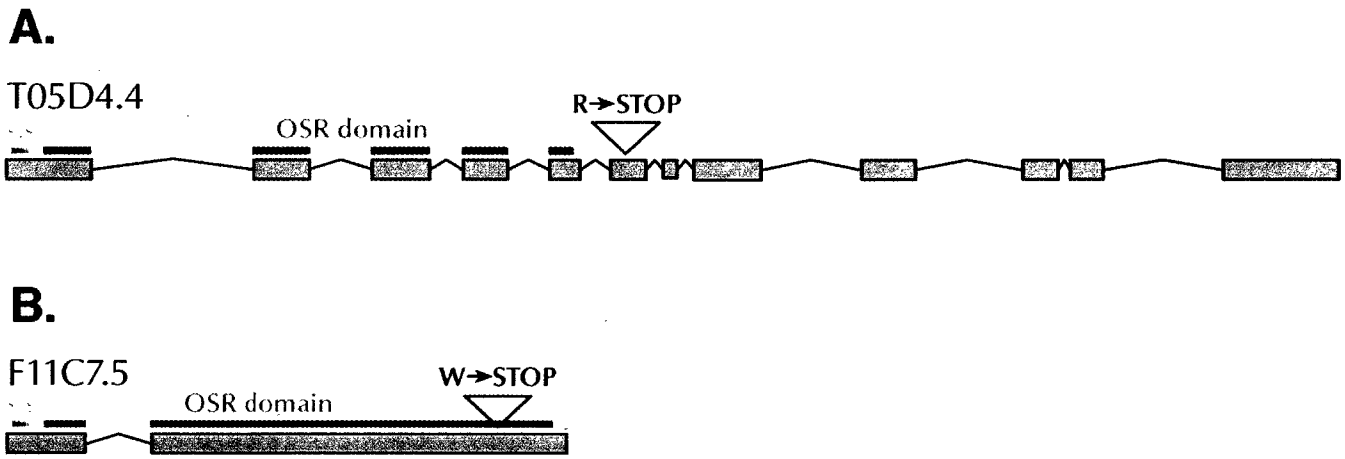


Figure 4. Exon structure of A. *dec-2/osm-7* and B. *osm-11*. The location of the OSR domain of each gene is marked in blue, and the signal sequences (s.s.) are marked in red. The locations of sequenced mutations are indicated with a triangle. Drawing is to scale.

A.

```
T05D4.4 GGDEESEGKPENFQGSANDYCDKFEQHFSYFCYG-----ETDQTDKNAYVIKKFCPSYKTACRHKAVTTSVSLTSWPTDPFAKEITVASRPLPST
CB18238 GGGNRGD-KIEHFDGGKDDYCDRFEQHFQYFCYG-----ETDKTDKNAYVIKKFCPSYKTACRHKAVTSSVSLTSWPTDPFEKEITVTSRPVSVG
F11C7.5 VLAQANPARVRRNEEMSEDYCIKHLDHYNKYCGD----NAGPIDRALFGKVAKFCPAYEKHCAVGK-AGLVELPDLGSPLVMPPVLPRGSDFAS-
CB15929 VLAQASPARVRR-DSASDDYCVKHMEHYNKYCGE----NAGPIDRALFGKVAKFCPAYEKHCAVGK-AGLVELPDLGSPLVMPPVLPRGSDFAS-
CB24197 GLAQANPARVRR--EAADIYCMKHFENFQKFCGE----DDFSATRDLLPKIIKFCPAYQKHCAVGK-AGVLELPDLGSDLVLPPSIDRATKYDRL
CB24198 GLTQANPVRVRR--EAADYYCIRHFEHFQKYCGD----ENFLATRDLLPKVVKFCPAYQKHCAVGK-SGVVELPDLGSQLVLPPSIERASKFDRL
KO2F3.7 SRSSTGEDKTYLYKGSKKEYCEKFSVNYEYYCYG-----NIEN----AEIFTKFCPSYKKAC--------VQER-VPANPFS--ASKGQIQSASG
CB24557 SDSTSSEDKTYLYKGTKKEYCEKFSVNYEYYCYG-----TIEN----AEIFTKFCPSYKKAC--------VTANQKPSNPFS--PSKGQTPVSSA
ZK507.4 FESHHSEPRLRTSNLDVGEYCATFHENYVYYCYGIWTAEKLLEHRSIMKNIGKFCPSYKSAC----------VTRTRIDPEEE
CB06935 FATCN---FIFQSTLDVGEYCAVYHENYVYYCYGIYTAEKLHKHRNIMENIGKFCPSYKAAC----------VTRTRGDPEED
```

```
T05D4.4 SGRTSHSRRRDSDEDSDSAEDEEVYYAELRKRYPCKPDCDKRIFAHCTEECKCDYIYPVVQRFCNPPPMP-MFLNTCRLWYHGCPKYGRYHYASQ
CB18238 RARPTP-RRRYSDEDSDSAEDEEIYYAELRKRYPCKPDCDKRIFAHCTEECKCDYIYPVVQRFCNPPPMP-LFLNTCRLWYHGCPKYGRYHYSSQ
F11C7.5 LDLPIADERKP-AHIHSSRTAPQTTRLTAAIVATCTPECTA---AHCTDECKCAHTHPKVHQMCNPPSSA-AMAETCQRWYSKCTMFTPVQY---
CB15929 LDLPIADERKP-IQHNAPRTVPQTTRLTAAIVATCTPECTA---PHCTDECKCAHTHPKVHQMCNPPSSA-SMAQTCQRWYSKCTMFAPVQY---
CB24197 LDSPIEKENEVFSSFKYPSSKPNQTRLTAAIVATCTPDCKA---PQCTDECKCAHTHPKVHSMCNPPSTA-TMAQTCQRWYAKCTMFAPVQY---
CB24198 LDLPMSDDKDIFTSNKFSPAKS--TRLTAAIVATCNPDCVD---PQCTDECKCAHTHPKVHATCNPPSSA-AMAQTCQRWYAKCTMFSPVQY---
KO2F3.7 -TSDFPDLDHPSIRKSSAASRR---AKLLKR-PCSADCDERIFPHCTKECKCDYDYPAVQKFCNPPPLP-LFLNTCRLWYYGCPKYGRYHYASQ
CB24557 STSDFPDVEHPSAGKSKSSRRR---GKKIKKLPCSIDCDERIFPHCTKQCKCDYDYPAVQKFCNPPPLP-MFLNTCRLWYYGCPKYGQYHYASQ
ZK507.4 RERKEDHKKELTDDVGDMAFDD--LMRKLEKIYPCRPNCNVSVHPHCTRQCKCEYEYHRMQKWCKPPRIEERFKYFCRIWYMSCSTYIEELDPEM
CB06935 REAKEEHQKELTDDVGDMLFDD--LMRKLEKIYPCRPNCNQSVHPHCTRRCKCEYEYHRMQKWCKPSRIEDRFKYFCKIWYMSCSTYIDEIDPDM
```

B.

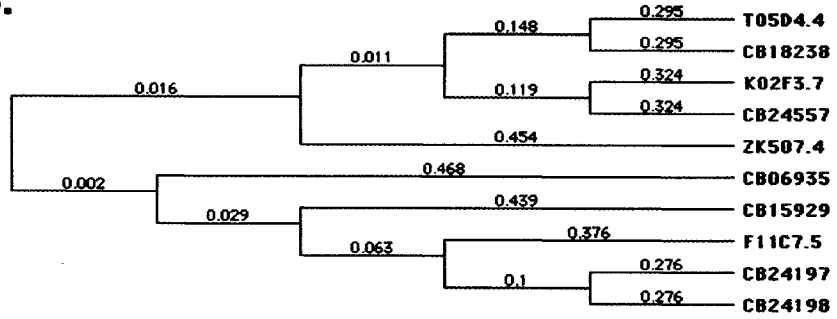


Figure 5. A novel gene family containing the OSR domain. A. Partial ClustalW alignment of T05D4.4 with its nearest homologues in *C. elegans* and *C. briggsae*. Only the region of highest similarity is shown. **B.** Phylogenetic tree of the relationships between T05D4.4 and its homologues.

