

THE EFFECTS OF CHANGING THE INTERSTIMULUS INTERVAL
DURING HABITUATION IN *CAENORHABDITIS ELEGANS*

by

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ABSTRACT

Although habituation is one of the simplest forms of non-associative learning, its underlying neural mechanisms are still not well understood. One factor that plays a key role in habituation is interstimulus interval (ISI). Understanding, at a behavioural level, the effects that ISI has on habituation may provide important insights into the cellular events involved in this form of learning.

The experiments in this thesis further explored the role of ISI in habituation of the reversal response of the nematode *Caenorhabditis elegans* by examining the effect of changing the ISI during habituation training. The effect of ISI change was examined in terms of both its impact on habituation and its impact on spontaneous recovery from habituation.

One type of ISI change tested was continual variation in the ISI used during habituation. When habituation stimuli were delivered at variable ISIs having an overall average of 10 s the recovery from habituation observed was slower than that seen when habituation stimuli were given at regular 10 s intervals. A comparison of fixed and variable stimulation during habituation with a 60-s ISI revealed no differences in recovery rate. Thus, the impact of variable ISIs during habituation on recovery from habituation was noticeable at a 10-s ISI, but not a 60-s ISI.

In a second experiment, the effect of shifting to a different ISI part-way through habituation training was explored. Whether the shift was from a 10-s ISI to a 60-s ISI or a 60- to a 10-s

ISI, in both cases the recovery rate (which is typically different for each ISI on its own) observed after habituation was primarily determined by the ISI given in the last half of the habituation treatment.

Examination of the impact on response patterns resulting from variation or change in ISI generated a model of how response potential may interact with ISI that can be used to further understand the relationship between ISI and response magnitude during habituation.

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Introduction

Habituation, defined as the decrease in an organism's responsiveness resulting from repeated stimulation, is probably the most simple and ubiquitous form of learning known (Groves & Thompson, 1970). It has been studied in a wide variety of creatures and preparations, ranging from protozoa (Wood, 1970) and isolated spinal neurons (e.g., Farel, Glanzman, & Thompson, 1973) to many different invertebrates and vertebrates, including humans (e.g., Sokolov, 1963). Many of these efforts, especially those conducted in the past 30 years, have aimed at elucidating the neural events that underlie this relatively simple form of plasticity, but, despite great technological advances in molecular biology and neurophysiology, habituation is still poorly understood.

In all organisms habituation is characterized by the form the response curve takes when response level is graphed over time. At the beginning of habituation there is a sharp decline in responsiveness, and this is followed by a flattening of the response curve, known as the asymptote, beyond which little further decrease in response level takes place. This decrement can be distinguished from processes not considered learning, such as sensory or motor fatigue, by the ability of a novel or noxious stimulus to restore responsiveness sooner than an organism would recover from fatigue. This phenomenon is known as dishabituation (Groves & Thompson, 1970).

Most of the progress made thus far has involved the use of a simple systems approach, whereby researchers study learning in either partially intact nervous systems or organisms with relatively few neurons, as is the case with many invertebrates. For example, Thompson and Spencer (1966) used data from a spinal cat preparation in their classic characterization of the main behavioural features of habituation, while Kandel and colleagues have attempted to elucidate the cellular mechanisms of habituation using the marine mollusc *Aplysia californica*. Extensive research on this organism by Kandel, Carew and many others (for a reviews see Hawkins, 1988, and Sahley & Carew, 1983) has indicated that habituation probably involves a decrease in available neurotransmitter (Bailey & Chen, 1988; Castellucci & Kandel, 1974), coupled with a decrease in calcium current (Klein, Shapiro, & Kandel, 1980) and possibly a decline in the efficiency of the neurotransmitter replenishment system (Bailey & Chen, 1988; Kandel, 1976). All of these processes would contribute to a gradual decrease in the amount of transmitter released, and, therefore, a drop in responsiveness. While work on *Aplysia* continues to be fruitful, other researchers have chosen to study invertebrates having nervous systems that are even less complex and, more importantly, more amenable to genetic and neuroanatomical analysis.

One such organism is the soil dwelling nematode *Caenorhabditis elegans*. This animal was originally isolated by

Brenner (1974) who felt it held great promise for studying the genetic basic of behaviour. Since that time, researchers have primarily concentrated on understanding its genome, which has now been almost completely mapped out (Coulson, Sulston, Brenner, & Karn, 1986; Hodgkin, Edgley, Riddle, & Albertson, 1988). The genome is only about half the size of that of *Drosophila melangoster*, containing only six small haploid chromosomes and 8×10^7 nucleotide base pairs (Sulston & Brenner, 1974). As well, a number of techniques have been developed for the purposes of isolating, maintaining, and studying genetic mutants (Wood, 1988). Most of these mutants are readily obtainable from a library of mutants located at the University of Missouri, Columbia (Hodgkin *et al.*, 1988).

C. elegans has only 302 neurons (Sulston, Schierenberg, White, & Thompson, 1983), all of which have had their putative synapses (electrical or chemical) located and their cellular lineage defined (Chalfie 1984; White, Southgate, Thomson, & Brenner, 1986). As well, the general function of many of these neurons in various behaviours has been ascertained, which has greatly aided efforts to examine the plasticity of these behaviours at the neural level.

In addition to being a promising link between behaviour and genes, *C. elegans* is also easy to maintain. These free-living nematodes are generally kept on nematode growth medium agar (Brenner, 1974), though they can also be frozen (often using

liquid nitrogen), a stasis from which they can be revived without ill-effect (Wood, 1988). They have a life cycle that lasts, on average, 12-14 days, during which time they can be sustained on a diet of *Escherichia coli*. Reproduction is primarily accomplished through self-fertilization, as *C. elegans* is typically hermaphroditic. Male *C. elegans* exist, but they arise only through chromosomal abnormality, and, therefore, are rare unless specifically bred for (Hodgkin, Horvitz, & Brenner, 1979).

Many of the behaviours studied in *C. elegans* involve locomotion. The worm usually lies on its side and moves in an undulating fashion made possible by alternating contractions of the dorsal and ventral muscles. When on agar, *C. elegans* will move forward most of the time, but it will occasionally move backward, either spontaneously, or when stimulated by vibration or a tactile stimulus delivered to the anterior region of its body.

Recently, using this reversal response to vibration, Rankin and associates have explored the ability of the worm to demonstrate learning. Rankin, Beck, and Chiba (1990) found that a mechanical tap delivered to the side of an agar-filled Petri dish caused a worm on the agar's surface to cease its forward movement and swim backward. Furthermore, they found that this tap-withdrawal response decreased in both frequency and magnitude with repeated stimulation. The pattern of this

response decrement was very similar to classic descriptions of habituation (Groves & Thompson, 1970). It was also shown that, once habituated, this reversal response could be dishabituated, which indicated that this behaviour was indeed learning, and not just fatigue. In addition to habituation, Rankin, Beck, and Chiba were also able to demonstrate other forms of non-associative learning such as sensitization and long-term habituation (Rankin, Beck, & Chiba, 1990; Rankin & Chiba, 1988).

An important part of the study of plasticity in the tap-withdrawal response has been the elucidation of the neural circuit underlying this behaviour. Through the use of laser microsurgery, genetic mutation, and electron microscopy, Chalfie *et al.* (1985) described a subset of neurons known as the touch withdrawal circuit. This circuit consists of 85 neurons (5 sensory cells, 5 pairs of interneurons, and 69 motor neurons) and underlies head-touch induced backward movement and tail-touch induced forward movement. Rankin and Chalfie (1989; see also Rankin & Wicks, 1991) demonstrated that this touch withdrawal circuit was also responsible for mediating the tap withdrawal response. The next step in a circuit analysis is to use laser ablation and mutation to localize where and how the learning is taking place.

Before a neural understanding of plasticity in the tap withdrawal response can truly be accomplished, however, it is essential to have a firm grasp of the characteristics of the

learning at a behavioural level. Knowledge of the descriptive characteristics of a form of learning is essential because any theory involving neural mechanisms must account for the ways in which that learning changes under different behavioural conditions and parameters. This forces theorists to expand or sharpen their theories to match behavioural observations.

Thus, Rankin and colleagues have continued their behavioural assessment of learning in *C. elegans*, concentrating particularly on habituation. Rankin and Broster (1990, 1992) have focussed their attention on the effect of interstimulus interval (ISI) on habituation of the tap withdrawal response. The importance of the ISI factor in habituation has been recognized for many years and observed with a variety of behaviours. Yerkes (1906), who studied habituation of the shadow withdrawal response in the serpulid *Hydroides dianthus*, was one of the first invertebrate researchers to note that habituation developed at a faster rate and to a greater extent when repeated stimulation was more frequent (shorter ISI). Similar results have been obtained with creatures as simple as the protozoan *Stentor coeruleus* (Wood, 1970) and the leech (Ratner, 1972). Even complex behaviours, such as territoriality in fish (e.g., Peeke & Peeke, 1973) and the orienting response in humans (e.g., Geer, 1966) appear to follow the the same pattern. In their study, Rankin and Broster (1992) found that *C. elegans* conformed to this trend; shorter interstimulus

intervals resulted in more rapid and complete habituation than longer ISIs.

One important issue that has been raised, however, concerning the evaluation of habituation is that the test used to measure the effects of a particular parameter on habituation is often confounded with the training protocol used to produce that habituation (Davis, 1970). For instance, direct comparison of response levels during the last stimulus of habituation for one animal habituated with a 5-s ISI and another habituated with a 60-s ISI is confounded by unequal periods of rest since the previous stimulus. Thus, the relative performance of these two animals may be more dependent on the amount of time since the last stimulus, as opposed to the actual amount of habituation that has taken place. To remedy this, Davis (1970) suggested that a better approach would be to test all groups at equal times by using a small battery of post-habituation test stimuli. For example, in his experiment, Davis tested all groups with a similar procedure 24 hours following habituation. Rankin and Broster (1992) adopted a similar protocol in that they tested all of their groups using spontaneous recovery from habituation. This was done by giving all animals probe stimuli at 30 s, 5 min, 10 min, and 20 min following habituation at either a 2-s, 10-s, 30-s, or 60-s ISI. The responses to these probe stimuli served as a reflection of the rate of recovery from habituation. Rankin and Broster (1992) found that, although shorter ISIs

produced more rapid and extensive habituation, such treatment also resulted, surprisingly, in a recovery rate that was faster and more complete than with longer ISIs.

There are very few other investigations of habituation that involve thorough examination of spontaneous recovery, and even fewer that have looked at the relationship between recovery and ISI. Most relevant research has instead looked at the related phenomenon of retention, which is often assessed by either rehabituation or brief tests given some time after the initial habituation. The majority of this research agrees, in principle, with the findings of Rankin and Broster in that the effects of habituation last longer when a long ISI is used. For instance, Davis (1970) found greater 24-hr retention of habituation (i.e., reduced response) in the acoustic startle response in the rat with longer ISIs than with shorter ISIs. Similarly, File (1973) reported better retention of habituation to lick suppression when rats had previously been habituated with longer ISIs.

One advantage that a recovery protocol might hold over a retention protocol is that, because recovery reflects the reversal of habituation, it may provide more direct clues as to how habituation develops in the first place. For example, the results of Rankin and Broster (1992) suggest that habituation is unlikely to be produced purely by depletion of neurotransmitter at key areas in the response circuit. If this were the case,

then treatment with shorter ISIs , which results in more complete habituation, should require longer recovery time, rather than the shorter recovery time that was observed. For these results to be explained, at least one other process in addition to reduced transmitter release must be involved in habituation and recovery. It may just be that a short ISI constrains the amount of recovery that can take place between stimuli, resulting in lower response levels during habituation, or it may be that the interplay of cellular mechanisms during habituation is different for different ISIs.

