A Developmental Analysis Of Habituation in *C. elegans*

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Abstract:

*C. elegans* is particularly well-suited for a developmental analysis of learning and memory. A developmental analysis of learning attempts to establish relationships between the emergence of particular forms of learning and memory, and the emergence of specific neural structures and circuits, and *C. elegans*' ontogeny is extremely well-characterized, including the complete developmental lineage of each somatic cell. Previous developmental research found that whereas the adult and young adult worms almost always reversed and swam backwards to tap, the larval worms only reversed about one-half of the time, and accelerated forwards the other half of the time. This response heterogeneity is a serious problem, because accelerations and reversals are two qualitatively different behavioral outcomes that cannot be easily compared. One solution to this problem is to laser ablate the PLM sensory neurons (which results in a worm that only reverses to tap) in one experiment, and ablate the ALM sensory neurons (which results in a worm that only accelerates forward in response to tap) in a second experiment so that in each experiment there is a single, homogeneous response type that can be compared across and within groups.

The focus of this thesis was to study the effect of repeated stimulation on accelerations and reversals in ablated worms across development. Habituation training was given to PLM-ablated and ALM-ablated animals at each of the 6 developmental stages (L1, L2, L3, L4, young adult, 4 day old adult), and at each of 2 ISIs (10 second and 60 second) using the tap-withdrawal paradigm. The results showed that habituation and recovery from habituation were both present in *C. elegans* at all stages, both ISIs, and both response types. There was also surprisingly little systematic variation in the characteristics of habituation and recovery over development, which further suggests that the basic neural machinery underlying this behavioral plasticity is present and functional as early as the L1 stage. These experiments represent the first systematic and quantitative investigation of learning and memory in *C. elegans* over the course of its development.
# Table of Contents:

Abstract ii

List of Tables v

List of Figures vi

Acknowledgements vii

I. Introduction 1
   A. Behavioral Plasticity and Simple Systems 1
   B. *C. elegans* as a Simple System 3
   C. Habituation of the Tap Withdrawal Response 6
   D. Toward a Developmental Analysis 7
   E. A Developmental Analysis of *C. elegans* 11
   F. The Tap Withdrawal Circuit 15
   G. Objectives 19

II. Method 21
   A. The Animals 21
   B. Laser Ablation 21
   C. Behavioral Testing 23
   D. Behavioral Scoring 24
   E. Data Analysis 26

III. Results 29
   A. Experiment One: The PLM-ablated Groups 29
   B. Experiment Two: The ALM-ablated Groups 47
   C. Comparisons Between PLM-ablated and ALM-ablated Groups 60

IV. General Discussion 63

V. References 71

VI. Appendix I 76
VII. Appendix II 77
VIII. Appendix III 78
IX. Appendix IV 79
List of Tables:

Table 1. Summary table of significant differences for all 24 groups.
List of Figures:

Figure 1. The life cycle of *C. elegans*.  
Figure 2. The simplified tap withdrawal circuit.  
Figure 3. The mean relative responsiveness of the PLM-ablated groups to the initial tap.  
Figure 4. The habituation curves for each of the PLM-ablated groups trained at a 10 second ISI (a. to f.).  
Figure 5. The habituation curves for each of the PLM-ablated groups trained at a 60 second ISI (a. to f.).  
Figure 6. The mean initial slope of each developmental stage at each ISI for the PLM-ablated animals.  
Figure 7. The mean relative responsiveness of the ALM-ablated groups to the initial tap.  
Figure 8. The habituation curves for each of the ALM-ablated groups trained at a 10 second ISI (a. to f.).  
Figure 9. The habituation curves for each of the ALM-ablated groups trained at a 60 second ISI (a. to f.).  
Figure 10. The mean initial slope of each developmental stage at each ISI for the ALM-ablated animals.
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I. INTRODUCTION

A. Behavioral Plasticity and Simple Systems

A fundamental question in studying the neuronal control of behavior is the investigation of the mechanisms underlying behavioral plasticity. Behavioral plasticity is the characteristic that allows organisms to modify their behavior over time, based on their experience, and it is thus critical to the survival of any organism in a changing environment. It gives organisms the power to adapt to new surroundings and to capitalize better on any novel situational variables that they may encounter. It is therefore an extremely important and ubiquitous behavioral phenomenon, and over the years has attracted much research attention in innumerable organisms and across several disciplines.

In attempting to describe the neural underpinnings of behavioral plasticity, neurobiologists have set for themselves a formidable challenge. They are confronted not only with the tremendous difficulty of describing the characteristics of plasticity at the behavioral level itself, but also with the problem of correlating and explaining the nature of the relationship between the behavior and the underlying complexity of neural circuitry. In the face of such a challenge, researchers have often fallen to the recourse of simplification--either simplify the behavior, simplify the underlying circuitry, or simplify both.

The behavior must always be simplified at least to the point that it can be suitably defined and controlled for study. Behavioral plasticity is a blanket term that refers to countless behavioral instances, and thus it is necessary to focus on some chosen subset of behaviors and define them for some chosen subset of contexts. Researchers of behavioral plasticity have studied a wide variety of behaviors, ranging from sensory adaptation and response fatigue, through associative and non-associative learning, to complicated problem solving, learned social interactions, and even human language. Not surprisingly, as the behaviors become more complicated, the protocols used to study them become less clearly defined; it becomes increasingly difficult to isolate specific behavioral components, and to control for confounding inter-relationships and contexts. As such, for the neurobiologist
hoping to correlate behavior with neuronal activity, the problem is more tractable if a more simple and well-defined form of plasticity is chosen for analysis--the better described and defined the behavior, the better a neural correlate can be matched to it.

Unfortunately, the range and richness of an organism's behavioral repertoire often comes at the expense of neural complexity. An organism to study is chosen not only for the range and richness of its behavioral repertoire and the ease and clarity with which its behaviors can be studied, but also for its own inherent neural and/or genetic simplicity, and its conduciveness to laboratory study. Again, the researcher is faced with no lack of choices--there is a long list of organisms and species that have been studied by neurobiologists, from protozoa, through worms and slugs and insects, and amphibians and lower mammals, to higher mammals and man. And again, if the goal is to correlate and explain behavior in terms of neural events, then simplicity imparts attractiveness.

However, even the simplest nervous system is terribly complex, and so every attempt is made to choose an organism that best combines its behavioral and neurobiological virtues into an ideal system. Over the last 30 years there has been a steady increase in so-called "simple-systems" learning research, which is characterized by concentrated efforts to capitalize on a particular system's relative behavioral and neurobiological simplicity. The general strategy of such a "simple-systems" approach is to first try to identify the neural circuits, cells, ion currents, channels, molecules, and/or genes underlying a selected behavior, and then attempt to determine the precise nature of their respective roles in producing the behavior.

Within several selected "simple" systems, researchers from a variety of fields including psychology, physiology, biochemistry, genetics, neurobiology, and molecular biology have integrated their knowledge and resources into inter-disciplinary investigations focused on the general goal of elucidating the cellular, molecular, and genetic basis of behavioral plasticity (for reviews, see Carew & Sahley, 1986; Byrne, 1987). These simple-system approaches have led to considerable progress in our understanding of the
mechanisms underlying behavioral plasticity in invertebrate systems as diverse as habituation and classical conditioning in *Aplysia californica*, and habituation and classical conditioning in *Drosophila melanogaster* (for an extensive list of references, see Carew & Sahley, 1986), and even in vertebrate systems such as eyeblink conditioning in the house-cat (Kim *et al.*, 1983; Woody *et al.*, 1983; Anderson & Steinmetz, 1994), and long-term-potentiation in the rat hippocampal slice (McNaughton *et al.*, 1978; Lynch & Baudry, 1984; Massicotte & Baudry, 1991). These varied systems, along with several others, continue to be used because they seem to satisfy best the set of demand characteristics associated with the particular research questions being asked—there is no one "ideal" system that can do it all. Unfortunately, because no such "ideal" system exists, researchers are faced with the reality of compromise. Each system proudly boasts its relative strengths, but each system also has its several inherent weaknesses, with any one system's advantage often being another's flaw.

The nematode, *Caenorhabditis elegans*, introduced to the learning literature by Rankin, Beck, and Chiba (1990), stands as a relatively new and attractive alternative for the study of behavioral plasticity. *C. elegans* has a comparatively simple nervous system, a rich array of clearly definable behaviors, and it has been extensively studied genetically. *C. elegans* is the only organism yet studied that is highly amenable to both circuit analysis and molecular genetic techniques. Its biggest disadvantage is that as yet, researchers can do no electrophysiology, although recent reports suggest that some electrophysiological techniques are currently being developed (Lockery, 1994) and may soon be available. Nonetheless, *C. elegans* is a new simple-system that offers some unique opportunities to explore the relationships between the cellular, molecular, and genetic mechanisms of behavioral plasticity.

**B. C. elegans as a Simple System**

Although relatively simple, *C. elegans' behavioral repertoire is still varied enough to provide a broad range of possibilities for study. In its natural environment, *C. elegans* is a
small, free-living, non-parasitic soil nematode. It spends its life in the lab swimming on the
two-dimensional surface of an agar-filled dish, eating (E. coli is its food), defecating, and
laying eggs (if hermaphrodite) or mating (if male). *C. elegans* has several different sense
modalities, including mechanosensation (Chalfie & Sulston, 1981; Croll, 1975),
chemosensation—"taste" (Dusenbury, 1974; Ward, 1973), chemosensation—"smell"
(Bargmann et al., 1993), thermosensation (Hedgecock & Russell, 1975), osmosensation
(Culotti & Russell, 1978), and perhaps galvanosensation (Sukul & Croll, 1978) and
photosensation (Burr, 1985). In addition, *C. elegans* has demonstrated responses to various
sensory input by either moving towards or away from the centers of prepared gradients of
chemical compounds, temperature, and electric fields. *C. elegans* also responds to
mechanical stimulation, such as touch and vibration, by either accelerating forwards, or by
reversing (swimming backwards) for some distance and then changing course and resuming
forward movement in a new direction.

*C. elegans* is one of the simplest multicellular organisms yet to be studied for
behavioral plasticity, and it offers the unique opportunity to elucidate the functions of all the
neurons involved in a given behavior. Its nervous system has only 302 neurons, and its
neuroanatomical map has been described completely, including its neuronal inter-
connectivity which has been defined to the synaptic level by serial section electron
microscopy (White *et al.*, 1986; Chalfie, 1984). Furthermore, the complete developmental
lineage of each somatic cell has been mapped using Nomarski microscopy on living worms
(Sulston *et al.*, 1983).

Similar to its simple nervous system, the genetics of *C. elegans* are also relatively
simple. Its genome is approximately half the size of the genome of *Drosophila*, and over
95% of it has been mapped and over 50% of it has been sequenced (Coulson, Sulston,
mode of reproduction (self-fertilization) allows for true breeding of mutants, and the
existence of males, which mate with the hermaphrodites, makes it possible for both
homozygous and heterozygous offspring to be produced. Its relatively short 3 day reproductive cycle is also convenient. Many mutant strains have already been isolated and the cryoviability of the worms has allowed for a central library of mutants to be established. In addition, several genetic and neurobiological techniques have been adapted for use with C. elegans, including in situ hybridization (Albertson, 1984), integrative transformation (Fire, 1986), and transposon tagging (Moerman, 1986).

C. elegans is also optically transparent, which allows for the visualization of nuclei of every cell body in the developing worm. As a result, a complete embryonic cell lineage is available for every cell in the larval animal (Sulston et al., 1983), and a complete post-embryonic cell lineage is available for every cell in the adult (Sulston & White, 1977). The transparency also makes C. elegans amenable to the use of laser ablation techniques: A laser microbeam can be focused down through the optics of a Nomarski microscope onto the nucleus of a specific, identified cell, and after intense or prolonged exposure the cell can be lesioned (Sulston & White, 1980). This laser ablation technique allows for the experimental manipulation of the nervous system to determine which of the anatomical connections are functional for a given behavior. In fact, the functions of neural circuits underlying certain behaviors, such as the touch (Chalfie et al., 1985) and tap (Wicks & Rankin, 1995a) withdrawal circuits, and thermotaxis (Mori & Oshima, 1995) have already been demonstrated, clearly establishing the link between behavior and anatomy. C. elegans has demonstrated behavioral plasticity in a number of protocols, and similar links have already been made between behavioral plasticity and the underlying neural circuitry it is hypothesized to be based on (Wicks & Rankin, 1995b).