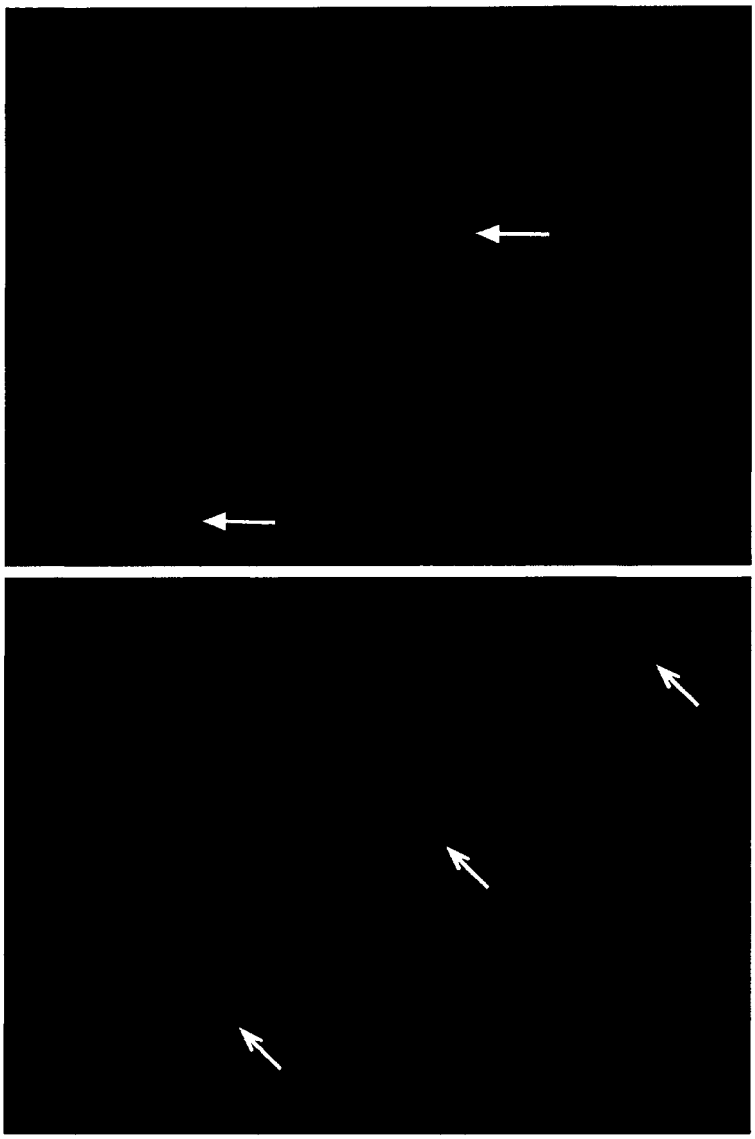


Figure 6. Expression of *dec-2*::GFP in the hypodermis. The bottom panel shows the midsection of an animal, with seam cells visible as non-GFP cells along the center (open arrows). The top panel shows the head region of another animal (anterior and posterior bulbs of pharynx, closed arrows).

Chapter Three:

osm-7/*dec-2* is a member of a novel gene family involved in the response to osmotic stress

Introduction

All living organisms have mechanisms that allow them to cope with changing environmental conditions such as temperature, food availability, the presence of toxic compounds, and osmolarity. When an organism or tissue is exposed to high osmolarity, intracellular water rapidly diffuses across cell membranes causing a decrease in volume and increased concentration of cellular contents. These changes lead to mechanical stress, disruption of the activity of proteins and DNA, and eventually shutdown of cellular function (Garner & Burg 1994). In order to avoid this outcome, cells accumulate physiologically compatible solutes which increase intracellular osmolarity without a concomitant disruption of ionic bonds due to increased salt concentration. For instance, mammalian kidney cells must endure large variation in solute concentrations, depending on the hydration state of the animal. In response, these cells increase production or transport of compatible solutes including sorbitol, inositol, betaine, and taurine (Burg *et al.* 1997).

Adaptation to osmotic stress has been studied extensively in yeast (Hohmann 2002), where glycerol is used as a compatible solute. The MAP kinase Hog1 is required for this response (Brewster *et al.* 1993), and indirectly activates Gpd1, a glycerol-3-phosphate dehydrogenase shown to be rate-limiting for glycerol accumulation (Albertyn *et al.* 1994, Remize *et al.* 2001). Mutants in the Hog1 pathway are sensitive to high osmolarity, but this sensitivity is alleviated by growth at high temperature (Siderius *et al.* 2000). The most likely explanation for this alleviation is the activation of a second pathway

involving protein kinase C (Pkc1), which responds to high temperature, cell wall damage, and osmotic stress (Gustin *et al.* 1998). In other systems, PKC is known to be activated by diacylglycerol produced by phospholipase C (PLC). While direct evidence of this interaction is lacking in yeast, it has been shown that yeast PLC (Plc1) is required for increased glycerol synthesis in response to osmotic stress (Lin *et al.* 2002).

While the response to high osmolarity in yeast is well understood, the response of the nematode *C. elegans* has only recently been examined and remains poorly understood. In the natural soil environment of *C. elegans*, osmolarity probably varies widely and changes rapidly. Because *C. elegans* has a high surface-to-volume ratio and water can pass through its cuticle, worms are particularly vulnerable to osmotic stress. The primary mechanism that *C. elegans* uses for protection from osmotic stress is locomotion away from an area of hyperosmolarity (Culotti & Russel 1978). Nematode locomotion is achieved by alternating contractions of body wall muscles that are connected to the cuticle. Internal hydrostatic pressure on the cuticle gives the animal rigidity, against which locomotory muscles work. Thus, the worm cuticle functions as a hydrostatic skeleton, and locomotion is dependent on turgor pressure to maintain the rigidity of the animal. Hyperosmotic conditions lead to loss of internal pressure by water efflux, collapse of the hydrostatic skeleton, and rapid paralysis of *C. elegans*. In order to avoid death induced by hyperosmotic shock, *C. elegans* has evolved mechanisms that maintain turgor pressure and allow for continued locomotion.

Similar to yeast, *C. elegans* increases glycerol synthesis in order to attenuate the damaging effects of high osmolarity. Worms that have been exposed to high levels of environmental NaCl contain high internal levels of glycerol, and show increased expression of the glycerol-3-phosphate dehydrogenase F47G4.3 (Lamitina *et al.* 2003). However, it has not been determined whether MAP kinase, PKC, or any other previously identified pathways are involved in this response. Mutations in one gene, *osr-1*, have previously been shown to cause resistance to high osmolarity (Solomon *et al.* 2004). OSR-1 is a novel protein expressed in the hypodermis and likely secreted. *osr-1* mutants have high basal levels of glycerol, even under normal growth conditions, and this is thought to be the reason for their osmotic resistance. The exact mechanism by which *osr-1* mutations lead to accumulation of glycerol is not known, but one component of the Osr phenotype (resistance to chronic exposure) may be suppressed by mutation of components of a nematode MAP kinase pathway (Solomon *et al.* 2004).

In this section I describe the finding that *dec-2(sa89)*, in addition to affecting the period of the *C. elegans* defecation cycle, also causes an extreme resistance to osmotic stress. These experiments shed light on the molecular function of *dec-2* and also on the mechanism *C. elegans* uses to sense and respond to osmotic stress.