A model of habituation and recovery in the gill-withdrawal reflex of *Aplysia* has been proposed by Gingrich and Byrne (1985) that both accounts for the results of Rankin and Broster (1992) and suggests an explanation for the critical role played by interstimulus interval. Their model, which focuses on the events that might take place during habituation and recovery in a single neuron, suggested that during short ISIs the cell does not have enough time to reduce the high intracellular calcium concentration resulting from influx during stimulation. As a result, calcium accumulates in the cell. Some of this excess calcium activates the neurotransmitter mobilization system. This enhances the ability of the neuron to re-stock transmitter at the terminal, which means that shorter ISIs can produce faster recovery.

The accuracy of this model remains to be demonstrated, but it does emphasize a trend that can be seen in many aspects of habituation and recovery and that is that there seem to be both short- and long-term processes at work. For instance, one thing that has often been noted about recovery is that up to 85% of the initial response amplitude can return within a relatively short time while the rest of the recovery process can take much longer (e.g., Pakula & Sokolov, 1973). Such a pattern was also evident in the *C. elegans* data reported by Rankin and Broster (1992). While the group treated with the shortest ISI (2 s) reached 100% recovery in the first 10-min post-habituation, all other groups exhibited rapid recovery up to 60 or 70% of baseline in the same time, but then improved very little or not at all in the 20-min time period afterward. It is easy to imagine that the first phase of recovery might represent one set of mechanisms that recover rapidly and are associated with short ISIs, which would explain the advantage that shorter ISI groups appear to have in the early part of recovery. Likewise, the later stages of recovery may involve the resetting of processes that are more strongly affected by habituation, particularly when longer ISIs are used.

A number of researchers (e.g., Davis, 1970; File, 1973) have suggested that the rapid and extensive response decrement seen during habituation with short ISIs may be due more to some sort of refractory phenomenon, rather than a process that is

more clearly habituation (such as that seen during long ISIs). Rankin and Broster (1992) have expressed the idea that a possible refractory process might be sensory adaptation. They suggested that greater involvement of sensory adaptation during short ISIs would substantially reduce response levels while protecting the neuron terminal from extensive neurotransmitter depletion. This savings would then be reflected in superior recovery once habituation stimulation had been terminated. Rankin and Broster further highlighted the impact of ISI on the processes that determine pattern of habituation and recovery by demonstrating that the level of habituation at asymptote did little to dictate the rate of subsequent recovery. More importantly, they also showed that increasing the number of stimuli given during habituation once asymptote had been reached had very little effect on the recovery that followed. For example, they found the recovery rate after 60 stimuli at a 10-s ISI to be very similar to the recovery rate after as few as 8 stimuli at the same ISI. Both of these findings highlight the apparently critical role played by the ISI used during the initial part of habituation. They also reinforce the idea that habituation may consist of two phases. The first phase, which appears to have the biggest impact on recovery, seems to occur during the initial sharp decline in response level prior to asymptote. The second phase, which appears to have little effect on recovery, would begin once asymptote is reached.

The following experiments were designed to further explore the role played by ISI in habituation and recovery from habituation in an attempt to clarify some of the issues that have been discussed. Given that the time between stimuli has such an important impact, I felt it would be interesting to explore the effects of switching the ISI during habituation.

In the first experiment, the importance of precise and regular ISIs was examined by comparing worms habituated with comparable fixed and variable ISI schedules. Little research has been done on the subject of ISI variation, and even fewer studies have dealt with its effects on recovery from habituation. Davis (1970) explored the effect of a variable ISI on retention of habituation in the acoustic startle response of the rat. He found that this treatment resulted in less retention 5 min after habituation than when a fixed interval was used. While less habituation with a variable ISI has often been reported (Laming & MCKinney, 1990; Mackworth, 1968; Ruchkin, 1965) there are few studies that have looked at its effects from a post-habituation frame of reference.

The other broad issue investigated in these experiments was the apparent importance of the first few stimuli of habituation, and how this position effect may interact with a shift in the ISI during habituation. This was explored by examining the effect on habituation and recovery of shifting from a long to a short ISI (and vice versa) part way through the habituation

procedure and observing which ISI, the initial or the final, had the most influence in determining the rate of recovery.

General Methods

Subjects

A total of 160 hermaphroditic adult *C. elegans* (Bristol strain N2) were used in these experiments. Until testing, all worms were stored at 20°C on 5-cm petri plates filled with 10 ml of NGM agar and streaked with *Escherichia coli* (strain op50; Brenner, 1974).

In these experiments recovery from habituation was assessed by comparing response levels of post-habituation test stimuli to the initial response in the habituation series. Because of this comparison to initial response levels a response criterion was used to screen subjects. To qualify as a subject each worm had to have an initial reversal of half a body length or more, and at least one of the two subsequent responses had to be a reversal. In these experiments about 90% of worms tested met this criterion.

Apparatus

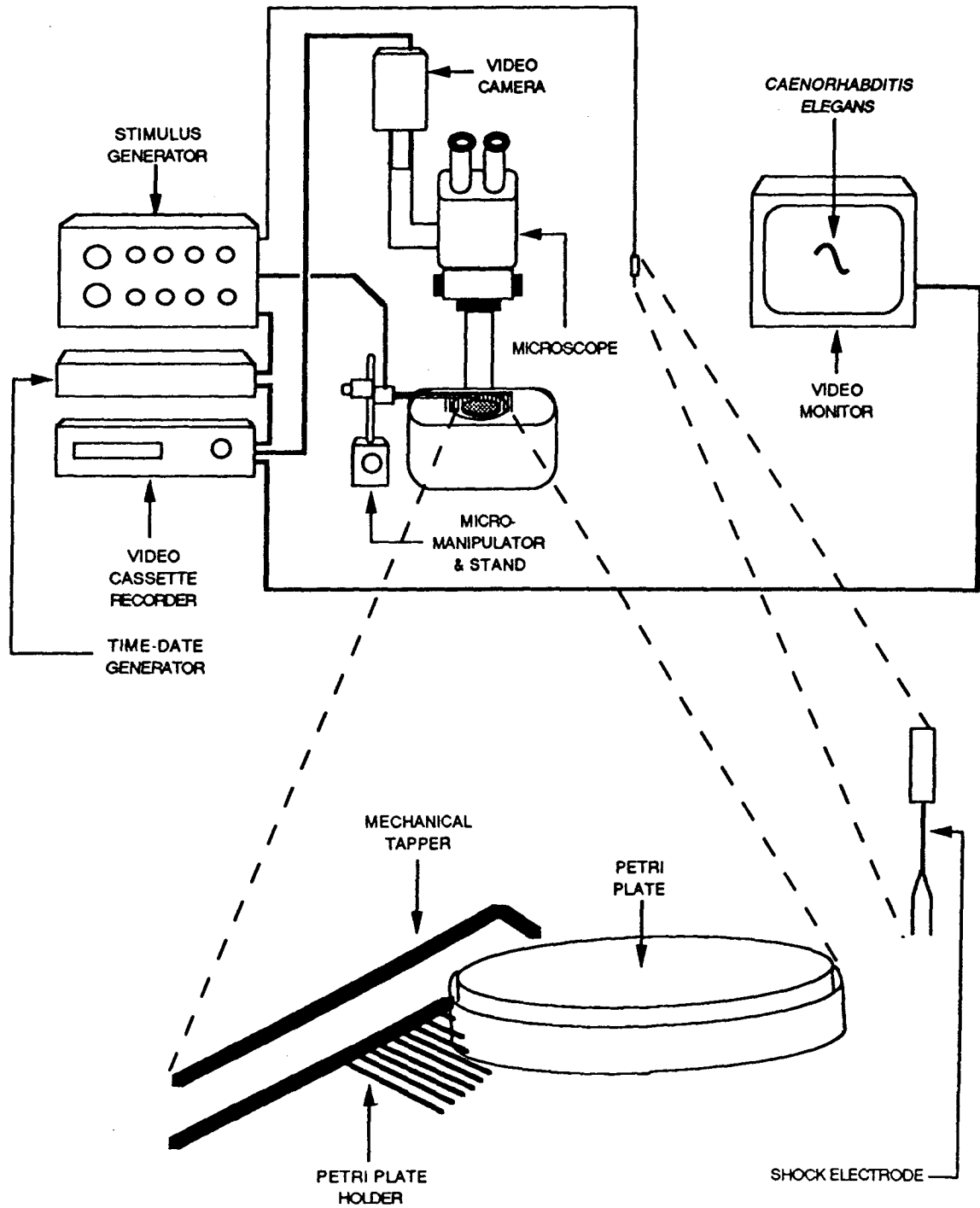
Individual worms were tested and observed on unstreaked petri plates using a stereomicroscope (Wild Leitz, Canada, Ltd., model M3Z) and attached videorecording equipment (Panasonic camera D5000, Panasonic AG-1960 VCR, JVC colour monitor). Stimulus delivery timing was aided by a time-date generator

(Panasonic 814) which superimposed a stop-watch onto the video image.

For testing, plates with single worms were placed in a holder made from a petri plate lid which was glued to a plastic rod. The other end of the rod was held by a Marzhauser micromanipulator (MM33) so that the plate could be moved smoothly when keeping the worm within the camera field. Also mounted on the rod is the mechanical tapper used to stimulate the worm (refer to Figure 1). The tapper consisted of an L-shaped copper wire arm (1.7 mm thick) attached at one end to the armature of an electromagnetic relay (6 V). The main arm of the tapper was 14 cm in length from the point where it was attached to the relay to where it bent at 90° to form the smaller arm. This smaller arm, which was 3.5 cm long and rubber tipped, was positioned perpendicular to, and halfway up, the wall of the petri dish. When the relay was activated, the tip of the tapper, which was touching the dish at rest, would oscillate at a peak amplitude of approximately 2.5 mm perpendicular to the tangent of the point where it contacted the dish. This contact created vibrations which were transmitted through the dish and the agar.

The stimulus used in these experiments was a brief (600-ms) train of six taps that delivered a peak force of 1.1 N (refer to Appendix I) to the side of the petri dish. To produce this stimulus the relay was electrically connected to a Grass

Fig. 1. The apparatus used to test and observe worms. Depicted in the top of the figure are the stereomicroscope, video equipment, and stimulator equipment used. In the lower part of the figure are the mechanical tapper and holder used for stimulating the worm. (From Mah, 1991).



S88 stimulator which was set to deliver a signal of six 25-ms pulses at 60 V and a rate of 8.5 pulses per second.

Procedure

In all experiments individual worms were transferred to a fresh agar-filled petri plate from a colony plate about 2 min prior to testing. In each of the following experiments a pre-determined number of trains was given according to a specified interstimulus interval schedule. To monitor spontaneous recovery from habituation, single stimuli were given at 30 s, 5 min, 10 min, and 20 min following the last habituation stimulus. Specific details for each experiment are described in the corresponding procedure section.

Response Analysis

The length of reversals (distance travelled while swimming backwards) given in response to trains of taps was the response measure that was used. Responses were scored by reviewing videotapes using stop-frame video analysis and tracing the path (the distance travelled) of each reversal onto an acetate sheet. The tracings were then digitized using a digitizing tablet (Summagraphics Bit Pad Plus) interfaced with a Macintosh SE microcomputer and Macmeasure software.

Reversals were considered to be caused by the stimulus only if they occurred within 1 s after the last tap in the train was delivered. If the worm appeared to be unaffected by a stimulus that response was given a score of zero. If the worm was in the

process of reversing when the stimulus occurred, or if it accelerated in response to it, the response was assigned a blank. About one in every five responses was scored as a blank, either for the reasons just mentioned, or because technical difficulties prevented the scoring of a response.