In C. elegans, as with most organisms, this behavioral plasticity can take several forms, ranging from sensory adaptation to learning, including both non-associative and associative conditioning. As alluded to above, the choice of the specific behavior to study is an important one. The chosen behavior should be clear, consistent, and measurable. It should be a behavior that best takes advantage of the strengths and weaknesses of the
organism, and any ongoing or previous research that has been done. Ideally, it would be a behavior that is well-correlated with the known underlying neural circuitry. In *C. elegans*, the behavior most studied, and best meeting the above criteria is habituation of the tap withdrawal response (Rankin *et al.*, 1990).

### C. Habituation of the Tap Withdrawal Response

Although there have been a few passing mentions of behavioral plasticity in *C. elegans* literature (for a review, see Gannon & Rankin, 1995), it was not until the development of the tap-withdrawal paradigm that learning and memory were directly and systematically studied in the worm (Rankin *et al.*, 1990). In the tap-withdrawal paradigm (described in detail in the Methods section), a single worm is first placed on a small agar-filled petri dish, where the worm can be tracked under a microscope while it swims along the agar surface. The side of the dish is then "tapped" by a small metal wire triggered by a mechanical stimulus generator, which ensures the standardized consistency of each tap. The tap causes the whole dish to vibrate momentarily, and this vibration stimulates adult worms to elicit a reversal response. The magnitude of this reversal response is measured by the distance that the animal reverses following a tap, which can be easily video-recorded and measured. It was found that the reversal response, as measured by this reversal distance, habituates after a number of successive stimuli (Rankin *et al.*, 1990).

Although the tap withdrawal paradigm has been used to demonstrate that *C. elegans* is capable of all forms of non-associative conditioning, including habituation, dishabituation, and sensitization, studies have primarily focused on analyses of habituation. Habituation is defined as a decrement in response due to the repeated application of a stimulus (Thompson and Spencer, 1966; Groves and Thompson, 1970). This decrement in responding is distinguished from sensory adaptation or motor fatigue on the basis that the subject habituates to a stimulus despite the fact that it remains fully capable of sensing the stimulus and of making the muscle movements required for the response (Groves & Thompson, 1970). This property can be demonstrated by dishabituation, in which the subject's
responsiveness is instantly recovered by the application of a single novel or noxious stimulus (Thompson and Spencer, 1966; Groves & Thompson, 1970). Habituation and recovery from habituation are also sensitive to ISI, and repeated short-term habituation often leads to long-term habituation, after a critical number of trials, again very dependent on the ISI (Groves & Thompson, 1970; Beck & Rankin, 1995).

The factors that affect the rate of habituation, the degree of habituation, and the rate of recovery from habituation of the tap withdrawal reflex in C. elegans have been thoroughly investigated (Rankin & Broster, 1992; Broster & Rankin, 1994). The habituation curves for reversal responses all show the standard habituation pattern of an initial, negative slope followed by a more gradual, almost flat slope which eventually reaches an asymptote. Results of studies on the effects of ISI show that habituation is more pronounced and faster with a short ISI (10 seconds) than with a longer ISI (60 seconds). Also, the more stimuli that are delivered, the more complete is the habituation, although it is currently unknown whether there is any effect of continuing stimulation after asymptote is reached (Rankin & Broster, 1992). Dishabituation was demonstrated by giving an habituated worm a brief electrical shock (Rankin et al., 1990). Normally, there is significant dishabituation, as evidenced by an increase in response amplitude to tap following the shock.

Rankin and Broster (1992) analyzed spontaneous recovery from habituation by monitoring the worm's response to some additional test stimuli presented at different, longer intervals (i.e. 30 seconds, 5 minutes, 10 minutes, and 20 minutes) following the last training stimulus. They showed that C. elegans spontaneously recovers from habituation, and the amount and nature of this recovery was dependent on the ISI used in the habituation training. Specifically, they demonstrated that spontaneous recovery from habituation is greater and more rapid with shorter ISIs (10 seconds) than with longer ISIs (60 seconds).

D. Towards a Developmental Analysis

One strategy for studying behavioral plasticity that has been used with success in the past, and that appears to take advantage of many of C. elegans' unique strengths is a
developmental strategy. It has long been appreciated that important insights into the neuronal control of behavior can be gained from a developmental perspective (Campbell & Spear, 1972; Shair et al., 1991). In many animals, there is substantial growth and development of the nervous system after birth, and as a result of these ontogenetic processes these animals become better able to successfully and independently interact with their environments as they approach maturity. A developmental analysis of learning focuses on the changes in learning capacity and any correlated neuronal changes that occur during or as a result of these periods of ontogenetic growth. The power of this approach is that it can reveal relationships that can be difficult or impossible to establish in the complex circuitry of fully mature adult animals.

The first goal of a developmental analysis is to identify age-related variation in the capacities of the functional system (Hyson & Rudy, 1984). The logic of analyzing the age-dependent properties of a system, such as the habituation of the tap withdrawal reflex, is the same as that used to isolate the contribution of any other organismic variable (e.g. area of the brain as defined by some focal lesion) to that system. In both cases, the role of organismic variation (e.g. age or region of brain) is assessed with a range of behavioral tasks that operationally define the functional system. This analysis informs us not only of any behavioral differences that relate to organismic variation, but also of any behavioral similarities that exist despite this variation. It is from this array of behavioral differences and similarities that one then constructs an interpretation of the contribution of organismic, or developmental, variation to the functional system (Hyson & Rudy, 1984).

A developmental analysis of behavioral plasticity has proven to be a valuable approach in establishing relationships between different forms of learning and memory and different stages of development in a number of mammalian systems (Campbell & Spear, 1972; Spear & Rudy, 1991; Galef, 1991). Much of this research has been done with the rat, an altricial animal whose nervous system matures substantially after birth. This literature shows clearly that the newborn rat can learn, and strongly suggests that there is a sequential emergence of simple detection and associative learning processes, which may reflect the operation of a
general principle of sensory system development (Moye & Rudy, 1985). Rudy and his colleagues found that, regardless of the time of developmental emergence of a sensory system, the newborn rat's capacity to learn using that system invariably follows the system's capacity to perform reflexive behaviors (Vogt & Rudy, 1984; Hyson & Rudy, 1984; Moye & Rudy, 1985). However, because of the inherent complexity of these mammalian systems, it is extremely difficult to implement this developmental approach at a cellular level, where direct comparisons of learning over the course of development are made in either anatomical or mechanistic terms. Such an analysis is more ideally suited to a system that not only exhibits behavioral plasticity early in development, but that also has a nervous system that is amenable to cellular investigation at the same early developmental stages (Nolen et al., 1987).

Invertebrate animals have proven to be particularly valuable for such an approach. The expression of new patterns of behavior during post-embryonic development depends on neuronal growth, the construction of new neural circuits, and on the modification of existing neural circuits (Edwards et al., 1994). In some invertebrate simple-systems it has been possible to specify these critical changes in the underlying neural circuitry during development, and thus attempt to establish relationships between the emergence of particular forms of learning and memory, and the emergence of these critical neural structures and circuits.

One example of a developmental analysis of learning in a simple-system is research that was done using *Aplysia* (Rankin & Carew, 1987). In this study, Rankin and Carew investigated the development of two forms of nonassociative learning, habituation and dishabituation, in the siphon withdrawal component of the organism's defensive reflex. They examined this reflex throughout the juvenile life of the animal (stages 9-12), since the reflex is not functionally intact until the siphon emerges in stage 9. They studied the development of habituation in stages 9-12 using tactile stimuli to the siphon delivered at 1, 5, 10, and 30 sec ISIs. They demonstrated habituation of siphon withdrawal at stage 9,
although it was produced only with a very short (1 sec) ISI. Approximately 4 days later, in stage 10, they demonstrated significant habituation with 1 and 5 sec ISIs. One to two weeks later, in stage 11, they demonstrated significant habituation with 1, 5, and 10 sec ISI. In stage 12 juveniles and adults (stage 13), they finally demonstrated significant habituation with the 30 sec ISI too. Therefore, there was a systematic developmental trend in the ability of the animals to habituate: Progressively older animals were capable of habituating to stimuli presented at progressively longer intervals. This systematic development of habituation was also evident by examining the amount of habituation exhibited to comparable ISIs by animals at different developmental stages. For all 4 ISIs examined, older animals showed significantly greater habituation than younger animals. Thus, their results showed that habituation is present as soon as the siphon response system emerges and that it then develops progressively throughout the juvenile life of the animal.

Another example of the successful application of a developmental strategy to study behavioral plasticity in a simple system is the recent research on the crayfish (Edwards et al., 1994). As they grow, the defensive strategy of crayfish changes. Juvenile (0.5 cm in length) crayfish are quick to escape from attack, whereas adult (8-12 cm) crayfish are more prone to turn and face their threat by raising their claws in a defensive display (Fricke, 1986; Lang et al., 1977). The best studied form of escape is the somersault tail flip that is reliably triggered by a single spike in the lateral giant (LG) interneurons in response to a sharp tap on the abdomen (Wine and Krasne, 1982). In juvenile crayfish shorter than 2 cm, LG tail flip is a low-threshold, highly reliable behavior that shows no change in response to repeated stimulation at frequencies as high as 2 Hz (Fricke, 1986). As the animal reaches 2-3 cm in length, repeated stimulation does habituate the LG tail flip response (Fricke, 1986). In adult crayfish, LG tail flip has a much higher stimulus threshold and is subject to habituation and several other modulatory effects, including inhibition, sensitization, and changes in arousal (see Wine & Krasne, 1982 for a review). Tail flip habituation occurs in adult crayfish in response to repeated stimulation at frequencies as low as once per 2 minutes.
The post-embryonic development of this LG tail flip response was studied with standard electrophysiological techniques in animals between 1 and 12 cm long (Edwards et al., 1994). They showed how the expression of habituation in the crayfish results from neuronal growth that changes a neuron's integrative properties. Tail flipping in juvenile crayfish was shown previously to be triggered by the reliable, non-habituating monosynaptic pathway directly from the mechanosensory afferents to LG, and not by the depression-prone disynaptic pathway (Fricke, 1984). The onset of response habituation occurs during development as the depression-prone disynaptic pathway replaces the reliable, monosynaptic pathway in providing the low-threshold input for firing LG.

E. A Developmental Analysis of *C. elegans*

*C. elegans*, due to its relative simplicity and intensively studied ontogeny, seems to be particularly well-suited for a developmental analysis of learning and memory. *C. elegans* develops rapidly, proceeding from egg through 4 larval molts to a reproducing adult in approximately three and a half days at 20 degrees C (see Figure 1) (Byerly et al., 1975). *C. elegans*’ life cycle has 7 well-characterized stages: the egg, the 4 larval stages (L1 (approx. 200 um long), L2 (approx. 370 um), L3 (approx. 480 um), and L4 (approx. 640 um)), the young adult (pre-egg laying) (approx. 850 um), and the adult (peak egg laying—defined as 4-day olds)(approx. 1 mm). As shown in Figure 1, each of the larval stages lasts a specified, predictable number of hours, and ends when the animal sheds its cuticle (i.e: it molts). The animal grows continuously between molts and the timing and pattern of cell-division between individuals under similar conditions is highly consistent (see Sulston, 1988, for summary).

