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Neural and molecular mechanisms underlying behavioral state modulation in *C. elegans*

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by

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The text of Chapter II is a reprint of the material as it appears in L. E. Waggoner *et al*. "The effect of a neuropeptide gene on behavioral states in *Caenorhabditis elegans* egg-laying," Genetics (2000). Laura Anne Hardaker was the secondary investigator and co-author of this paper. She made Figures 1-2, 1-3b, 1-3c, 1-6a, 1-6c, and contributed data to Table 1-1.

The results presented in Chapter III are being revised for publication in a paper entitled "Serotonin modulates locomotory behavior and coordinates egg-laying and movement in *Caenorhabditis elegans,*" (Hardaker, L.A., Singer, E., Kerr, R., Zhou, G, and Schafer, W. R.) Laura Anne Hardaker was the primary investigator and author of this paper.

ABSTRACT OF THE THESIS

Neural and molecular mechanisms

underlying behavioral state modulation in *C. elegans*

by

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In order to understand the neural and molecular mechanisms underlying behavioral state modulation, and more specifically, the mechanisms through which the decision to execute a given motor program is influenced by sensory information and the activity of other motor pathways, I have studied how different neural circuits are coupled to the egg-laying circuitry in *Caenorhabditis elegans*. First, I analyzed how sensory input leads to motor output, by studying the molecular mechanisms by which food cues are transmitted to the egg-laying circuitry to modulate egg-laying behavior. I found evidence that the neuropeptides encoded by the gene *flp-1* are important neuromodulators of egg-laying, and that they may be released when the animal encounters food, acting in a hormonal mechanism to affect egg-laying behavior. Second, I investigated how one motor output affects another, by analyzing the correlation between egg-laying and locomotion. Here, I found evidence that the egg-laying circuitry back-modulates the brain to affect locomotion. Through these studies, I have gained important insights into how motor patterns can be regulated by sensory circuits, and how the activity of one motor pathway can influence another.

CHAPTER I INTRODUCTION

The study of behavior, how and why an organism behaves in a certain manner, has long been an interest in neurobiology. An animal must have mechanisms in place in order to respond to its environment and behave in a manner beneficial to its survival and the survival of its progeny. Many behaviors, ranging from feeding and locomotion in simple organisms, to sleep and mood in the more complex, involve fluctuations between discrete alternative behavioral states. At the most basic level, these various behavioral states result from differences in the functional properties of the neurons and muscle cells in the circuits that produce the behavior. The regulation of switching between these functional states occurs mainly by the action of molecules known as neuromodulators. An important goal of reductionist neuroscience is to understand how specific proteins act within the context of the neuronal circuitry to control an animal's behavior.

To understand a vertebrate nervous system at the molecular and cellular level is difficult due to the extreme complexity of vertebrate brains. However, for animals with less complex nervous systems, such as the free-living soil nematode *Caenorhabditis elegans*, understanding the molecular and cellular basis of behavior is a realizable goal.

C. elegans is particularly well-suited to genetic and molecular studies of nervous system function for a number of reasons: 1) it is a simple organism, with roughly 1000 cells, 2) it has a small genome, which has been mapped, 3) it has a short life cycle, about three days, 4) it is easily maintained in the laboratory, grown on agar plates seeded with bacteria, 5) it is a self-fertilizing hermaphrodite, although males can occur for mating, 6) there are only 302 neurons, all of which have known invariable locations and lineage [Sulston and Horvitz 1977; Sulston *et al.* 1983], 7) it is transparent, and since each neuron can be identified by position, it

is possible to infer the roles of individual neurons in nervous system function by cell-specific laser ablation, and determine the effect of the ablation on behavior [Bargmann and Avery 1995], 8) it uses a wide variety of neuromodulators [Rand and Nonet 1997]. Although a simple organism, *C. elegans* is capable of perceiving and responding to a wide range of environmental conditions, including heavy and light touch, temperature, volatile odorants, osmotic and ionic strength, food, and other nematodes. Each of these sensory modalities in turn regulates many aspects of the animal's behavior, including the rate and direction of movement, the rates of feeding, egg-laying, defecation, and the process of mating. *C. elegans* is amenable to classical, molecular, and developmental genetic studies; thus, isolation, phenotypic characterization, and molecular analysis of behavioral mutants provides a promising avenue toward identifying the molecular events that underlie the animal's behavior.

Using these genetic and cell biological approaches, it has been possible to obtain many important insights into the molecular and cellular basis of behavior. For example, studies of chemotaxis-defective and touch-insensitive mutants have provided important information about the molecular mechanisms underlying sensory transduction in olfactory and mechanosensory neurons. Genetics and cell ablation experiments have also provided detailed information about the molecular basis for several simple motor behaviors, including egg-laying, feeding and defecation, which involve regulation of the contractile properties of a single specialized muscle group. However, because genes and neurons that affect higher-level aspects of nervous system function tend to have only subtle effects on behavior, much less has been learned about the neural basis for more complex motor patterns such as those involved in locomotion. Likewise, the mechanisms through which the decision to execute a given motor program are influenced by sensory information and the activity of other motor pathways are not well understood even in this simple organism.

My lab studies the complex behavior of egg-laying in *C. elegans*, in order to discover the underlying molecular mechanisms of behavioral control. I will begin with a brief introduction to *C. elegans* egg-laying, and I will discuss how we analyze and quantify the behavior in the laboratory.

Egg-laying occurs when embryos are expelled from the uterus through the contraction of 16 vulval and uterine muscles [White *et al.* 1986]. In order to analyze this behavior, we have used an automated tracking system that is able to record an individual animal's egg-laying over a long period of time. By analyzing the recordings, we can determine the egg-laying pattern and calculate the parameters for egg-laying [Waggoner *et al.* 1998]. This is a quantitative way to assess egg-laying behavior.

In the presence of abundant food, wild-type animals lay eggs in a specific temporal pattern. There is a burst of egg-laying activity, called an active phase, followed by a period of inactivity, called an inactive phase. Both the onset of the active phase and egg-laying within the active phase are aperiodic and model closely as Poisson processes with distinct rate constants [Waggoner *et al.* 1998]. In this egg-laying pattern, animals fluctuate between discrete inactive, active, and egg-laying states. Although this egg-laying pattern, the neuronal circuitry, and pharmacology affecting egg-laying has already been discovered, there is still much to understand regarding how the animal controls egg-laying in response to sensory cues, as well as how egg-laying behavior may be correlated to other complex behaviors such as locomotion.

In this thesis, I have focussed on the following two questions:

1) How does sensory input lead to motor output?
2) How does one motor output affect another?

To address the first question, I have studied how food presence is relayed to the egg-laying circuitry. To address the second, I have studied how the behaviors of egg-laying and locomotion are correlated.

Through these studies, I have gained important insights into how the motor patterns of egg-laying are regulated by information sent from sensory circuits in the brain to the egg-laying neural circuitry, and how the egg-laying circuitry in turn back-modulates the brain to affect locomotion. The combination of these two processes may be beneficial to the nematode in a natural environment, as they ensure that eggs are laid in a disperse manner within regions of food availability.

CHAPTER II THE EFFECT OF A NEUROPEPTIDE GENE, *FLP-1*, ON EGG-LAYING IN *C. ELEGANS*

ABSTRACT

Egg-laying behavior in the nematode *Caenorhabditis elegans* involves fluctuation between alternative behavioral states: an inactive state, during which eggs are retained in the uterus, and an active state, during which eggs are laid in bursts. We have found that the *flp-1* gene, which encodes a group of structurally related neuropeptides, functions specifically to promote the switch from the inactive to the active egg-laying state. Recessive mutations in *flp-1* caused a significant increase in the duration of the inactive phase, yet egg-laying within the active phase was normal. This pattern resembled that previously observed in mutants defective in the biosynthesis of serotonin, a neuromodulator implicated in induction of the active phase. Although *flp-1* mutants were sensitive to stimulation of egg-laying by serotonin, the magnitude of their serotonin response was abnormally low. Thus, the *flp-1*-encoded peptides and serotonin function most likely in concert to facilitate the onset of the active egg-laying phase. Interestingly, we observed that *flp-1* is necessary for animals to down-regulate their rate of egg-laying in the absence of food. Since *flp-1* is known to be expressed in interneurons that are post-synaptic to a variety of chemosensory cells, the FLP-1 peptides may function to regulate the activity of the egg-laying circuitry in response to sensory cues.

INTRODUCTION

Many aspects of behavior, including mood, aggression, sleep, and sexual arousal, involve discrete, alternative behavioral states. At the most basic level, these different

behavioral states result from differences in the functional properties of the neurons and muscle cells in the circuits that produce the behavior. The regulation of switching between these functional states occurs largely through the action of molecules known as neuromodulators. In general, neuromodulators function by activating signaling pathways that regulate the activity of receptors and ion channels in excitable cells. A wide variety of molecules are known to function as neuromodulators, including biogenic amines (e.g. dopamine, serotonin, norepinephrine, and histamine), adenosine, glutamate, acetylcholine (through their action at muscarinic receptors), and a diverse array of neuropeptides. Identification of the mechanisms by which neuromodulators influence behavior at the molecular, cellular, and circuit levels is essential for understanding the function of both simple and complex nervous systems.

We have employed a genetic approach to a simple animal, the nematode *Caenorhabditis elegans,* to investigate the molecular mechanisms by which neuromodulators control behavioral states. *C. elegans* is particularly well-suited to molecular studies of nervous system function. It has a simple nervous system consisting of 302 neurons, and the position, cell lineage, and synaptic connectivity of each of these neurons is precisely known [White *et al.* 1986; Sulston and Horvitz 1977; Sulston *et al.* 1983]. Because a particular neuron can be positively identified based on its position, it is possible to evaluate the function of an individual neuron or group of neurons through single cell laser ablation [Bargmann and Avery 1995]. Moreover, because of their short generation time, small genome size, and accessibility to germline transformation, these animals are highly amenable to molecular and classical genetics [Wood 1988]. Thus, in *C. elegans*, it is relatively easy to identify genes involved in specific behaviors, and to characterize the functions of their products using molecular, behavioral, and immunocytochemical analyses. Although its nervous system contains relatively few neurons, *C. elegans* makes use of a surprisingly broad array of neuromodulators,

including a large number of putative neuropeptides and the biogenic amines serotonin, dopamine, and octopamine [Rand and Nonet 1997]. In addition, it exhibits a number of easily assayed and quantifiable behaviors that are affected by a wide range of neuroactive substances [Rand and Johnson 1995]. For these reasons, it is an excellent model organism for studying the molecular and cellular basis of neuromodulator action.

The largest class of neuromodulators in *C. elegans* are the FMRFamide-related peptides, or FaRPs. These peptides are characterized by a common carboxy-terminal motif of Arg-Phe-NH_2, and generally range in size from 4-20 amino acids. FaRPs are the predominant family of neuropeptides in invertebrates, where they have been shown to play a role in cardioregulation, learning, and the modulation of muscle contraction [Maule *et al.* 1996]. FaRPs have also been identified in vertebrates, where they have been implicated in the regulation of pain responses [Yang *et al.* 1985]. In *C. elegans*, immunocytochemical experiments have shown that at least 30 neurons and gland cells contain peptides which have the C-terminal RFamide epitope characteristic of the FaRP family [Schinkmann and Li 1992]. The *C. elegans* genomic sequence contains at least 20 genes (designated *flp*- genes, for FMRFamide-like peptide deficient) which encode predicted polypeptide precursors of more than 50 distinct FaRPs [Nelson *et al.* 1998a]. Most of these *flp* genes have subsequently been shown to be expressed in larval and/or adult *C. elegans*, and in some cases the predicted peptide products have actually been purified from *C. elegans* extracts. At present, only one *flp* gene, *flp-1*, has been extensively characterized at the molecular and genetic level [Rosoff *et al.* 1992; Nelson *et al.* 1998b]. *flp-1* is expressed in a specific subset of head neurons, and encodes two alternatively spliced products that can be processed to give rise to seven closely related FaRPs. *flp-1* loss-of-function mutants exhibit a number of behavioral abnormalities, including hyperactive locomotion, nose touch insensitivity, and defective osmotic avoidance [Nelson *et al* 1998b].

Although many questions remain concerning the molecular and cellular mechanisms through which the *flp-1* encoded peptides influence these behaviors, it is clear that the peptides encoded by *flp-1* have many specific effects on behavior which are at least partially distinct from the other *C. elegans* FaRPs.

In this study, we have investigated the role of *flp-1* in another behavior --egg-laying. *C. elegans* hermaphrodites are self-fertile, and continuously produce embryos and retain them in their uterus for at least two days following the adult molt. Egg-laying in *C. elegans* occurs when embryos are expelled from the uterus through the contraction of 16 vulval and uterine muscles [White *et al.* 1986]. In the presence of abundant food, wild-type animals lay eggs in a specific temporal pattern: egg-laying events (i.e., contractions of the egg-laying muscles leading to the expulsion of one or more eggs) tend to be clustered in short bursts, or active phases, which are separated by longer inactive phases during which eggs are retained. Both the onset of the active phase and egg-laying within the active phase are aperiodic, and model closely as Poisson processes with distinct rate constants (Figure 1a-c). This egg-laying pattern can be accurately modeled as a three-state probabilistic process, in which animals fluctuate between discrete inactive, active, and egg-laying states [Waggoner *et al.* 1998]. This process has three parameters: the rate constant for the duration of the inactive phase (λ_2), the rate constant for egg-laying within the active phase (λ_1), and the probability of remaining in the active phase after an egg-laying event (p). Using a maximum-likelihood algorithm, it is possible to estimate these egg-laying parameters from real behavioral data, and thereby compare the egg-laying patterns of wild-type and mutant strains [Zhou *et al.* 1997].

Genetic, pharmacological, and cell ablation studies have provided important insight into the roles of particular neurons and neurotransmitters in the control of egg-laying [Horvitz *et al.*

1982; Trent *et al.* 1983; Weinshenker *et al.* 1995]. Two classes of motorneurons make extensive synapses with the vulval muscles: the 2 HSNs and 6 VCs, each of which expresses multiple neurotransmitters and neuromodulators. For example, the HSNs express serotonin, acetylcholine, and one or more FaRPs, while the VCs express acetylcholine, FaRPs, and possibly a biogenic amine [Desai *et al.* 1988; Schinkmann and Li 1992; Rand and Nonet 1997]. Both serotonin and acetylcholine have been shown pharmacologically to increase the overall rate of egg-laying [Trent *et al* 1983; Weinshenker *et al.* 1995]. By characterizing the egg-laying patterns of mutant and ablated animals, it has been possible to distinguish neurons and genes that modulate the switching between behavioral states from those that promote egg-laying within the active phase. For example, in both HSN-ablated animals and serotonin-deficient mutants, the inactive egg-laying phase is abnormally long, whereas egg-laying within the active phase is unimpaired [Waggoner *et al.* 1998]. Thus, serotonin released from the HSNs apparently stimulates egg-laying by facilitating the switch from the inactive to the active egg-laying state. Similar experiments have implicated acetylcholine, released from both the HSNs and VCs, in the induction of egg-laying events within the active phase [Waggoner *et al.* 1998].