Statistical Analysis

Many of the statistical methods that were used on these data have already been established through other research examining changes in habituation in *C. elegans* (e.g., Beck & Rankin, in press; Mah & Rankin, in press; Rankin & Broster, 1992). In general, data involving response magnitude was analyzed using t-tests or ANOVAs with Fisher's protected least significant difference planned comparisons (PLSD) when statistical significance was achieved. All between-group comparisons were made using data that were standardized by dividing all responses of a given animal by its initial response. All within-group analyses were conducted with the data untransformed. Any time multiple t-tests were employed the type I error rate was adjusted for each test to keep the family-wise error rate below .05.

The specific characteristics of habituation and spontaneous recovery that were examined included the following: presence of habituation, rate of habituation, level of habituation, and extent of recovery. The presence of habituation was tested by

comparing the first response to the average of the last four responses of habituation within each group. Differences between groups in rate of habituation were examined by comparing mean slopes of the first few (12 or 25, depending on the ISI used and the protocol) responses in the habituation series. Differences between groups in response level prior to recovery (i.e., at asymptote) were assessed by comparing the average of the last four habituation responses.

With regard to recovery, in almost all situations there were no significant differences between the 5-min, 10-min and 20-min responses for a particular group, therefore, these points were usually pooled. The resulting mean was used in any comparisons involving recovery. Within each group, an overall repeated measures ANOVA involved the initial response, the mean habituated response, and the mean recovery response. The extent of recovery was assessed by comparing overall recovery to both the asymptote level (to determine whether significant recovery took place) and the initial response level (to determine whether responsiveness returned to baseline levels).

In Experiment 2 the mixed ISI habituation curves consisted of two components, one for each ISI. To assess the effects of ISI transition, separate mean habituated responses were computed from the last four responses at each ISI, and these averages were included in the overall ANOVA. Also included were the initial responses for each ISI.

Experiment 1: Variable vs. fixed interstimulus intervals

The aim of this experiment was to investigate the effect of continuous ISI variation on habituation and recovery in *C. elegans*. This was done by comparing two groups habituated with the same average ISI, one given a wide range of intervals in an irregular order, and another given the same interval on a regular basis. Sokolov (1963), in his stimulus-model comparator theory of habituation, suggested that regularity of stimulus timing is an important part of learning not to respond to that stimulus. It was expected that varying the ISI might, therefore, slow the rate of habituation. Because Davis (1970) found that animals treated with variable ISIs had less retention of habituation, it was also expected that such treatment would influence the extent of recovery observed. Because the outcome might depend on whether the average ISI was long or short, this experiment was carried out at both a 10- and 60-s ISI.

Method

A total of 80 worms were used. During habituation one group of 20 worms received variable stimulation at, on average, a 10-s ISI (scheduled intervals ranging from 2 s to 40 s; see Appendix II) and another 20 animals received stimuli at precisely a 10-s ISI. An additional group of 20 worms were

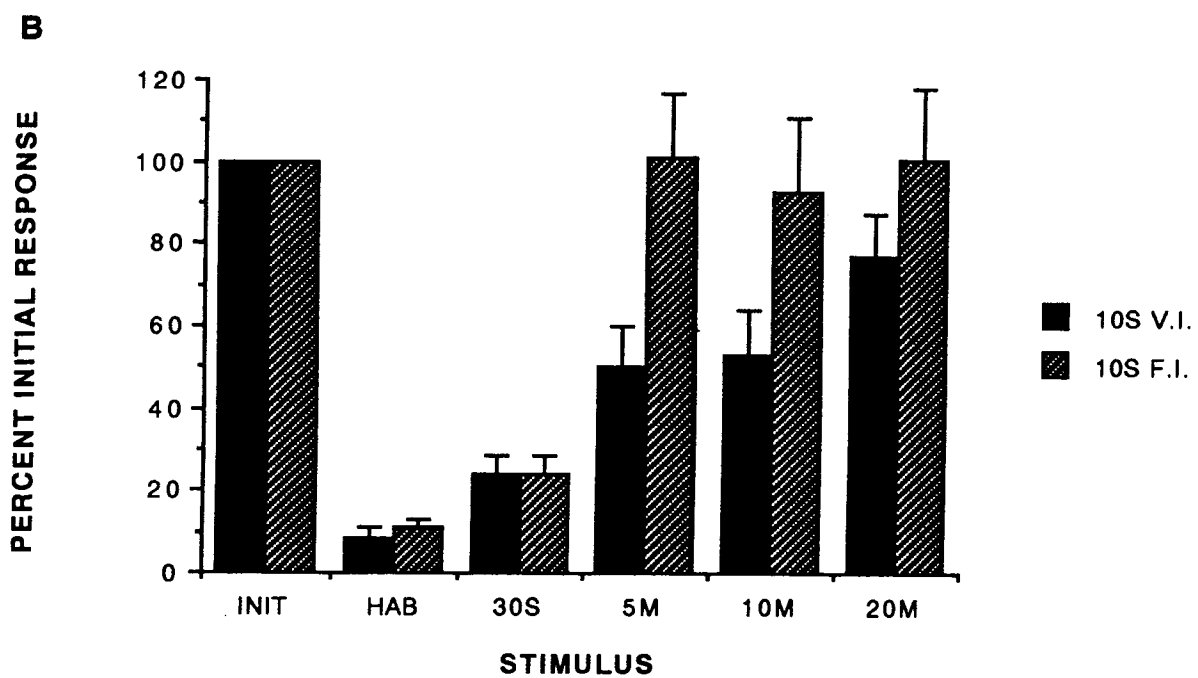
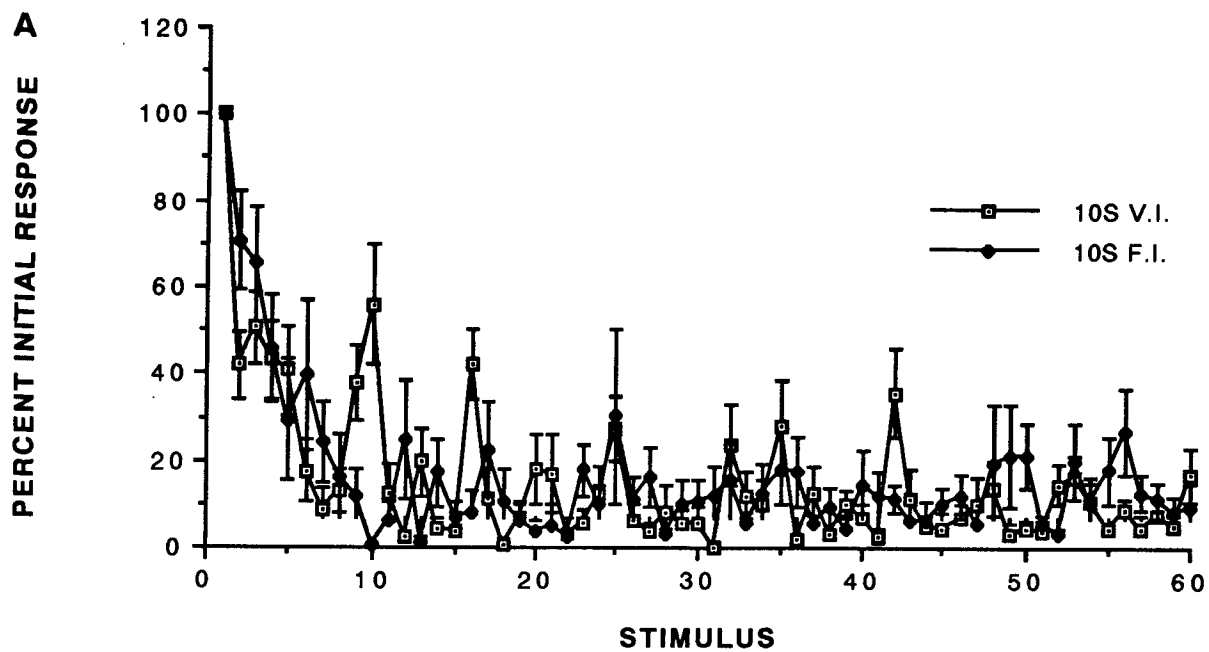
given stimulation at regular intervals of 60 s, and a corresponding group of 20 animals were stimulated at scheduled variable intervals having an average of 60 s (minimum of 5 s to a maximum of 4 min; see Appendix II). All worms received a total of 60 stimuli during habituation, and were given four additional stimuli to test for recovery. These stimuli were administered at 30 s, 5 min, 10 min, and 20 min after the last habituation stimulus.

Results and Discussion

10-s ISI fixed vs. variable interval. For the fixed-interval group, an overall repeated-measures ANOVA with Fisher's PLSD comparisons indicated that there was a significant decrease in response level from the beginning to the end of habituation training, $F(2, 38) = 88.039$, $p = .0001$ (refer to Figure 2). This analysis also showed that there was significant recovery from the habituated level (the mean of the last four habituation responses), however, there wasn't enough recovery to return response levels to baseline. For this test, recovery level was represented by taking the mean of the 5-, 10-, and 20-min post-habituation responses for each animal. An ANOVA found no significant differences between these points even when each of the blank cells had been replaced by the group mean.

The variable-interval group showed similar habituation but a slightly different recovery pattern. There were significant

Fig. 2. A comparison between the 10-s ISI fixed and variable treatment groups during habituation and recovery. Response magnitude (\pm SEM) is expressed in terms of a percentage of each worm's initial response ($n = 20$ for each group). A) Habituation: 60 stimuli at, on average, a 10-s ISI. B) Recovery: Recovery stimuli were given at 30 s, 5 min, 10 min, and 20 min after the last habituation stimulus. INIT is the response to the first habituation stimulus. HAB is the average of the last four habituation stimuli.



differences between the initial response, the mean habituated response, and the mean recovery response, $F(2, 38) = 72.07$, $p = .0001$. A difficulty with this analysis was that the 5-min, 10-min, and 20-min recovery points were found to be significantly different, $F(2, 38) = 6.59$, $p = .0035$, and, therefore, it was not appropriate to pool them into a recovery mean. When the analysis was recalculated with the recovery separated into a 20-min point and a pooled 5- and 10-min point, the pattern of significant differences was very similar; the 20-min point was still significantly below baseline, $F(3, 39) = 35.55$, $p = .0001$.

When the variable- and fixed-interval groups were directly compared the data used were standardized to initial response (see Statistical Analysis). Unpaired t-test results showed there to be no significant differences between these two groups at habituation asymptote. There was, however, a significant difference between the fixed-interval mean overall recovery level and the variable-interval 5- & 10-min mean recovery level, $t(37) = 3.20$, $p = .0028$. After 20 min of recovery time had passed this difference disappeared. Thus, although neither group recovered back to baseline, the fixed-interval group showed more complete recovery earlier (at 5 and 10 min post-habituation) than those animals habituated with variable-intervals.