Materials and Methods

C. elegans strains

Strains used in this study were N2 Bristol wild type, JT89 *osm-7(sa89)*, MT3564 *osm-7(n1515)*, MT3643 *osm-11(n1604)*, RB1032 *osr-1(ok959)*, BE93 *dpy-2(e8)*, CB128 *dpy-10(e128)*, CB88 *dpy-7(e88)*, CB61 *dpy-5(e61)*, CB184 *dpy-13(e184sd)*, CB266 *unc-43(e266)*, MT2605 *unc-43(n1186 n498sd)*, CX4998 *nsy-1(ky397)*, KU4 *sek-1(km4)*, JT28 *egl-8(sa28)*, JT734 *goa-1(sa734)*, PS2444 *dpy-20(e1282ts); egl-30(syIs36)*, MJ500 *tpa-1(k501)*, RB781 *pkc-1(ok563)*, VC127 *pkc-2(ok328)*, JT7428 *osm-7(sa89); unc-43(e266)*, JT10096 *osm-7(sa89); unc-43(n1186 n498sd)*, JT11473 *nsy-1(ky397); osm-7(sa89)*, JT11474 *osm-7(sa89); sek-1(km4)*, JT11475 *osm-7(sa89); egl-8(sa28)*, JT11476 *osm-7(sa89); pkc-1(ok563)*, JT11477 *osm-7(sa89); tpa-1(k501)*, and JT11478 *osm-7(sa89); pkc-2(ok328)*. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources.

Genetics and strain constructions

I confirmed that *sa89* and *n1515* are alleles of the same gene by a complementation test. *n1515/+* males were crossed to *sa89; dpy-9(e12)* hermaphrodites, and half of the resulting cross progeny were Dec.

Double mutant strains of *osm-7* and various candidate suppressors were made using the following general scheme: *osm-7* was crossed to a marker mutation tightly linked to the

candidate suppressor. This marked double was used to make heterozygous males, which were then crossed to candidate suppressor hermaphrodites. From plates that segregated both Dec and marker phenotypes, Dec non-marker progeny were picked. I confirmed that the marker segregated from these individuals (indicating that the parent is heterozygous for the marker over the candidate suppressor), then picked non-marker progeny. Animals that no longer segregated the marker were presumed to be homozygous for both *osm-7* and the candidate suppressor.

Behavioral assays

For all defecation assays, worms were raised and scored at 20°C. For each strain, ten L4 animals were picked to a fresh NGM plate seeded with OP50 and assayed as adults the following day for 10 minutes each. Observations were recorded using the Etho program developed by J. Thomas.

High salt plates were poured three days before use, and seeded with OP50 one day before use in order to minimize variation in salt concentration due to evaporation. Worms raised on standard 50mM NGM plates were picked onto high salt plates and assayed by touch 10 minutes later. Response to touch was counted as any attempt at locomotion, even weak movements. For each strain, at least 70 individuals were assayed.

Sequencing and RNAi knockdown of F11C7.5

Sequencing of the *n1604* mutation was performed using bulk PCR product generated from genomic DNA and BigDye Terminator reagents (Applied Biosystems). The full

length of all *osm-11* exons was sequenced, and the *n1604* mutation was identified in multiple independent PCR reactions. The bacterial strain used for knockdown of F11C7.5 was obtained from the Ahringer RNAi library (MRC Geneservice), and the RNAi protocol was performed as previously described (Fraser 2000).

Determination of glycerol content

Each assay was done in triplicate on at least two separate days. Well-fed worms were rinsed off plates with M9, spun down, then rinsed with fresh M9 and resuspended in 600µl M9. Worms were sonicated 2 x 30sec and disruption of the cuticle was confirmed using a dissecting microscope. Insoluble material was removed by centrifugation for 5 minutes at maximum speed. The supernatant from each sample was divided, 50µl used for measurement of protein content with a BCA protein assay kit (Pierce) and 200µl used for measurement of glycerol content with an enzymatic assay (R-Biopharm). In both cases, the assays were performed as described in the product instructions. Prior to measurement of glycerol content, a Carrez clarification was performed to remove protein: the sample was brought up to 600µl with water, then 50µl each of 85mM ferrocyanide and 250mM zinc sulfate were added. The pH was adjusted to 8.0 using 1M NaOH, and the final volume brought up to 1ml. Proteins were pelleted by centrifugation for 15 minutes at maximum speed.

Results and Discussion

Molecular identification of *osm-7* and *osm-11*

osm-7(n1515) was identified in a screen for mutants with defective osmotic avoidance (Osm) (J. Thomas, unpublished results). It was later noticed that *osm-7* worms are also resistant to high osmolarity (Osr) (K. Mase and M. Koga, personal communication). *osm-7* encodes T05D4.4, and *osm-7(n1515)* contains the same nonsense mutation as *dec-2(sa89)* (K. Mase and M. Koga, personal communication). In addition, I have confirmed that *n1515* and *sa89* are alleles of the same gene by complementation testing. Since the *n1515* mutation was isolated before *sa89*, this gene will henceforth be referred to as *osm-7*.

osm-11(n1604) was identified in the same screen as *osm-7(n1515)*, based on its osmotic avoidance defect, and like *osm-7* also displays the Osr and Dec phenotypes. *osm-11* had been previously mapped to a region containing F11C7.5, which encodes a protein related to *osm-7* (Figure 5). I observed that knockdown of F11C7.5 by RNAi produced a slow-growing, Dec phenotype identical to that of *osm-11(n1604)*. Sequencing of this gene in the *osm-11(n1604)* strain revealed a nonsense mutation in the coding sequence (Figure 4B). Thus, I have shown that at least two members of this novel gene family cause the same phenotypes when mutated, suggesting that they have similar but nonredundant functions in the animal.

Osmotic resistance phenotypes

Table 6 shows the osmotic resistance phenotype of several Osr strains. Upon exposure to 500mM NaCl, wild-type *C. elegans* ceased egg-laying and became noticeably deflated. Within minutes, the loss of water by osmosis led to complete paralysis. In sharp contrast to N2, *osm-7* and *osm-11* worms moved normally and laid eggs at 500mM NaCl. *osr-1* animals were somewhat resistant and continued to move, but appeared slightly deflated and sluggish. At 800mM NaCl, the difference between *osr-1* and *osm-7/osm-11* was more pronounced, with a majority of *osr-1* animals paralyzed. This difference is interesting because *osr-1* was isolated specifically for its Osr phenotype (Solomon *et al.* 2004), and yet *osm-7* and *osm-11* display a much stronger resistance to osmotic stress.

The cuticle collagen mutants *dpy-2* and *dpy-10* were previously reported to exhibit the Osr phenotype (Solomon *et al.* 2004) and thus were included in this study. In my assay, *dpy-10* mutants were resistant to 500mM NaCl, but like *osr-1* were less tolerant of 800mM NaCl. *dpy-2* and *dpy-10* mutations have been shown to affect the localization of DPY-7, another cuticle collagen (McMahon *et al.* 2003). *dpy-7* mutants also displayed resistance to 500mM NaCl, whereas *dpy-5* and *dpy-13* mutants did not (Table 6). This indicates that not all types of cuticle defect can cause osmotic resistance. *dpy-2, dpy-7,* and *dpy-10* are known to be required for proper formation of circumferential furrows, called annuli, on the surface of the *C. elegans* cuticle. *dpy-5* and *dpy-13* do not affect the structure of the annuli, suggesting the hypothesis that the annuli are specifically involved in the osmotic stress response. This hypothesis is discussed in more detail below.

High glycerol levels in Osr strains

Since *C. elegans* is known to upregulate glycerol synthesis in response to osmotic stress, and since *osr-1* animals were previously shown to have high basal levels of glycerol, I hypothesized that other Osr strains would have high glycerol levels as well. Indeed, all tested Osr strains have elevated levels of glycerol (Table 6), and the elevation in glycerol is roughly correlated with the severity of the Osr phenotype. *osr-1* and *dpy-10*, which had lower glycerol levels than *osm-7* and *osm-11*, were also less resistant to osmotic stress. These results are consistent with the hypothesis that high basal levels of glycerol are the cause of osmotic stress resistance in these strains. The Osr strains are pre-adapted to survive osmotic stress, and the level of internal glycerol determines the level of osmolarity a particular strain can tolerate.