In this study, we show that the peptides produced by the *flp-1* gene function in the regulation of egg-laying behavior. Specifically, the *flp-1* encoded peptides appear to promote the onset of the active phase of egg-laying, an activity that is at least partially independent of the HSN motorneurons. In addition, we provide evidence that these peptides may participate in the regulation of egg-laying by sensory cues.

RESULTS

flp-1 affects the transition between behavioral states involved in egg-laying

To test the possible involvement of FLP-1-encoded peptides in the modulation of egg-laying behavior, we analyzed the egg-laying patterns of *flp-1* mutants. We first analyzed the egg-laying patterns of mutants carrying recessive loss-of-function mutations in the *flp-1* gene. These animals were not grossly defective in the ability to lay eggs, and their egg-laying patterns were qualitatively similar to wild-type animals: egg-laying events were still clustered in active phases, and both the switch into the active phase and the laying of eggs within the active phase still modeled as Poisson processes. However, the duration of the inactive phase was substantially longer in the *flp-1* mutants (λ_2 for *flp-1(yn2)* and *flp-1(yn4)* were 4.7 and 7.0 x 10^{-4} s^{-1}, respectively) than in wild-type animals (λ_2 was 1.4 x 10^{-3} s^{-1}; Table 1-1) (Figure 1-2a, b). In contrast, egg-laying within the active phase was unimpaired in *flp-1* mutants; in fact, the intra-cluster time constants were actually faster in the *flp-1(yn2)* and *flp-1(yn4)* deletion mutants than in wild-type (Table 1-1, Figure 1-2a). Thus, loss of *flp-1* function appeared to specifically decrease the probability of switching from the inactive to the active phase of egg-laying, suggesting that the function of the wild-type *flp-1* gene products is to promote the onset of the active egg-laying state.

flp-1 and serotonin function in concert to promote the active egg-laying phase

The egg-laying defect exhibited by *flp-1* mutants--longer-than normal inactive phase but rapid egg-laying within the active phase--was quantitatively and qualitatively similar to the defect seen in serotonin-deficient mutants. In principle, these two modulators could function in a common biological pathway, or they could affect distinct parallel pathways. To examine these possibilities, we constructed a double mutant carrying loss-of-function mutations in both

flp-1 and *cat-4*, a gene required for serotonin biosynthesis [Loer and Kenyon 1993]. Simple measurements of egg-laying rates indicated that the severity of the egg-laying defect in the double mutant was comparable to that of *flp-1* or *cat-4* single mutants (Figure 1-3a). Moreover, both the inactive phase rate constant λ_2 and the inter-cluster time constant of the double mutant were essentially identical to those of either single mutant (Table 1-1). These results supported the hypothesis that serotonin and *flp-1* most likely function in the same pathway to induce the active egg-laying state.

To further explore the relationship between the effects of these two modulators on the regulation of egg-laying, we assayed the responses of *flp-1* mutants to exogenous serotonin. We first measured the ability of serotonin to stimulate egg-laying under conditions which are normally inhibitory for egg-laying (i.e., in the hypertonic salt solution M9). In this assay, we observed that *flp-1* loss-of function mutants were still responsive to serotonin (Figure 1-3b). Moreover, the serotonin sensitivity of *flp-1* mutants, as measured by the concentration of serotonin that gave half-maximal stimulation, was comparable to that of wild-type animals. However, the magnitude of their response (i.e., the number of eggs laid when stimulated by serotonin) was reduced relative to wild-type. Thus, while a functional *flp-1* gene did not appear to be essential for serotonin to stimulate egg-laying, neither was exogenous serotonin able to completely bypass the effect of *flp-1* on egg-laying. To further investigate the effect of *flp-1* mutations on the egg-laying response to serotonin, we analyzed the egg-laying patterns of wild-type and *flp-1* mutants in the presence of exogenous serotonin. In wild-type animals, treatment with serotonin not only increased rate of egg-laying, it also caused eggs to be laid in a monophasic pattern resembling a simple Poisson process. This pattern implied that in the presence of exogenous serotonin, wild-type animals were mostly in the active egg-laying phase. In *flp-1* mutants, we observed that serotonin treatment affected egg-laying behavior in

a similar manner; eggs were laid at a higher rate and in a more monophasic pattern in the presence of serotonin than on drug-free medium, though the rate of egg-laying in the presence of serotonin was slower in one of the *flp-1* mutants than that in wild-type (Figure 1-3c). Taken together, these experiments led us to conclude that *flp-1* is not necessary for the stimulation of egg-laying by serotonin, though a functional *flp-1* product is required for a maximal serotonin response.

HSN-independence of the *flp-1* mutant phenotype

The serotonergic neurons most strongly implicated in the control of egg-laying are the HSN motorneurons. Although the HSNs themselves do not express *flp-1*, a number of *flp-1* expressing cells in the head lie in close proximity to, and in some cases actually make synapses with, the dendrite of the HSN in the nerve ring. This raised the possibility that the effects of *flp-1*-encoded peptides might be mediated through modulation of the HSNs. Alternatively, given the small size of *C. elegans*, it was also possible that *flp-1* could regulate egg-laying through a neuroendocrine mechanism that bypassed the HSNs. To distinguish between these models, we tested the effect of a cell-specific ablation of the HSNs on the egg-laying behavior of *flp-1* mutants. We observed that HSN-ablated *flp-1* animals were no more severely egg-laying defective than HSN-ablated wild-type animals: both their overall egg-laying rates and their egg-laying patterns were essentially identical (Figure 1-4a). This result was consistent with our earlier observations indicating that serotonin, a neuromodulator known to be released from the HSNs, affects the same aspect of egg-laying behavior as *flp-1*. However, when we analyzed the behavior of HSN-ablated *flp-1* animals in the presence of exogenous serotonin, we found that they laid eggs significantly more slowly than HSN-ablated wild-type animals under the same condition (Figure 1-4b). Thus, the ability of *flp-1* to potentiate the stimulation of egg-laying by serotonin did not appear to require the HSNs.

Thus, the effects of the FLP-1 peptides on egg-laying, in particular their ability to facilitate egg-laying in response to serotonin, were at least partially independent of the HSNs.

flp-1 is necessary for regulation of egg-laying by food signals

What functional role might the FLP-1 peptides play in the control of egg-laying behavior? Among the neurons that express *flp-1* are several pairs of interneurons, which are major recipients of synaptic input from sensory cells and have been implicated in processing and relaying integrated sensory information to motor circuits. The expression of *flp-1* within these cells raised the possibility that *flp-1*-encoded peptides might be involved in the regulation of egg-laying behavior by sensory cues. Egg-laying is affected by a number of environmental conditions, including the presence or absence of a bacterial food source. To determine if *flp-1* affects the regulation of egg-laying by food, we tested the effects of *flp-1* loss-of-function mutations on the ability of animals to control their egg-laying rate in response to the presence or absence of a bacterial lawn. We observed that wild-type animals maintained on agar plates seeded with *E. coli* laid eggs at a significantly higher rate than animals maintained on agar plates that lacked food. Strikingly, however, we observed that *flp-1* loss-of-function mutants laid eggs at essentially the same rate in the presence of a bacterial lawn as in the absence of a lawn (Figure 1-5). This defect in food regulation of egg-laying was fairly specific to *flp-1* mutants, and was not merely a consequence of their general egg-laying defect. For example, egg-laying behavior in *egl-1* mutants, which lacked HSN neurons as a result of inappropriate programmed cell death, was strongly regulated by food, even though their egg-laying rate and pattern in the presence of food was similar to that of *flp-1* mutants. Thus, *flp-1* appeared to function specifically in mediating the control of egg-laying behavior in response to the availability of food.

goa-1 functions independently from _flp-1_ and serotonin in the control of egg-laying

What genes might function downstream of *flp-1* and serotonin in the control of egg-laying? One possible candidate is *goa-1*, which encodes a G_o homologue that has been hypothesized to mediate the effects of serotonin [Segalat *et al.* 1995] and the FLP-1 peptides [Nelson *et al.* 1998b] on locomotion. *goa-1* is expressed in the egg-laying neurons as well as the vulval muscles, and mutations in *goa-1* have been shown to enhance (in the case of recessive alleles) or inhibit (in the case of dominant gain-of-function alleles) egg-laying behavior [Mendel *et al.* 1995; Segalat *et al.* 1995]. Thus, the *goa-1* gene product was a plausible candidate for a gene that might function downstream of *flp-1* and/or serotonin as a negative regulator of egg-laying.

To investigate this possibility, we analyzed the egg-laying patterns of *goa-1* mutants. We observed that the inactive phase was substantially shorter in *goa-1* recessive mutant animals than in wild-type (Table 1-1; see also Figure 1-6a, b), implicating GOA-1 as a negative regulator of the switch into the active phase. Since this effect was roughly converse to the effect of mutations in *flp-1* and *cat-4* , one possible interpretation of this result was that GOA-1 activity might be negatively regulated by serotonin and/or *flp-1*. Alternatively, *goa-1* could function independently from, and antagonistically to the pathway(s) activated by serotonin and *flp-1*. To distinguish these possibilities, we constructed double mutants carrying recessive mutations in *goa-1* and either *flp-1* or *cat-4* and analyzed their egg-laying behavior. In each case, the double mutant showed a phenotype intermediate between that of the two single mutants (Figure 1-6a, b; Table 1-1). For example, in the case of *flp-1*, both the inactive phase rate constant λ_2 (.0014 s^{-1}) and the inter-cluster time constant (1950 s) for the *goa-1*; *flp-1* double mutant were intermediate between those of the *goa-1* single mutant (.0031 s^{-1}; 890 s)

and the *flp-1* single mutant (.0005; 3840 s). Similarly, both λ_2 and the inter-cluster time constant for the *cat-4*; *goa-1* double mutant were intermediate between the *goa-1* single mutant and the *cat-4* single mutant. Pharmacological experiments also supported the hypothesis that serotonin, *flp-1* and *goa-1* functioned independently. For example, *goa-1* loss-of function mutants responded to serotonin at abnormally low threshold concentrations, an effect that was suppressed by mutations in *flp-1* (Figure 1-6c). Together, these results suggested that *goa-1* defined a new pathway, independent of the ones activated by *flp-1* and serotonin, regulating the switch into the active egg-laying phase.

Pharmacological experiments have suggested that GOA-1 functions in both neurons and muscle cells to inhibit egg-laying [Mendel *et al.* 1995]. In principle, GOA-1 might control the onset of the active egg-laying phase by negatively regulating the activity of the HSNs; alternatively, it might negatively regulate the response of the vulval muscles to modulatory inputs from neurons. To distinguish between these models, we analyzed the egg-laying behavior of HSN-ablated *goa-1* mutant animals. Surprisingly, we observed that the inactive phase in HSN-ablated *goa-1* mutants was no shorter than in HSN-ablated wild-type animals (Figure 1-6d; Table 1-1). This indicated that the shortening of the inactive phase by *goa-1* mutations was dependent on the HSNs, and suggested that GOA-1 controls the switch into the active phase by directly or indirectly modulating HSN function. HSN-ablation was not completely epistatic to mutations in *goa-1*; the number of eggs laid within a given active phase (a function of the clustering parameter p) was higher in HSN-ablated *goa-1* mutants than in HSN-ablated wild-type (Table 1-1). Since *goa-1* recessive mutants appeared to have longer active phases, this implied that the function of GOA-1 in the vulval muscles may be to promote the switch from the active egg-laying phase back to the inactive phase.

DISCUSSION

Modulation of egg-laying behavioral states by FaRPs

Egg-laying behavior involves switching between two alternative behavioral states: an active state, during which eggs are laid in bursts, and an inactive phase, during which eggs are retained in the uterus. We observed that loss-of-function mutants defective in the gene *flp-1*, which encodes a set of FMRFamide-related peptides, displayed a specific abnormality in their temporal pattern of egg-laying: the inactive phase was abnormally long, whereas egg-laying within the active phase was normal. Thus, the *flp-1* gene products appeared to function specifically to facilitate the switch from the inactive to the active egg-laying phase. Previous work had shown that the serotonergic HSN motorneurons also were specifically required to promote the onset of the active phase [Waggoner *et al.* 1998]. Nonetheless, at least some of the effects of *flp-1* on egg-laying appeared to be HSN-independent: *flp-1* mutant animals whose HSNs had been eliminated through laser ablation had more severe egg-laying defects, and responded less strongly to serotonin, than HSN-ablated wild-type animals. These results were perhaps surprising, since all the chains of synaptic connections between the *flp-1*-expressing neurons in the head and the vulval muscles involve the HSNs [White *et al* 1986]. In fact, *flp-1* mutations only slightly enhanced the egg-laying defect of HSN-ablated animals, suggesting that some of the effects of the FLP-1 peptides are likely to be HSN-dependent. Thus, the FLP-1 peptides may regulate the egg-laying muscles both through modulation of the HSNs as well as through an HSN-independent humoral mechanism (Figure 1-7).

Many questions remain about the cellular mechanism through which FLP-1 peptides regulate egg-laying. *flp-1* expression has been detected in a number of neurons in the head, including AIA, AIY, AVA, AVK, AVE, RIG, and RMG [Nelson *et al.* 1998b]. Based on the

results presented here, the simplest hypothesis is that humoral release of FLP-1 peptides from one or more of these neuronal classes modulates the egg-laying muscles directly. Alternatively, it is possible that some or all of the effects of the FLP-1 peptides on egg-laying could be indirect. For example, FLP-1 peptides could modulate the activity of other neurosecretory cells in the head, affecting the release of a hypothetical neurohormone that modulates the egg-laying muscles. Some of the effects of *flp-1* on egg-laying might also involve the VC neurons, although the fact that *flp-1* mutations lengthen the inactive phase much more than ablations of the VCs do [Waggoner *et al.* 1998] argues that the VCs are not the primary target of the FLP-1 peptides. Laser ablations of various combinations of *flp-1*-expressing neurons, as well as neurons postsynaptic to these cells, may provide more detailed information about the cellular basis for the effect of *flp-1* on egg-laying behavior.

Interactions between *flp-1* and serotonin in the control of egg-laying

The effects of *flp-1* on egg-laying are quite similar to the effects of another neuromodulator, serotonin. We observed here that loss of *flp-1* function did not confer resistance to the effects of serotonin on egg-laying, though it did significantly reduce the magnitude of the serotonin response. Therefore, the *flp-1*-encoded peptides appear to stimulate egg-laying at least in part by enhancing the response of the egg-laying muscles to serotonin. This hypothesis is consistent with previously published work, which demonstrated that synthetic peptides identical to the shared carboxy-terminus of the 7 *flp-1* peptides (FLRF-NH_2) increased the average number of eggs laid in response to serotonin [Schinkmann and Li 1992]. Serotonin and FLP-1 appear to function synergistically not only in the stimulation of egg-laying, but in other *C. elegans* behaviors as well. For example, both serotonin and FLP-1 inhibit locomotion, and the ability of serotonin to inhibit movement has been shown to require a functional *flp-1* gene [Nelson *et al.* 1998b]. Thus, for locomotive as well FLP-1 peptides

appear to be necessary to potentiate the effects of serotonin on locomotion. Although the molecular pathways through which serotonin and *flp-1* control egg-laying are likely to differ in some respects from those involved in locomotion, it is tempting to speculate that the parallel actions of these two modulators on these two different behaviors might depend on a conserved molecular mechanism. Since co-modulation of neuromuscular activity by biogenic amines and FaRPs is observed in many organisms [Scott *et al.* 1997; Klein *et al.* 1986], the molecular interactions between the *flp-1* and serotonin-activated signaling pathways in the egg-laying cells may provide a useful model for similar processes in other animals.