Although capable of eating and swimming and living independently, the newly hatched L1 animal has a nervous system substantially different from that of the adult. The L1 animal has only 60% of the adult complement of somatic nuclei, and 10% of these cells are somatic blast cells that will undergo further cell division, contributing substantially to the hypodermis, nervous system, musculature, and somatic gonadal structures (Wood, 1988).
Figure 1: The life cycle of *C. elegans*. There are seven developmental stages: the egg, the four larval stages (L1, L2, L3, L4), the young adult (YAD), and the fully mature adult at four-days-old (4D). The developmental time-course is measured in hours, with the onset of each stage very reliably predicted (the time-line given is for worms raised at 20 degrees C).
For example, the newly hatched L1 animal has only 22 of the 76 ventral cord motor neurons that it will have as an adult, and only 3 of the 8 types of motor neurons that it will have as an adult (Sulston, 1976; Sulston & Horvitz, 1977). Changes begin in the larval nervous system 3 hours after hatching, when cell division is re-initiated. Within the next 10 hours, most of the cells that will comprise the adult nervous system are generated, although not necessarily functional, because some neurons need more time to migrate and/or extend processes to their appropriate targets before becoming fully integrated into the nervous system (Walthall & Chalfie, 1988). Importantly, some of these developmental changes include elements of the adult tap withdrawal circuit, which is described in some detail below. Perhaps most significantly, one of the head-touch sensory neurons, AVM, does not become fully integrated into the circuit until the L4 stage (after approx. 46 - 51 hours post-hatching) (Chalfie et al, 1985). The occurrence of such large developmental changes in the animal's relevant neural circuitry strongly suggests that there could be corresponding changes in the animal's behavior, and indeed, some of these developmental differences have already been revealed (Chiba & Rankin, 1990).

Chiba and Rankin (1990) carried out a developmental analysis of spontaneous and reflexive reversals in C. elegans, with interesting results. First, Chiba and Rankin (1990) examined the frequency of spontaneous reversals across development, and found that only the young adult group differed significantly from the other stages, showing significantly more spontaneous reversals than all the other stages. They also examined the general level of locomotor activity across stages and so determined that the increase in spontaneous reversals in the young adult stage was not the result of a general increase in locomotor activity at that stage; only the L1's were significantly different in that they spent less time active than the other stages. Second, they confirmed the findings of Chalfie and Sulston (1981), that the head-touch withdrawal reflex (where the head of a worm is lightly stroked by a small hair), does not appear to change over development—regardless of developmental stage, the worm almost always reverses.
Interestingly, Chiba and Rankin (1990) did find some significant ontogenetic differences when they studied the worms' response to tap across development. Whereas the adult and young adult worms almost always reversed and swam backwards to tap (95% of the time), the larval worms only reversed about one-half of the time, and accelerated forwards the other half of the time (Chiba & Rankin, 1990). This observed behavioral change coincides with known periods of neuroanatomical change in the underlying touch withdrawal circuit. Specifically, the addition of the late-developing head-touch sensory neuron, AVM, is hypothesized to underlie the shift from accelerations to reversals in response to tap observed in larval animals and adults (Chiba & Rankin, 1990)

Because accelerations (increasing forward speed in response to tap) and reversals (swimming backwards in response to tap) are two qualitatively different outcomes that cannot easily be compared, an "apples and oranges" sort of problem arises for ontogenetic studies using the tap withdrawal paradigm. It is extremely difficult to quantitatively compare the magnitude of an acceleration with the magnitude of a reversal. The problem is then compounded by the fact that the ratio of accelerations to reversals varies with the developmental stage of the worm, with a shift in the tendency for the worm to reverse to tap with age. One solution to this measurement problem that will allow an ontogenetic analysis of learning draws from our extensive knowledge and understanding of the tap-withdrawal circuit which underlies both the acceleration and the reversal responses (Figure 2) (Wicks & Rankin, 1995a&b).

F. The Tap Withdrawal Circuit

A first step in determining the cellular mechanisms of the habituation of the tap withdrawal response is to identify the neuronal circuitry underlying the behavior. In *C. elegans* this neural circuitry can be identified by laser-ablating putative circuit cells and noting the effects of the ablation on the animal's behavior. Neural circuits mediating behaviors as varied as pharyngeal pumping (Avery & Horvitz, 1989), chemotaxis (Bargmann *et al.*, 1989), thermosensation (Mori & Oshima, 1995), and mechanosensation (Chalfie *et al.*, 1989)
Figure 2: The simplified tap withdrawal circuit. The circuit which mediates the tap withdrawal reflex consists of seven sensory neurons (squares), nine interneurons (circles) and two motor neuron pools (not shown) which produce forward and backward locomotion (triangles) (Wicks & Rankin, 1995a). Chemical connections are indicated by arrows, with the number of synaptic contacts being proportional to the width of the arrow. Gap junctions are indicated by dotted lines. Modified from Wicks & Rankin (1995a).
1985; Kaplan & Horvitz, 1993; Wicks & Rankin, 1995a) have been identified using this technique. The touch withdrawal circuit described by Chalfie et al. (1985) mediates head-touch induced backward movement and tail-touch induced forward movement and thus served as an excellent starting point to elucidate the circuit underlying the worm's response to vibration (tap).

Wicks and Rankin (1995a) determined that the tap withdrawal reflex is mediated by five sensory neurons (2 ALMs, 2 PLMs, and AVM) and 11 interneurons (AVAs, AVBs, AVDs, PVCs, PVDs, and DVA) (see Figure 2). In general, the tap withdrawal circuit can be roughly divided into 2 subcircuits which appear to functionally inhibit each other: One circuit is designed to integrate anterior (head-touch) sensorimotor input to produce backward movement, and the other is designed to integrate posterior (tail-touch) sensorimotor input, to produce forward movement. Specifically, the subcircuit sensitive to head-touch includes the sensory neurons (2 ALMs & AVM) that drive the reversal response (i.e. the worm swims backward to head-touch), and the subcircuit sensitive to tail-touch includes the sensory neurons (2 PLMs) that drive the acceleration response (i.e. the worm accelerates forward to tail-touch). It is important to understand that vibration simultaneously stimulates both sets of sensory neurons and therefore both subcircuits are activated and mutually inhibit each other (i.e. a tap stimulates a worm to reverse and accelerate at the same time, but the responses are mutually exclusive). The tap withdrawal circuit integrates this competing information to produce a single behavioral outcome.

Given this knowledge of the neural circuit, one solution to the "apples and oranges" measurement problem is to use the laser to selectively ablate one of the sets of sensory neurons. If the PLM sensory neurons are ablated (PLM-), then the animal will always reverse to tap because there is no competing input from the tail to drive a forward response. If the ALM sensory neurons are ablated (ALM-), then the animal will always accelerate to tap because there is no competing input from the head to drive the reversal response. Thus,
following either set of ablations, we are left with an easily measurable and quantifiable set of homogeneous responses which can be easily compared across the developmental stages.

Using this laser technique to selectively ablate either set of sensory neurons, Wicks and Rankin (1995b) have studied the effects of repeated stimulation on each of these two subcircuits. They have demonstrated that both response types, accelerations and reversals, do habituate, although they show different dynamics of the habituation process, especially at short ISIs. At a 10 sec ISI, some acceleration responses (AVMALM-animals) first show an initial rise in the magnitude of acceleration before they eventually habituate, whereas reversal responses simply habituate, as with intact (unablated) adult worms. Groves and Thompson (1970) suggested that both a decrementing process and a facilitating process might underlie the net behavior of an animal during habituation. For the purposes of this discussion, facilitation is defined as an increase in responsiveness following tap. Wicks and Rankin (1995b) developed a test for identifying such facilitation, by using a double exponential curve fitting procedure (described below in Methods). Using this technique, they found significant facilitation in the ALMAVM-ablated animals trained at a 10 second ISI. Wicks and Rankin went on to suggest how such a dual process model, in the context of the acceleration and reversal responses, could explain various characteristics known about habituation in C. elegans, including the differential effect of ISI on the initial slope of the habituation of the reversal response.

G. Objectives

The focus of this thesis is the study of the effect of repeated stimulation on accelerations and reversals in ablated animals across development. Habituation training was given to PLM-ablated and ALM-ablated animals at each of the 6 developmental stages (L1, L2, L3, L4, young adult (YAD), & 4 day old adult (4D)), and at each of 2 ISIs (10 second and 60 second) using the tap withdrawal paradigm. These experiments are the first systematic and quantitative investigation of learning and memory in C. elegans over the
course of its development (preliminary reports of these findings have been reported in

The goal of this research is to investigate any developmental changes in C. elegans'
ability to show habituation, and thus the previous behavioral work on habituation in C.
elegans should prove invaluable. It is first necessary to thoroughly understand and
characterize a behavior, before any systematic search for developmental anomalies and their
anatomical correlates can begin. The in-depth behavioral analysis of the habituation of the
adult tap withdrawal reflex offers several possible directions in which to look for
developmental variation. The general shape and dynamics of habituation to long and short
ISIs is well-characterized, as well as the nature of the animal's spontaneous recovery from
habituation (see above) (Rankin & Broster, 1992; Broster & Rankin, 1994), and so
comparisons along these dimensions will guide the analysis. It will be interesting to
compare the interaction between development and ISI in C. elegans to the systematic trend
found between development and ISI in Aplysia (discussed above) (Rankin et al., 1987).

The characterization of the habituation dynamics of both the acceleration and the
reversal responses across development should also prove invaluable in understanding the
nature of these different responses themselves, and thus the nature of their interaction in the
fully intact animal. It will be interesting to investigate the complementary development of
both the reversal and acceleration responses, and to specifically examine the effects of AVM
growing into the tap-withdrawal circuit, to better define its specific role in the facilitatory
process. Again, it is important to remember that the final behavior produced by the intact
animal, including any habituated behavior, reflects a compromise between the inputs of the
two competing circuits underlying the behavior.

The eventual objective of this research is to establish strong relationships between the
emergence of these particular forms and characteristics of behavioral plasticity, and the
emergence of the specific neural structures and circuits referred to above. In addition, it is
hoped this research will offer valuable insights into the nature of the cellular and molecular
processes involved in habituation, and thus serve as a starting point for further investigations into these underlying mechanisms. The key is to use the behavior as a guide. In this case, it can be correlated with development and prove to be an invaluable tool in provoking interesting new questions and directing future lines of research.

II. METHOD

A. The Animals

The worms used in these experiments were *C. elegans* var. Bristol (strain N2) maintained on NGM agar at room temperature (20 degrees C) with *E. coli* (strain OP50) as food (techniques described by Brennar, 1974). Synchronous age groups (or colonies) were established by allowing approx. fourteen 4-Day-Old adult worms to lay eggs on *E. coli* seeded plates for about 1 to 3 hours. To control for any genetic variability over time, a new colony of worms of the original strain was thawed at the beginning of every month. The agar plates that the worms were grown on were freshly poured every 1 to 2 weeks.

There were approximately 20 animals in each test group (one group had 19 animals, 3 groups had 21 animals, 20 groups had 20 animals) for a total of 482 subjects (2 animal types (ALM- & PLM-) x 6 stages (L1, L2, L3, L4, young adult, 4 day old adult) x 2 ISIs (10 sec and 60 sec) x approx. 20 animals per group). These groups were subdivided into two main groups (ALM- and PLM-) which formed the basis of the two experiments documented in the Results section below.

B. Laser Ablation

In order for animals to have their neurons ablated they were isolated as early L1s and mounted on microscope slides. This isolation procedure began by washing the synchronized colony plate with M9 buffer to obtain a heterogeneous population of animals and eggs in a test tube. This population was then treated with alkaline sodium hypochlorite (as described in Wood et al., 1988), for the removal of bacteria) which kills all the post-hatching animals. The resulting homogeneous population of only eggs was then spun in a tabletop centrifuge and the resulting pellet was washed again in M9 buffer to remove any residual cleaning
solution and placed onto an unseeded (i.e. no *E. coli*) agar plate. After approx. 2 to 5 hours, approximately 10-12 larval worms were collected from the plate with sterile M9 buffer and placed on a wet agar pad containing 10 mM sodium azide (an anesthetic; Wood, 1988) on a microscope slide. Animals were then covered with a 12 mm round glass coverslip.