An interesting side effect of this high glycerol phenotype is cryoresistance. *C. elegans* strains can be stored at –80°C for many years if the worms are suspended in a solution containing 30% glycerol. I observed that approximately 5% of *osm-7* mutant worms were able to survive freezing without addition of glycerol, whereas wild-type worms never survived this treatment (data not shown).

Correlation between Dec and Osr phenotypes

Since *osm-7(sa89)* was known to alter the defecation cycle period, I examined the defecation phenotype of the other Osr mutants. As shown in Figure 7A, all of the known Osr mutants have an altered defecation cycle period. The correlation between the Osr and Dec phenotypes suggests that they are mechanistically linked. Therefore, I

hypothesized that exposure to high osmolarity might alter the defecation cycle in wild-type *C. elegans*. Indeed, I found that adult worms exposed to 150mM NaCl exhibited a modest increase in cycle period (Figure 7B). If raised on high-salt plates, wild-type *C. elegans* adapt to osmotic stress by accumulating glycerol (Lamitina *et al.* 2003). In this adapted state, the animals are resistant to subsequent osmotic challenge and can survive exposure to even higher levels of osmotic stress. I observed that worms raised on 200mM NaCl to permit adaptation and then shifted to 500mM had cycle periods as long as *osm-7* and *osm-11* (Figure 7B). Thus, the period of the defecation rhythm is altered in response to osmotic stress, and the length of the cycle is correlated with the osmolarity of the growth medium.

While it seems clear that high internal glycerol is the cause of the osmotic resistance phenotype in the Osr mutants, the relationship between these phenotypes and the defecation phenotype is not clear. Glycerol levels are not directly correlated with the cycle period, as illustrated by the *dpy-10* worms, which have intermediate glycerol levels and a very long cycle period. Thus, glycerol is probably not a direct regulator of the defecation clock. It is possible that the same signaling molecules that act downstream of *osm-7* to trigger glycerol accumulation also interact with the clock in the intestine. Alternatively, the response to high osmolarity may trigger a more general stress response in parallel to the specific glycerol response, and this general stress response may be responsible for alteration of the defecation cycle. This would be consistent with my observation that RNAi knockdown of ribosomal genes causes a Dec phenotype, suggesting that the defecation clock is affected by general metabolic stress. The energy

expended in increased glycerol production in Osr strains must be at a high cost to the animal, and probably has an effect similar to that of starvation. It is possible that the high glycerol phenotype of *osm-7* and *osm-11* is the cause of the slow growth and scrawny appearance of these strains.

Interaction with MAP kinase and PKC pathways

In both yeast and mammals, a MAP kinase pathway has been shown to mediate the response to osmotic stress by increasing the production of physiologically compatible solutes (Burg *et al.* 1997, Hohmann 2002). In yeast, a parallel pathway involving protein kinase C and phospholipase C has also been shown to respond to osmotic stress (Brewster *et al.* 1993, Lin *et al.* 2002). I hypothesized that if either of these pathways is required for the nematode response, it should be possible to suppress the phenotypes of *osm-7* using mutants in these pathways. *nsy-1* and *sek-1* are worm homologues of MAPKKK and MAPKK, respectively, and function in both pathogen resistance and neuronal cell fate (Kim *et al.* 2002, Sagasti *et al.* 2001). *egl-8* encodes phospholipase C, and mutants display defects in locomotion, egg-laying, and defecation (Miller *et al.* 1999). *C. elegans* has four protein kinase C homologues, *tpa-1, pkc-1, pkc-2*, and *pkc-3*, which are homologues of the mammalian PKCδ/θ, PKCε, PKCα/β and PKCζ respectively (Tabuse 2002). *pkc-3* has an essential function in embryogenesis and thus was not included in this study. *unc-43* encodes the only CamKII in *C. elegans*, and has been shown to function upstream of both *nsy-1* and *egl-8* pathways (Sagasti *et al.* 2001, Robatzek and Thomas 2000).

If the defects in *osm-7* mutants are caused by constitutive signaling through osmotic stress response pathways, then it should be possible to restore osmotic sensitivity by elimination of downstream pathway members. The *unc-43/nsy-1/sek-1* MAPK pathway was previously shown not to be required for the acute osmotic stress resistance of *osr-1* animals (Solomon *et al.* 2004). In agreement with these results, I found that *unc-43, nsy-1,* and *sek-1* mutations did not suppress the Osr and high glycerol phenotypes of *osm-7.* Thus, the *nsy-1* pathway is not required for the accumulation of glycerol that leads to resistance to acute osmotic stress. Of course, it remains possible that the *nsy-1* pathway functions in parallel to multiple redundant pathways, each capable of upregulating glycerol synthesis independently.

nsy-1 pathway mutations were reported to suppress the chronic osmotic stress resistance of *osr-1* mutants (Solomon *et al.* 2004) and thus I also tested the ability of my double mutants to survive 24 hours at 500mM NaCl. All candidate suppressor mutations examined in this study did not affect the chronic osmotic stress resistance of *osm-7* (data not shown). This may indicate that *osm-7* and *osr-1* activate different downstream signaling molecules, or it may reflect the difference in the severity of the Osr phenotype in these strains. Since the molecular basis for resistance to chronic osmotic stress is not understood, this result is difficult to interpret.

I also looked for evidence of interaction between *osm-7* and PLCβ/PKC signaling, which acts in parallel to the Hog1 MAPK pathway for osmotic resistance in yeast. Double mutants between *osm-7* and any of *egl-8* (PLCβ), *tpa-1, pkc-1,* or *pkc-2* had the same

phenotype as the *osm-7* single mutant. I also examined *goa-1(sa734)* and *egl-30(syIs36)* single mutants, which are predicted to cause constitutive activation of the *egl-8* pathway, but neither exhibited an Osr phenotype (Table 6). This indicates that these genes are not required for signaling downstream of *osm-7*, but it remains possible that this pathway functions redundantly in parallel to the *nsy-1/sek-1* pathway or another unidentified pathway. In addition, *C. elegans* has four PKC homologues, whereas yeast only has one, and thus it is possible that they have redundant functions in worm. Based on the situation in yeast, it is likely that the pathways regulating the response to changing osmotic conditions are complex and overlapping.

A molecular model for OSM-7 and OSM-11 function

dpy-2, dpy-7 and *dpy-10* encode collagens that are required for proper formation of circumferential furrows, which delineate ridges called annuli, on the surface of the *C. elegans* cuticle (McMahon *et al.* 2003). The observation that these mutants also display Osr and Dec phenotypes is intriguing, since it suggests that defects in the cuticle can trigger the same response as osmotic stress. Furthermore, cuticle defects that do not affect the annuli (*dpy-5* and *dpy-13*) (McMahon *et al.* 2003) do not cause Osr or Dec phenotypes, suggesting that annuli are specifically involved in osmotic stress response. I suggest a model in which the circumferential bands of proteins that form the annular furrows act as stretch sensors that monitor the turgor pressure of the cuticle (Figure 8). In this model, *osm-7* and *osm-11* are secreted from the hypodermis and associate with proteins at the annular furrows. An increase in environmental osmolarity would cause efflux of water, and loss of turgor pressure would cause a decrease in tension on the

furrow bands. If *osm-7* and *osm-11* interact directly with proteins that form the furrow bands, a change in the conformation of this structure might release *osm-7* and *osm-11* from the cuticle to perform a signaling function, such as the derepression of a kinase that activates the glycerol synthesis pathway. The absence of *osm-7* or *osm-11* at the cuticle would have the same effect as null mutations in these genes – activation of the glycerol synthesis pathway. Thus, I propose that *osm-7* and *osm-11* are responsible for transducing a stretch signal from the annuli.