Insights into the regulation of egg-laying by sensory information

The analysis of the *flp-1* mutants also revealed a role for the FLP-1 peptides in the control of egg-laying behavior by sensory cues. We observed that whereas wild-type worms laid eggs at a much slower rate in the absence of a bacterial food source, *flp-1* loss-of-function mutants laid eggs at the same rate in either presence or absence of food. This insensitivity to the presence of bacteria was not merely a consequence of the *flp-1* animals' generally slower egg-laying rate, as other egg-laying defective animals (e.g., *egl-1* mutants which lacked the HSN motorneurons) still showed significant regulation of egg-laying by food availability. Therefore, the *flp-1*-encoded peptides may be specifically dedicated to relaying signals of food abundance to the egg-laying circuit. In the absence of bacteria, levels of FLP-1 release could be low, leading to long inactive phases and slow egg-laying, whereas abundant food would lead to increased FLP-1 release and more active egg-laying. Other aspects of the *flp-1* mutant phenotype are consistent with FLP-1 functioning as an indicator of food availability. For example, when nematodes, maintained in the absence of food, encounter a lawn of bacteria, they slow their rate of movement [Sawin 1996]. Both the hyperactive locomotion and the "wandering" behavior previously noted in *flp-1* recessive mutants [Nelson *et al.* 1998b] could

plausibly stem from a defect in this response to food. Thus, *flp-1* may function quite generally to facilitate a variety of behavioral patterns that are appropriate for conditions of food abundance.

The expression pattern of *flp-1* in the *C. elegans* nervous system is well suited for a gene that encodes a food signal. The presence of bacteria in the environment is thought to be detected primarily through olfactory or chemosensory cues [Bargmann and Mori 1997]. The primary route through which nematodes gather chemosensory information is by using a pair of polymodal sense organs known as amphids. Synaptic output from the amphid sensory neurons is relayed to four pairs of amphid interneurons: AIA, AIB, AIY and AIZ [White *et al.* 1986]. In thermotaxis behavior, the amphid interneurons have been shown to be an important site for integrating and processing sensory information that is used to modulate behavioral outputs [Mori and Ohshima 1995]. Both AIA and AIY express *flp-1*; thus a simple model for how egg-laying behavior could be controlled by food signals is that under conditions favorable to egg-laying (i.e., abundant food), the AIA and AIY neurons release FLP-1 peptides, switching the animal into the active egg-laying state. This release of FLP-1 peptides from the amphid interneurons could likewise switch the animal into a more inactive state with respect to locomotion**.**

Evidence that *goa-1* modulates neural states involved in egg-laying behavior

In addition to the pathways activated by FLP-1 and serotonin, a third pathway, defined genetically by the *goa-1* gene, also appears to regulate the onset of the active phase of egg-laying, in a manner antagonistic to and apparently independent of both *flp-1* and serotonin. A recessive mutation in *goa-1*, which encodes the *C. elegans* homologue of the G_o alpha subunit [Mendel *et al.* 1995; Segalat *et al.* 1995], increased the rate of egg-laying by shortening the

inactive phase. Genetic analysis indicated that *goa-1* probably functions to regulate egg-laying in a pathway distinct from the ones activated by *flp-1* and serotonin. Interestingly, the effect of *goa-1* on the onset of the active phase appeared to be completely dependent on the HSNs. Thus, *goa-1* may function by negatively regulating release from the HSNs of a neuromodulator that facilitates the switch from the inactive to the active egg-laying state.

An interesting implication of this hypothesis is that the behavioral states involved in egg-laying may correspond not only to functional states of the egg-laying muscles themselves, but also to distinct functional states of neurons (such as the HSNs) dedicated to egg-laying. Our earlier studies led to the hypothesis that the active egg-laying state depends on a functional activation of the vulval muscles, which allows the excitatory transmitter acetylcholine to readily induce muscle contraction. Our analysis of *goa-1* mutants suggests that the active and inactive egg-laying states may also correspond to functional states of the HSNs--an inactive state in which the HSNs release neurotransmitter with low probability, and an active state in which the probability of neurotransmitter release is high. According to this model, activated GOA-1 may inhibit the switch of the HSNs into this active state; thus, when GOA-1 is inactive or absent, the switch into the active state becomes more frequent. The likely involvement of GOA-1 in controlling HSN activity implies that additional neuromodulators, possibly released from neurons in the head, may regulate egg-laying behavior by controlling GOA-1 activity.

Regulation of egg-laying muscle activity by multiple neuromodulators

Although the effect of *goa-1* on the switch into the active phase was dependent on the HSNs, it was not completely dependent on serotonin. These observations imply that the HSNs contain another neuromodulator, whose release may be regulated by GOA-1, that facilitates

the switch into the active phase. Although at present we can only speculate as to the identity of such a molecule, there appear to be a number of candidates. For example, the HSNs almost certainly contain one or more non-*flp-1* encoded FaRPs, since they contain FaRP immunoreactivity which is not eliminated by deletions of the *flp-1* gene [Schinkmann and Li 1992; Nelson *et al.* 1998b]. In addition, pharmacological experiments indicate that muscarinic acetylcholine agonists stimulate egg-laying [Weinshenker *et al.* 1995]. Since the HSNs are cholinergic, acetylcholine released from the HSNs might modulate egg-laying through muscarinic receptors. In principle, GOA-1 regulated release of any of these molecules could facilitate the onset of the active egg-laying state. The diversity of neurotransmitter usage in the HSNs is also a hallmark of other neurons and gland cells that participate in the control of egg-laying. For example, non-*flp-1*-encoded FaRPs are present in both the VC motorneurons and the uv1 uterine gland cells [Schinkmann and Li 1992]. In addition, the VCs contain acetylcholine and, since they express the vesicular monoamine transporter, possibly an unidentified biogenic amine as well [Duerr *et al.* 1999]. Thus, egg-laying behavior is likely to be regulated by a surprisingly diverse array of neurotransmitters and neuromodulators, which are likely to activate complex, interacting signaling pathways in the vulval muscle cells. The elucidation of these signaling mechanisms represents an important challenge for future studies, and may be a useful model for the functional interaction of neuromodulatory pathways in other organisms.

METHODS

Strains and Genetic Methods

Routine culturing of *Caenorhabditis elegans* was performed as described [Brenner 1974]. The chromosomal locations of the genes studied in these experiments are as follows: LGI: *goa-1;* LGIV: *flp-1 ;* LGV: *cat-4, egl-1*. Unless otherwise indicated, all mutant strains are in the N2 genetic background. Behavioral assays were performed at room temperature (approximately 22^{o} C). Serotonin (creatinine sulfate complex) was obtained from Sigma. The *flp-1(yn2)* allele was chosen for use in double mutant constructions because its phenotype and behavior in genetic crosses suggests that it causes a more severe loss of gene function [Nelson *et al.* 1998b]. *goa-1(n1134)* was used for behavioral analysis and double mutant construction because it encodes a putatively non-functional product, and because the near sterility conferred by the *n363* deficiency allele (brood size is approximately 35 [Segalat *et al.* 1995]) makes embryo production rather than egg-laying muscle contraction limiting for egg-laying in *n363* mutant animals.

Egg-laying Assays

Unless otherwise stated, nematodes were grown and assayed at room temperature on standard NGM seeded with *E. coli* strain OP50 as a food source. For dose response experiments, individual young, gravid hermaphrodites were placed in microtiter wells containing liquid M9 and the indicated concentration of drug. After a 1 hour incubation at room temperature, the eggs laid by each animal were counted. Experiments measuring egg-laying rate on standard growth medium were performed as described above, and after 1 hour, the eggs laid by each animals were counted. Plates in which the animal had crawled off the agar surface before the end of the assay period were not included in the analysis.

Egg-laying behavior of individual animals on solid media (NGM agar) was recorded for 4-8 hours as described [Waggoner *et al.* 1998] using an automated tracking system. For tracking experiments on serotonin, 5-hydroxytryptamine (creatinine sulfate complex, Sigma) was added to NGM agar at 7.5 mM. Our tracking system was unable to record the behavior of animals on plates lacking a bacterial lawn, since the animals were prone to crawl to the edge of the plate where our system could not follow them.

Analysis of egg-laying patterns

Intervals between egg-laying events were determined from analysis of videotapes obtained using the automated tracking system. Quantitative analysis of the egg-laying pattern using this interval data was performed as described [Zhou *et al.* 1997]. Briefly, egg-laying events in *C. elegans* are clustered, with periods of active egg-laying, or active phases, separated by long inactive phases during which eggs are retained. Both the duration of the inactive phases ("inter-cluster intervals") and the duration of intervals between egg-laying events in a cluster ("intra-cluster intervals") model as exponential random variables with different time constants [Waggoner *et al.* 1998]. Thus, the probability density function for the intervals between events is

$$f_X(x) = k_1\ \lambda_1\ e^{-\lambda_1 x} + k_2\ (p\lambda_2)\ e^{-(p\lambda_2)x}, \qquad x \geq 0,$$

$$k_1 = \frac{p(\lambda_1 - \lambda_2)}{\lambda_1 - p\lambda_2}, \qquad k_2 = \frac{\lambda_1(1-p)}{\lambda_1 - p\lambda_2}.$$

, where the intra-cluster time constant is $1/\lambda_1$ and the inter-cluster time constant is $1/p\lambda_2$. The parameters were determined using the maximum likelihood estimation technique described previously [Zhou *et al.* 1997]. The expected variance of estimated parameters and time constants was determined by generating 100 independent sets of simulated egg-laying data using the model probability

density function, and computing the standard deviation of the parameters estimated from these simulations. All data in Table 1-1 were obtained and analyzed in this manner.

For analysis of egg-laying patterns on serotonin (Table 1-2), a single exponential time constant was estimated using a weighted least-squares linear regression to the log tail distribution [Waggoner *et al.* 1998]. The expected variance of these time constants was determined by generating 100 independent sets of simulated egg-laying data using a simple exponential probability density function, and by computing the standard deviation of the parameters estimated from these simulations.

Construction of Double Mutant Strains

For *flp-1; cat-4* double mutants, *cat-4* and *flp-1* single mutants were mated, and double mutant progeny were identified in the F2 generation by scoring for bleach sensitivity (*cat-4*[Loer 1995]) and the presence of a diagnostic PCR product using sequence specific primers (*flp-1* [Nelson *et al.* 1998]). For the *goa-1; cat-4* and *goa-1; flp-1* double mutants, single mutants were crossed as above, and *goa-1* homozygotes were identified in the F2 generation as hyperactive, egg-laying constitutive animals. These were picked individually and then allowed to self-fertilize; those F2s that were heterozygous for *cat-4* or *flp-1* segregated double mutant progeny that could be identified using the bleach or PCR assays described above.

This chapter is, in full, a reprint of material as it appears in L. E. Waggoner et al. (1999) “The effect of a neuropeptide, *flp-1*, on behavioral states in C. elegans egg-laying” (in press). The thesis author was the secondary researcher and the co-author of this paper. She made Figures 1-2, 1-3b, 1-3c, 1-6a, 1-6c, and contributed data to Table 1-1.

Table 1-1: Egg-laying behavior of mutant and ablated animals

Animal type (#, hrs, intervals)	p	λ_1 (s^{-1})	λ_2 ($s^{-1} \times 10^{-3}$)	Intra-cluster time constant ($1/\lambda_1$; s)	Inter-cluster time constant ($1/p\lambda_2$; s)
N2	0.537	0.055	1.4	18	1340
(12, 61, 305)	±0.031	±0.005	±0.18	±2	±140
flp-1(yn2)	0.554	0..079	0.47	13	3820**
(6, 37, 50)	±0.086	±0.022	±0.20	±3	±1170
flp-1(yn4)	0.651	0.090	0.75	11	2050*
(8, 37, 131)	±0.049	±0.016	±0.20	±2	±460
cat-4(e1141)	0.609	0.023	0.36	43	4550**
(12, 52, 63)	±0.073	±0.007	±0.13	±9	±1240
flp-1(yn2); cat-4(e1141)	0.772	0.063	0.30	16	4320
(7, 35, 60)	±0.058	±0.014	±0.15	±3	±1520
goa-1(n1134)	0.357	0.028	3.1	36	890**
(3, 27, 98)	±.055	±.009	±0.81	±16	±160
goa-1(n1134); flp-1(yn2)	0.377	0.174	1.4	6	1950*
(6, 23, 66)	±0.069	±0.138	±0.38	±2	±420
goa-1(n1134); cat-4(e1141)	0.515	0.141	1.4	7	1380*
(6, 32, 101)	±0.056	±0.038	±0.30	±1	±270
N2; HSN-ablated	0.534	0.125	0.55	8	3400**
(9, 44, 66)	±0.069	±0.019	±0.16	±2	±900
flp-1(yn2); HSN-ablated	0.382	0.041	1.1	25	2440
(5, 54, 77)	±0.068	±0.018	±0.29	±8	±440
goa-1(n1134); HSN-ablated	0.720	0.072	0.27	14	5140
(4, 24, 51)	±0.062	±0.020	±0.10	±3	±2060

*/** Long intervals (> 120s) statistically different either from wild-type (for single mutants) or from both constituent single mutants (for double mutants) according to the Mann-Whitney rank test. * indicates significance at .05>p>.01; ** indicates significance at p < .01 or less.