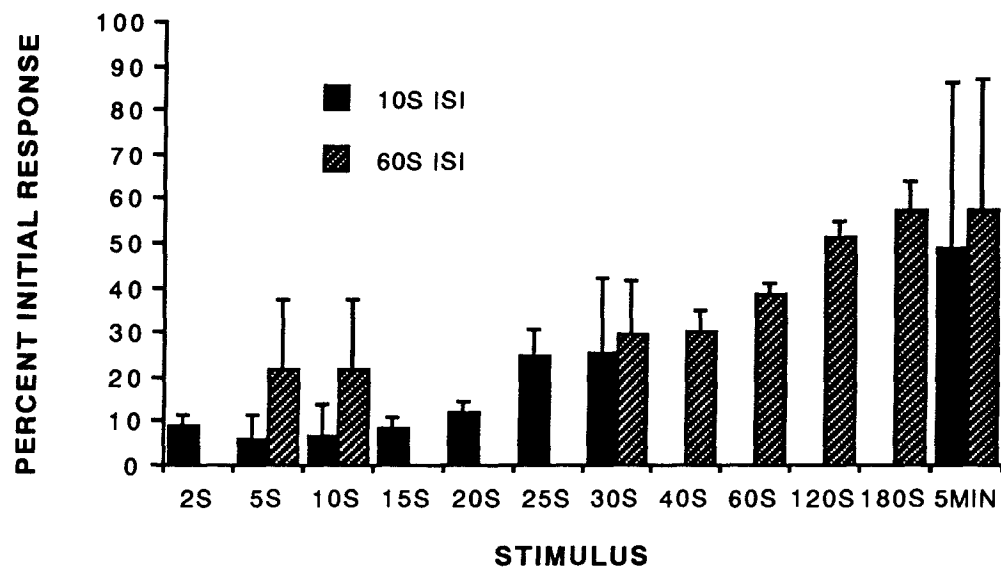
Slope analysis revealed that these groups also differed in their rate of habituation prior to reaching asymptote. A slope for each animal was derived from the regression line that best fit the first 12 responses. It was decided that, for the 10-s ISI group, the first 12 stimuli were best for assessing the rate of habituation because by the 12th stimulus both groups had spent exactly the same amount of time being tested, and both groups had reached asymptotic response levels. A t-test revealed that the fixed-interval group had a steeper slope, indicating a faster rate of habituation than the variable-interval group, $t(38) = 2.54$, $p = .0153$.

Two other characteristics of these groups were explored. First, it was suspected that the asymptotic portion of the habituation curve of the fixed-interval group might contain some sort of periodic variation of response level that could not be explained by any variation in the time between each stimulus. This periodicity was a property investigated purely for the sake of interest, and therefore is described in Appendix III.

A second issue explored was the extent to which each mean response during habituation of the variable-interval group could be correlated with the amount of time elapsed since the previous stimulus. It was hoped that this might permit a better understanding of how time interval can affect the response outcome of any given stimulus. A correlation taken over the entire habituation process resulted in $r(59) = .401$, $p < .005$.

Interestingly, there was a tendency for this interval-response correlation to vary, depending on whether the data involved was taken from the period prior to or during asymptote. When separate correlations were done for before (approximately the first 15 intervals) and during (the remaining 44 intervals) asymptote, there was a noticeably, but not significantly, larger correlation during asymptote, $r(15) = .376$, $p < .10$, and $r(44) = .582$, $p < .0005$. To determine how responsiveness was related to time since the last stimulus, the average response of each animal for each interval type was calculated and plotted (see Figure 3). This idea was prompted by the work of Davis (1970), who found response amplitude to be proportional to interval length. The intervals used for this calculation were taken only from the asymptotic portion of the curve (the last 44 intervals). Intervals during pre-asymptote were excluded because there was unequal representation of them during this phase, which, considering the large magnitude of many of those responses, might have biased the results. These data should, therefore, be thought of as a reflection of the interval-response relationship that exists only once a fairly stable habituation level, using a variable ISI protocol, has been established. For a comparison, these data are plotted next to similar asymptotic response data from the 60-s ISI variable group.

Fig. 3. A plot of the average response magnitude for different interval lengths during habituation for both the 10-s and 60-s variable ISI groups ($n = 20$ for each group). Responses used did not include those occurring prior to asymptote (i.e. the first 15 stimuli for the 10-s ISI group and the first 23 stimuli for the 60-s ISI group).

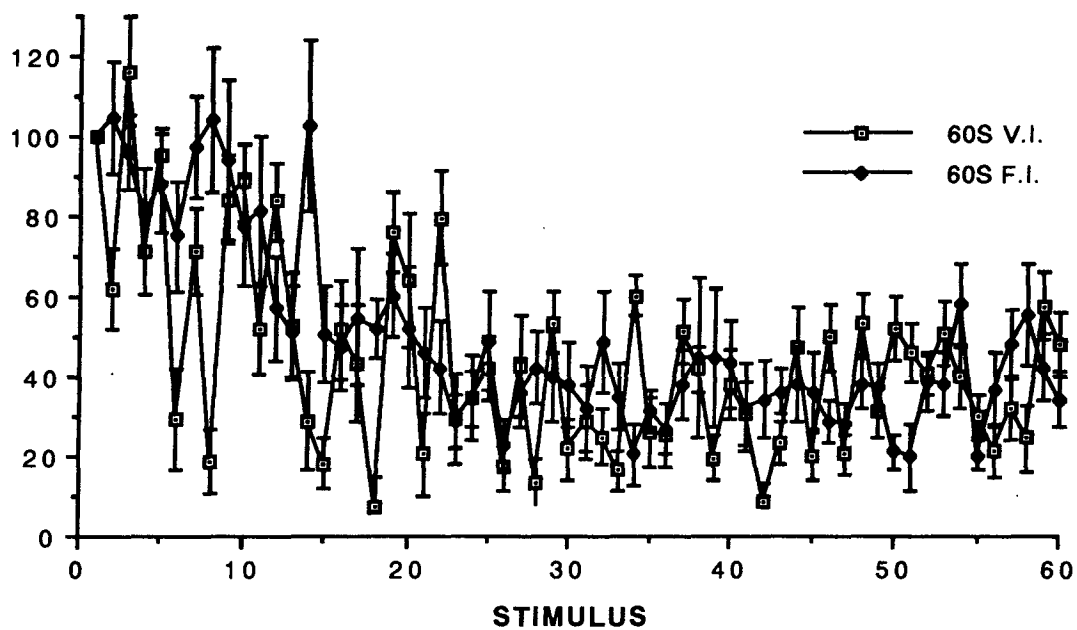
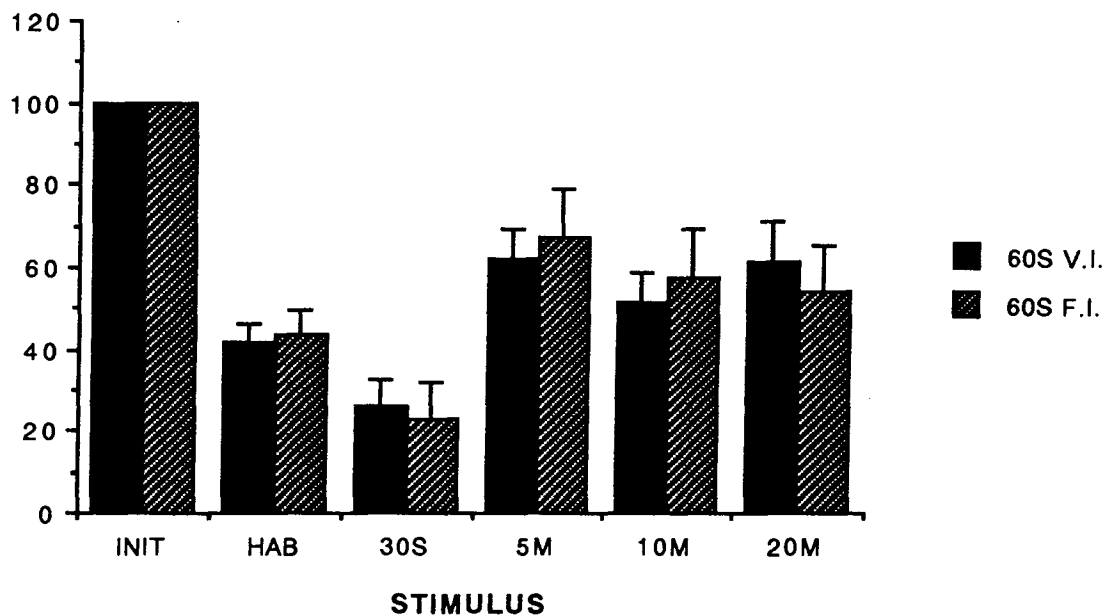


60s-ISI fixed vs. variable interval. Analysis of the fixed-interval 60-s ISI group indicated that there was a significant decrease in response level from the initial response to the mean habituated response (the average of the last four habituation responses), $F(2, 38) = 26.605$, $p = .0001$ (refer to Figure 4). The mean of the 5-, 10-, and 20-min recovery responses, which a separate ANOVA showed were not significantly different, and, therefore, could be pooled, was found to be significantly greater than the habituated level. Recovery did not, however, return to baseline.

Analysis of the variable-interval group showed a very similar pattern of habituation and recovery, $F(2, 38) = 47.277$, $p = .0001$. There was significant habituation and significant recovery from habituation, but not enough recovery to reach the initial response level. As with most of the other groups in this experiment, most of the recovery took place in the first 5 min post-habituation, with no significant gain in recovery occurring at 10- or 20-min points.

T-tests, with the alpha-level adjusted downward to keep the overall error rate below .05, were used to directly compare these two groups. There were no significant differences between mean habituated response levels or between mean recovery levels. To analyze relative rates of habituation, slopes were calculated over the first 25 responses for each animal. The first 25 stimuli were used because by that point both groups had been

Fig. 4. A comparison between the 60-s ISI fixed and variable treatment groups during habituation and recovery. Response magnitude (\pm SEM) is expressed in terms of a percentage of each worm's initial response ($n = 20$ for each group). A) Habituation: 60 stimuli at, on average, a 60-s ISI. B) Recovery: Recovery stimuli were given at 30 s, 5 min, 10 min, and 20 min after the last habituation stimulus. INIT is the response to the first habituation stimulus. HAB is the average of the last four habituation stimuli.

A
PERCENT INITIAL RESPONSE**B**
PERCENT INITIAL RESPONSE

tested for the same amount of time, and both groups were within asymptotic response levels. The fixed-interval group was found to have a significantly steeper slope, reflecting a more rapid rate of habituation, than the variable-interval group, $t(38) = 2.26$, $p = .0296$.

As with the 10-s ISI variable group, a correlation coefficient was calculated on the 60-s ISI variable interval data to determine the extent to which the response magnitude for each stimulus was dependent on the length of the interval which preceded it. This correlation was found to be significantly different from zero, $r(59) = .672$, $p < .0001$.

Figure 3 shows how response amplitude varies as a function of the time since the last stimulus. For this graph, response averages for each interval were calculated without inclusion of the first 22 intervals, because the unequal representation of these intervals prior to asymptote biased the results. Figure 3 also shows a comparison of the 60-s variable ISI data with the 10-s variable ISI data. Included were the two 5 min recovery responses, to assess how the response level for each of these groups compared over very long intervals. A repeated-measures two-factor ANOVA conducted on the common intervals (5 s, 10 s, 30 s, and 5 min) revealed that there were significant differences between the two variable ISI groups at the two shortest intervals (5 and 10 s), $F(2, 38) = 9.82$, $p = .0033$. Although there is a strong correlation between ISI and response

magnitude, these data indicate that the ISI alone does not control response amplitude. Here the same intervals are producing different response amplitudes. The response magnitude to short intervals appears to be influenced by the cumulative effect of previous stimulation. Something about the 10-s variable ISI treatment has reduced the mean response level, or, alternatively, something about the 60-s variable ISI treatment has facilitated the mean response level, for these intervals during asymptote. The longer intervals appear more similar to each other.

In general, for both a 10- and 60-s ISI, treatment with a fixed interval during habituation appeared to result in a sharper decline in response levels prior to asymptote than that seen when a variable interval was used. There were, however, differences between the two ISIs in the effect that regularity of stimulus delivery had on extent of recovery. While no difference in recovery levels was observed for the 60-s fixed and variable groups, the 10-s fixed interval group recovered to a greater extent during the early part of the recovery phase (at 5 and 10 min post-habituation).

An additional point to mention is that the results of this experiment are not incompatible with an associative interpretation of habituation (e.g., see Whitlow & Wagner, 1984). In Instrumental Learning stimuli delivered at variable intervals produce slower acquisition rates and longer retention

than stimuli delivered at fixed intervals. This is similar to the effect of habituating stimuli delivered at variable and fixed ISIs, especially the 10 s ISI group.