This model is consistent with my finding that *osm-7* is expressed in the hypodermis, and with the evidence that it is a secreted protein. *osr-1* was also shown to be expressed in the hypodermis, and to contain a signal sequence (Solomon *et al.* 2004). Furthermore, another putative secreted protein, T19B10.2, was recently shown to be required for the osmotic resistance of *age-1* mutants (Lamitina & Strange 2005). Taken together, this evidence indicates that there may be a large number of yet unidentified proteins secreted from the hypodermis that are important for sensing external conditions and mediation of stress responses. It is not yet understood why so many proteins are required for the response to osmotic stress. Because loss of function of either *osm-7* or *osm-11* causes upregulation of the glycerol synthesis pathway, I conclude that these proteins act as nonredundant negative regulators of this pathway. One possibility is that different sensor proteins interact with different proteins in the annuli structure. The ability to respond to osmotic stress rapidly is critical for survival of the animal, and multiple sensor proteins may provide a more robust response system.

This model is analogous to the case in yeast, where the systems used for sensing cell wall integrity and osmotic stress overlap. Cell wall defects activate the PKC1 pathway (de Nobel *et al.* 2000) and overexpression of cell wall biosynthesis genes alleviates the growth defect of Hog1 MAPK pathway mutants (Alonso-Monge *et al.* 2001). In addition, the yeast genes Wsc1 and Mid2 function upstream of Pkc1 as sensors of osmotic stress at the cell surface (Hohmann 2002). Both Wsc1 and Mid2 have an extracellular domain that interacts with the cell wall, and a cytoplasmic domain that interacts with signaling molecules responsible for activating the PKC1 pathway (Philip & Levin 2001, Vay *et al.* 2004). These yeast sensors do not show sequence similarity to any metazoan proteins, and it remains to be seen whether functional homologues will be identified.

Although I favor the above model as the simplest explanation for the phenotypes of the Osr mutants, alternative models are also plausible. Since the functional location of OSM-7 has not been identified, but is only inferred from sequence information and the GFP expression pattern, it is possible that OSM-7 functions within the hypodermal cells, or that it is secreted and transported to some location other than the cuticle. In addition, because OSM-7 does not exhibit similarity to any previously studied protein family or functional domain, it is conceivable that it might have any imagined molecular function. For instance, it might be a structural component of the cuticle that alters its permeability. One such protein (BIS-1) has been identified, which localizes to the cuticle and defects in which alter the resistance of the animal to certain drugs but do not affect the gross morphology of the animal (Watanabe *et al.* 2005). Alternatively, if OSM-7 functions

intracellularly, it might act as a scaffold for other signaling cascade molecules, or even as a transcription factor. Further experiments should easily distinguish between these various possibilities. Identification of additional molecules that contribute to glycerol accumulation in response to osmotic stress will allow for placement of *osm-7, osm-11* and other Osr genes in a genetic pathway. More detailed characterization of the expression pattern of OSR domain-containing proteins will identify their site of action and narrow the range of possible molecular functions. Characterization of additional cuticle mutants will clarify the role of the annular furrows in the response to osmotic stress. Future work will address the question of whether the mechanisms for survival of osmotic stress are conserved between yeast and worms, or if the common solution (glycerol accumulation) might be a result of convergent evolution.

Conclusions

I have described mutations in two genes, *osm-7* and *osm-11*, that cause resistance to high osmolarity by production of high basal levels of glycerol. In addition, I have shown that the defecation rhythm of Osr worms is altered in a manner similar to that of worms exposed to chronic osmotic stress. This observation explains why *osm-7* was isolated in screens for both defecation and osmotic avoidance behaviors. Both *osm-7* and *osm-11* are members of a novel gene family in *C. elegans*, and are likely to encode secreted proteins. I have shown that, in contrast to findings from yeast, signaling components of the MAP kinase and PKC pathways are not required for the phenotypic changes observed in *osm-7* mutants. Future work should include suppressor screens to identify signaling

components downstream of *osm-7*. Finally, I have described the observation that defects in the annuli of the *C. elegans* cuticle also cause an Osr phenotype, and discuss a model wherein the annuli act as stretch sensors capable of responding to an osmotic challenge. Further experimental support for this model could be obtained by immunolocalization of OSM-7, to see if it is physically associated with the cuticle, or by immunoprecipitation to identify binding partners of OSM-7.

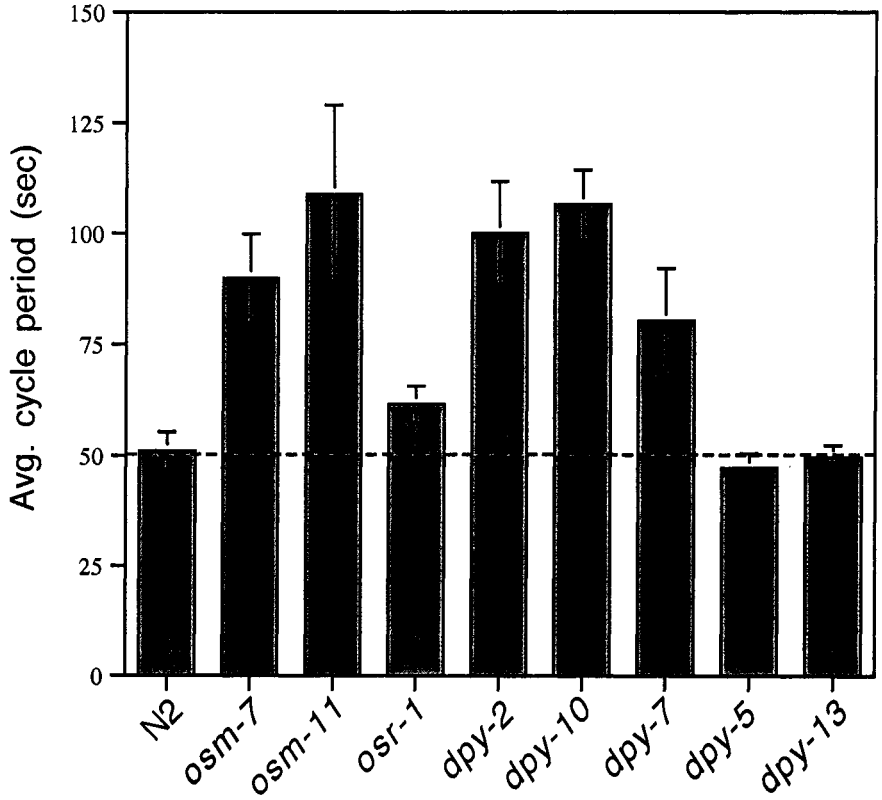
Table 6. Osmotic resistance phenotype of several strains.

Strain	Response to touch on 500mM NaCl	Response to touch on 800mM NaCl	Glycerol (ng/mg protein ± s.d.)
N2	3%	0%	3.2 ± 0.7
osm-7	100%	100%	159.8 ± 28.9
osm-11	100%	100%	141.8 ± 6.6
osr-1	97%	27%	42.4 ± 4.9
dpy-2	96%	n.d.	n.d.
dpy-10	97%	6%	42.5 ± 15.9
dpy-7	100%	10%	n.d.
dpy-5	6%	0%	n.d.
dpy-13	10%	0%	n.d.
goa-1	3%	0%	n.d.
egl-30(gf)	4%	0%	n.d.