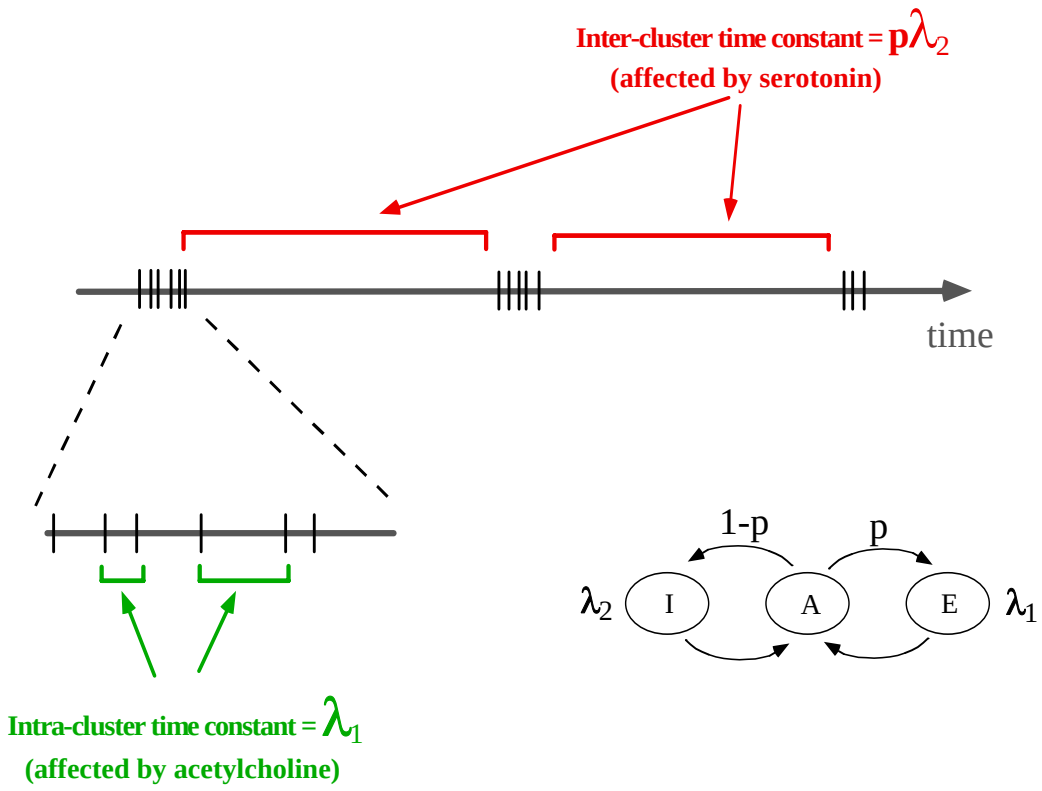


Figure 1-1a. Temporal pattern of egg-laying. A more detailed description of the egg-laying pattern of *C. elegans* and its analysis is described in [Waggoner *et al.* 1998]. Shown is a representative egg-laying pattern, with egg-laying events indicated by hash marks. According to the model, the animal can exist in one of three states: an inactive state (I), an active state (A), and an egg-laying state (E). Eggs are laid upon entry into the E state. λ_1 is the rate constant for the egg-laying state, λ_2 is the rate constant for the inactive state, and p is the probability that after a given egg-laying event, another egg will be laid before the animal enters the inactive phase. Short intervals (resulting from a single visit to the A state) are governed by the time constant $1/\lambda_1$. Long intervals result from one or more visits to the I (inactive) state (i.e., from E to A to I to A to E, or from E to A to I to A to I to A to E, etc.); the time constant for these intervals is equal to $1/p\lambda_2$ (for proof, see [Zhou *et al.* 1997]).

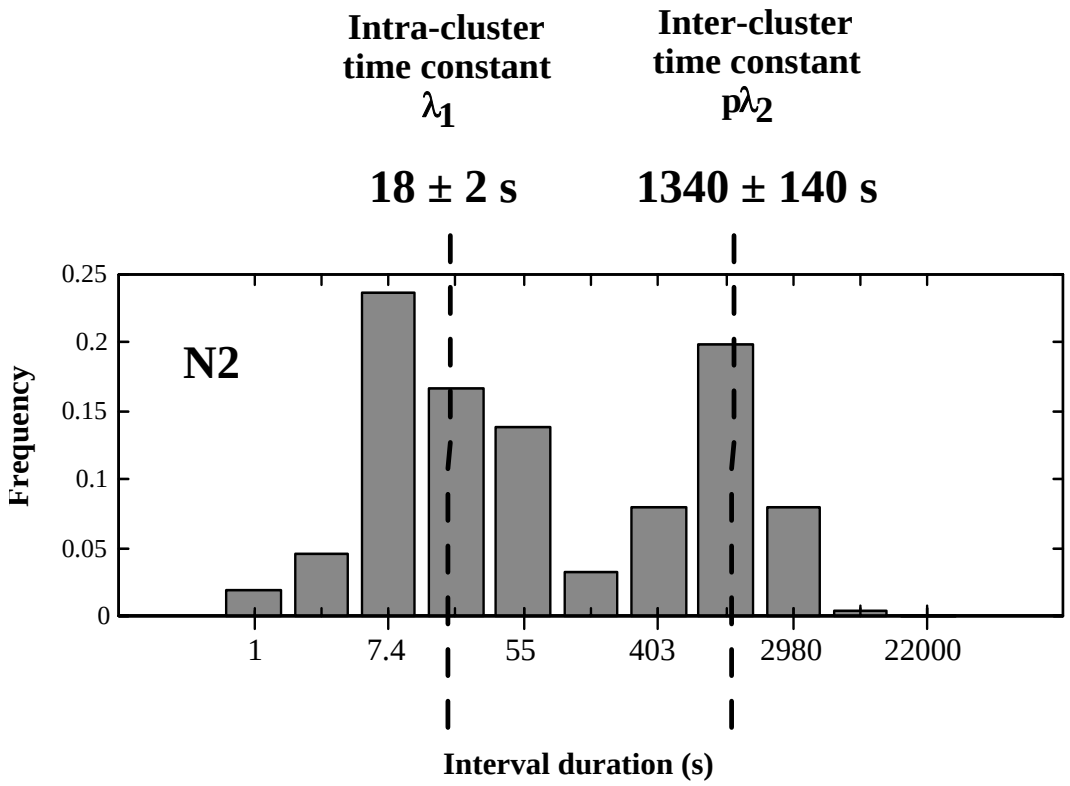


Figure 1-1b. Histogram of log interval times. Shown is a histogram of intervals between egg-laying events recorded from wild-type animals; the bins are on a log scale. According to the coupled-Poisson process model, the location of the two peaks corresponds to the intra-cluster and inter-cluster time constants. Values for these time constants were estimated using maximum likelihood analysis (see Methods).

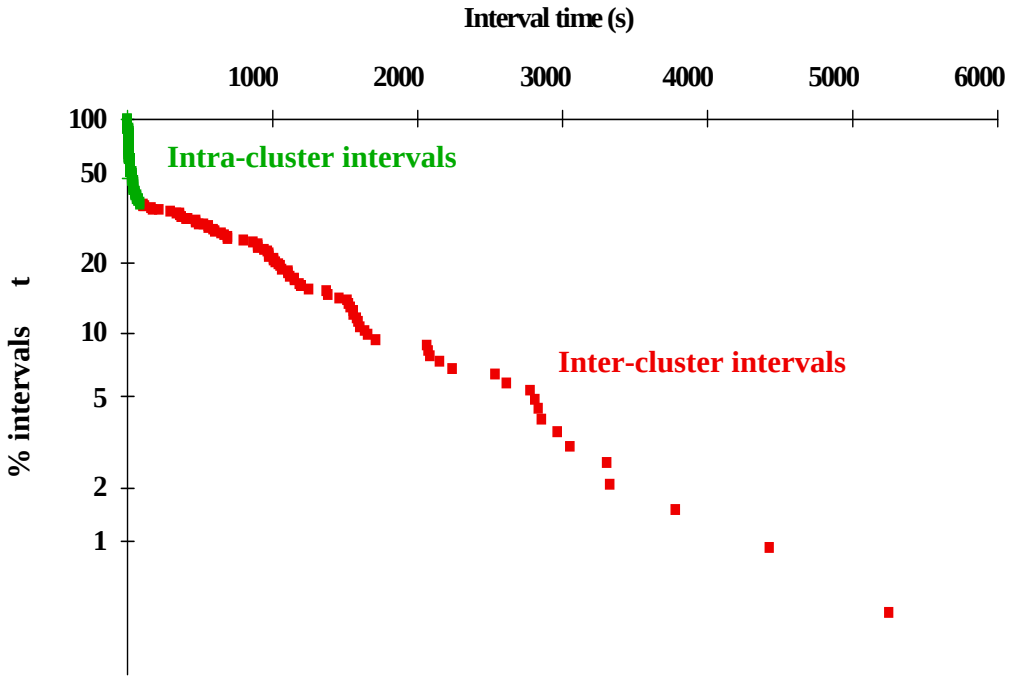
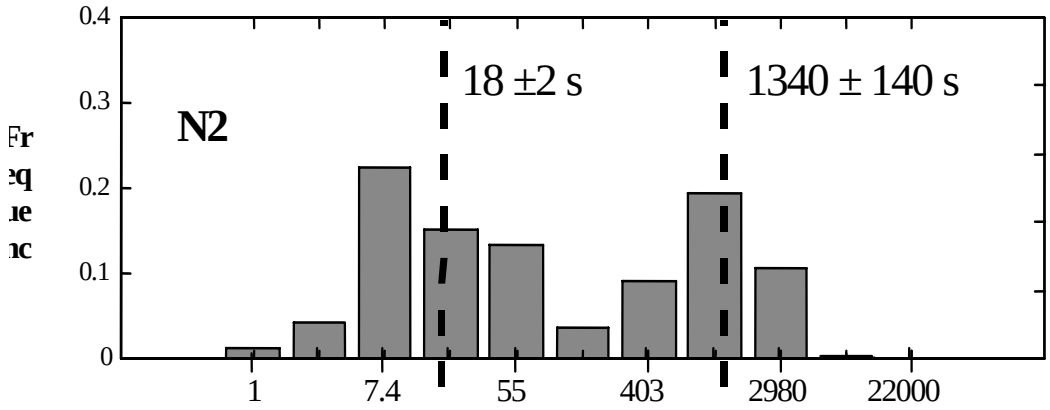
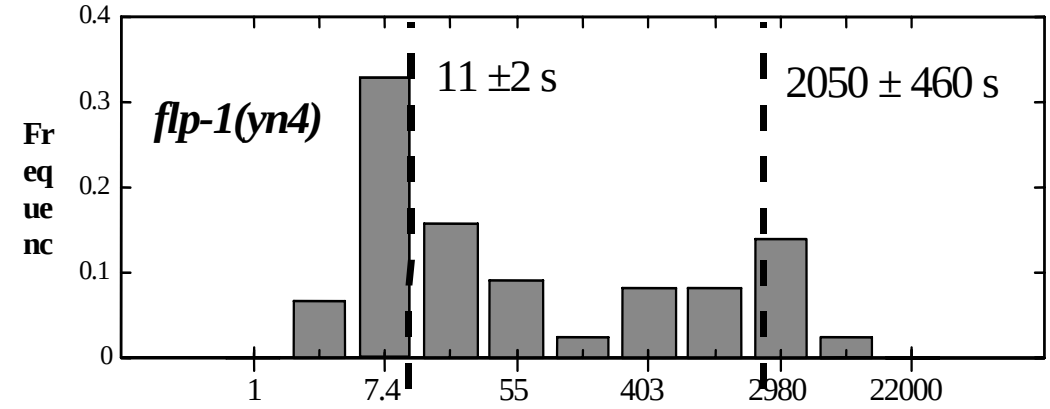
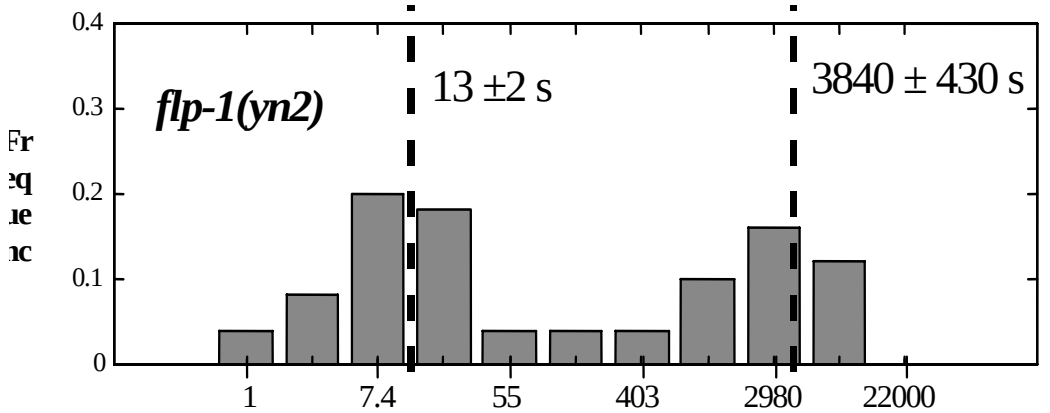


Figure 1-1c. Log-tail distribution of egg-laying intervals. The distribution of egg-laying intervals from wild-type animals is shown. The model predicts that this distribution should be biphasic, with the steep part of the curve corresponding to the intra-cluster intervals and the more gradual part to the inter-cluster intervals. The slope of the long intervals (i.e., the right part of the curve) is equal to the inter-cluster rate constant.

Figure 1-2a. Effect of *flp-1* recessive mutations on the pattern of egg-laying. *flp-1* loss-of-function mutations increase the duration of the inactive phase. For these and other tracking experiments, the numbers of animals tracked, hours observed, and intervals analyzed, along with the estimated model egg-laying parameters, are in Table 1-1**.** Shown is a histogram of intervals between egg-laying events (log scale); intra-cluster and intra-cluster time constants estimated using maximum likelihood analysis are indicated. Both the *yn2* and *yn4* alleles of *flp-1* specifically lengthen the inter-cluster constant (indicated by the location of the right-most peak). The long intervals (>120 s) were significantly longer in *yn2* ($p < .001$) and *yn4* ($p < .05$) according to the Mann-Whitney rank sum test.







Interval duration (s)

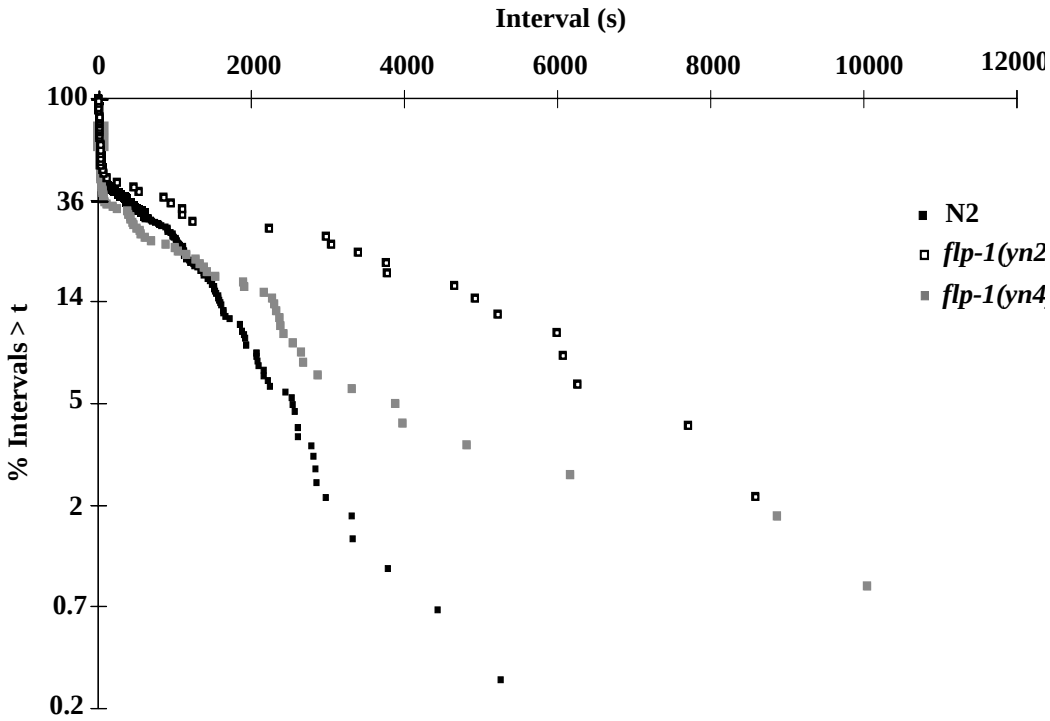


Figure 1-2b. Effect of *flp-1* recessive mutations on inactive phase duration. Shown are the log tail distributions of egg-laying intervals for wild-type and *flp-1* mutant animals. The more gradual slopes of the *yn2* and *yn4* curves indicate longer inter-cluster time constants for these mutants.