All the animals on a given slide were then systematically bilaterally ablated for either ALM, or for PLM. On any given slide, all the worms had the same neurons ablated. To ablate these neurons the laser pulses were delivered by a VSL-377 nitrogen laser (Laser Science, Inc., Cambridge, MA). The beam was directed through a laser dye module (Laser Science, Inc., Cambridge, MA) containing a coumarin 440 dye (Laser Science, Inc., Cambridge, MA) that re-emitted with a peak gain of 437 nm. Single cell ablations were performed under 100x oil immersion lens mounted on a Zeiss Axiostop equipped with Nomarski (differential interference contrast) optics (Karl Zeiss, Canada). The beam was directed down through the optics of the microscope with a semi-silvered mirror and targeted into the plane of optical focus with a beam expander (Laser Science, Inc., Cambridge, MA). The intensity of the laser beam was attenuated by interposing glass microscope slides between the laser and the microscope such that when the beam was focused in the plane of the coverslip it would just barely damage the glass coverslip (this intensity was ideal because single laser pulses do little damage to a cell, but repeated pulses would destroy the cell). The nucleus of the targeted neuron was then located within the animal according to its unambiguous positioning relative to various "landmarks" (such as the gonad) and other cells in the area. All damage was monitored visually. Any animals in which the damage was considered either incomplete or extra-neuronal, and any animals in which the targeted cell was not clearly and unambiguously identified were destroyed. The successfully ablated animals were then removed from the slide in M9 buffer and individually placed one-to-a-plate on some *E. coli* seeded agar and then stored in an incubator at 20 degrees C.
C. Behavioral Testing

The experimental apparatus consisted of a stimulus generator, an electromagnetic relay that drives a wire tapper, a resting plate connected to a plastic resting arm, a micromanipulator, a dissecting microscope with under lighting, a video camera, a VCR, a color monitor, and a time-date generator (Rankin et al., 1990). A 4 cm petri plate filled with 10 ml of NGM agar was placed onto the resting plate (which is an inverted petri plate lid). The resting plate was connected by a plastic resting arm to the micromanipulator (Marzhauser model MM33), which enabled the experimenter to make smooth, precise movements and adjustments to the plate's position under the microscope (Wild M3Z, Wild Leitz Canada) using fine and coarse dials. The relay that drove the tapper was connected to a Grass S88 stimulus generator which allowed for easy regulation of stimulus delivery and timing. The wire tapper was connected to the relay and was positioned halfway up the side wall of the dish to deliver vibratory stimuli (of approximately 1 to 2 Newtons per tap) which were transmitted through the dish and the agar to stimulate the worm. For the tap stimulus used in these experiments, the generator was set to deliver a 25 ms pulse to the relay.

Behavioral testing of these animals was done on the same plates on which the animals were individually placed following the laser ablation. As a consequence, the animals were tested in the presence of a bacterial lawn (E. coli), which is their source of food. The animals were selected for their developmental stage on the basis of the ontogenetic time-course (Byerly, 1975) in Figure 1. At the time of selection, the animals were also visually examined to double-check that they were of the proper developmental stage. The L1 animals were allowed at least 3 hours to recover from the laser surgery before testing, and no animal of any stage was tested within 1 hour on either side of the standard molting time given in Figure 1. Due to the nature of the isolation procedure there is a small time range (approx. 2 to 5 hours—the amount of time the eggs were given to hatch after they were isolated) of possible ages for any given worm, and therefore, to ensure that worms were in the desired developmental stage, the extremes of this possible age range were used to define the
functional stage boundaries. Thus, most animals were run within a relatively short time range around the temporal "center" of each stage.

Each ablated worm was run in one of 24 experimental groups (2 ablations x 2 ISIs x 6 stages), with 20 worms per group. The stimuli, taps to the side of the dish the worm is on, were presented to the worm and the worm's behavior was recorded. The 30 habituation stimuli were delivered at either a short ISI (10 seconds between each tap) or at a long ISI (60 seconds between each tap). The timing of the stimulus was controlled manually for the 60 sec ISI condition and it was automated by the Grass S88 generator for the 10 sec ISI condition. For all groups, every worm received 30 taps to the side of its dish to produce the habituation, and then every worm in every group received 4 stimuli to test for spontaneous recovery from the habituation. These recovery stimuli consisted of a 31st tap at 30 seconds post-habituation, a 32nd tap at 5 minutes post-habituation, a 33rd tap at 10 minutes post-habituation, and a 34th tap at 20 minutes post-habituation.

D. Behavioral Scoring

In response to the tap, the worm either accelerated, reversed, paused/froze, showed no response, or some combination thereof. As the worm moved along the agar surface, it was manually tracked so that its behavior was recorded by a video camera which is connected to the microscope. The video camera (Panasonic Digital 5100) was connected, through the VCR (Panasonic AG1960), to a 10 inch color monitor (NEC Model No. PM-1271A) in such a way that the recorded image appeared "live" on the screen. The time-date generator (Panasonic WJ-810) was used to superimpose a digital stopwatch and time-date display on the video record, which was important for marking the presentation of each stimulus. The image of the worm was then kept centered on the screen through the adjustments of the micromanipulator (all of these adjustments were made during the intervals between each successive tap--never during the tap itself).

The experiment was run and video-recorded and then the behavior was scored using stop-frame video analysis. Each worm's behavior was scored by tracing its body from the
video-image, onto a transparent acetate sheet, going frame by frame through the tape. The PLM- worms (lacking tail sensory input) almost always responded to the tap with a reversal response, with the size and number of the responses decreasing as stimuli were repeated through the habituation run. The ALM- worms (lacking the majority of head sensory input) almost always responded to the tap with an acceleration response, with the size and number of the accelerations decreasing as the taps were repeated over the habituation run.

Due to the fact that accelerations and reversals are qualitatively different behaviors (as discussed above), they must be scored differently. In a typical reversal response, a stopped or forward-moving worm moved backwards for a distance (usually less than 1 or 2 worm lengths) and then either remained still or re-initiated forward movement in a new direction. To score a reversal response, the pre- and post-tap positions of the worm were noted, and then the total distance the worm reversed (i.e. track length) was traced onto the acetate sheet. In a typical acceleration response, a stopped or forward-moving worm either initiated forward movement or increased its speed, respectively. An acceleration was scored by measuring the distance the worm moved (i.e. track length) during the 1-sec interval before the tap and subtracting it from the distance the worm moved during the 1-sec interval after the tap. In PLM- worms, any acceleration responses were scored as missing data points, and in ALM- worms, any reversal responses were scored as missing data points. Also, some other responses must be scored as missing data points due to technical difficulties in visually discriminating the behavior of some worms (due to the small size of the larval worms, they are sometimes obscured upon entering particularly thick growth patches of E. coli). These missing data points totaled approximately 10% of the actual responses that were recorded over the course of these experiments.

The "scored" tracings of both reversals and accelerations were then digitized using a digitizing tablet (Summa graphics Bit Pad Plus) that was interfaced with a Macintosh SE microcomputer and Mac Measure software, or they were scanned directly into a computer file from the acetate with the Hewlett Packard Scanjet 3c into a Macintosh Centris 650. These
values, representing the reversal/acceleration magnitude of each response, were then directly transported into a statistical package for data analysis.

E. Data Analysis

1) Initial Responsiveness Analysis. The first step in the analysis was to compare the initial point of the habituation curve across all stages for a given response type. This initial point was the average response level given to the first tap an animal of a particular stage received. This comparison showed whether there were differences in the initial responsiveness of the animals across stages. For this analysis, the magnitude of these responses was measured in units of wormlength (i.e. the absolute response magnitude was divided by the animal's wormlength) to control for the differences in size between the worms of different stages (because the worms were of different sizes and the worm-images from which the measurements were taken were of different magnifications). A one-way factorial ANOVA was then run on these standardized initial responses with follow-up comparisons using Fisher's PLSD post-hoc tests (All statistics used in this thesis were done with STATVIEW, Abacus Concepts, Inc., Berkeley, CA).

2) Habituation Analyses. Since it was likely that many characteristics of habituation were going to be related to the initial response magnitude, and since there would be comparisons of characteristics of habituation between groups that were most likely going to have different initial levels of responding, all the data were standardized as a percentage of the mean initial point (Wicks & Rankin, 1995b). This standardization also allowed for quantitative comparisons between the characteristics of habituation of accelerations and reversals. Thus, for the following comparative analyses, all habituation scores were expressed as a percent decrement from initial levels.

Comparisons were made using paired t-tests, with missing data points being replaced by the mean level of responding for that stimulus and group. These missing data points were responses that could not be properly scored, most often due to difficulties in "seeing" the worms, because they were occasionally temporarily obscured by thick patches of food on
the agar plate (A total of 49 [3.4%] of the 1428 responses used in the following 3 comparisons were replaced in this manner). Three comparisons were made in each group: the first tested whether the final level of habituation (defined below) was significantly lower than the initial level of responding (1-tailed test), the second comparison tested whether the final level of recovery (defined below) was significantly higher than the final level of habituation (1-tailed test), and the third comparison tested whether the final level of recovery was different than the initial level of responding (2-tailed test). Because there were three comparisons being made in each group, the alpha level was reduced by dividing by the number of comparisons (0.05 / 3 = 0.017).

**a) Final Level of Habituation.** Once the data were standardized, the final asymptotic level of habituation was defined as the average response level over the last three responses (taps #28, #29, & #30). The average of the last three habituation responses was chosen to provide a more consistent measure of the asymptote of habituation, and thus also decrease the variability. This final level of habituation was compared with the initial point using a paired one-way t-test to test whether the final level of habituation was significantly less than the initial level of responding. A factorial ANOVA was also run on the final level of habituation across all stages, ISIs, and ablations, with follow-up comparisons using Fisher's PLSD post-hoc tests.

**b) Spontaneous Recovery from Habituation.** The first step in the analysis of spontaneous recovery from habituation was to examine the nature of the 4 recovery points, and test for any significant differences among them. It was expected that at least the first recovery point (at 30 seconds post-habituation) would be significantly different from the other 3 (i.e. the worm will not have yet recovered), whereas it was not clear whether there would be any differences between the other recovery points, or whether there would be any effect of stage or ISI. Therefore an ANOVA was done on the four recovery points at each ISI for each response type. The results showed that only the first recovery point (30 seconds post habituation) was significantly different from the rest of the recovery points, at both ISIs
for both response types. Therefore, the final three recovery points (5 minutes, 10 minutes, and 20 minutes post habituation) were averaged to obtain the mean recovery for a particular group, which was used as the final level of spontaneous recovery from habituation. This final level of recovery, or mean recovery, was then compared to the final level of habituation, as described above using a one way paired t-test to determine whether there was significant recovery. In addition, this mean recovery was compared to the initial response level using a two-way paired t-test to measure the full extent of the recovery. A factorial ANOVA was also run on the mean recovery minus the final level of habituation, across all stages, ISIs, and ablations, with follow-up analyses using Fisher's PLSD planned post-hoc comparisons.

c) Rate of Habituation. The dynamics of habituation were also analyzed by using a double-exponential curve fitting procedure (Wicks & Rankin, 1995b). For this analysis, a curve of the general form,

\[ F(x) = A(\exp)k_1 x + B(\exp)k_2 x, \]

where \( A, B, k_1, \) and \( k_2 \) are free parameters and \( x \) is the trial number, was used to produce best-fit lines which can then be used to determine the initial slope of the habituation curve. A double exponential curve of this form can not be linearized (i.e. there is no unique solution which can be derived conveniently) and therefore the best-fit curve for each group was determined using a least-squares non-linear curve fitting algorithm (Microsoft Excel). The initial rate of habituation was estimated by calculating the initial slope from the first 2 points of this function. Since the best-fit curve is smooth and continuous, this value was considered to be characteristic of the initial slope of the habituation curve (Wicks & Rankin, 1995b).

In order to maintain a measure of variance in the data-set, a best-fit line and a corresponding initial habituation slope were determined separately for each animal, and then a mean initial rate of habituation was derived for each group and compared using factorial ANOVAs. Each individual slope was also tested for being significantly positive or negative.
by using a two-tailed one sample t-test to determine if the slope was significantly different from zero. The least-squares solution of the double-exponential equation given above was highly sensitive to the initial values of the four free parameters (k1, k2, A, and B), and thus the values of the parameters derived from the equation which represents the best-fit to the group data were used as the initial values in the derivation of the individual best-fit curves (as in Wicks & Rankin, 1995b).