Experiment 2: The effect of mixed ISIs during habituation

In this experiment the ways that ISI exerts its powerful effects on habituation and recovery were further investigated by using two different ISIs, each for an equal number of stimuli, during habituation. Of interest was the effect that such treatment would have on the pattern of habituation and the extent of subsequent recovery. The two ISIs used, 10 and 60 s, were chosen because previous research (Rankin & Broster, 1992) has shown them to be distinctly different, both in terms of their habituation pattern and their recovery rate. It was felt that these differences would make it easier to determine which ISI was having a bigger impact on habituation and recovery response levels. Based on the findings of Rankin and Broster (1992) it was hypothesized that the order of presentation (i.e., which ISI was given during the first half of habituation and which was given in the second half) might be an important factor.

Method

A total of 80 worms were used for this experiment. One

group of 20 worms was given 15 stimuli at a 10-s ISI followed by 15 stimuli at a 60-s ISI. Another group of 20 worms was given 15 stimuli at a 60-s ISI followed by 15 stimuli at a 10-s ISI. Both groups then received four recovery stimuli, each one given at 30 s, 5 min, 10 min, and 20 min, respectively, after the 30th stimulus of habituation.

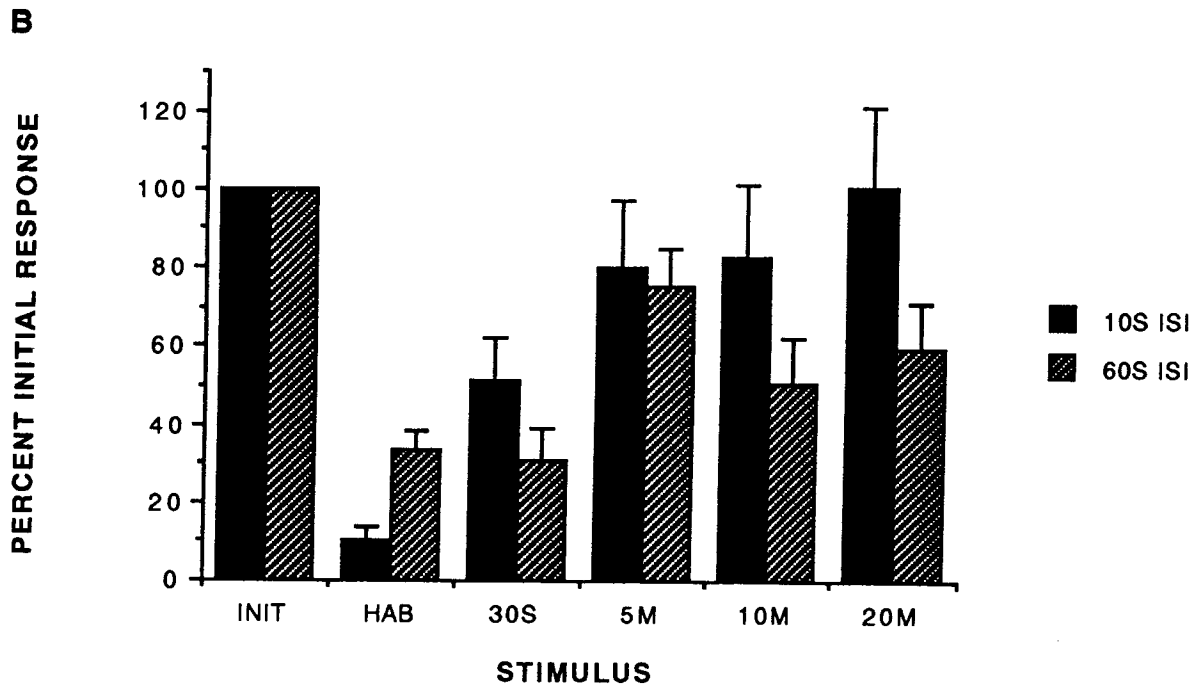
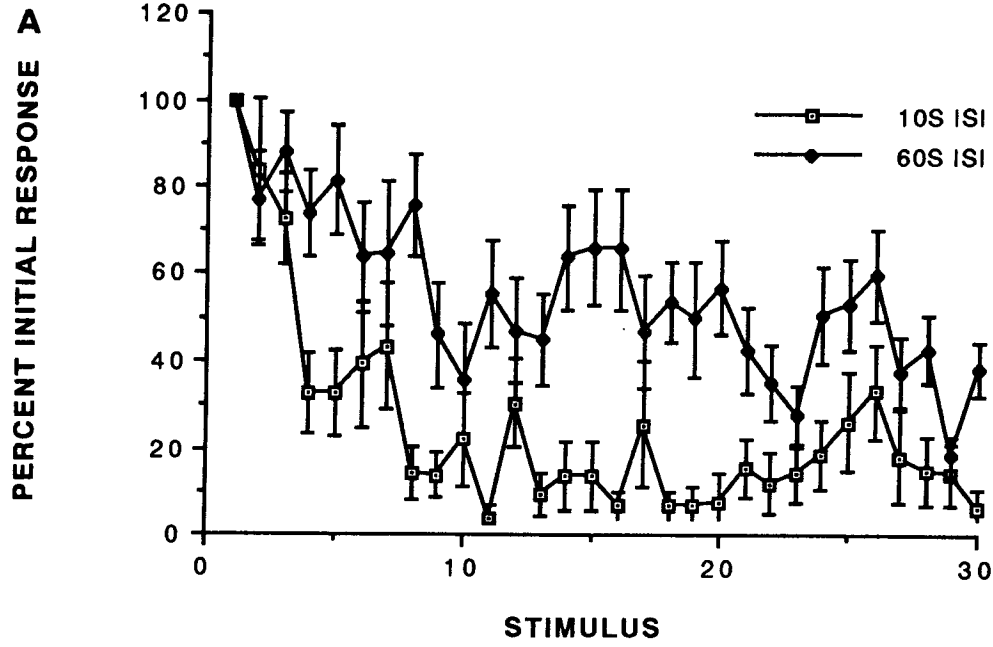
For controls, one group of 20 worms was habituated with 30 stimuli at a 10-s ISI, and another group of 20 worms was given the same number of stimuli at a 60-s ISI. Both groups then received recovery test stimuli at 30 s, 5 min, 10 min, and 20 min after habituation.

Results and Discussion

Single ISI control groups. In order to have a baseline condition to compare with the experimental groups, the results of the two control groups were examined first. Within each of these groups there were no significant differences between the 5-, 10-, and 20-min recovery points, thus, any further recovery analysis employed a mean of these three responses.

A repeated-measures ANOVA of the 10-s ISI control group showed that animals were significantly less responsive by the end of habituation (as represented by the average of the last four responses), compared to the start, $F(2, 38) = 32.11$, $p = .0001$ (refer to Figure 5). Recovery was not only significantly above the mean habituated level, it was extensive enough to be not significantly different from the initial response level.

Fig. 5. A comparison of the two 30 stimuli control groups (10-s and 60-s ISI; $n = 20$ per group). Response magnitude is expressed in terms of a percentage of each worm's initial response, and includes \pm SEM. A) Habituation: 30 stimuli at either a 10-s or a 60-s ISI. B) Recovery: Recovery stimuli were given at 30 s, 5 min, 10 min, and 20 min after the last habituation stimulus. INIT is the response to the first habituation stimulus. HAB is the average of the last four habituation stimuli.



The 60-s control group also showed a significant decrease in response level during the course of habituation, $F(2, 38) = 28.66$, $p = .0001$, but, unlike with the 10-s group, there was no significant recovery. The mean recovery level was significantly below the initial response level and was also not significantly different from the mean habituated level.

These two 30-stimuli control groups were compared in terms of their rate of initial habituation prior to asymptote by examining differences in the mean slopes of the regression lines calculated for the first 12 stimuli of each group. Using this method, the 60-s ISI group was found to habituate at a significantly slower rate than the 10-s ISI group. Once habituated to asymptote, the 60-s group also showed less overall decrement in responsiveness compared to the 10-s group, $t(38) = 4.19$, $p = .0002$. Thus, these two groups were different in many aspects of their response curves; the 60-s ISI group was characterized as having habituation that was slower and less extensive and recovery that also was slower and less extensive compared to the 10-s ISI group.

Mixed ISI experimental groups. For both the 10-to-60-s ISI and the 60-to-10-s ISI groups initial response levels, the mean habituated response levels for each ISI given (the average of the last four responses given in each series), the response level after the ISI was shifted, and the mean recovery response level (which, in both cases was pooled after no significant

difference was found between the 5-, 10-, and 20-min points) were analyzed using an overall repeated-measures ANOVA and Fisher PLSD comparisons.

The 10-to-60-s group (refer to Figure 6) was characterized by a sharp and significant decrease in response level that is typical of habituation with a 10-s ISI. When the ISI was switched to 60 s there was an immediate and significant increase in response level over the 10-s ISI habituated level, but not enough to return to baseline, $F(4, 76) = 47.02$, $p = .0001$. Over the course of the 15 stimuli given at a 60-s ISI there was a significant decrease in responsiveness, similar to that seen with other 60-s ISI groups. The mean response level at the end of the 60-s ISI part of the habituation curve was significantly higher than the mean response level at the end of the 10-s ISI treatment. Recovery was similar to that seen with the 60-s ISI control group in that there was no significant improvement in responsiveness from the 60-s habituated level.

With the 60-to-10-s ISI group (refer to Figure 7) there was a slow, but significant, decrease in responsiveness over the course of the 15 stimuli given at a 60-s ISI, $F(4, 76) = 44.47$, $p = .0001$. Additional multiple comparisons showed that as soon as the ISI was shifted to 10 s there was an immediate and significant drop in the response level that appeared to take the group down to the level of a typical 10-s ISI asymptote, as was indicated by the finding that the mean of the first two 10-s

Fig. 6. The effect on reversal response magnitude of shifting from a 10-s to a 60-s ISI during habituation ($n = 20$). Response magnitude (\pm SEM) is expressed in terms of a percentage of each worm's initial response. A) Habituation: The first 15 stimuli were given at a 10-s ISI; the latter 15 stimuli were given at a 60-s ISI. B) Recovery: Four recovery stimuli were given at 30 s, 5 min, 10 min, and 20 min following the last habituation stimulus.