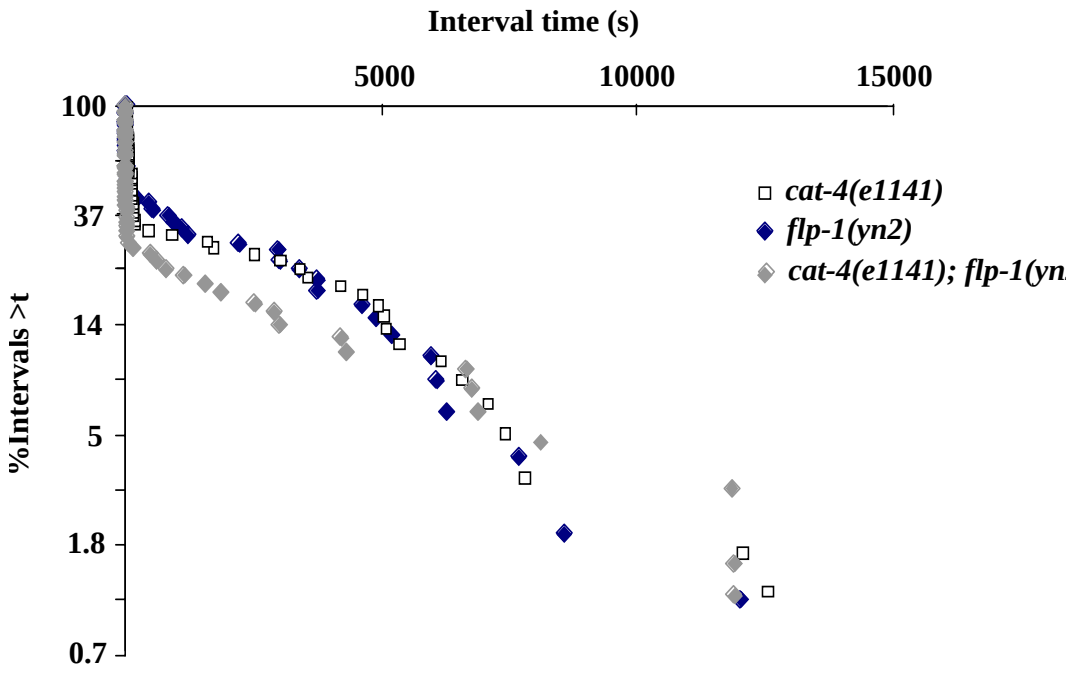


Figure 1-3a. Egg-laying pattern of serotonin-deficient *flp-1* animals. Shown are log tail distributions of egg-laying interval times for *flp-1*, *cat-4* , and *flp-1; cat-4* double mutant animals. The inter-cluster time constants (indicated by the curve tails) were essentially identical (see Table 1-1). Egg-laying rates for each strain were: *flp-1* = 1.94 ± 0.50 (n=6), *cat-4* = 2.35 ± 0.40 (n=12) , *flp-1; cat-4* = 3.03 ± 0.26 (n=7).

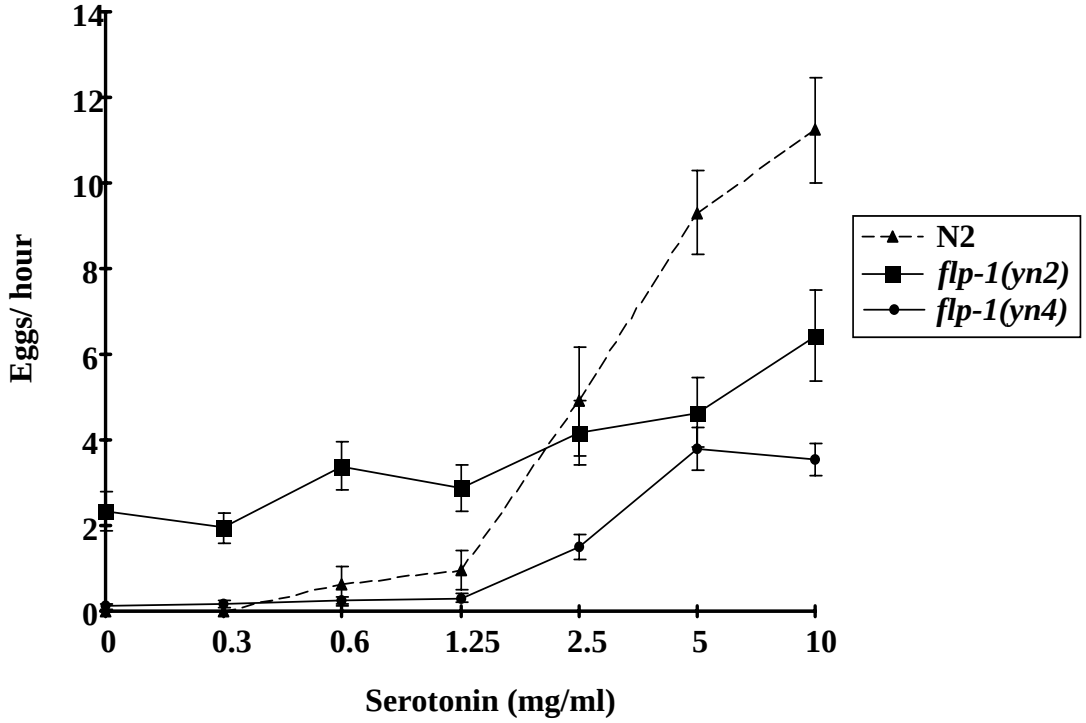
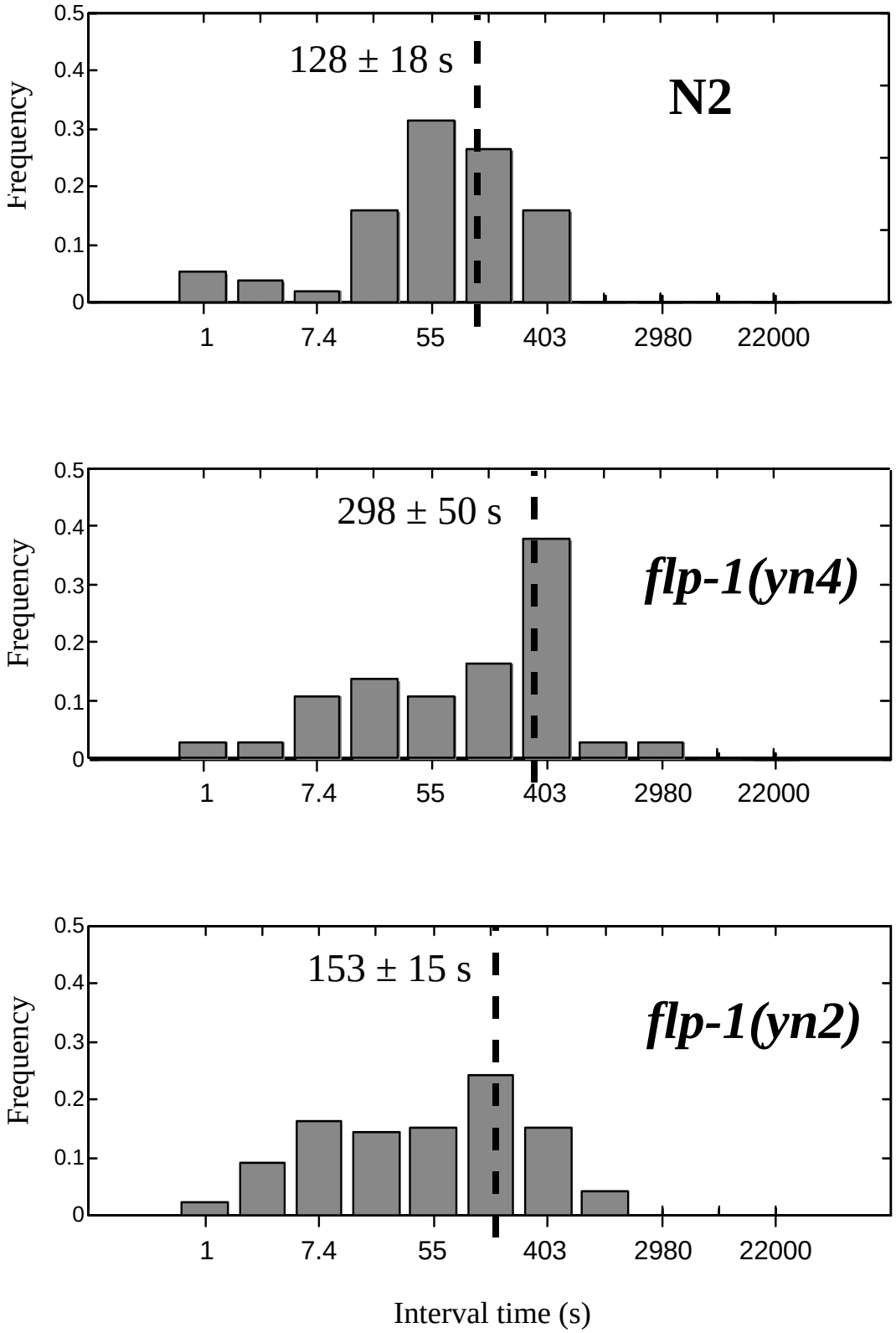


Figure 1-3b. Serotonin response of *flp-1* mutants. Egg-laying responses to serotonin were determined for wild-type and *flp-1* mutants by placing individual animals in liquid M9 at the indicated concentration of serotonin, and counting the number of eggs laid by each animal after 1 hour. Individual points and error bars indicate the mean and SEM of the following numbers of trials: N2= 20, *yn2*= 58, *yn4*= 45. For both *flp-1* mutants, the number of eggs laid at 10 mg/ml was significantly higher than in the no drug control ($p < .001$), and significantly lower than in wild-type at the same concentration ($p< .002$) according to the Mann-Whitney rank sum test.

Figure 1-3c. Egg-laying patterns of *flp-1* mutants on serotonin.

The histograms show the distribution of interval times for wild-type and *flp-1* mutants on 7.5 mM serotonin; bins are on a log scale. A single exponential time constant for each data set (indicated by the dashed line) was estimated as described in Methods. Intervals for *flp-1(yn4)* were significantly ($p < .05$) longer than for wild-type according to the Mann-Whitney rank sum test.


128 ± 18 s
N2
298 ± 50 s
flp-1(yn4)
153 ± 15 s
flp-1(yn2)
Frequency
0.5
0.4
0.3
0.2
0.1
0
1
7.4
55
403
2980
22000
Interval time (s)

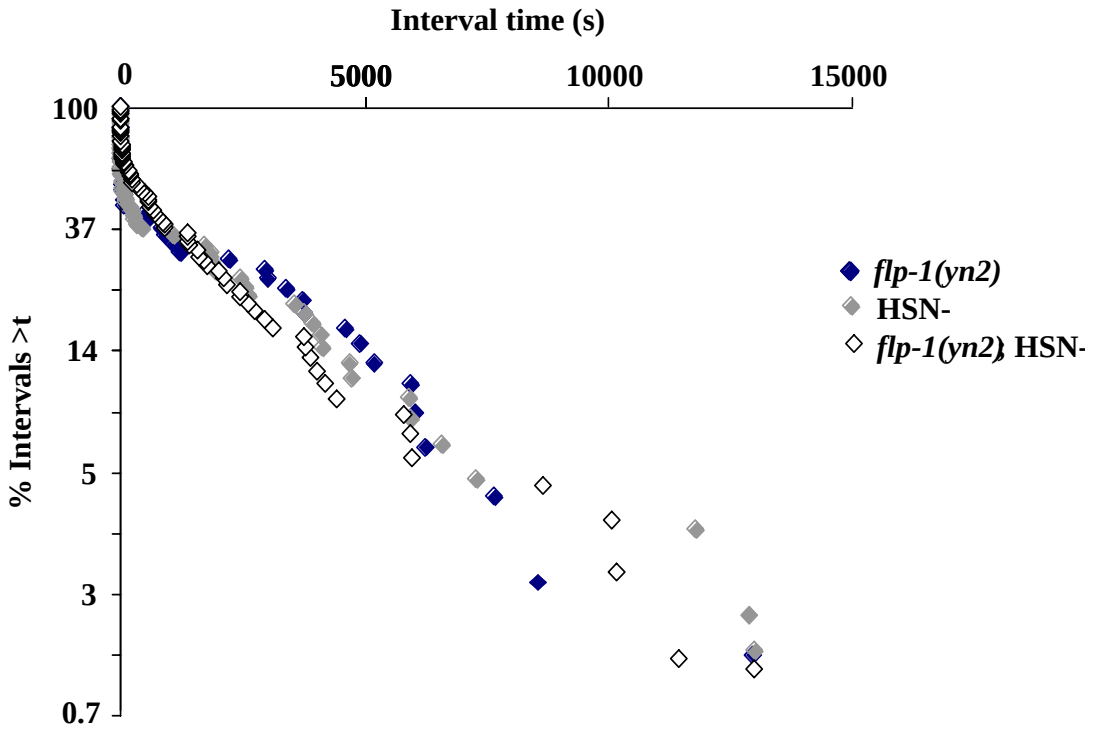


Figure 1-4a. Egg-laying pattern of HSN-ablated *flp-1* animals. Shown are the log tail distributions of egg-laying interval times for HSN-ablated wild-type and *flp*- mutant animals. The inter-cluster time constant (indicated by the slope of the curve tails) was no higher in the ablated mutant animals than in ablated wild-type or unablated mutant animals (see Table 1-1). Egg-laying rates were also measured independently on NGM agar by counting eggs laid by individual animals in an hour; mean and SEM of these experiments were (in eggs/hr): HSN-ablated wild-type= 1.35 ± 0.25 (n=4), unablated *flp-1*= 1.94 ± 0.50 (n=6), HSN-ablated *flp-1*= 1.05± 0.41 (n=11).