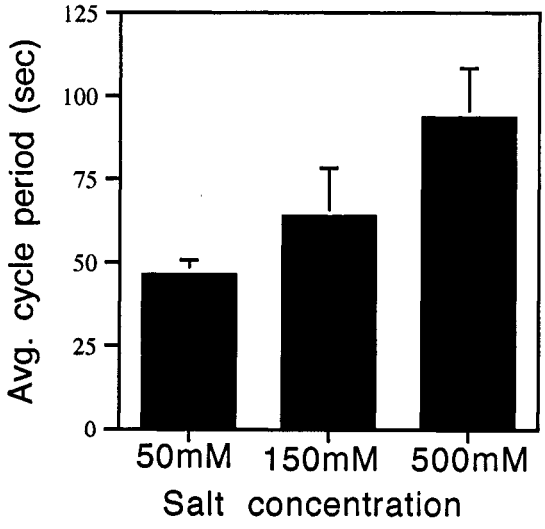
Table 7. *osm-7* double mutant phenotypes.

Strain	% response to touch on 800mM NaCl	Glycerol (ng/mg protein ± s.d.)	Avg. defecation cycle period (sec) ± s.d.
N2	0%	3.2 ± 0.7	51.0 ± 4.5
osm-7	100%	159.8 ± 28.9	89.8 ± 10.0
osm-7; unc-43	100%	174.8 ± 33.8	96.5 ± 16.7
osm-7; nsy-1	100%	180.6 ± 5.9	89.2 ± 9.0
osm-7; sek-1	100%	145.9 ± 6.4	85.3 ± 9.9
osm-7; egl-8	100%	166.9 ± 25.8	82.1 ± 18.8
osm-7; pkc-1	100%	177.9 ± 36.1	74.2 ± 8.4
osm-7; pkc-2	100%	n.d.	101.1 ± 15.3
osm-7; tpa-1	100%	n.d.	79.9 ± 4.1

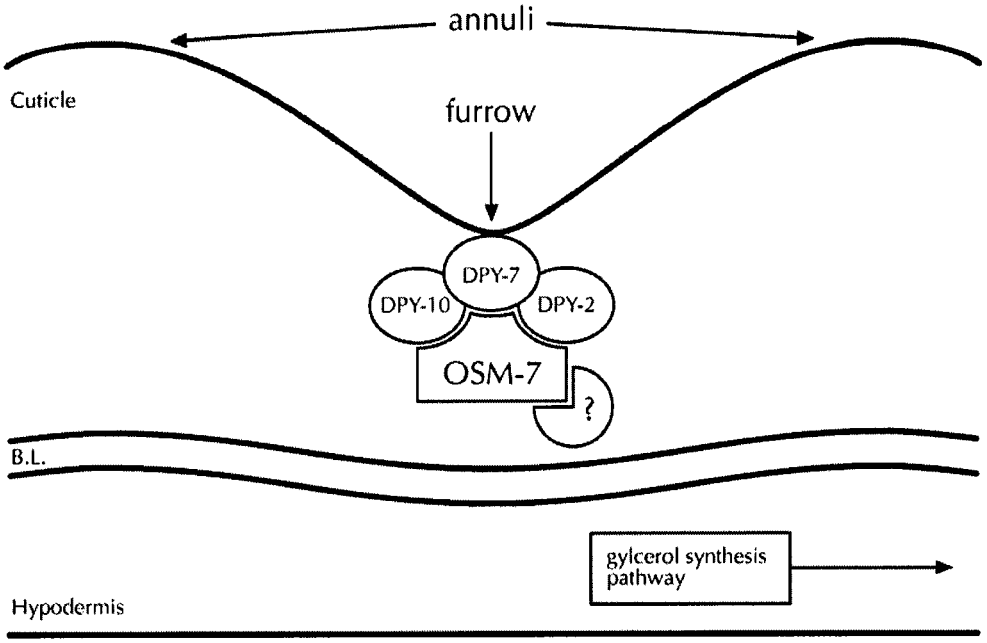
A.


Avg. cycle period (sec)
150
125
100
75
50
25
0
N2
osm-7
osm-11
osr-1
dpy-2
dpy-10
dpy-7
dpy-5
dpy-13

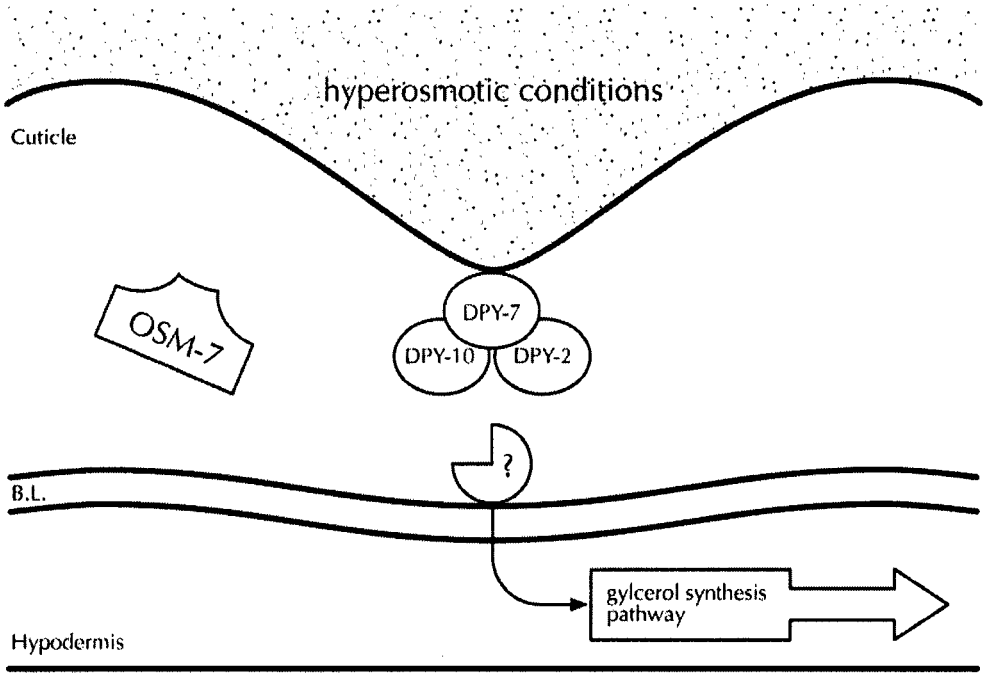
B.


Avg. cycle period (sec)
125
100
75
50
25
0
50mM
150mM
500mM
Salt concentration

A.


annuli
Cuticle
furrow
DPY-7
DPY-10
DPY-2
OSM-7
?
B.L.
gylcerol synthesis pathway
Hypodermis

B.


hyperosmotic conditions
Cuticle
OSM-7
DPY-7
DPY-10
DPY-2
?
B.L.
gylcerol synthesis pathway
Hypodermis

Bibliography

Albertyn, J., Hohmann, S., Thevelein, J. M., and Prior, B. A. (1994). GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol Cell Biol *14*, 4135-4144.

Alonso-Monge, R., Real, E., Wojda, I., Bebelman, J. P., Mager, W. H., and Siderius, M. (2001). Hyperosmotic stress response and regulation of cell wall integrity in *Saccharomyces cerevisiae* share common functional aspects. Mol Microbiol *41*, 717-730.

Avery, L. (1993) The genetics of feeding in *Caenhorabditis elegans*. Genetics *133*, 897-917.

Bany, I. A., Dong, M. Q., and Koelle, M. R. (2003). Genetic and cellular basis for acetylcholine inhibition of *Caenorhabditis elegans* egg-laying behavior. J Neurosci *23*, 8060-8069.

Bargmann, C. I. (1998). Neurobiology of the *Caenorhabditis elegans* genome. Science *282*, 2028-2033.

Bargmann, C. I., Hartwieg, E., and Horvitz, H. R. (1993). Odorant-selective genes and neurons mediate olfaction in *C. elegans*. Cell *74*, 515-527.

Bastiani, C. A., Gharib, S., Simon, M. I., and Sternberg, P. W. (2003). *Caenorhabditis elegans* Gαq regulates egg-laying behavior via a PLCβ-independent and serotonin-dependent signaling pathway and likely functions both in the nervous system and in muscle. Genetics *165*, 1805-1822.