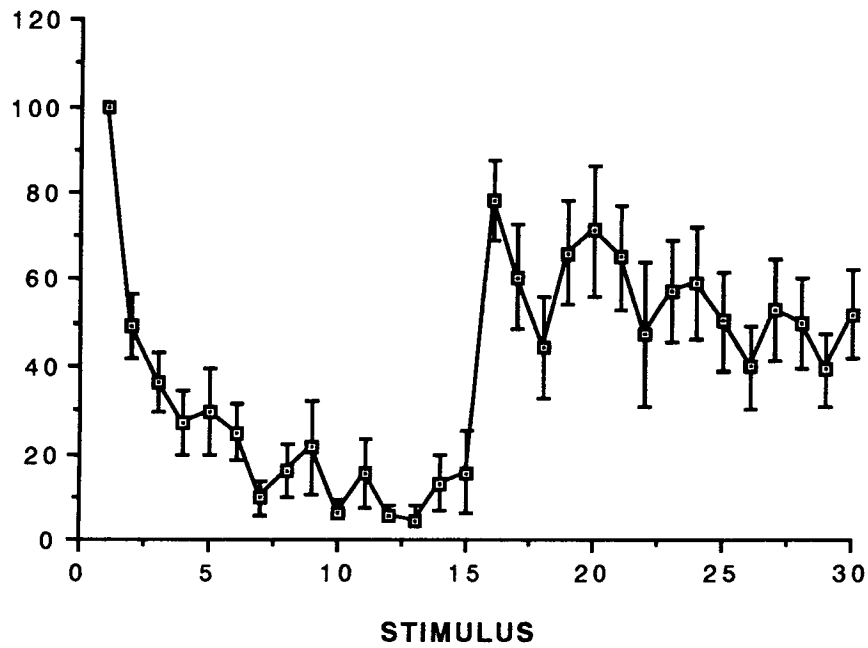
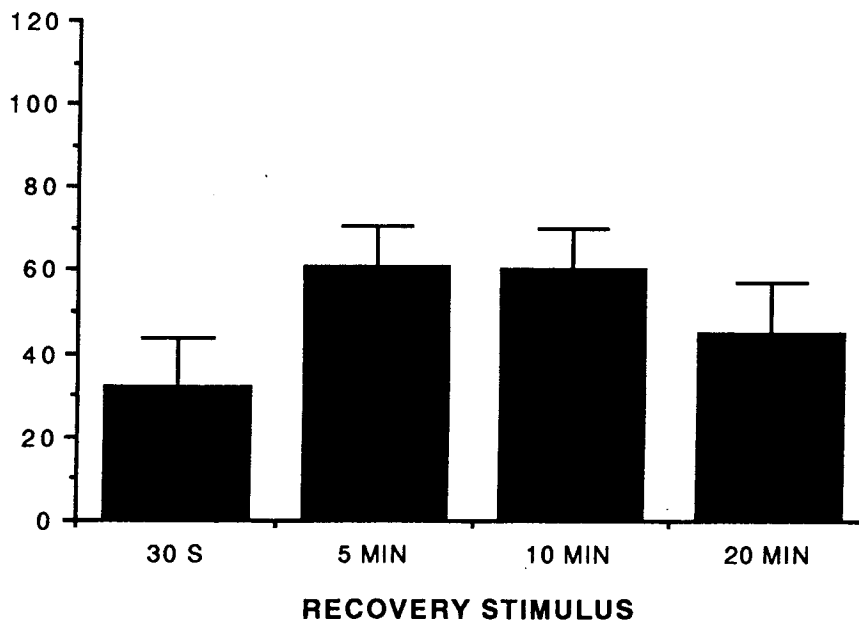
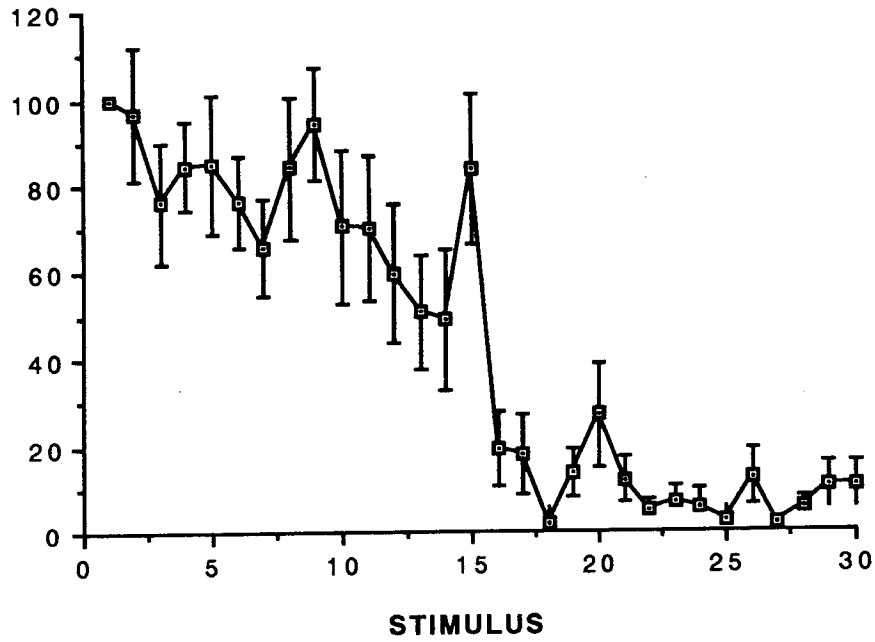
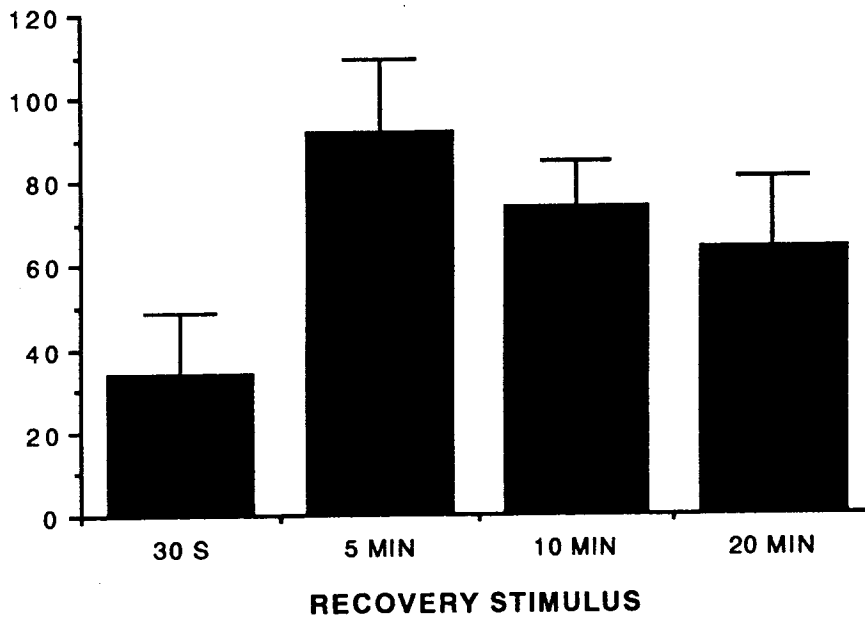
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Fig. 7. The effect on reversal response magnitude of shifting from a 60-s to a 10-s ISI during habituation ($n = 20$). Response magnitude (\pm SEM) is expressed in terms of a percentage of each worm's initial response. A) Habituation: The first 15 stimuli were given at a 60-s ISI; the latter 15 stimuli were given at a 10-s ISI. B) Recovery: Four recovery stimuli were given at 30 s, 5 min, 10 min, and 20 min following the last habituation stimulus.

A
PERCENT INITIAL RESPONSE**B**
PERCENT INITIAL RESPONSE

responses was no different from the mean of the last four. The recovery of this group more closely resembled that of the 10-s ISI control group in that it was significantly above both the 60-s and 10-s habituated levels. Recovery did not return to baseline, perhaps due to a lingering effect of the 15 60-s ISI stimuli. The overall recovery of this group was higher than that of the 10-to-60-s ISI group, $t(38) = 2.13$, $p = .0397$.

For each of the ISI transition groups, recovery rate seemed to be primarily determined by the ISI most recent to the onset of recovery, as opposed to the ISI to which the animals were first habituated. It is unclear whether the early ISI had any effect at all on recovery rate, though there is the suggestion that initial habituation with a 60-s ISI may have slightly reduced the amount of recovery that took place after subsequent habituation with a 10-s ISI. With respect to any effect habituation with the first ISI may have had on habituation with the second ISI, initial treatment with a 60-s ISI seemed to have influenced the habituation pattern observed with a 10-s ISI. There is no evidence that initial habituation with a 10-s ISI had any effect on the habituation with a 60-s ISI.

The ways that habituation with one ISI were affected by prior habituation with another ISI were further explored by testing whether the rate and level of habituation were dependent on whether an ISI came first or second in the habituation series. Within each ISI, t-tests were used to test for

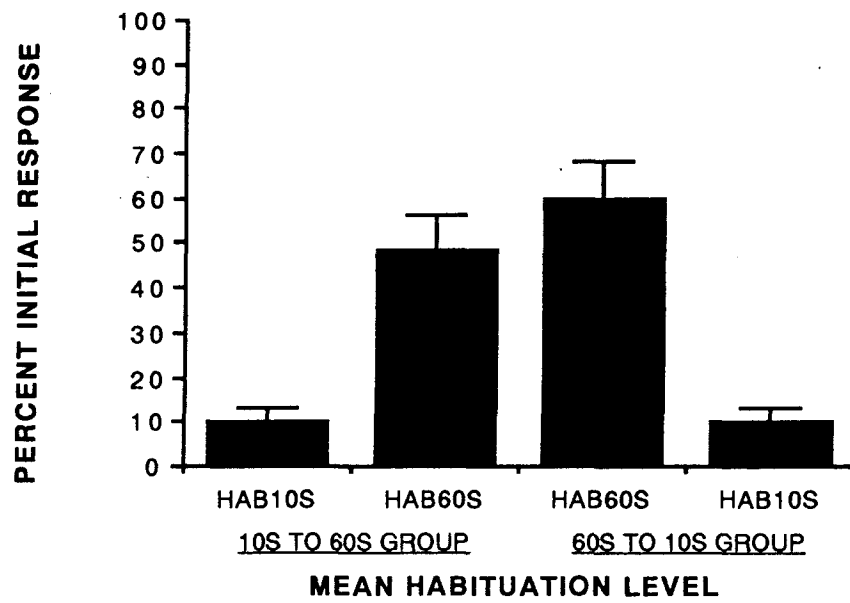
differences between the mean response level at the end of 15 stimuli for the group that received the ISI first and the group that received it second. For both the 10- and 60-s ISIs there was no difference in the respective habituated (asymptotic) response levels. Thus, the asymptotic response level achieved at a 60-s ISI was not significantly affected by the 15 10-s ISI stimuli that preceded it, and, likewise, the asymptotic response level achieved at a 10-s ISI was not significantly affected by prior stimulation at a 60-s ISI.

For each block of 15 stimuli, t-tests were used to determine whether there was significant decrement in response magnitude from the 2nd to the 15th stimulus by comparing the mean of the first two responses following the first interval at that ISI (i.e., stimuli 2 & 3, and stimuli 16 & 17) with the mean of the last four habituation stimuli at that ISI. There was an effect of prior stimulation on how quickly the asymptote was reached, but it was only seen during the 10-s ISI habituation when it followed habituation at a 60-s ISI. In the 10-s portion of the 10-s to 60-s ISI habituation, asymptote was not reached by the second or third stimulus, $t(16) = 5.479$, $p = .0001$, and the same was true for the second (60-s ISI) half of the habituation, $t(19) = 2.83$, $p = .0108$. For the 60-s to 10-s habituation, the results were the same, asymptote was not reached by the second or third stimulus during the 60-s ISI half of habituation, $t(19) = 2.738$, $p = .0131$; however, with the 10-s

ISI habituation that followed, asymptote was reached rapidly, as there were no significant differences between the first two and the last four 10-s ISI habituation responses.

These findings support the idea that there was some transfer of habituation from the 60-s ISI to the 10-s ISI, but not vice-versa. It is also interesting to note that asymptotic level, once established, was unaffected by previous stimulation (refer to Figure 8), but, the rate of response decrement was affected when 10-s ISI habituation that was preceded by 15 stimuli at a 60-s ISI. This suggests that the response decrement phase of habituation, prior to asymptote, is more sensitive to prior habituation than the asymptotic phase itself. This supports the hypothesis that one or more processes in the pre-asymptote phase are different from the processes of the asymptotic phase of habituation.

Fig. 8. A comparison of the mean habituated response levels for each of the mixed ISI groups. HAB 10S is the mean of the last four response at a 10-s ISI. HAB 60S is the mean of the last four response at a 60-s ISI. The first pair of bars are from the 10-s to 60-s ISI group ($n = 20$) and the second pair of bars are from the 60-s to 10-s ISI group ($n = 20$). Response magnitude (\pm SEM) is expressed as a percentage of each worm's initial response.