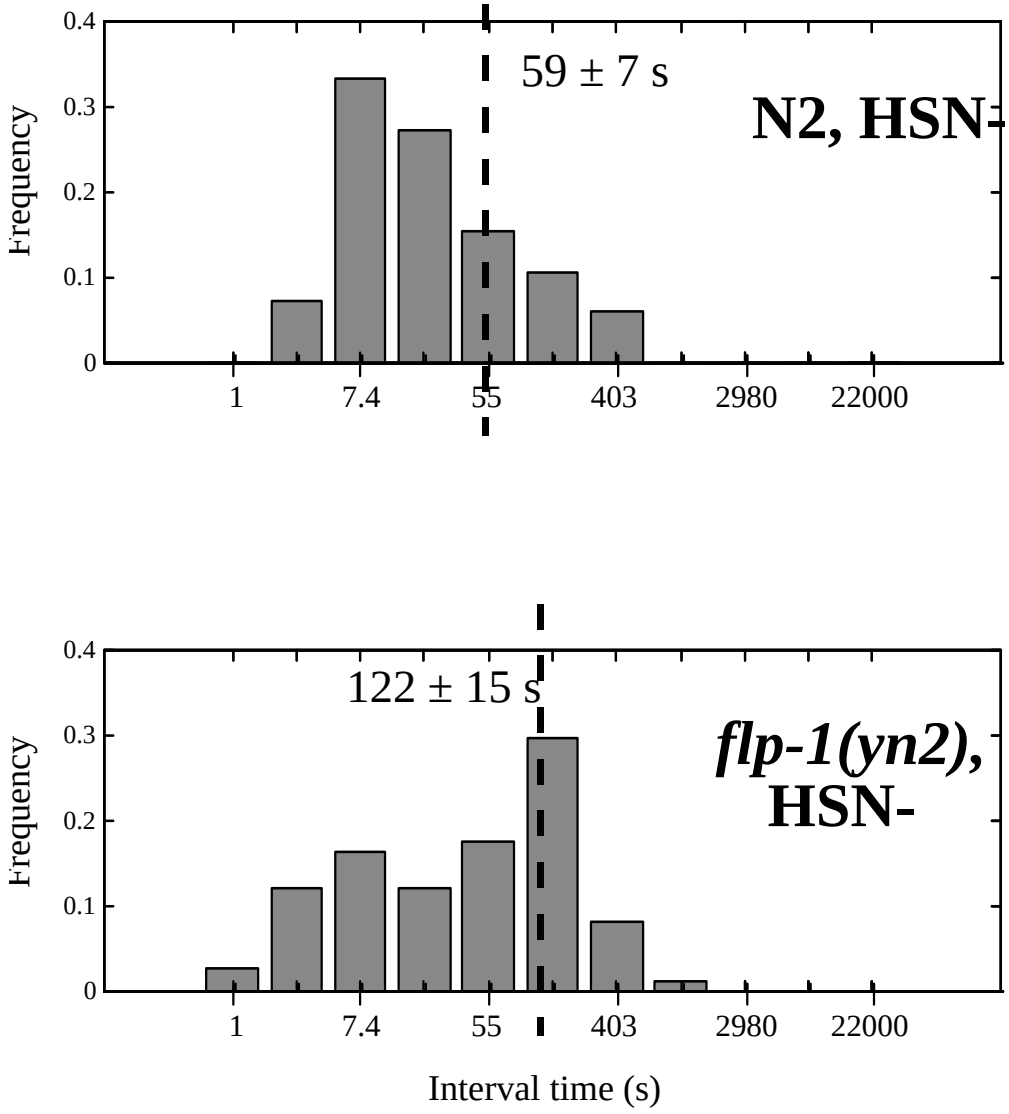


Figure 1-4b. Egg-laying pattern of HSN-ablated *flp-1* mutants on serotonin. The histograms show the distribution of interval times for HSN-ablated wild-type and *flp-1* mutants on 7.5 mM serotonin; bins are on a log scale. A single exponential time constant for each data set was estimated as described in Methods. Intervals for HSN-ablated *flp-1(yn2)* were significantly ($p < .001$) longer than for HSN-ablated wild-type according to the Mann-Whitney rank sum test.

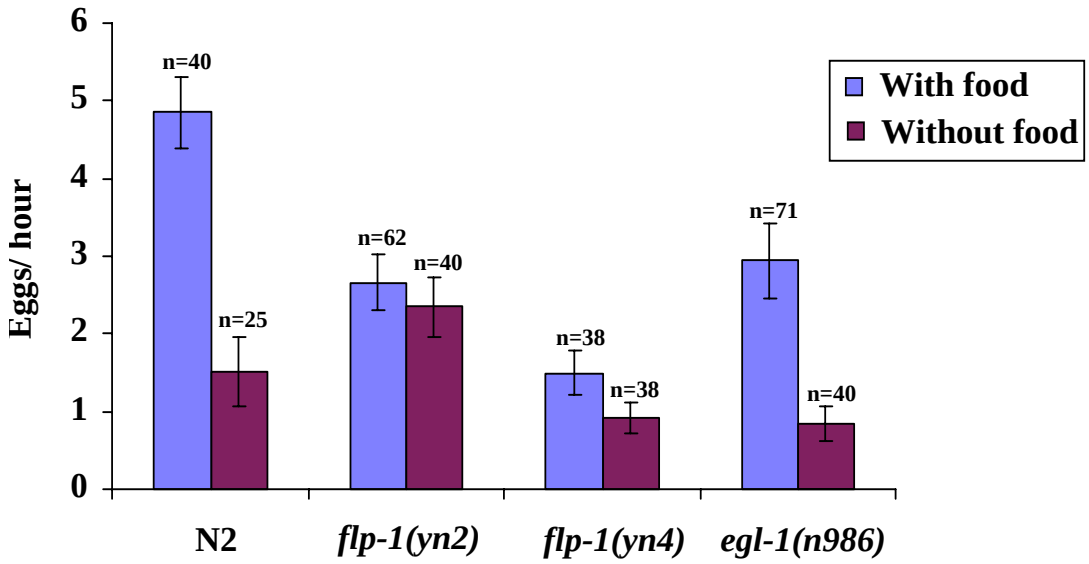


Figure 1-5. Effect of *flp-1* mutations on regulation of egg-laying by food. The histogram shows the mean egg-laying rates of wild-type, *egl-1* and *flp-1* mutant animals in the presence and absence of food. Error bars indicate the SEM; the number of independent trials under each condition are indicated. The wild-type and *egl-1* mutant strains showed a significantly lower ($p < .001$ for wild-type, $p < .02$ for *egl-1*) egg-laying rate in the absence of food according to the Mann-Whitney rank sum test. In contrast, there was no statistically significant difference between the egg-laying rates in the presence or absence of food for either *flp-1* mutant ($p > .5$ for *yn2*, $p > .2$ for *yn4*).

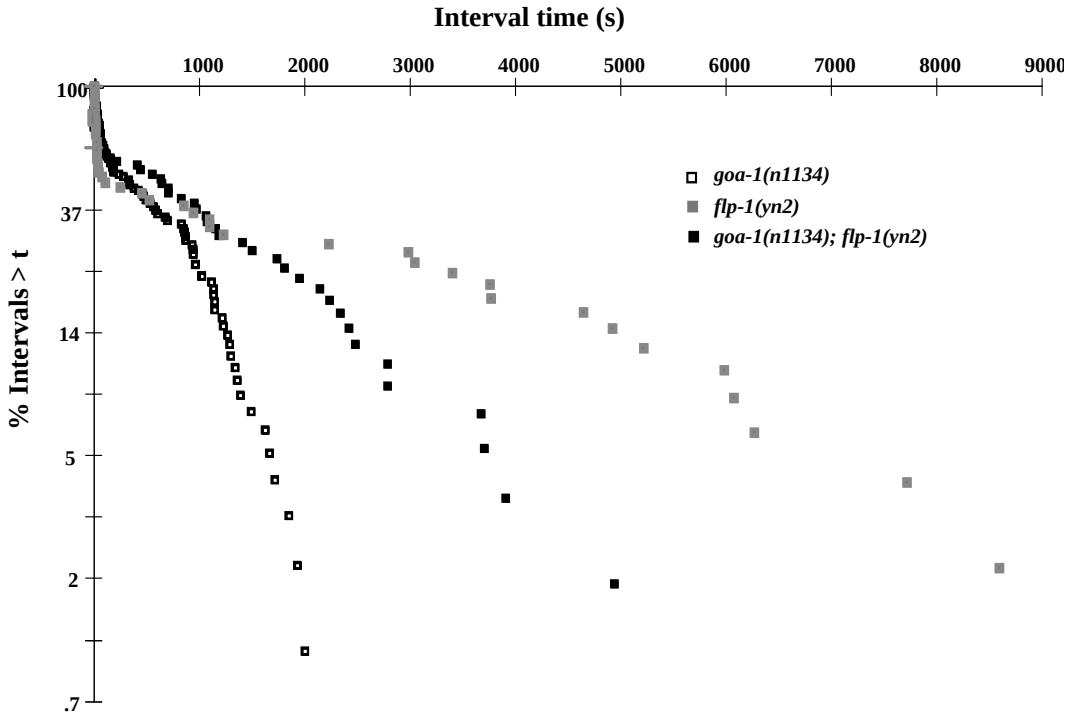


Figure 1-6a. Independence of *flp-1* and *goa-1* egg-laying phenotypes. Shown are the log tail distributions of egg-laying intervals for *flp-1* and *goa-1* mutant animals. The inter-cluster time constant of the *goa-1; flp-1* double mutant curve (indicated by the slope of the curve) is intermediate between that of the *flp-1* and *goa-1* single mutants (see Table 1-1). The long intervals (> 120s) in the double mutant were significantly shorter than in the *flp-1* single mutant ($p < .05$) and significantly longer than in the *goa-1* single mutant ($p < .002$) according to the Mann-Whitney rank sum test.

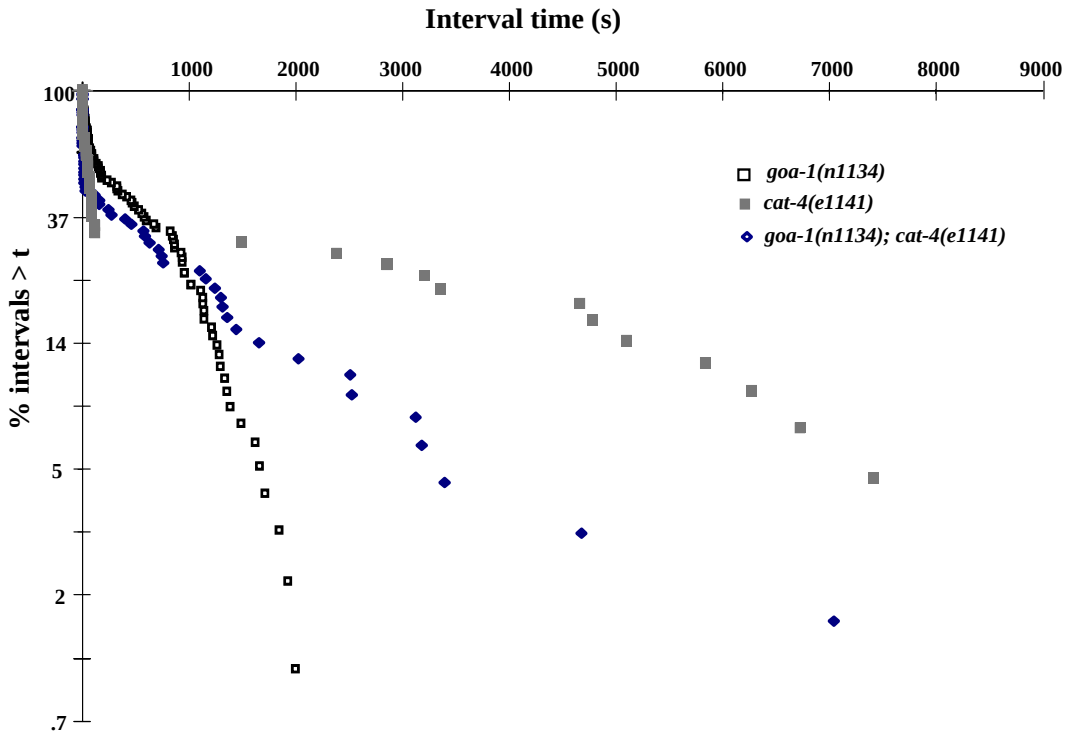


Figure 1-6b. Independence of *goa-1* and *cat-4* egg-laying phenotypes. Shown are the log tail distributions of egg-laying intervals for *cat-4* and *goa-1* mutant animals. The inter-cluster time constant of the *goa-1; cat-4* double mutant curve (indicated by the slope of the curve) is intermediate between that of the *cat-4* and *goa-1* single mutants. The long intervals (> 120s) in the double mutant were significantly shorter than in the *cat-4* single mutant ($p < .001$) and significantly longer than in the *goa-1* single mutant ($p < .02$) according to the Mann-Whitney rank sum test.

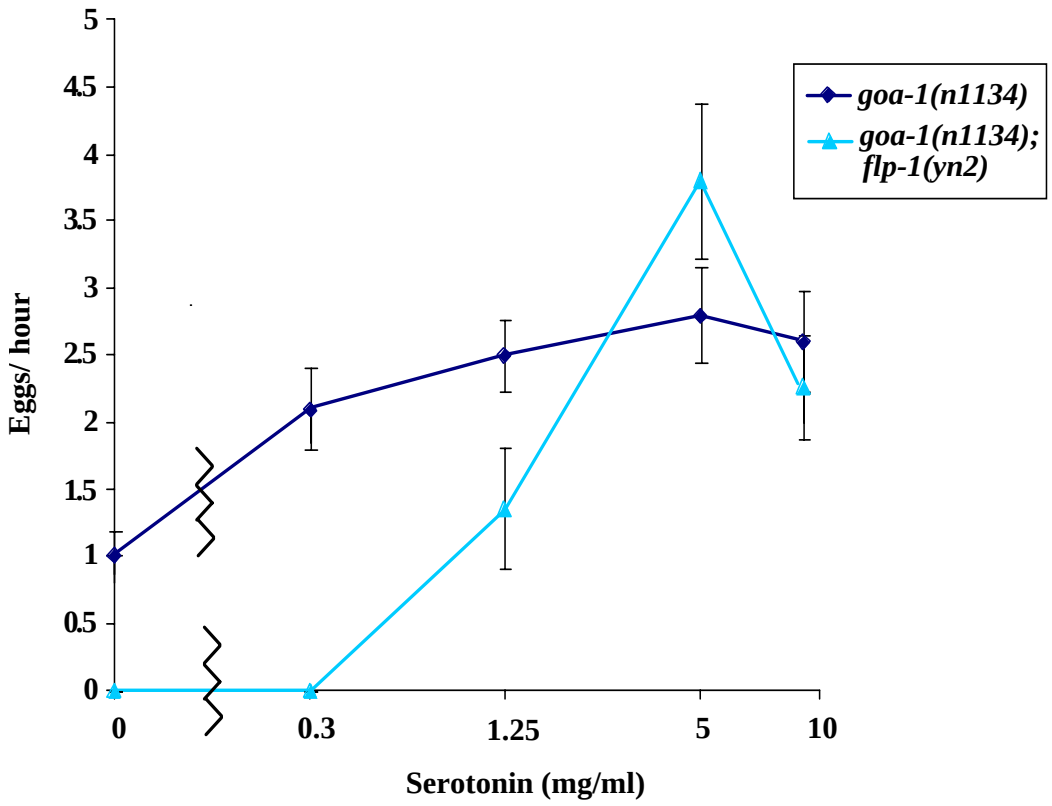


Figure 1-6c. Serotonin responses of *goa-1* mutants. Egg-laying responses to serotonin were determined for *goa-1* and *goa-1; flp-1* mutants as described in Figure 1-3. Individual points and error bars indicate the mean and SEM of the following numbers of trials: *goa-1(n1134)*= 40, *goa-1(n1134); flp-1(yn2)*= 25.