III. RESULTS

A. Experiment One: The PLM-Ablated Groups

In this experiment, all the worms had their tail-touch sensory neurons (the 2 PLM cells) laser-ablated and thus always responded to tap with the reversal response (described above). These worms were trained with the tap-withdrawal paradigm at each of the six developmental stages, at both a 10 second and a 60 second ISI. Three worms out of 239 were not included in any of the analyses because they responded consistently with accelerations, implying that the function of one or both of the PLM neurons survived the laser surgery. All other worms that underwent the tap-withdrawal training are reported, and all the responses were counted as recorded (except where explicitly mentioned below). The final number of worms per group was as follows: L1, 60 sec ISI (19); L2, 60 sec ISI (18); L4, 60 second ISI (19); all other groups had 20 animals per group.

1) Initial Responsiveness. This analysis was done to determine whether worms of different stages were differentially responsive to tap. This relative responsiveness was measured by the relative response magnitude of worms of different stages to the first tap, and thus was determined by averaging the response to the first stimulus during habituation training at both ISIs for each stage. Groups that were trained with a 10 second and a 60 second ISI were collapsed together, since the magnitude of this first response was independent of ISI. For this analysis, to control for the differences in size and magnification between the worms of different ages, the magnitude of each worm's initial response was standardized into units relative to wormlength (i.e. the absolute response magnitude was
divided by the animal's wormlength). Due to the inherent difficulties in scoring animals, some data points, either for wormlength or for the initial response, were missing. These animals were not included in this analysis (in total 18 of the 236 animals were not included). The data for the relative responsiveness analysis are displayed in Figure 3.

The results of the ANOVA for relative responsiveness of initial responses for the six stages studied (collapsed across ISI) indicated that there was a significant effect of stage on the initial responsiveness of the animals, $F(5, 212)=2.726, p < .05$. There appeared to be a general trend towards increased responsiveness as the animals progressed through their life cycle, although Fisher's planned post-hoc comparisons indicated that the only comparisons that were significantly different were L1 and Young adult ($p < .05$), L2 and Young adult ($p < .002$), and L2 and 4-Day old adult ($p < .005$).

2) Stage by Stage Analysis. The individual habituation curves, showing the data as a percentage of the mean initial response (as described above), for each developmental stage and ISI are shown in Figure 4 (10 second ISI) and Figure 5 (60 second ISI). For all stages, the final level of habituation was determined by averaging the 28th, 29th, and 30th points (i.e. the last three habituation points) as a mean measure of the asymptote of the habituation curve. An ANOVA was done on the four recovery points at each ISI (10 second ISI: $F(3, 432)=12.713; p<.0001$ and 60 second ISI: $F(3, 433)=4.072; p=.0072$). The post-hoc tests showed that only the first recovery point (30 seconds post habituation) was significantly different from the rest of the recovery points (with $p<.0001$ for all comparisons involving first recovery point at a 10 second ISI, and with $p=.0071$, $p=.0474$, and $p=.0011$ for comparisons between the first recovery point and the second, third, and fourth recovery points respectively, at a 60 second ISI). Therefore, the final three recovery points (5 minutes, 10 minutes, and 20 minutes post habituation) were averaged to obtain the mean recovery for a particular group.

The following stage by stage analyses also include comparisons between the 10 second and 60 second ISI groups of the same stage. Based on the findings of previous
Figure 3: The mean relative responsiveness of the PLM-ablated groups to the initial tap (all response magnitudes were divided by wormlength).
studies (discussed above), there was a predicted effect of ISI on the final level of habituation: Worms trained with a 10 second ISI were expected to habituate more deeply (i.e. they would have a lower final level of habituation) than worms trained with a 60 second ISI. If this predicted effect occurred, then in order to make meaningful comparisons about the amount of recovery between worms trained with different ISIs, the recovery was considered as the increase in responding above the final level of habituation. To do this the final level of habituation was subtracted from the mean recovery, and the resulting measure was called the mean difference of recovery minus habituation. This measure was then used in all comparisons of recovery between groups of worms trained at different ISIs, and from different developmental stages.

a) Stage L1:

**Habituation.** The newly hatched L1 worms habituated to tap when trained at either a 10 second or a 60 second ISI. For the worms trained at a 10 second ISI (Figure 4a), the final level of habituation was 37% of the initial response, which was significantly different from the initial point (t=4.765, p<.0001). For the worms trained at a 60 second ISI (Figure 5a), the final level of habituation was 31% of the initial response, which also was significantly different from the initial point (t=6.646, p<.0001). These final levels of habituation at both ISIs were compared with each other and were found to be not significantly different (F(1,37)=.306; n.s.).

**Recovery.** For the L1 worms trained at a 10 second ISI (Figure 4a), the mean recovery was 75% of the initial response, which was significantly different from the final level of habituation (t=-3.313, p<.0018), but its difference from the initial point just missed significance (t=2.143, p=.045). At a 60 second ISI (Figure 5a), the mean recovery was 54% of initial response, which was significantly different from the final level of habituation (t=-2.728, p=.0069) and from the initial point (t=3.970, p=.0009). The mean differences of recovery minus habituation were compared between both ISI groups and it was found that they were not significantly different (F(1,37)=1.084; n.s.).
Figure 4: The habituation curves for each of the PLM-ablated groups trained at a 10 second ISI (a. to f.). The first 30 points (on the x-axis) are the 30 habituation stimuli (marked S1 and so on), and the last 4 points (marked R1 to R4) denote the 4 recovery stimuli, at 30 seconds, 5 minutes, 10 minutes, and 20 minutes post-habituation.
Figure 5: The habituation curves for each of the PLM-ablated groups trained at a 60 second ISI (a. to f.). The first 30 points (on the x-axis) are the 30 habituation stimuli (marked S1 and so on), and the last 4 points (marked R1 to R4) denote the 4 recovery stimuli, at 30 seconds, 5 minutes, 10 minutes, and 20 minutes post-habituation.
b) Stage L2:

**Habituation.** The L2 worms also habituated to tap when trained at either a 10 second or a 60 second ISI. For the worms trained at a 10 second ISI (Figure 4b), the final level of habituation was 22% of the initial response, which was significantly different from the initial point ($t=6.98, p<.0001$). For the worms trained at a 60 second ISI (Figure 5b), the final level of habituation was 38% of the initial response, which also was significantly different from the initial point ($t=4.221, p=.0003$). These final levels of habituation at both ISIs were compared with each other and were found to be significantly different ($F(1,36)=4.192; p=.048$).

**Recovery.** For the L2 worms trained at a 10 second ISI (Figure 4b), the mean recovery was 92% of the initial response, which was significantly different from the final level of habituation ($t=-4.568, p=.0001$), but its difference from the initial point was not significant ($t=.417, n.s.$). At a 60 second ISI (Figure 5b), the mean recovery was 70% of the initial response, which was significantly different from the final level of habituation ($t=-3.847, p=.0006$), but just missed significance for its difference from the initial point ($t=2.126, p=.0484$). The mean differences of recovery minus habituation were compared between both ISI groups and it was found that they were significantly different ($F(1,36) = 4.557; p=.04$).

c) Stage L3:

**Habituation.** The L3 worms also habituated to tap when trained at either a 10 second or a 60 second ISI. For the worms trained at a 10 second ISI (Figure 4c), the final level of habituation was 16% of the initial response, which was significantly different from the initial point ($t=5.588, p<.0001$). For the worms trained at a 60 second ISI (Figure 5c), the final level of habituation was 38% of the initial response, which also was significantly different from the initial point ($t=4.851, p<.0001$). These final levels of habituation at both ISIs were compared with each other and were found to be significantly different ($F(1,35)=10.228; p=.003$).
Recovery. For the L3 worms trained at a 10 second ISI (Figure 4c), the mean recovery was 63% of the initial response, which was significantly different from the final level of habituation ($t=-2.849, p<.003$), but just missed significance from the initial point ($t=2.408, p=.0264$). At a 60 second ISI (Figure 5c), the mean recovery was 58% of the initial response, which was not significantly different from the final level of habituation ($t=-1.612, n.s.$), but was significantly different from the initial point ($t=2.856, p=.0101$). The mean differences of recovery minus habituation were compared between both ISI groups and it was found that they were not significantly different ($F(1, 38)=.404; n.s.$).

d) Stage L4:

Habituation. The L4 worms also habituated to tap when trained at either a 10 second or a 60 second ISI. For the worms trained at a 10 second ISI (Figure 4d), the final level of habituation was 40% of the initial response, which was significantly different from the initial point ($t=4.422, p=.0001$). For the worms trained at a 60 second ISI (Figure 5d), the final level of habituation was 34% of the initial response, which also was significantly different from the initial point ($t=5.323, p<.0001$). These final levels of habituation at both ISIs were compared with each other and were found to be not significantly different ($F(1,37)=.889; n.s.$).

Recovery. For the L4 worms trained at a 10 second ISI (Figure 4d), the mean recovery was 76% of the initial response, which was significantly different from the final level of habituation ($t=-2.664, p=.0077$), but its difference from the initial point was not significant ($t=1.654, n.s.$). At a 60 second ISI (Figure 5d), the mean recovery was 59% of the initial response, which was significantly different from the final level of habituation ($t=-3.856, p=.0006$) and from the initial point ($t=3.167, p=.0053$). The mean differences of recovery minus habituation were compared between both ISI groups and it was found that they were not significantly different ($F(1, 37)=.465; n.s.$).
e) Stage Young adult (YAD):  

**Habituation.** The young adult (YAD) worms also habituated to tap when trained at either a 10 second or a 60 second ISI. For the worms trained at a 10 second ISI (Figure 4e), the final level of habituation was 38% of the initial response, which was significantly different from the initial point (t=6.284, p<.0001). For the worms trained at a 60 second ISI (Figure 5e), the final level of habituation was 41% of the initial response, which also was significantly different from the initial point (t=7.222, p<.0001). These final levels of habituation at both ISIs were compared with each other and were found to be not significantly different (F(1,38)=.089; n.s.).  

**Recovery.** For the young adult (YAD) worms trained at a 10 second ISI (Figure 4e), the mean recovery was 75% of the initial response, which was significantly different from the final level of habituation (t=-3.467, p<.0013) but was not significantly different from the initial point (t=1.617, n.s.). At a 60 second ISI (Figure 5e), the mean recovery was 66% of initial response, which was significantly different from the final level of habituation (t=-3.545, p=.0011) and from the initial point (t=2.981, p<.0077). The mean differences of recovery minus habituation were compared between both ISI groups and it was found that they were not significantly different (F(1,38)=.809; n.s.).

f) Stage 4-day-old adult (4D):  

**Habituation.** The full-grown 4 day old adult worms also habituated to tap when trained at either a 10 second or a 60 second ISI. For the worms trained at a 10 second ISI (Figure 4f), the final level of habituation was 44% of the initial response, which was significantly different from the initial point (t=5.391, p<.0001). For the worms trained at a 60 second ISI (Figure 5f), the final level of habituation was 53% of the initial response, which also was significantly different from the initial point (4.615, p<.0001). These final levels of habituation at both ISIs were compared with each other and were found to be not significantly different (F(1,37)=.579; n.s.).
Recovery. For the 4-day-old adult worms trained at a 10 second ISI (Figure 4f), the mean recovery was 108% of the initial response, which was significantly different from the final level of habituation ($t=-6.173$, $p<.0001$), but not significantly different from the initial point ($t=-.665$, n.s.). At a 60 second ISI (Figure 5f), the mean recovery was 73% of initial response, which was significantly different from the final level of habituation ($t=-2.691$, $p=.0072$) and from the initial point ($t=2.726$, $p<.0134$). The mean differences of recovery minus habituation were compared between both ISI groups and it was found that they were significantly different ($F(1, 37) = 10.031; p=.003$).

g) Summary of PLM-ablated Worms:

In summary (see Table 1), worms of all stages showed significant habituation at each of the 10 second and 60 second ISIs. Although there was a general tendency for worms trained at a 10 second ISI to habituate more deeply than worms trained at 60 second ISI (the mean of the final level of habituation for PLM worms trained at a 10 sec and 60 sec ISI was 33% and 39% of the initial response, respectively) an ANOVA was run on the effect of ISI on the final level of habituation (collapsed across stages) and it failed to reach significance ($F(1,230)=2.408$; n.s.). Also, only comparisons between the final level of habituation of L2's and L3's reached significance when compared between both ISIs.