General Discussion

There is a wealth of research that has emphasized the importance of the effect that interstimulus interval has on habituation. It is highly conceivable that one of the reasons the neural underpinnings of this form of learning have thusfar been so elusive is that the underlying mechanisms may be different for habituation to different ISIs. One way of approaching this problem at a behavioural level is to first understand the overall effect a particular ISI protocol has, and then look at how each individual stimulus event might have contributed to this effect. Understanding the relationship between these two aspects of habituation in terms of behavioural dynamics may, in turn, make it easier to elucidate the cellular mechanisms underlying them.

These two experiments have made it easier to apply this approach by employing protocols in which the ISI changes during the habituation procedure. By examining the effect that these changes had on recovery from habituation it was possible to assess them in terms of their overall effect. By examining the immediate effect during habituation of these ISI variations it was possible to come to a better understanding of how ISI might be interacting with events on a stimulus-by-stimulus basis.

In the first experiment, the importance of the regularity of ISI timing was explored. It was found that, with a 10-s ISI, fixed time intervals during habituation produced faster recovery

than if the intervals were varied. No difference in recovery was noted when the average interval was 60 s. That the outcome is dependent on which ISI the experiment is conducted with might indicate that animals are more affected by ISI variability when shorter, rather than longer, intervals are used. The difference seen in recovery with the 10-s ISI version of this experiment differs from the results of Davis (1970), who found that there was more pronounced retention of habituation after treatment with fixed intervals than with variable intervals. This contradiction is probably due to differences in the post-habituation test protocol used. Davis used a large battery of post-habituation tests, rather than just a few probe stimuli, and this may have made his protocol more like rehabituation. The results of Davis (1970) might, therefore, be replicated if Experiment 1 were re-run using rehabituation instead of recovery.

Another overall effect examined was the impact that irregular ISIs had on rate of habituation. With both the 10- and 60-s comparisons, slope analysis revealed a faster rate of habituation when the stimulation was given at regular intervals. Laming and MCKinney (1990) also used slope analysis to assess rate of habituation to light in the goldfish, and they too found that it was slower when there was variation in the ISI. One hypothesis is that regularity of stimulation might make it easier for the animal to learn more quickly that a stimulus is

not relevant. In general, though, the literature on this subject is mixed, with results often changing with subtle differences in protocol and method of analysis.

An important issue concerning the interpretation of these data is that it is difficult to determine whether the effects observed are due to the presence of regularity (or the lack of it), or an imbalance between the effects of the longer intervals and the shorter intervals that are included in the treatment. For instance, the slower recovery seen with the 10-s ISI variable group may be there because any long-term inhibition of recovery contributed by one type of interval (for example, the longer ones) may outweigh any facilitation of recovery that might be provided by another type of interval (for example, the shorter ones). It is unlikely that the effects of overall regularity can be disentangled from the specific contribution made by intervals of different length until the latter is better understood.

Rankin and Broster (1992) have speculated that very short ISIs might enhance recovery, or at least spare it from being restricted, by producing some sort of effect, such as sensory adaptation, that reduces the amount of transmitter lost from the nerve terminal. From this, one might have predicted that the inclusion of very short intervals in the 10-s ISI variable schedule would have contributed to faster recovery for the variable group. The results of Experiment 1 do not support this

prediction, but they also can not rule out the idea because any effect that short ISIs might have been outweighed by an opposite effect brought about by the inclusion of much longer ISIs in the variable habituation schedule.

In Experiment 2, the use of just two different ISIs during habituation made for easier interpretation. The ISI used most recent to the onset of recovery was the one that had the largest influence on rate of recovery. This is an interesting finding, especially when analyzed in light of the findings of Rankin and Broster (1992), who found that as few as eight stimuli at an ISI were enough to produce a recovery curve that was typical for that ISI, and that further stimulation did little to influence the shape of that curve. Their results seemed to indicate that recovery rate had become, in a sense, fixed once habituation to asymptote had occurred. The present experiments have demonstrated that recovery rate can be changed substantially even once asymptote has been established. For instance, animals first habituated to asymptote at a 10-s ISI and then treated with a 60-s ISI exhibited the limited recovery that is usually seen after full habituation with a 60-s ISI. This suggests that habituation stimuli during the asymptote are capable of both maintaining recovery rate once it has been established (if the same ISI is used throughout habituation) and changing it if it has been previously set by some other ISI. Thus, it is likely that each stimulus during habituation plays some role in

determining recovery rate. Depending on what has preceded the stimulus, this contribution can either help set, change, or maintain the recovery rate.

Another important aspect of Experiment 2 is the effect that the ISI change had on habituation itself. This should first be examined in terms of the general effect that the first ISI had on the second, i.e., whether habituation with the first ISI carried over to the second. When the ISI was switched from 10 s to 60 s there was an elevation in response level that brought the group almost back to baseline, and the subsequent habituation curve was much like a typical 60-s ISI habituation curve. So, in this case, although the 10-s ISI habituation substantially reduced the response level prior to the change, it had minimal impact on habituation at a 60-s ISI.

In contrast, the effect of initial habituation with 15 stimuli at a 60-s ISI seemed to carry over to the 10-s ISI habituation. When the ISI was switched from 60 to 10 s, the response level went from a 60-s ISI asymptotic habituation level to being not significantly different from a 10-s ISI habituation level. That a substantial transfer effect was observed with the 60-to-10-s ISI protocol and not the reverse protocol suggests that the underlying mechanisms responsible for habituation at the two ISIs may be linked in such a way that the events occurring during habituation at a 60-s ISI can potentially mimic some of the events that occur during habituation at a 10-s ISI.

This contrast between the 10-s-to-60-s change and the 60-s-to-10-s change could be explained in terms of two types of habituation processes, one type that is transient and more associated with shorter ISIs and another type that is longer lasting and more associated with longer ISIs. According to this model (Staddon, personal communication) habituation at a short ISI may involve processes that result in a rapid and pronounced response decrement during habituation as well as faster and more complete recovery following habituation. In addition, habituation at a long ISI may involve processes that cause a slow and shallow habituation that takes a long time to recover from. During the change from a 10-s to a 60-s ISI the first 60 s interval may have provided enough time for most of the 10-s ISI habituation effects to recover. During the change from a 60-s to a 10-s ISI there would not have been enough time to recover from the initial habituation at a 60-s ISI, and, therefore, the effects of this treatment would be carried over to the 10-s ISI habituation. If one considers the slow recovery after habituation stimuli at a 60-s ISI to be indicative of the amount of time that the effects of habituation at this ISI last, then it is interesting to note that there was little trace of these long-lasting effects in the recovery of the group that received 2.5 min of 10-s ISI stimulation between 60-s ISI habituation and recovery. This suggests that 10-s ISI stimuli may be actively reversing the effects of 60-s ISI stimulation.

Assuming that the long-lasting effects of 60-s ISI habituation were somehow reduced by 10-s ISI habituation, there may be one or more processes that can improve, or facilitate, (up to a point) rate of recovery. This idea has been expressed by others, such as Gingrich and Byrne (1985), who suggested that an abnormally high increase in calcium levels at key areas during high frequency (short ISI) stimulation may temporarily improve the ability of a neuron to replenish depleted transmitter, hence, generating faster recovery.

At this point it may be useful to approach any further speculation about the results of these experiments by generating a model that gives some account of what, in general, is occurring between each individual interval during habituation stimulation. This model makes the assumption that, immediately after a stimulus occurs, the ability of the circuit to respond to another stimulus is essentially zero. As time goes on, the response potential of the circuit, i.e., its ability to respond to another stimulus, rises. (The interval-response plots shown in Figure 3 would be examples of how response potential may vary with time since the last stimulus). The rate at which the response potential rises (or, recovers) may depend on what sort of stimulation treatment was received prior to the previous stimulus. The response amplitude of the next stimulus could be predicted by observing how high the response-potential curve is for the ISI that has just been experienced.

Habituation data suggests that the shape of the response potential curve changes with repeated stimulation. If it was always the same after each stimulus then, with regular stimulation at a single ISI, one would observe a drop in response level when the second stimulus was given (assuming this was done with a reasonably short ISI, that wasn't long enough to prevent interstimulus recovery back to baseline), but, there would be no further drop in response level, as long as subsequent stimuli were given at the same ISI. Further stimulation would always interrupt the response-potential curve at the same point and, therefore, if the shape of the curve didn't change, roughly the same level of response would result each time stimuli were given at that ISI. What actually happens with the first few stimuli is very different from this, as most habituation graphs initially show a steady decrease in response level with repeated stimulation, at least until the asymptote is reached.

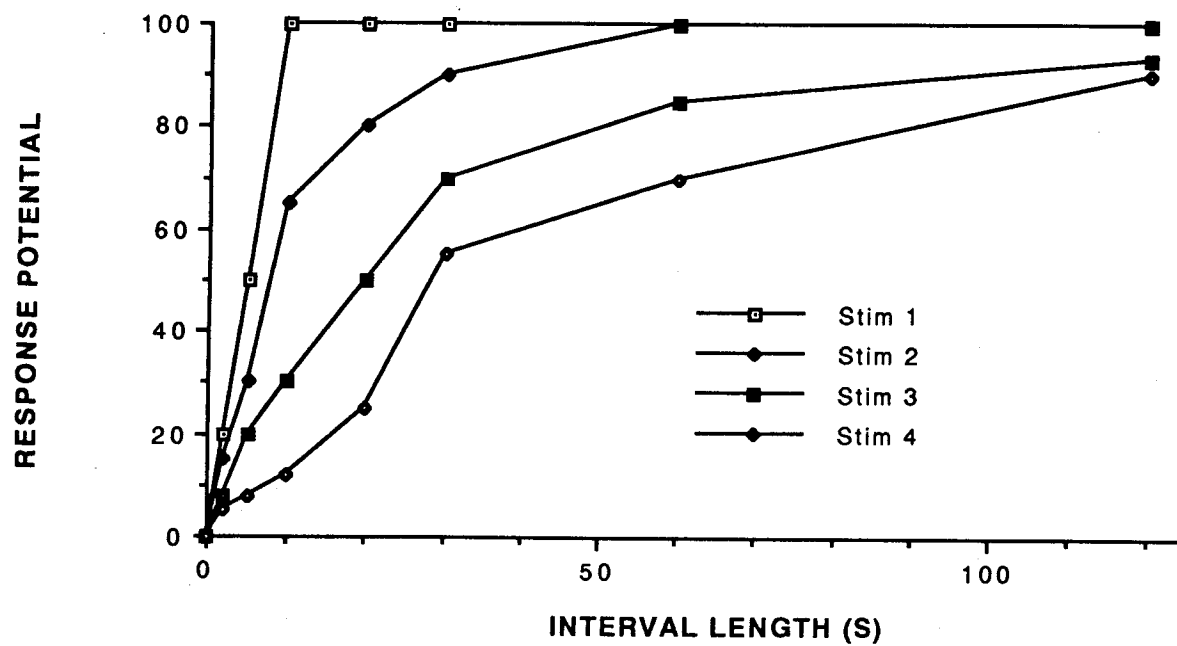
This observed pattern may reflect a gradual slowing of the response-potential curve, with each stimulus during the pre-asymptote phase of habituation further decreasing the speed of inter-stimulus recovery of response-potential. Once asymptote is reached, the observed response level flattens out, suggesting that the response-potential curve is no longer being modified within the time window of the ISI given; each new stimulus at that ISI produces roughly the same level of response

as the previous stimulus. An illustration of this is shown in Figure 9.