Bell-Pedersen, D., Cassone, V. M., Earnest, D. J., Golden, S. S., Hardin, P. E., Thomas, T. L., and Zoran, M. J. (2005). Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat Rev Genet *6*, 544-556.

Benian, G. M., Tinley, T. L., Tang, X., and Borodovsky, M. (1996). The *Caenorhabditis elegans* gene *unc-89*, required fpr muscle M-line assembly, encodes a giant modular protein composed of Ig and signal transduction domains. J Cell Biol *132*, 835-848.

Branicky, R., Shibata, Y., Feng, J., and Hekimi, S. (2001). Phenotypic and suppressor analysis of defecation in *clk-1* mutants reveals that reaction to changes in temperature is an active process in *Caenorhabditis elegans*. Genetics *159*, 997-1006.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. Genetics *77*, 71-94.

Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E., and Gustin, M. C. (1993). An osmosensing signal transduction pathway in yeast. Science *259*, 1760-1763.

Burg, M. B., Kwon, E. D., and Kultz, D. (1997). Regulation of gene expression by hypertonicity. Annu Rev Physiol *59*, 437-455.

Burgess, J., Hihi, A. K., Benard, C. Y., Branicky, R., and Hekimi, S. (2003). Molecular mechanism of maternal rescue in the *clk-1* mutants of *Caenorhabditis elegans*. J Biol Chem *278*, 49555-49562.

Chalfie, M. (1993). Touch receptor development and function in *Caenorhabditis elegans*. J Neurobiol *24*, 1433-1441.

Colbert, H. A., and Bargmann, C. I. (1997). Environmental signals modulate olfactory acuity, discrimination, and memory in *Caenorhabditis elegans*. Learn Mem *4*, 179-191.

Culotti, J. G., and Russell, R. L. (1978). Osmotic avoidance defective mutants of the nematode *Caenorhabditis elegans*. Genetics *90*, 243-256.

Dal Santo, P., Logan, M. A., Chisholm, A. D., and Jorgensen, E. M. (1999). The inositol trisphosphate receptor regulates a 50-second behavioral rhythm in *C. elegans*. Cell *98*, 757-767.

de Nobel, H., Ruiz, C., Martin, H., Morris, W., Brul, S., Molina, M., and Klis, F. M. (2000). Cell wall perturbation in yeast results in dual phosphorylation of the Slt2/Mpk1 MAP kinase and in an Slt2-mediated increase in FKS2-lacZ expression, glucanase resistance and thermotolerance. Microbiology *146 (Pt 9)*, 2121-2132.

Deitmer, J. W., Verkhratsky, A. J., and Lohr, C. (1998). Calcium signalling in glial cells. Cell Calcium *24*, 405-416.

Dempsey, C. M., Mackenzie, S. M., Gargus, A., Blanco, G., and Sze, J. Y. (2005). Serotonin (5HT), fluoxetine, imipramine and dopamine target distinct 5HT receptor signaling to modulate *Caenorhabditis elegans* egg-laying behavior. Genetics *169*, 1425-1436.

Felkai, S., Ewbank, J. J., Lemieux, J., Labbe, J. C., Brown, G. G., and Hekimi, S. (1999). CLK-1 controls respiration, behavior and aging in the nematode *Caenorhabditis elegans*. Embo J *18*, 1783-1792.

Fraser, A. G., Kamath, R. S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., and Ahringer, J. (2000). Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. Nature *408*, 325-330.

Garner, M. M., and Burg, M. B. (1994). Macromolecular crowding and confinement in cells exposed to hypertonicity. Am J Physiol *266*, C877-892.

Goulding, M., and Pfaff, S. L. (2005). Development of circuits that generate simple rhythmic behaviors in vertebrates. Curr Opin Neurobiol *15*, 14-20.

Grillner, S. (2003). The motor infrastructure: from ion channels to neuronal networks. Nat Rev Neurosci *4*, 573-586.

Gustin, M. C., Albertyn, J., Alexander, M., and Davenport, K. (1998). MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev *62*, 1264-1300.

Hasegawa, K., Saigusa, T., and Tamai, Y. (2005). *Caenorhabditis elegans* opens up new insights into circadian clock mechanisms. Chronobiol Int *22*, 1-19.

Hohmann, S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev *66*, 300-372.

Iwasaki, K., Liu, D. W., and Thomas, J. H. (1995). Genes that control a temperature-compensated ultradian clock in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A *92*, 10317-10321.

Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., *et al.* (2003). Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature *421*, 231-237.

Kiehn, O., and Kullander, K. (2004). Central pattern generators deciphered by molecular genetics. Neuron *41*, 317-321.

Kim, D. H., Feinbaum, R., Alloing, G., Emerson, F. E., Garsin, D. A., Inoue, H., Tanaka-Hino, M., Hisamoto, N., Matsumoto, K., Tan, M. W., and Ausubel, F. M. (2002). A conserved p38 MAP kinase pathway in *Caenorhabditis elegans* innate immunity. Science *297*, 623-626.

Kippert, F. (2001). Cellular signalling and the complexity of biological timing: insights from the ultradian clock of *Schizosaccharomyces pombe*. Philos Trans R Soc Lond B Biol Sci *356*, 1725-1733.

Klevecz, R. R., Bolen, J., Forrest, G., and Murray, D. B. (2004). A genomewide oscillation in transcription gates DNA replication and cell cycle. Proc Natl Acad Sci U S A *101*, 1200-1205.

Kniazeva, M., Sieber, M., McCauley, S., Zhang, K., Watts, J. L., and Han, M. (2003). Suppression of the ELO-2 FA elongation activity results in alterations of the fatty acid composition and multiple physiological defects, including abnormal ultradian rhythms, in *Caenorhabditis elegans*. Genetics *163*, 159-169.

Lamitina, S. T., Morrison, R., Moeckel, G. W., and Strange, K. (2004). Adaptation of the nematode *Caenorhabditis elegans* to extreme osmotic stress. Am J Physiol Cell Physiol *286*, C785-791.

Lamitina, S. T., and Strange, K. (2005). Transcriptional targets of DAF-16 insulin signaling pathway protect *C. elegans* from extreme hypertonic stress. Am J Physiol Cell Physiol *288*, C467-474.

Lin, H., Nguyen, P., and Vancura, A. (2002). Phospholipase C interacts with Sgd1p and is required for expression of GPD1 and osmoresistance in *Saccharomyces cerevisiae*. Mol Genet Genomics *267*, 313-320.

Lints, R., and Emmons, S. W. (1999). Patterning of dopaminergic neurotransmitter identity among *Caenorhabditis elegans* ray sensory neurons by a TGFβ family signaling pathway and a Hox gene. Development *126*, 5819-5831.

Liu, D. W., and Thomas, J. H. (1994). Regulation of a periodic motor program in *C. elegans*. J Neurosci *14*, 1953-1962.

Loros, J. J., and Dunlap, J. C. (2001). Genetic and molecular analysis of circadian rhythms in *Neurospora*. Annu Rev Physiol *63*, 757-794.

Maeda, I., Kohara, Y., Yamamoto, M., and Sugimoto, A. (2001). Large-scale analysis of gene function in *Caenorhabditis elegans* by high-throughput RNAi. Curr Biol *11*, 171-176.

Maricq, A. V., Peckol, E., Driscoll, M., and Bargmann, C. I. (1995). Mechanosensory signalling in *C. elegans* mediated by the GLR-1 glutamate receptor. Nature *378*, 78-81.

McKay, J. P., Raizen, D. M., Gottschalk, A., Schafer, W. R., and Avery, L. (2004). *eat-2* and *eat-18* are required for nicotinic neurotransmission in the *Caenorhabditis elegans* pharynx. Genetics *166*, 161-169.