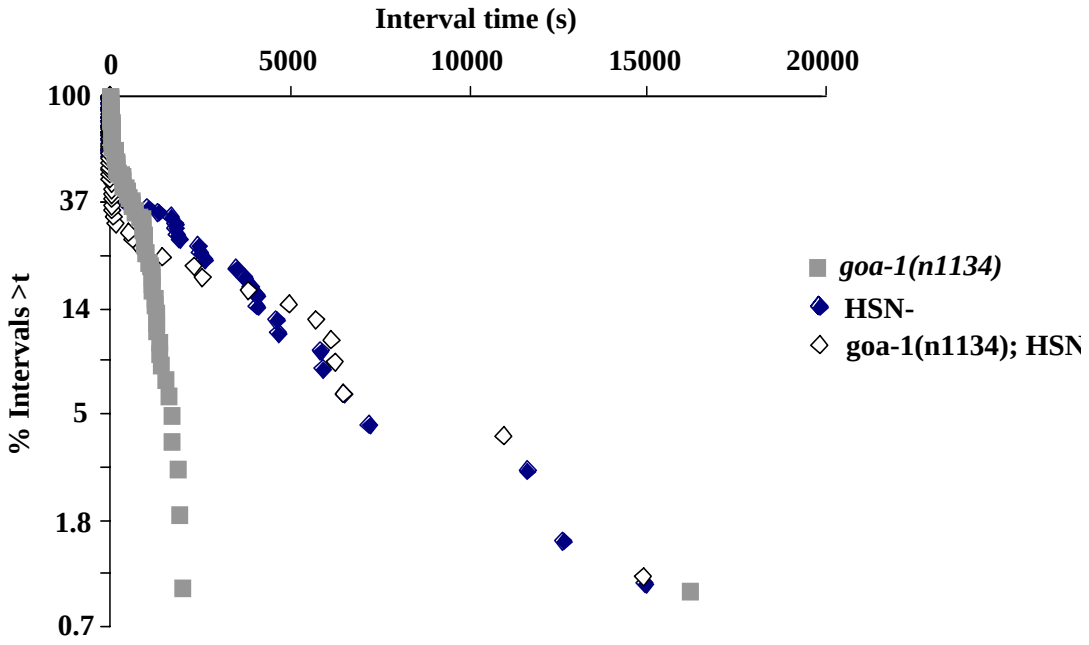


Figure 1-6d. HSN-dependence of the *goa-1* hyperactive egg-laying phenotype. Shown are the log tail distributions of egg-laying intervals for HSN-ablated and *goa-1* mutant animals. The inter-cluster time constant of the HSN-ablated *goa-1* mutant curve (indicated by the slope of the curve) is as long or longer than that of HSN-ablated wild-type.

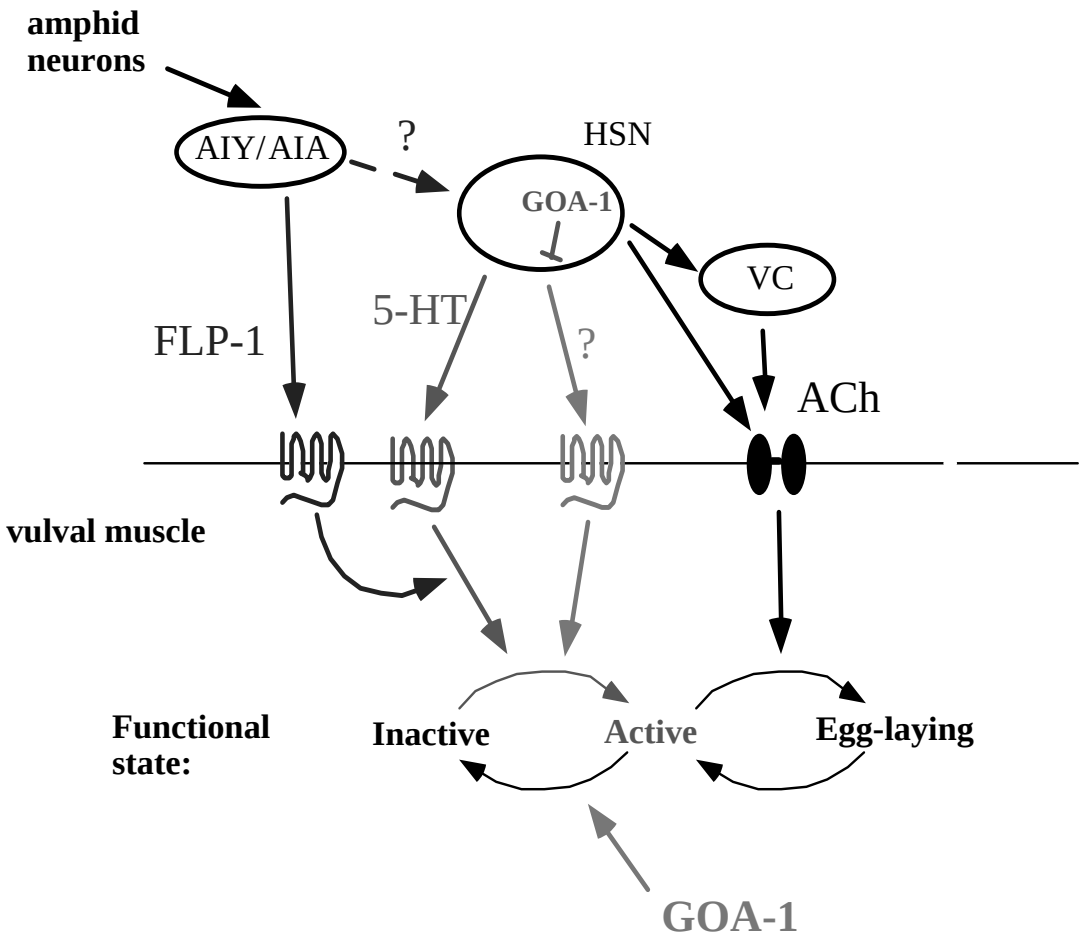


Figure 1-7. Model for neural and molecular regulation of egg-laying. Egg-laying in *C. elegans* is affected by at least two parameters: the rate of switching from the inactive to the active phase, and the rate of egg-laying within the active phase. FLP-1 peptides, released from neurons in the head, specifically regulate the switch into the active phase, a process also regulated by serotonin and possibly another neuromodulator whose release from the HSNs is controlled by GOA-1. The FLP-1 peptides may also modulate the activity of HSNs. The activity of the FLP-1 pathway may be sensitive to chemosensory cues indicating the abundance of food.

CHAPTER II APPENDIX EGG-LAYING BEHAVIORAL ANALYSIS OF TYPE II DAF-C MUTANTS

INTRODUCTION

Chapter Two showed that *flp-1* encoded peptides may function in the regulation of egg-laying by sensory cues. To summarize the model, food is sensed by the amphid chemosensory neurons, which send a signal to the AIA and AIY interneurons onto which they synapse. These neurons then release FLP-1 peptides which thereby modulate egg-laying. Although we believe that the effects of FLP-1 may be mediated, at least in part, by a hormonal mechanism, the exact cellular and molecular mechanisms through which they work is still unknown. We were interested in elucidating this pathway, and thus began looking for possible candidates. One possibility is that FLP-1 peptides are involved in the type II TGF-β pathway responsible for dauer formation.

Under unfavorable environmental conditions, *C. elegans* larvae can enter an alternative third-larval stage called the dauer larva. The dauer larva is developmentally arrested and is adapted for long term survival under harsh conditions [Cassada and Russell 1975]. It is thin and dense, as well as being resistant to detergent treatment. Although it does not feed, it survives at least four to eight times longer than the normal two-week life span of *C. elegans* [Klass and Hirsh 1976]. When environmental conditions improve, dauer larva will recover and resume its normal life cycle.

The decision whether to enter the dauer state is made during the very early larva stages in response to chemosensory cues, such as presence of a *Caenorhabditis*-specific pheromone, abundance of food, and temperature [Golden and Riddle 1984]. Many genes mediate dauer formation, and numerous branched genetic pathways which represent a network of overlapping functions have been constructed [Riddle and Albert 1997]. One such pathway

is the type II TGF-β pathway, which is one of two parallel genetic pathways corresponding to two partially distinct neuronal signaling pathways responsible for mediating the dauer formation [Thomas *et al.* 1993].

Not only is the TGF-β pathway known to function in sensory neurons [Riddle and Albert, 1997], but the type II *daf* (dauer formation) mutants been found to display defects in egg-laying [Daniels *et al* 2000]. Thus, it seems to be a reasonable candidate to be involved in the regulation of egg-laying by sensory cues.

RESULTS

To investigate the possibility that the TGF-β pathway could be involved with *flp-1*, we first analyzed the egg-laying patterns of the type II temperature sensitive Daf-constitutive mutants (*daf-1, daf-4, daf-7,* and *daf-8*). Interestingly, we found that the egg-laying patterns of these mutants are similar to that of *flp-1*: all have a lengthened inactive period of egg-laying, with normal egg-laying within the active phase (Figure 1-8a). (The egg-laying parameters of *daf-4* were unable to be calculated, as these mutants lay so few eggs, perhaps due to their small brood size and result of also having the Sma phenotype, that it was not possible to obtain enough egg-laying interval data.) These results are consistent with the idea that FLP-1 may be involved in the *daf* pathway; however it is still possible that they could be working through two separate hormonal pathways. We next performed egg-laying food response assay on the *daf* mutants to determine if they, too, were unable to modulate their egg-laying in response to food. We found that *daf-4* and *daf-8*, but not *daf-1* or *daf-7*, displayed food modulation defects similar to *flp-1* (Figure 1-8b). *daf-4* encodes a type II TGF-β receptor, whereas *daf-8* encodes a smad protein downstream in the TGF-β signal transduction pathway. *daf-1* encodes the type I TGF-β receptor that is recruited by DAF-4, after it binds to

DAF-7, the TGF-β ligand expressed in ASI neurons. It is presently unclear as to why *daf-4* and *daf-8*, but not *daf-1* or *daf-7*, display the food modulation defect.

It is known that the Daf-c phenotype (but not the Sma phenotype) of *daf-4* can be suppressed by the Daf-defective mutation in *daf-3* (another smad protein). We were interested to see if it also suppressed the food modulation defect, and we found that it did (Figure 1-9a), although the egg-laying rates both on food and off of food were dramatically decreased, probably as a result of the small brood size of *daf-4*. This result confirms that *daf*-3 is downstream in the sensory pathway for both dauer formation and egg-laying response to food.

We wanted to explore the possibility that the effects of FLP-1 might be mediated through the TGF-β pathway. To investigate the possibility that *daf-3* could also suppress the egg-laying and food modulation defects of *flp-1*, we constructed *flp-1;daf-3* double mutants. The results were not clear-cut. Although *daf-3* did seem to suppress the food modulation defect of *flp-1*, the egg-laying defect was only partially suppressed (Figure 1-9b). The partial suppression suggests that there may actually be two hormonal pathways involved with relaying sensory cues to the egg-laying circuitry, one involving *flp-1* and the other involving the Daf-c genes.

DISCUSSION

The results presented here do not confirm or disprove either theory (one pathway or two), and more work needs to be done on this project to further investigate how the Daf pathway may be connected to egg-laying. However, the complexity of the Daf pathways makes this a difficult task. For the time being, this project has been set aside. Nevertheless, the following future experiments are possible and might be helpful in elucidating more information: analyzing other alleles of the Daf-c genes for egg-laying and food-modulation

defects, constructing more double mutants to study the epistatic relationships, such as *flp-1;daf-4* and *daf-1;daf-3,* and performing serotonin dose response assays on the *daf* mutants as well as the *daf* double mutants.

METHODS

Strains and genetic methods

Routine culturing of *C. elegans* was performed as described [Brenner 1974]. The chromosomal locations of the genes studied in these experiments are as follows: LGI, *daf-8;* LGIII, *daf-4, daf-7;* LGIV, *flp-1, daf-1;* LGX, *daf-3*. Unless otherwise indicated, all mutant strains are in the N2 genetic background. Behavioral assays were performed at room temperature (~22°). The *flp-1(yn2)* allele was chosen for use in double mutant constructions because its phenotype and behavior in genetic crosses suggests that it causes a more severe loss of gene function [Nelson *et al.* 1998b]. The *daf-8(e1393), daf-4(m63), daf-7(e1372), daf-1(m40),* and *daf-3(e1376)* alleles were chosen for analysis of Daf phenotype, as these were the alleles available from the Caenorhabditis Genetics Center.

Egg-laying assays

Unless otherwise stated, nematodes were grown and assayed at room temperature on standard nematode growth medium (NGM) seeded with *Escherichia coli* strain OP50 as a food source. The Daf-c mutants (*daf-1, daf-4, daf-7, daf-8*) were grown at 15° (to allow them to recover from dauer larva) until the L4 stage, at which time they were allowed to grow at room temperature until the assays were performed.

Egg-laying behavior of individual animals on solid media (NGM agar) was recorded at room temperature (22°) for 4-8 hr as described [Waggoner *et al.* 1998] using an automated tracking system.

Experiments comparing egg-laying rates in the presence or absence of food were performed as described [Waggoner *et al.* 2000].

Analysis of egg-laying patterns

Intervals between egg-laying events were determined from analysis of videotapes obtained using the automated tracking system. Quantitative analysis of the egg-laying pattern using this interval data was performed as described [Zhou *et al.* 1997; Waggoner *et al.* 2000].

Construction of double mutant strains

For the *flp-1;daf-3* double mutants, *daf-3* homozygous single mutant hermaphrodites were crossed to *flp-1* heterozygous single mutant males, and the male progeny (which were all *daf-3* loss-of-function, as *daf-3* is located on the X chromosome) were then crossed to *daf-3* homozygous single mutant hermaphrodites, producing either *daf-3* homozygous single mutant hermaphrodites or *daf*-3 homozygous; *flp-1* heterozygous double mutant hermaphrodites. These were picked individually and allowed to self-fertilize, and then animals that carried the *flp-1* mutant allele could be identified by scoring for the presence of a diagnostic PCR product using sequence-specific primers (*flp-1*; Nelson *et al.* 1998b). For the *daf-4;daf-3* double mutants, the matings were performed as above, and *daf-4* homozygotes were identified in the F_2 generation as dauer formation-constitutive.