This hypothetical response-potential model may be useful for investigating habituation in a number of ways. One of the most fundamental issues that can, and should, be examined using it is whether or not the properties of the response-potential curve are affected in different ways by different ISIs, and, if so, how. A comparison of the 10-s and 60-s ISI recovery curves observed in these experiments (e.g., the 30 stimuli control groups) indicates that, by 5 min after the last habituation stimulus, the 10-s ISI group has recovered very rapidly, both in terms of absolute recovery and net recovery (recovery subtract asymptote), compared to the 60-s ISI group. This implies that there is a difference in the long-range portion of the response-potential curve for the two ISI groups after 30 stimuli. By 5 min after the last stimulation, the response-potential curve for the 10-s ISI group would appear to be at a higher point than that for the 60-s ISI group. Thus, it would appear, in the context of this model, that habituation at a 60-s ISI probably produces some sort of long-lasting inhibition of response-potential, and/or, habituation at a 10-s ISI probably causes some limited facilitation of recovery.

An overview of the research that has been done on habituation and recovery in *C. elegans* suggests that it may be best to distinguish between those changes in the

Fig. 9. An illustration of hypothetical trends in the response-potential model. The response-potential curve may change with repeated stimulation at a constant ISI. Each point represents potential response amplitude for a stimulus delivered at the post-stimulus interval shown on the x-axis. The curve of recovery of response potential for the first stimulus is relatively fast, but it becomes slower with each successive stimulus.



response-potential curve that are seen in the early post-stimulus period and those that are seen in the late post-stimulus period. As discussed above, assessment of recovery responses indicates that there are different ISI effects on the late post-stimulus part of the response-potential curve; habituation with a 60-s ISI seems to depress it, and habituation with a 10-s ISI may facilitate it to some degree. Furthermore, the research of Rankin and Broster (1992) suggests that the effects of each ISI on this portion of the response-potential curve are fully realized by the time asymptotic response levels have been reached, as groups habituated just to asymptote exhibit typical recovery curves for the ISI they were treated with. This evidence reinforces the idea that the response-potential curve is stabilized by asymptote.

With regard to any ISI differences in the early post-stimulus portion of the response potential curve, it is more difficult to speculate about what may be happening. A hint of ISI differences here is given in the 10-s variable ISI vs. 60-s variable ISI plot depicted in Figure 3. During the 60-s variable ISI treatment, the shorter ISIs (5 and 10 s) resulted in significantly higher responses than were seen at the same intervals during 10-s variable ISI treatments. One possible explanation of this result is that the use of generally shorter ISIs inhibits the early part of the response-potential curve.

There are, of course, other explanations of these data, including the possibility that the 60-s variable ISI treatment produces some sort of facilitation of response potential during this period, or, that something about the irregularity of these two groups is responsible for the differences between them.

If different ISIs during habituation affect the response-potential curve in different ways, the next logical question to ask is to what extent does each stimulus contribute to these differences. The results reported here suggest that it may take relatively few stimuli at a particular ISI before the specific cumulative effects on the response-potential curve are maximized. As mentioned before, the results of Experiment 2 suggest that stimuli can either set, change, or maintain the asymptotic habituation level, as well as the rate of recovery. Also, it appears that it takes relatively few stimuli (15 or less) for the second ISI to alter both the level of habituation and the pattern of recovery that had been, presumably, already set by the first ISI. The dynamic nature of these data underscores the importance of the role played by individual stimuli. The cellular events that an individual stimulus precipitates are likely the same every time, but their effect on response level is probably more a function of the interaction that these events have with the cumulative effect of other events and factors, as reflected by the state of the response-potential curve at the time. For example, the

inhibition of the early portion of the post-stimulus response-potential curve that might be occurring in short ISI habituation may reflect the presence of a transient inhibitory process that lasts for a few seconds after every stimulus (regardless of ISI), but which remains unseen unless several short ISIs in a row allow for an accumulation of the effects of such a process.

It would be interesting to follow up this research with experiments that test how few stimuli are needed to change a recovery pattern that had already been set by one ISI. For instance, if five stimuli at one ISI were to follow 15 stimuli at another ISI, would that be enough to convert the recovery pattern observed? Perhaps the most important follow-up experiments to be conducted, though, would be ones that more systematically explored the relationship between length of the ISI and the response magnitude observed. Such experiments might start by looking at the effect of the interjection of a single interval that is different from the ISI used during the rest of habituation. The effect of this interval may change depending on when during habituation it is given, what the absolute length of the interval is, and how its length compares to the other ISI used. Experiments exploring the effect of one different interval could then be followed by ones that looked at the effects of having two interjection stimuli in a row, and then three in a row, and so on. Experiments involving single

interjection stimuli offer two useful pieces of information. On one hand, they may permit a full plotting of the response-potential curve for a given habituation protocol, as long as a range of different single ISIs are used. Secondly, they may clarify the amount of impact that a single stimulus interval can have, and how this impact varies with the treatment that follows or precedes the stimulus. Experiments involving a few interjection stimuli in a row may reveal the cumulative effects of this impact.

The present experiments have produced a better understanding of the role that ISI plays in habituation, and, more importantly, have introduced ways of viewing ISIs that may prove very useful in the future. By understanding what may be happening between each stimulus, and how that relates to the cumulative effects of ISI it may be possible to more readily connect these observations at a behavioral level with the molecular events that might be responsible for them.

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Appendix I

The force imparted by the mechanical tapper was measured using a Showa strain-guage that was electrically connected to a 6 V DC power supply, a wheatstone bridge and an oscilloscope. The strain guage was mounted on a metal cantilever such that any deformation of the cantilever created by a force applied to one end of it resulted in a deviation from zero of the oscilloscope reading. The peak amount of deviation created by the tapper was then matched by calibrated masses suspended from the cantilever (at the same point where the tapper force was applied) by a carriage of negligible weight. The mass that matched the tapper deflection was multiplied by 9.81 m/s^2 to give a force value in Newtons. The average value obtained over several readings was 1.1 N.

Appendix II

The order of the variable intervals (as listed below) for each of the variable-ISI groups was chosen with the following considerations in mind. The first and last 5-7 stimuli in each set were chosen to be as varied in length as possible to avoid a possible effect where predominance of one type of interval could have a large impact on either the initial rate of habituation or the rate of recovery. Also, any sort of regular pattern (e.g., a larger interval every 4 or 5 stimuli) was avoided. Finally, having more than two short or long intervals in a row was also avoided.

10s ISI Group Variable Schedule: 10s, 2s, 15s, 5s, 10s, 5s, 10s, 2s, 40s, 10s, 5s, 2s, 10s, 5s, 30s, 5s, 5s, 10s, 2s, 25s, 5s, 10s, 2s, 25s, 5s, 10s, 2s, 15s, 2s, 5s, 25s, 2s, 10s, 20s, 5s, 2s, 10s, 20s, 5s, 2s, 25s, 5s, 15s, 5s, 20s, 2s, 15s, 5s, 10s, 2s, 30s, 5s, 15s, 5s, 20s, 2s, 15s, 5s, 10s.

60s ISI Group Variable Schedule: 10s, 3m, 30s, 2m, 5s, 40s, 10s, 60s, 4m, 5s, 2m, 30s, 10s, 20s, 40s, 30s, 10s, 2m, 30s, 5s, 4m, 10s, 30s, 60s, 5s, 40s, 10s, 2m, 20s, 40s, 5s, 60s, 3m, 10s, 40s, 2m, 30s, 5s, 60s, 30s, 10s, 40s, 2m, 5s, 60s, 30s, 3m, 10s, 4m, 40s, 60s, 2m, 40s, 60s, 5s, 10s, 3m, 60s.

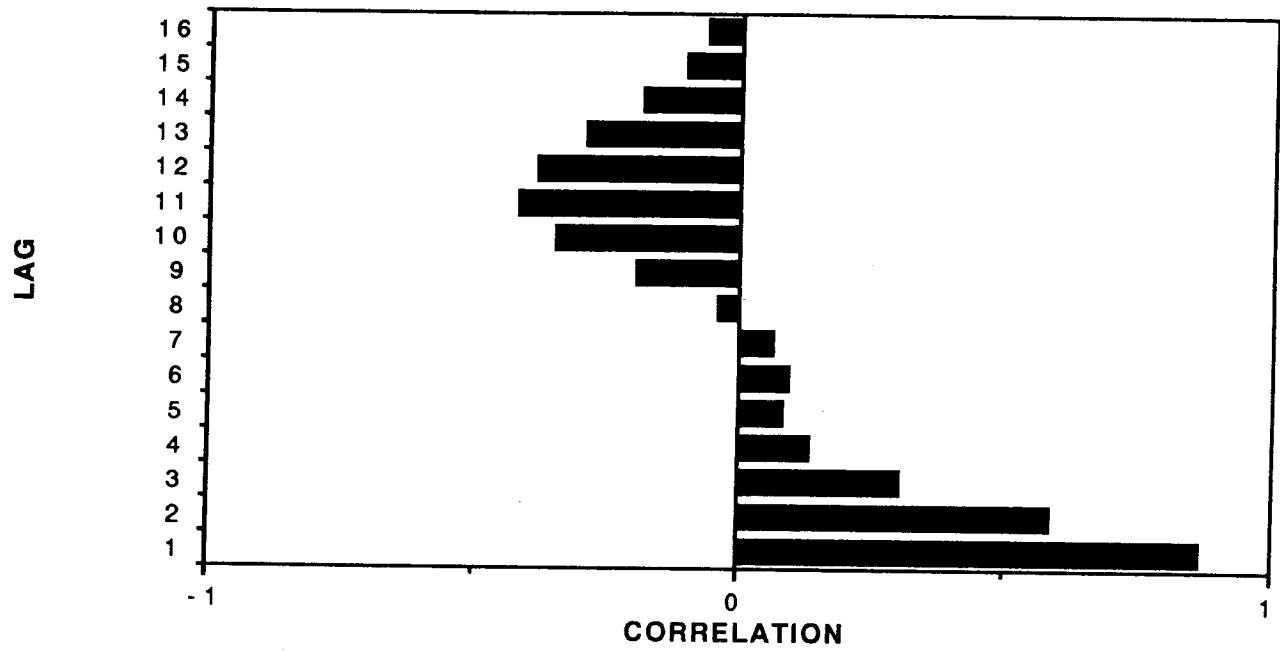
Appendix III

There appeared to be some degree of cyclicity in the fluctuation in response level during habituation of the fixed-interval 10-s ISI group. That is, the response level seemed to slightly rise and fall in periodic fashion above and below the response level that would have been predicted by a straight regression line running through the data points. This phenomenon was further explored by performing an autocorrelation on the mean response levels for each stimulus during asymptote (stimuli 13 through 60). Only asymptote data was used, since this made the analysis simpler. An autocorrelation done on the raw data indicated that there was some measure of cyclicity in the data. To make the period clearer, the data was smoothed, and the autocorrelation was recalculated (refer to Velleman & Hoaglin, 1981). The results are shown in Figure 10A.

As can be seen in this graph, any one observation is highly correlated with the one that immediately follows it (lag of 1), and, with each subsequent stimulus the correlation goes down until about seven or eight stimuli removed, where the correlation is near zero. Through the eight stimuli that follow this point (lag of 9 through 16) the correlation becomes more negative, before once again heading back toward zero. Thus, the full period describing these data appears to be about 16 stimuli long, with peak autocorrelation values ranging from 0.867 to

-0.418. The periodicity was also evident in a plot of these responses once they were smoothed (refer to Figure 10B).

Fig. 10. A) Autocorrelation values (between each response and those that follow it) for smoothed data from the last 48 responses during habituation of the 10-s ISI fixed-interval group ($n = 20$). The lag is the number of stimuli removed for which the correlation has been calculated. B) A plot of the smoothed data from the asymptotic habituation stimuli of the 10-s ISI fixed-interval group in Experiment 1. These data are based on the last 48 stimuli of habituation. Reversal response magnitude is expressed in terms of raw mm.

A**B**