McMahon, L., Muriel, J. M., Roberts, B., Quinn, M., and Johnstone, I. L. (2003). Two sets of interacting collagens form functionally distinct substructures within a

Caenorhabditis elegans extracellular matrix. Mol Biol Cell *14*, 1366-1378.

Merris, M., Wadsworth, W. G., Khamrai, U., Bittman, R., Chitwood, D. J., and Lenard, J. (2003). Sterol effects and sites of sterol accumulation in *Caenorhabditis elegans*: developmental requirement for 4α-methyl sterols. J Lipid Res *44*, 172-181.

Miller, K. G., Alfonso, A., Nguyen, M., Crowell, J. A., Johnson, C. D., and Rand, J. B. (1996). A genetic selection for *Caenorhabditis elegans* synaptic transmission mutants. Proc Natl Acad Sci U S A *93*, 12593-12598.

Miller, K. G., Emerson, M. D., and Rand, J. B. (1999). Goα and diacylglycerol kinase negatively regulate the Gqα pathway in *C. elegans*. Neuron *24*, 323-333.

Mori, I. (1999). Genetics of chemotaxis and thermotaxis in the nematode *Caenorhabditis elegans*. Annu Rev Genet *33*, 399-422.

Nguyen, M., Alfonso, A., Johnson, C. D., and Rand, J. B. (1995). *Caenorhabditis elegans* mutants resistant to inhibitors of acetylcholinesterase. Genetics *140*, 527-535.

Perkins, L. A., Hedgecock, E. M., Thomson, J. N., and Culotti, J. G. (1986). Mutant sensory cilia in the nematode *Caenorhabditis elegans*. Dev Biol *117*, 456-487.

Philip, B., and Levin, D. E. (2001). Wsc1 and Mid2 are cell surface sensors for cell wall integrity signaling that act through Rom2, a guanine nucleotide exchange factor for Rho1. Mol Cell Biol *21*, 271-280.

Rand, J. B. (1989). Genetic analysis of the *cha-1-unc-17* gene complex in *Caenorhabditis*. Genetics *122*, 73-80.

Ranganathan, R., Sawin, E. R., Trent, C., and Horvitz, H. R. (2001). Mutations in the *Caenorhabditis elegans* serotonin reuptake transporter MOD-5 reveal serotonin-dependent and -independent activities of fluoxetine. J Neurosci *21*, 5871-5884.

Remize, F., Barnavon, L., and Dequin, S. (2001). Glycerol export and glycerol-3-phosphate dehydrogenase, but not glycerol phosphatase, are rate limiting for glycerol production in *Saccharomyces cerevisiae*. Metab Eng *3*, 301-312.

Robatzek, M., and Thomas, J. H. (2000). Calcium/calmodulin-dependent protein kinase II regulates *Caenorhabditis elegans* locomotion in concert with a G(o)/G(q) signaling network. Genetics *156*, 1069-1082.

Sagasti, A., Hisamoto, N., Hyodo, J., Tanaka-Hino, M., Matsumoto, K., and Bargmann, C. I. (2001). The CaMKII UNC-43 activates the MAPKKK NSY-1 to execute a lateral signaling decision required for asymmetric olfactory neuron fates. Cell *105*, 221-232.

Sawin, E. R., Ranganathan, R., and Horvitz, H. R. (2000). *C. elegans* locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway. Neuron *26*, 619-631.

Schibler, U., and Naef, F. (2005). Cellular oscillators: rhythmic gene expression and metabolism. Curr Opin Cell Biol *17*, 223-229.

Selverston, A. I. (2005). A neural infrastructure for rhythmic motor patterns. Cell Mol Neurobiol *25*, 223-244.

Siderius, M., Van Wuytswinkel, O., Reijenga, K. A., Kelders, M., and Mager, W. H. (2000). The control of intracellular glycerol in *Saccharomyces cerevisiae* influences osmotic stress response and resistance to increased temperature. Mol Microbiol *36*, 1381-1390.

Simmer, F., Moorman, C., van der Linden, A. M., Kuijk, E., van den Berghe, P. V., Kamath, R. S., Fraser, A. G., Ahringer, J., and Plasterk, R. H. (2003). Genome-wide RNAi of *C. elegans* using the hypersensitive *rrf-3* strain reveals novel gene functions. PLoS Biol *1*, E12.

Solomon, A., Bandhakavi, S., Jabbar, S., Shah, R., Beitel, G. J., and Morimoto, R. I. (2004). *Caenorhabditis elegans* OSR-1 regulates behavioral and physiological responses to hyperosmotic environments. Genetics *167*, 161-170.

Stanewsky, R. (2003). Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals. J Neurobiol *54*, 111-147.

Stein, L. D., Bao, Z., Blasiar, D., Blumenthal, T., Brent, M. R., Chen, N., Chinwalla, A., Clarke, L., Clee, C., Coghlan, A., *et al.* (2003). The genome sequence of *Caenorhabditis briggsae*: a platform for comparative genomics. PLoS Biol *1*, E45.

Sze, J. Y., Victor, M., Loer, C., Shi, Y., and Ruvkun, G. (2000). Food and metabolic signalling defects in a *Caenorhabditis elegans* serotonin-synthesis mutant. Nature *403*, 560-564.

Tabuse, Y. (2002). Protein kinase C isotypes in *C. elegans*. J Biochem (Tokyo) *132*, 519-522.

Take-Uchi, M., Kawakami, M., Ishihara, T., Amano, T., Kondo, K., and Katsura, I. (1998). An ion channel of the degenerin/epithelial sodium channel superfamily controls the defecation rhythm in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A *95*, 11775-11780.

Take-uchi, M., Kobayashi, Y., Kimura, K. D., Ishihara, T., and Katsura, I. (2005). FLR-4, a novel serine/threonine protein kinase, regulates defecation rhythm in *Caenorhabditis elegans*. Mol Biol Cell *16*, 1355-1365.

Thomas, J. H. (1990). Genetic analysis of defecation in Caenorhabditis elegans. Genetics *124*, 855-872.

Vay, H. A., Philip, B., and Levin, D. E. (2004). Mutational analysis of the cytoplasmic domain of the Wsc1 cell wall stress sensor. Microbiology *150*, 3281-3288.

Watanabe, M., Mitani, N., Ishii, N., and Miki, K. (2005). A mutation in a cuticle collagen causes hypersensitivity to the endocrine disrupting chemical, bisphenol A, in *C. elegans*. Mutat Res *570*, 71-80.

Watts, J. L., Phillips, E., Griffing, K. R., and Browse, J. (2003). Deficiencies in C20 polyunsaturated fatty acids cause behavioral and developmental defects in *Caenorhabditis elegans fat-3* mutants. Genetics *163*, 581-589.

VITA

1998-2005	**Ph.D. in Genetics** Department of Genome Sciences University of Washington, Seattle, WA
1994-1998	**B.A. in Biology** Kalamazoo College, Kalamazoo, MI
1996-1997	Study Abroad Program University of Aberdeen, Scotland

Awards and Honors

1999	Honorable Mention, NSF Graduate Fellowship
1998	Research Fellowship, American Heart Association
1998	Magna Cum Laude, Kalamazoo College
1994-1998	Dean's List and Kalamazoo College Honors Scholarship
1994	National Merit Scholar

Teaching and Mentoring

2002	Teaching Assistant, Dept. of Genetics, Gene Structure and Function, Prof. Leo Pallanck
2000	Teaching Assistant, Dept. of Genetics, Introductory Genetics, Prof. MK Raghuraman
2000-2005	Training and supervising of undergraduates working in the Thomas lab.
2001-2003	Mentor in Making Connections program for girls in science.
2001-2005	Guided hands-on laboratory tours for high school groups through outreach program at UW Medical Center.

Future Plans

Jeanna will continue her work in behavioral genetics at the Oregon Health Sciences University in Portland, where she will conduct postdoctoral research on alcohol addiction behaviors in mice.