All groups of worms trained at a 10 second ISI also showed significant spontaneous recovery from habituated levels, with each stage recovering to initial response levels (i.e. the mean recovery was not significantly different from the initial point). Similarly, all groups of worms trained at 60 second ISI also showed significant spontaneous recovery above the habituated level, except for the L3 stage, and no stage recovered back to initial levels of responding (the L2's just missed being significantly different from the initial level). An ANOVA was run on the effect of ISI on the mean recovery minus habituation (collapsed across stages) and it showed that the groups trained at a 10 second ISI recovered to a significantly higher level than those groups trained at a 60 second ISI ($F(1, 233)=11.894; p=.0007$). In addition, there were significant differences between the mean difference of
Table 1. Summary table of significant differences for all 24 groups. The stages are denoted in the first column by L1, L2, L3, L4, YAD, and 4D. The second column denotes the comparison being tested: HAB denotes the test for a significant difference between the initial level of responding and the final level of habituation; REC denotes the test for a significant difference between the final level of habituation and the mean recovery; INIT denotes the test for a significant difference between the mean recovery and the initial level of responding. A YES indicates that the test was significant for that particular comparison and group, a NO indicates that the test was not significant for that particular comparison and group, and a *NO indicates that the comparison just missed significance.
<table>
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<th>Stage</th>
<th>PLM-10s ISI</th>
<th>PLM-60s ISI</th>
<th>ALM-10s ISI</th>
<th>ALM-60s ISI</th>
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* indicates no data available.
recovery minus habituation between the 10 second and 60 second ISI groups of both the L2 and 4D stages.

The effect of stage on the final level of habituation was significant over all PLM-animals (F(5, 226)=4.624, p=.0005) and at a 10 second ISI (F(5, 111)=4.724; p=.0005), suggesting a tendency toward higher final levels of habituation in the older stages. The effect of stage on the final level of habituation was not significant at a 60 second ISI (F(5, 109)=1.308; n.s.). The effect of stage on the mean recovery minus habituation was not significant over all PLM-animals (F(5, 229)=1.738; n.s.), or at a 10 second (F(5, 114)=1.946; n.s.) or a 60 second ISI (F(5, 109)=.213; n.s.).

3) Rate of Habituation

The best-fit curves for the mean data were derived and then used to generate the best-fit curves for each individual animal (see Appendix 1 and Appendix 2). The initial slope of each of these individual best-fit curves was calculated (based on the rise/run for the first 2 points on each curve) and then averaged to determine the mean initial slope for each developmental stage and ISI. All individual worm slopes that were greater than two standard deviations from the mean (for a total of 6 outliers) were removed from the data. The effect of stage was significant for the set of these mean initial slopes (see Figure 6) for the 10 second ISI groups (F(5, 107)=6.843; p<.0001) and the 60 second ISI groups (F(5, 104)=1.011; n.s.).

There was a significant effect of ISI on the mean slopes (F(1, 221)=15.042; p=.0001). For worms trained at a 10 second ISI (Figure 6a), the mean initial slopes of the L1, L2, L3, and 4D stages were negative (-20.7, -11.2, -16.5, and -10.5 respectively), as would be predicted for normal adult habituation. The L4 and YAD stages however, both have positive mean initial slopes of 13.7 and 11.4 respectively. Upon visual inspection of the habituation curves (see Figure 4), there is the suggestion of a trend towards more gradual (i.e. less steep) initial slopes as the worms progress through their life-cycle. T-tests were also done to test whether the mean initial slopes were either significantly positive or
Figure 6: The mean initial slopes of each developmental stage at each ISI for the PLM-ablated animals.
a. PLM 10 second ISI

![Graph showing Initial Slope vs Developmental Stage for 10 second ISI](image)

b. PLM 60 second ISI

![Graph showing Initial Slope vs Developmental Stage for 60 second ISI](image)
significantly negative, with all groups demonstrating significance except for the L4, 10 second animals. For worms trained at a 60 second ISI (Figure 6b), all stages had negative mean initial slopes (the L1's (-21.8), the L2's (-28.9), the L3's (-15.7), the L4's (-19.8), the YAD's (-16.5), and the 4D's (-11.5)) and all stages showed significance on the t-tests. There is the suggestion of a trend towards more gradual (i.e. less steep) initial slopes as the worms progress through their life-cycle.

B. Experiment Two: The ALM-Ablated Groups

In this experiment, the 2 ALM head-touch sensory neurons were laser-ablated and thus (as described previously) almost all of the worms accelerated to tap, rather than reversed to tap like the PLM-ablated worms. The protocol for this experiment was otherwise the same as for experiment one. Three worms out of 243 were not included in the analyses because they responded consistently with reversals, implying that the function of one or both of the ALM neurons survived the laser surgery. All other worms that underwent the tap-withdrawal training are reported, and all the responses were counted as recorded (except where explicitly mentioned below). The final number of worms per group was as follows: L1, 60 sec ISI (19); L2, 60 sec ISI (19); L3, 10 sec ISI (19); L3, 60 sec ISI (21); YAD, 10 second ISI (21); YAD, 60 second ISI (21); all other groups had 20 animals per group.

1) Initial Responsiveness. The results of the ANOVA for relative responsiveness to the initial tap for the six stages of ALM-ablated animals studied (collapsed across ISI) indicated that, unlike the PLM-ablated animals, there was no significant effect of stage on the initial response sensitivity of the animals, F(5, 208)=1.475, p=n.s. (26 of 240 animals had missing values and were not included in this analysis). Nevertheless, there was a similar general trend towards increased response sensitivity as the animals progressed through their life cycle since every stage except 4D showed an increase in responsiveness from the previous stage (see Figure 7).

2) Stage by Stage Analysis. The individual habituation curves for the ALM-ablated worms at each developmental stage and ISI are shown in Figure 8 (10 second ISI) and
Figure 7: The mean relative responsiveness of the ALM-ablated groups to the initial tap (all response magnitudes are divided by wormlength).
Figure 9 (60 second ISI). For all stages, as in the PLM-ablated groups, the final level of habituation was determined by averaging the 28th, 29th, and 30th points (i.e. the last three with the PLM-ablated worms, an ANOVA was done on the four recovery points at each ISI (see Figure 10). The results showed that only the first recovery point (30 seconds post habituation) was significantly different from the rest of the recovery points, at both ISIs (for the 10 sec ISI groups: F(3, 413)=7.124; p=.0001, with comparisons significant for rec1 vs. rec2 [p=.0017], rec1 vs. rec3 [p<.0001], rec1 vs. rec4 [p=.0002], and all other comparisons n.s.; for the 60 sec groups: F(3, 410)=10.368; p<.0001, with all comparisons involving the first recovery point significant at p<.0001, and all other comparisons n.s.). Therefore, as with the PLM-ablated animals, the final three recovery points (5 minutes, 10 minutes, and 20 minutes) were averaged to obtain the mean recovery for a particular group.

For the same reasons as noted above for the PLM-ablated worms, before any meaningful comparisons between worms trained with different ISIs could be made regarding the extent of spontaneous recovery from habituation, the final level of habituation was subtracted from the mean recovery. This difference was called the mean difference of recovery minus habituation and was also used in all comparisons of recovery between groups of ALM-ablated worms trained at different ISIs.

a) Stage L1:

**Habituation.** The newly hatched L1 worms habituated to tap when trained at either a 10 second or a 60 second ISI. For the worms trained at a 10 second ISI (Figure 8a), the final level of habituation was 46% of the initial response, which was significantly different from the initial point (t=2.338, p=.0152). For the worms trained at a 60 second ISI (Figure 9a), the final level of habituation was 42% of the initial response, which also was significantly different from the initial point (t=4.791, p<.0001). These final levels of habituation at both ISIs were compared with each other and were not found to be significantly different (F(1, 37)=.193; n.s.).
Figure 8: The habituation curves for each of the ALM-ablated groups trained at a 10 second ISI (a. to f.). The first 30 points (on the x-axis) are the 30 habituation stimuli (marked S1 and so on), and the last 4 points (marked R1 to R4) denote the 4 recovery stimuli, at 30 seconds, 5 minutes, 10 minutes, and 20 minutes post-habituation.
Figure 9: The habituation curves for each of the ALM-ablated groups trained at a 60 second ISI (a. to f.). The first 30 points (on the x-axis) are the 30 habituation stimuli (marked S1 and so on), and the last 4 points (marked R1 to R4) denote the 4 recovery stimuli, at 30 seconds, 5 minutes, 10 minutes, and 20 minutes post-habituation.
Recovery. For the L1 worms trained at a 10 second ISI (Figure 8a), the mean recovery was 68% of the initial response, which was significantly different from the final level of habituation (t=-3.348, p=.0017), but its difference from the initial point was not significant (t=.265, n.s.). At a 60 second ISI (Figure 8b), the mean recovery was 63% of initial response, which just missed being significantly different from the final level of habituation (t=-2.260, p=.0182), but was significantly different from the initial point (t=3.180, p=.005). The mean differences of recovery minus habituation were compared between both ISI groups and it was found that they were not significantly different (F(1, 37)=2.35; n.s.).

b) Stage L2:

Habituation. The L2 worms failed to show significant habituation to tap when trained at both a 10 second and a 60 second ISI. For the worms trained at a 10 second ISI (Figure 8b), the final level of habituation was 59% of the initial response, which just missed being significantly different from the initial point (t=2.030, p=.0283). For the worms trained at a 60 second ISI (Figure 9b), the final level of habituation was 73% of the initial response, which also just missed being significantly different from the initial point (t=1.577, p=.0661). These final levels of habituation at both ISIs were compared with each other and were not found to be significantly different (F(1, 37)=1.205; n.s.).

Recovery. For the L2 worms trained at a 10 second ISI (Figure 8b), the mean recovery was 53% of the initial response, which was significantly different from the final level of habituation (t=-2.866, p=.0049), but its difference from the initial point was not significant (t=.349, n.s.). At a 60 second ISI (Figure 9b), the mean recovery was 92% of the initial response, which just missed being significantly different from the final level of habituation (t=-2.176, .0216) and was not significantly different from the initial point (t=.462, n.s.). The mean differences of recovery minus habituation were compared between both ISI groups and it was found that they were not significantly different (F(1, 36)=.738; n.s.).
c) Stage L3:

**Habituation.** The L3 worms also habituated to tap when trained at either a 10 second or a 60 second ISI. For the worms trained at a 10 second ISI (Figure 8c), the final level of habituation was 34% of the initial response, which was significantly different from the initial point ($t=5.224, p<.0001$). For the worms trained at a 60 second ISI (Figure 9c), the final level of habituation was 47% of the initial response, which also was significantly different from the initial point ($t=2.32, p=.0155$). These final levels of habituation at both ISIs were compared with each other and were found to be significantly different ($F(1, 38)=4.793; p=.03$).

**Recovery.** For the L3 worms trained at a 10 second ISI (Figure 8c), the mean recovery was 77% of the initial response, which was significantly different from the final level of habituation ($t=-6.48, p=.0001$) but not from the initial point ($t=1.646, n.s.$). At a 60 second ISI (Figure 9c), the mean recovery was 84% of the initial response, which just missed significance for its difference from the final level of habituation ($t=-2.029, p=.028$) but was not significantly different from the initial point ($t=.878, n.s.$). The mean differences of recovery minus habituation were compared between both ISI groups and it was found that this comparison just missed significance ($F(1, 35)=3.488; p=.07$).

d) Stage L4:

**Habituation.** The L4 worms also habituated to tap when trained at either a 10 second or a 60 second ISI. For the worms trained at a 10 second ISI (Figure 8d), the final level of habituation was 52% of the initial response, which was significantly different from the initial point ($t=3.741, p=.0007$). For the worms trained at a 60 second ISI (Figure 9d), the final level of habituation was also 52% of the initial response, which also was significantly different from the initial point ($t=3.844, p=.0005$). These final levels of habituation at both ISIs were compared with each other and were not found to be significantly different ($F(1, 37)=.0001; n.s.$).
Recovery. For the L4 worms trained at a 10 second ISI (Figure 8d), the mean recovery was 80% of the initial response, which was significantly different from the final level of habituation (t=-3.393, p=.0007), but its difference from the initial point was not significant (t=1.673, n.s.). At a 60 second ISI (Figure 9d), the mean recovery was 70% of the initial response, which was significantly different from the final level of habituation (t=-2.786, p=.0059) and from the initial point (t=2.779, p=.0120). The mean differences of recovery minus habituation were compared between both ISI groups and it was found that they were not significantly different (F(1, 35)=.317; n.s.).

e) Stage Young adult (YAD):

Habituation. The young adult (YAD) worms also habituated to tap when trained at a 10 second ISI, but just missed significance when trained at a 60 second ISI. For the worms trained at a 10 second ISI (Figure 8e), the final level of habituation was 44% of the initial response, which was significantly different from the initial point (t=4.585, p<.0001). For the worms trained at a 60 second ISI (Figure 9e), the final level of habituation was 76% of the initial response, which just missed being significantly different from the initial point (t=1.630, p=.0593). These final levels of habituation at both ISIs were compared with each other and were found to be significantly different (F(1, 40)=10.342; p=.003).

Recovery. For the young adult (YAD) worms trained at a 10 second ISI (Figure 8e), the mean recovery was 62% of the initial response, which was significantly different from the final level of habituation (t=-2.402, p=.0131) and from the initial point (t=3.635, p=.0016). At a 60 second ISI (Figure 9e), the mean recovery was 79% of initial response, which was not significantly different from the final level of habituation (t=-.289, n.s.) and just missed significance from the initial point (t=1.951, p=.0652). The mean differences of recovery minus habituation were compared between both ISI groups and it was found that they were not significantly different (F(1, 38)=.898; n.s.).
f) Stage 4-day-old adult (4D):

**Habituation.** The full-grown 4 day old adult worms also habituated to tap when trained at either a 10 second or a 60 second ISI. For the worms trained at a 10 second ISI (Figure 8f), the final level of habituation was 61% of the initial response, which was significantly different from the initial point ($t=3.971$, $p=.0004$). For the worms trained at a 60 second ISI (Figure 9f), the final level of habituation was 80% of the initial response, which was not significantly different from the initial point ($t=1.028$, n.s.). These final levels of habituation at both ISIs were compared with each other and their difference just missed significance ($F(1, 38)=3.620; p=.07$).

**Recovery.** For the 4-day-old adult worms trained at a 10 second ISI (Figure 8f), the mean recovery was 66% of the initial response, which was not significantly different from the final level of habituation ($t=-.611$, n.s.), but was significantly different from the initial point ($t=3.992$, $p=.0008$). At a 60 second ISI (Figure 9f), the mean recovery was 117% of initial response, which was significantly different from the final level of habituation ($t=-3.752$, $p=.0007$), but was not significantly different from the initial point ($t=-.922$, n.s.). The mean differences of recovery minus habituation were compared between both ISI groups and it was found that they were significantly different ($F(1, 38) = 5.772; p=.02$).

g) Summary of ALM-ablated Worms:

In summary (see Table 1), all of the ALM-ablated groups trained at a 10 second ISI showed significant habituation to tap, except the L2's (which just missed significance). The worms trained with a 60 second ISI only showed significant habituation in the L1, L3, and L4 groups, although the L2 and YAD groups just missed significance, and the 4D group was in the right direction. An ANOVA testing the effect of ISI on the final level of habituation (collapsed across stages) showed that groups trained at a 10 second ISI had a significantly lower final level of habituation than groups trained at a 60 second ISI ($F(1, 238)=9.947; p=.0018$). At every stage except for the L1's, the final level of habituation was the same or
lower for groups trained at 10 second ISI than for groups trained at a 60 second ISI, and this difference was significant in both the L3 and YAD groups.

For animals trained at a 10 second ISI, all stages showed significant recovery from habituation, except the 4D stage, and all the larval stages had a mean recovery that was not significantly different from initial levels of responding, whereas the adult stages remained significantly below their initial response levels. For worms trained at a 60 second ISI, only the L4 and 4D stage groups showed significant recovery from habituation (although the L1 stage just missed significance), although animals from the 4D, YAD, L2, and L3 stages all had a mean recovery that was not significantly different from initial levels of responding. An ANOVA was run testing the effect of ISI on the mean recovery minus habituation (collapsed across stages), but it failed significance (F(1, 229)=1.912; n.s.), although the mean difference of recovery minus habituation was significantly different in the between ISIs in the 4D group, and just missed significance in the L3 group.

The effect of stage on the final level of habituation was significant over all ALM-animals (F(5, 234)=3.087, p=.0102) and at a 60 second ISI (F(5, 114)=3.126; p=.0111), although no clear trend was found. The effect of stage on the final level of habituation was not significant at a 10 second ISI (F(5, 114)=1.813; n.s.). The effect of stage on the mean recovery minus habituation was significant at a 10 second ISI (F(5, 111)=2.914; p=.0165), and showed a tendency toward lower recovery over the course of development. The effect of stage on the mean recovery minus habituation was not significant at a 60 second ISI (F(5, 108)=1.419; n.s.).

3) Rate of Habituation

The best-fit curves for the mean data were derived and then used to generate the best-fit curves for each individual animal (see Appendix 3 and Appendix 4). The initial slope of each of these individual best-fit curves was calculated (based on the rise/run for the first 2 points on each curve) and then averaged to determine the mean initial slope for each developmental stage and ISI. All individual worm slopes that were greater than two standard
deviations from the mean (for a total of 7 outliers) were removed from the data. The effect of stage was significant for the set of these mean initial slopes (see Figure 10) for the 10 second ISI groups (F(5, 109)=6.051; p<.0001) and the 60 second ISI groups (F(5, 106)=6.230; p<.0001). There was no significant effect of ISI on the mean slopes (F(1, 225)=.010; n.s.).

A series of two-tailed t-tests also was done to determine whether these mean initial slopes were significantly positive or negative, by comparing each individual mean initial slope to zero. At a 10 second ISI, both the L1 and L4 groups reached significance for a positive slope (L1: t=3.554; p=.002; L4: t=2.124, p=.0478) and the YAD and 4D group reached significance for a negative slope (YAD: t=-2.451, p=.0241; 4D: t=-3.726, p=.0015). At a 60 second ISI, the L3 (t=-4.314; p=.0005), the L4 (t=-2.902; p=.01), and the YAD (t=-3.41; p=.0031) groups all had significantly negative initial slopes, and the 4D group (t=3.031; p=.0072) had a significantly positive initial slope.

C. Comparisons Between PLM-ablated and ALM-ablated Groups

The mean magnitude of the initial point divided by worm length was analyzed for each of the 12 groups (the 6 developmental stages X the 2 response types). Collapsed over response type, the worms showed a significant developmental effect on their responsiveness to the first tap (F(5,420)=3.464; p=.004). There was a significant difference on the response magnitude of the worms to the initial tap between ablations (ALM vs. PLM) (F(1, 420) = 316; p<.0001): The measured magnitude of the mean reversal of the PLM worms was significantly higher than the measured magnitude of the mean acceleration of the ALM worms.

An ANOVA was run on the effect of ablation on the final level of habituation and it showed that PLM-ablated groups habituated to significantly lower levels of responding than ALM-ablated groups (F(1, 467)=42.468; p<.0001). An ANOVA on the effect of ablation on the final level of habituation was also significant at 10 sec (F(1, 235)=15.637; p=.0001) and 60 sec (F(1, 230) = 28.387; p<.0001) ISIs. There was no significant main effect of ISI
Figure 10: The mean initial slopes of each developmental stage at each ISI for the ALM-ablated animals.
a. ALM-10 second ISI

![Graph showing initial slope for different developmental stages with error bars for ALM-10 second ISI.]

b. ALM-60 second ISI

![Graph showing initial slope for different developmental stages with error bars for ALM-60 second ISI.]

Developmental Stage:
- L1
- L2
- L3
- L4
- YAD
- D4
on the final level of habituation in the PLM-ablated ($F(1,230)=2.4; p=\text{n.s.}$) groups, although there was a significant difference in the ALM-ablated groups ($F(1, 238)=9.947; p=.0018$). There was a significant effect of ablation on the mean recovery minus habituation, collapsed across ISI ($F(1, 464)=6.568; p=.0107$), and at a 10 second ISI ($F(1, 235)=7.084; p=.0083$), but not at a 60 second ISI ($F(1, 227)=.639; \text{n.s.}$).

There was a significant effect of ablation on the mean recovery minus habituation, collapsed across ISI ($F(1, 464)=6.568; p=.0107$), and at a 10 second ISI ($F(1, 235)=7.084; p=.0083$), but not at a 60 second ISI ($F(1, 227)=.639; \text{n.s.}$).

There was a significant effect of stage on the final level of habituation when all worms were collapsed across ablation and ISI ($F(5, 466)=3.873; p=.0019$), and when only collapsed across ablation at a 10 second ISI ($F(5, 231)=2.937; p=.0136$) and at a 60 second ISI ($F(5, 229)=3.649; p=.0034$). There was the suggestion of a general tendency toward higher final levels of habituation over the course of development. There was no significant effect of stage on the mean recovery minus final level of habituation when collapsed across ablation and ISI ($F(5, 466)=1.624; \text{n.s.}$), or when collapsed only across ablation at either the 10 second ($F(5, 231)=1.294; \text{n.s.}$) or the 60 second ($F(5, 223)=.735; \text{n.s.}$) ISI.

IV. GENERAL DISCUSSION

The goal of this research was to investigate the development of habituation and spontaneous recovery from habituation in *C. elegans*. To this end, habituation training was given to PLM-ablated and ALM-ablated animals at each of 6 developmental stages (L1, L2, L3, L4, YAD, & 4D) and at each of 2 ISIs (10 second and 60 second) using the tap withdrawal paradigm. Although the general shape and time-course of habituation shows remarkable consistency and stability as the organism develops, several developmental changes and trends were isolated from these results.

The mean magnitude of the initial point divided by wormlength was analyzed for each of the 12 groups (the 6 developmental stages X the 2 response types). Collapsed over response type, the worms showed a significant developmental effect on their responsiveness to the first tap. It appears that both the ALM-ablated and PLM-ablated animals tend to become more responsive to tap as they progress through the developmental stages (see Figures 3 and 7). The fact that both response types, reversals and accelerations, exhibit this
tendency suggests that the responsible neurodevelopmental changes may occur independent of the mechanosensory neurons. In particular, one possibility is the almost four-fold increase in the number of ventral cord motor neurons from the 22 functioning at L1, to the 76 functioning when the worm fully matures. In addition, from L1 to adult, the worm develops 5 more distinctive types of motor neurons which also could be responsible for the increased responsiveness of the worms to tap. Further investigation into the role of these emerging neurobiological changes is necessary, since the increased responsiveness may not be associated with neurodevelopment, but rather may be the result of something as basic as increased surface area on the contact space of the cuticle and the agar surface on which it propels itself along.

Not surprisingly, there was a significant difference in the initial response magnitudes of ALM-ablated and PLM-ablated groups. Given their qualitatively different characters, no claims can be made regarding the actual relative responsiveness of the two groups. It is merely an artifact of two different types of measurement: the reversals are measured by the distance a worm swims backward after a tap, and accelerations are measured by the change in velocity from the second just prior to the tap to the second just after. This fact, especially when coupled with the apparent developmental effect on responsiveness mentioned just above, makes clear the necessity of standardizing all responses into a percentage of the mean initial response, to allow for relatively unbiased comparisons across stages and ablations.

The habituation kinetics of *C. elegans* response to tap were analyzed in all 24 different groups, across stage, ISI, and ablation (or response type). One important finding was that all groups of worms trained at a 10 second ISI showed significant habituation, regardless of stage or response type (except the ALM, 10 second ISI, L2 group which just missed significance for habituation, p=.028). This result implies the worms possess the competence to habituate to short ISIs at hatching, and it demonstrates that the basic machinery underlying habituation is present and functional as early as the L1 stage in development. Cast in an ethological context, this result makes good sense for the survival of
the animal, considering that the worms are born from eggs, and are completely on their own for survival. Certainly, it is not surprising that any mechanism for behavioral plasticity which could increase the organism's chances for reproductive success would be present as early as possible, and at least in *C. elegans*, this seems to be the case.

At a 60 second ISI, all PLM-ablated and three ALM-ablated groups (L1, L3, & L4) also showed significant habituation, and two of the remaining three ALM groups (L2 & YAD) just missed significance. Add to that the fact that the only other group which failed to reach significance was the 4-day-old adults, which is a group that has demonstrated significant habituation previously (Wicks & Rankin, 1995b) and still showed a decrease of 27% from initial levels of responding, and it can cautiously be concluded that habituation is present at both ISIs and in each competing neural subcircuit of the tap withdrawal reflex throughout the complete post-embryonic course of *C. elegans' development.* One likely explanation for the differing levels of habituation seen between the ALM-ablated adult group reported here, and the one reported in Wicks and Rankin (1995b) is that the latter group was trained with 40 stimuli, and thus it is possible that for at least the ALM-ablated, 60 second ISI, 4-day-old group, the asymptote may not be reached until more than 30 taps have been given. If this is true of the 4D group then it also could explain the lack of significant habituation in the L2 and YAD groups, and may be an inherent characteristic of all ALM groups. Future habituation studies using the acceleration response should be run with 40 taps, and then tested to determine whether any other acceleration response-type groups have the same inherently slower time-course. Indeed, there was a significant effect of ablation on the final level of habituation across ALM-ablated groups, and at both the 10 second and 60 second ISIs individually.

Wicks and Rankin (1995b) compared the habituation kinetics of the reversal and acceleration responses in adult worms. They found that the acceleration response showed an initial facilitation at a 10 second ISI, which slowed the time-course of habituation and eventually resulted in the same higher final level of habituation found in this study for the
ALM-ablated groups. Although each of the acceleration groups that they studied (ALM-ablated, and ALMAVM-ablated) showed the suggestion of facilitation at a 10 second ISI, only the ALMAVM-group demonstrated a significantly positive mean initial slope with the curve-fitting algorithm described above.

There was a similar suggestion of facilitation from the habituation curves of the ALM-ablated groups in the present study. The first observation is that facilitation was not restricted to a 10 second ISI: Significant facilitation was found in the 4D, 60 second ISI, ALM-ablated group, and there was no significant difference between the mean initial slopes across ISIs. The second observation is that the facilitation is present as early as L1: The L1, 10 second ISI group also showed significant facilitation. The fact that other ALM-ablated groups, at both ISIs, showed significantly negative slopes suggests that other conditions, as yet unidentified, may be necessary to produce facilitation, or that other processes can act to obscure the facilitation at the behavioral level.

The third observation is that facilitation is not particular to the acceleration responses: The YAD, 10 second ISI, PLM group had a significant positive initial slope, and there was the suggestion of facilitation in L4 and 4D 10 second PLM groups. In the PLM groups the mean initial slope of the 60 second ISI groups was significantly lower than the mean initial slope of the 10 second ISI groups, and so ISI seems to play some role in facilitation (there was no facilitation found in any of the 60 second ISI PLM groups). Also, the facilitation in the PLM groups may have a developmental component. The L1, L2, and L3 groups trained at a 10 second ISI all showed significantly negative initial slopes, and there was no hint of facilitation until the L4 stage. Coupled with the observation that the L1 and L2 groups also had the largest (absolute value) negative mean initial slopes at a 60 second ISI, it seems likely that facilitation, at least in the PLM-ablated animals, is either weaker or not present at all until later in development. Taken together with the observations on initial slope from the ALM-ablated groups, these findings show a clear need for further research into factors influencing the rate of habituation.
One objective of this study was to indirectly investigate the role of AVM in the habituation of accelerations and reversals by correlating its emergence into the tap-withdrawal circuit with any changes in the behavioral characteristics of habituation. AVM is the late-developing sensory neuron which does not become fully integrated into the tap-withdrawal circuit until some time around the L4 stage. It is not obvious what, if any, the effect AVM growing into the circuit might have on the final level of habituation for either of the ALM and PLM groups. Ablating AVM in an unhabituated animal results in a decrease in both the frequency and magnitude of the reversal response (Wicks & Rankin, 1995a), and an increase in the magnitude of a worm's acceleration response. It is possible that in later development the presence of AVM might provide enough anterior stimulation to affect the magnitude of both reversals (increasing their magnitude) and accelerations (decreasing their magnitude).

It was therefore hypothesized that AVM's effect on the habituation of accelerations and reversals might also show a complementarity between the two ablation groups. Investigating the effect of ablation over the course of development may be a sensitive measure of AVM's role, because if AVM affects both the accelerations and reversals in a complementary manner, then its impact could be made more obvious in an analysis that considers the behavior of both accelerations and reversals at the same time. Thus, the effect of ablation on the final level of habituation was tested at each stage to see whether there were any noticeable differences between the larval stages and the adult stages when AVM becomes fully functional. No clear change or trend was found.

Another approach to look for the developmental effects of AVM's late emergence into the circuit might be to look at the final levels of habituation across stages, but within each ablation group separately. There was a significant effect of stage on the final level of habituation in both ablation groups, and both groups showed interesting developmental trends. In the ALM-ablated groups, there was a tendency for higher final levels of habituation in the adult, with the YAD and 4D groups having the third highest and highest
final levels of habituation, respectively. Similarly, the YAD and 4D stages also show the highest final levels of habituation for the PLM-ablated groups. Although it was hypothesized that any effect that AVM would have on one response would have the complementary effect on the other response, the above results do not support it, and suggest the opposite—that there was an increase in the final level of habituation in both reversals and accelerations at around the time when AVM enters the circuit. To further elucidate the role of AVM it would be interesting to study the developmental effects of the ALMAVM-co-ablation done by Wicks and Rankin (1995b). It would also be interesting to compare the effects of that ablation with the effects of a PLMAVM-ablation over development, and then compare these co-ablations with the results of the ALM- and PLM-ablations presented here.

Somewhat surprisingly, unlike with intact adult animals (Rankin & Broster, 1992) there was no significant main effect of ISI on the final level of habituation in the PLM-ablated groups, although this effect did reach significance in the ALM-ablated groups. Also, there appears to be at least the tendency for worms trained at a 10 second ISI to habituate to lower levels of responding in the PLM-ablated groups, since the mean final level of habituation was lower in the 10 second ISI groups (33%) than in the 60 second groups (39%). One factor that may contribute to the lack of a significant ISI effect on final level of habituation in the PLM-groups is that the PLM-groups already show a significant effect of ablation on their final levels of habituation as shown by Wicks and Rankin (1995b). Wicks and Rankin found that the PLM-groups showed significantly less habituation (they had a higher final level of habituation) than intact worms. It may be that since the ablations already act to lessen the extent of habituation, these ablation groups are no longer as sensitive to other effects such as the effect of different ISIs on the final level of habituation. This hypothesis allows for the fact that ISI still may have the fundamentally same effect at a neurobiological level, but that our behavioral assays are no longer as sensitive to these changes after ablation. Of course, the ALM-group has an even more shallow final level of habituation than the PLM-group, and yet it does show a significant effect of ISI, which
suggests that if there is any effect of the final level of habituation on a group's sensitivity to ISI effects, then it is unique to the PLM- group.

The above results also demonstrate that unlike *Aplysia*, where there was a significant systematic interaction found between development and ISI, in *C. elegans* even animals from the youngest developmental stages were capable of habituation to long ISIs. Despite the fact that in *Aplysia*, a 10 second ISI is considered "long", and wasn't completely learned until the juvenile stage, it is possible that habituation in *C. elegans* has a very different time-course. It is important to note that only 2 ISIs were tested in these experiments, and so it is possible that any developmental-ISI interaction is only sensitive at still higher ISIs. It may be interesting to test larval animals at the longest possible ISI's known to produce habituation in adults, to more conclusively test the worm's ability to habituate to longer ISIs at all developmental stages. Another important consideration is that, in the *Aplysia* study, the interaction between development and ISI was studied using the siphon-withdrawal reflex, before the siphon was fully functional. The tap-withdrawal reflex, on the other hand, is a necessary, functional reflex for *C. elegans* throughout development. Perhaps, there is no need for habituation to become fully functional, until the organ or reflex itself becomes functional and necessary.

There was a significant effect of ISI on the mean recovery minus habituation in the PLM-ablated groups, but this effect was not significant for the ALM-ablated worms. The PLM-ablated worms behaved as was predicted from the behavior of intact adult worms: Animals trained at a 10 second ISI tended to recover more completely from habituation than animals trained at a 60 second ISI. The ALM- worms also showed that basic trend but it was not significant. Interestingly, the PLM- animals showed a significant ISI effect on recovery, but not on the final level of habituation, and the ALM- groups showed a significant ISI effect on final level of habituation, but not on recovery. In addition, there was an effect of ablation on the mean recovery minus habituation at a 10 second ISI, but not at a 60 second
ISI, suggesting that there are some ISI sensitive differences in the processes guiding recovery between the two response types.

Again, similar to the effect of ISI on the final level of habituation, no significant interaction or trend was found between ISI effects and the development of the animal. The finding that PLM- and ALM- animals as young as stage L1 both show significant recovery from habituation is an important finding, because it indicates that the basic mechanism underlying spontaneous recovery at a 10 second ISI seems to be present throughout development in C. elegans. Although the PLM-ablated animals trained at a 60 second ISI also showed significant recovery as early as the L1 stage, the ALM-ablated animals did not. In fact, of the ALM-ablated animals trained at a 60 second ISI, the only developmental stage to express significant recovery from habituation was the 4-day-old adult, which suggests a possible developmental interaction, although the L1, L2, and L3 stages each just miss significant recovery. Taken together these data suggest that the basic processes underlying recovery from habituation are likely to be present and functional at both ISIs for all stages.

This conclusion regarding the developmental time-course of recovery from habituation echoes closely the conclusions reached regarding the final level of habituation as well, and indeed, stands as the main finding of this thesis. The fact that C. elegans demonstrates habituation and recovery from habituation in all developmental stages, and that the characteristics and dynamics of these assays are surprisingly consistent and robust, suggests that the same basic underlying neural mechanisms responsible for the behavior are present in the worms at hatching, and continue to function through to adulthood. This conclusion is particularly interesting given the extensive morphological changes in the nervous system of the organism after it hatches.
REFERENCES


Appendix 1: Mean best-fit curves for PLM- 10 second ISI

a. PLM- L1 10 sec ISI

b. PLM- L2 10 sec ISI

c. PLM- L3 10 sec ISI

d. PLM- L4 10 sec ISI

e. PLM- YAD 10 sec ISI

e. PLM- D4 10 sec ISI
Appendix 2: Mean best-fit curves for PLM- 60 second ISI

a. PLM- L1 60 sec ISI

b. PLM- L2 60 sec ISI

c. PLM- L3 60 sec ISI
d. PLM- L4 60 sec ISI

e. PLM- YAD 60 sec ISI

f. PLM- 4D 60 sec ISI
Appendix 3: Mean best-fit curves for ALM- 10 second ISI

a. ALM-L1 10 sec ISI

![Graph a](image1)

b. ALM-L2 10 sec ISI

![Graph b](image2)

c. ALM-L3 10 sec ISI

![Graph c](image3)

d. ALM-L4 10 sec ISI

![Graph d](image4)

e. ALM-YAD 10 sec ISI

![Graph e](image5)

f. ALM-4D 10 sec ISI

![Graph f](image6)
Appendix 4: Mean best-fit curves for ALM- 60 second ISI

a. ALM- L1 60 sec ISI

b. ALM- L2 60 sec ISI

c. ALM- L3 60 sec ISI

d. ALM- L4 60 sec ISI

e. ALM- YAD 60 sec ISI

f. ALM- 4D 60 sec ISI