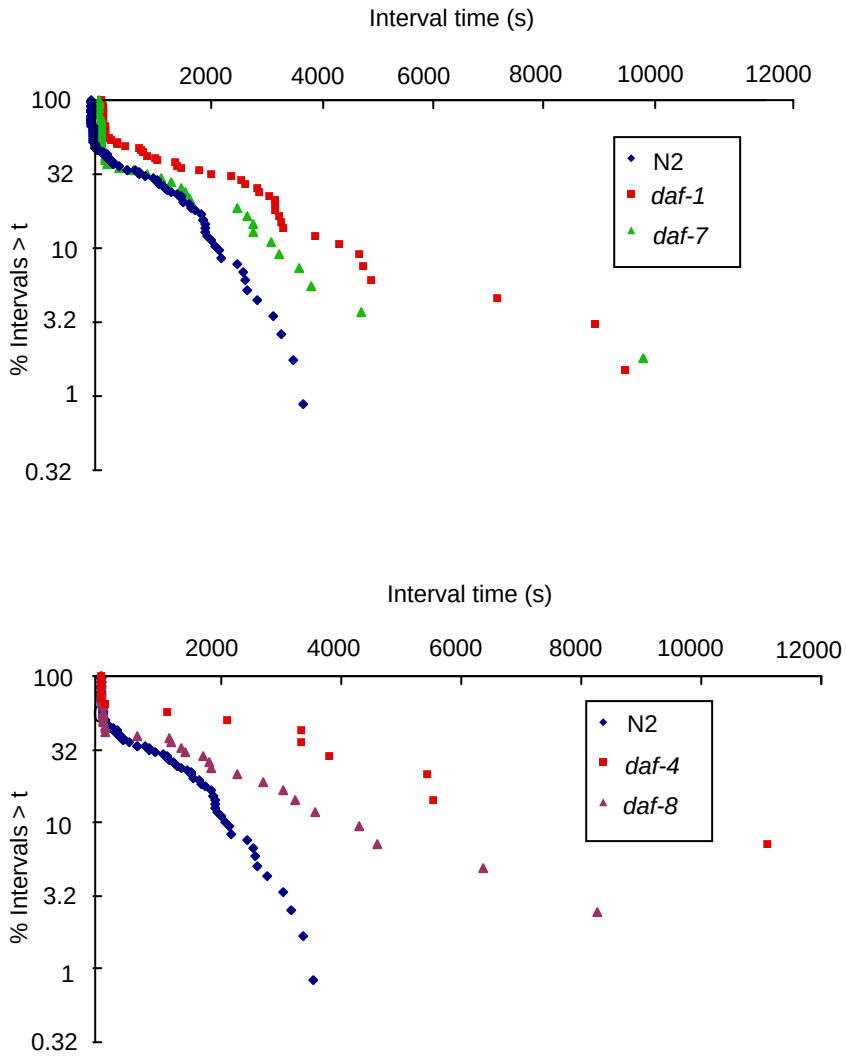


Figure 1-8a. Effect of type II Daf-c mutations on inactive phase duration
Loss-of-function mutations in *daf-1*, *daf-7*, *daf-4*, and *daf-8* all increase the duration of the inactive phase of egg-laying. Shown are the log-tail distributions of egg-laying intervals for wild-type and type II Daf-c mutant animals. The more gradual slopes of the Daf-c mutant curves indicate longer inter-cluster time constants for these mutants.

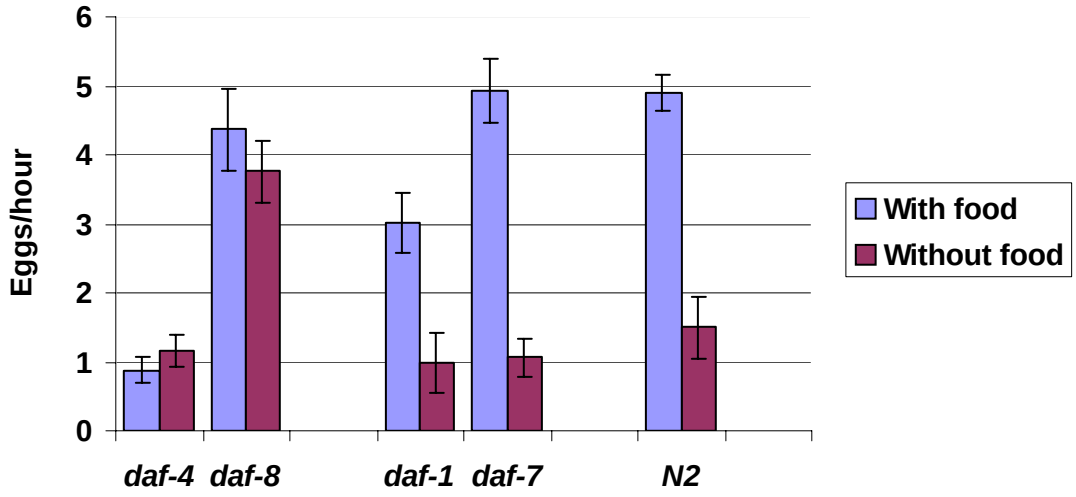


Figure 1-8b. Effect of Daf-c mutations on the regulation of egg-laying by food
The histogram shows the mean egg-laying rates of *daf-4*, *daf-8*, *daf-1*, *daf-7*, and wild-type animals in the presence and absence of food. Error bars indicate the SEM; the number of independent trials under each condition are between 50 and 60. The wild-type, *daf-1*, and *daf-7* show a lower egg-laying rate in the absence of food. However, the two other type II Daf-c mutants, *daf-4* and *daf-8*, do not have this food modulation of egg-laying. There was no significant difference between the egg-laying rates in the presence or absence of food for these animals.

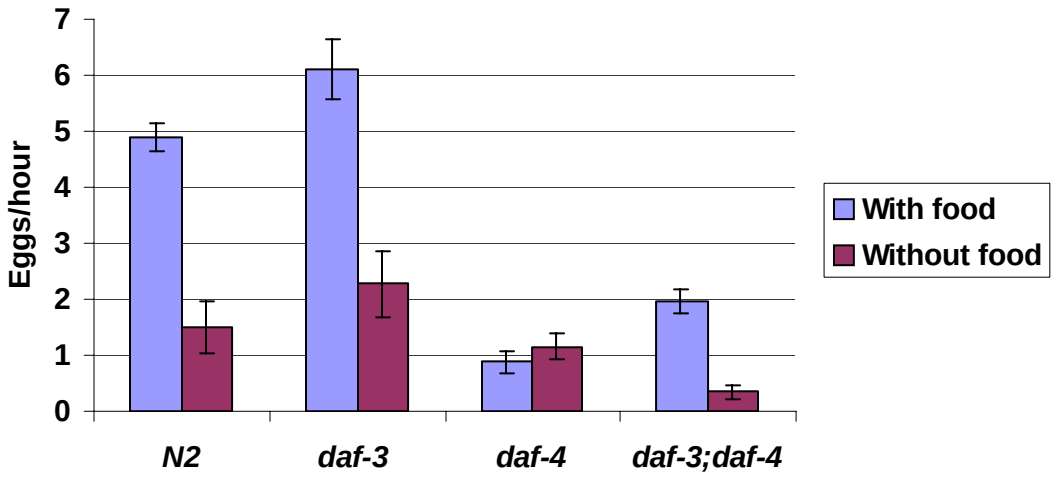


Figure 1-9a Effect of *daf-3* mutation on the food modulation defect of *daf-4*
The histogram shows the mean egg-laying rates of wild-type, *daf-3*, *daf-4*, and *daf-3;daf-4* mutant animals in the presence and absence of food. Error bars indicate the SEM; the number of independent trials under each condition are between 50 and 60. The wild-type, *daf-3*, and *daf-3;daf-4* show a lower egg-laying rate in the absence of food. In contrast, there was no significant difference between the egg-laying rates in the presence or absence of food for *daf-4*.

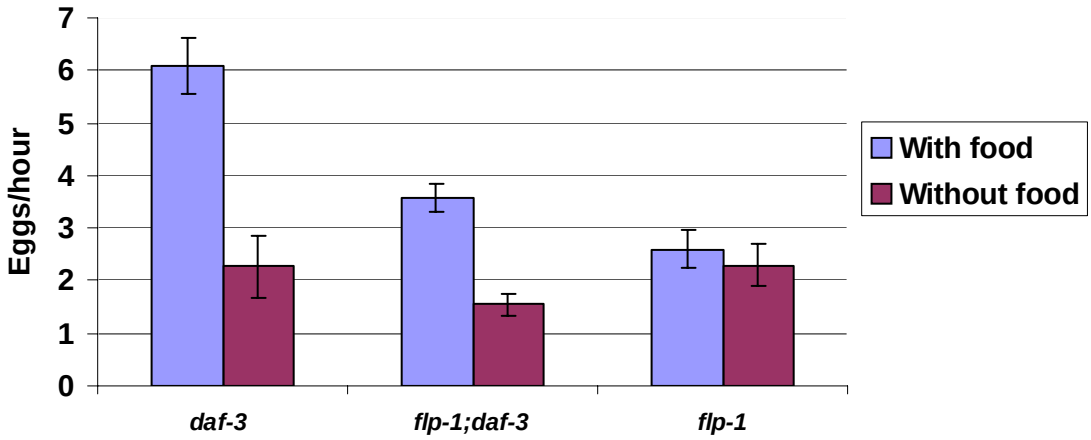


Figure 1-9b Effect of *daf-3* mutation on the food modulation defect of *flp-1*
The histogram shows the mean egg-laying rates of *daf-3*, *flp-1;daf-3*, and *flp-1* mutant animals in the presence and absence of food. Error bars indicate the SEM; the number of independent trials under each condition are between 50 and 60. The *daf-3* and *flp-1;daf-3* show a lower egg-laying rate in the absence of food. In contrast, there was no significant difference between the egg-laying rates in the presence or absence of food for *flp-1*.

CHAPTER III SEROTONIN MODULATES LOCOMOTORY BEHAVIOR AND COORDINATES EGG-LAYING AND MOVEMENT IN *CAENORHABDITIS ELEGANS*

INTRODUCTION

An important goal of reductionist neuroscience is to understand how specific proteins act within the context of the neuronal circuitry to control an animal's behavior. To understand a vertebrate nervous system at the molecular and cellular level is extremely difficult due to the extreme organizational complexity of vertebrate brains. However, for animals with less complex nervous systems, such as the nematode *Caenorhabditis elegans*, understanding the molecular and cellular basis of behavior is a realizable goal. The *C. elegans* nervous system is extremely simple and well characterized at the anatomical level: an adult hermaphrodite contains only 302 neurons, each with a precisely determined and invariant position and cell lineage [Sulston and Horvitz 1977; Sulston *et al.* 1983]. This small nervous system is capable of perceiving and responding to a wide range of environmental conditions, including heavy and light touch [Driscoll and Kaplan 1997], temperature, volatile odorants, osmotic and ionic strength, food, and other nematodes [Bargmann and Mori 1997]. Each of these sensory modalities in turn regulates many aspects of the animal's behavior, including the rate and direction of movement, the rates of feeding, egg-laying, and defecation, and the process of mating [Avery and Thomas 1997]. *C. elegans* is amenable to classical, molecular, and developmental genetic studies; thus, isolation, phenotypic characterization, and molecular analysis of behavioral mutants provides a promising avenue toward identifying the molecular events that underlie the animal's behavior [Bargmann 1993]. In addition, since each neuron can be identified by position, it is possible to infer the roles of individual neurons in nervous

system function by cell-specific laser ablation, and determine the effect of the ablation on behavior [Bargmann and Avery 1995].

Using these genetic and cell biological approaches, it has been possible to obtain many important insights into the molecular and cellular basis of behavior. For example, studies of chemotaxis-defective and touch-insensitive mutants have provided important information about the molecular mechanisms underlying sensory transduction in olfactory and mechanosensory neurons. Genetics and cell ablation experiments have also provided detailed information about the molecular basis for several simple motor behaviors, including egg-laying, feeding and defecation, which involve regulation of the contractile properties of a single specialized muscle group. However, because genes and neurons that affect higher-level aspects of nervous system function tend to have only subtle effects on behavior, much less has been learned about the neural basis for more complex motor patterns such as those involved in locomotion. Likewise, the mechanisms through which the decision to execute a given motor program are influenced by sensory information and the activity of other motor pathways are not well understood even in this simple organism.

In this study, we have characterized the long-term pattern of locomotory behavior in *C. elegans* and identified neurons that correlate these patterns with the activity of the egg-laying motor program. Specifically, we demonstrate that two aspects of locomotor function, velocity and directional reversals, are random processes whose occurrence is temporally coordinated with the onset of egg-laying events. We also provide evidence that this temporal link between egg-laying and locomotion results from the modulation of decision-making brain interneurons by serotonin.

RESULTS

Analysis of long-term patterns of movement

Previous studies of the temporal pattern of egg-laying indicated that egg-laying events are highly clustered in time, yet examination of the egg distribution on a culture plate suggests that egg-laying events are spatially dispersed. To gain insight into how this spatial dispersal could occur, we first attempted to obtain a quantitative description of the pattern of locomotion in wild-type *C. elegans*. To obtain information about the pattern of locomotion in *C. elegans*, we used an automated tracking system to follow the movements of individual animals. Using this system, it was possible to obtain a record of the animal's body position at periodic intervals and derive from this time-coded positional data a number of characteristics of the animal's movement over a long time course.

We focused our attention on two aspects of locomotory behavior: velocity and directional reversals. Reversals represent an important feature of locomotory behavior in *C. elegans*, and changes in reversal probability have been shown to underlie behavioral responses to a number of sensory stimuli. To obtain a quantitative description of the reversal pattern, we used the positional data collected by our system to measure the timing of reversal events in wild-type worms under constant environmental conditions. Analysis of these data indicated that reversal events were aperiodic and mutually independent. Furthermore, the distribution of reversal intervals indicated a temporal pattern that could be modeled as a stochastic process of a specific type: a random process with a lognormal probability density (Figure 2-1a). A lognormal random variable has two parameters: the logarithmic mean and logarithmic variance. These two parameters can be straightforwardly estimated from real data; thus, the reversal pattern of a given nematode strain can be effectively described in terms of these two model parameters.

We also analyzed the pattern of fluctuations in the animal's rate of movement across the plate. To compute an animal's translational velocity, positional data were sampled over intervals of constant time, and the distance traveled over each interval was divided by the interval duration. In this way, we obtained velocity measurements for wild-type animals over the course of several long recordings. When we analyzed the distribution of these velocity measurements, we again observed an approximately lognormal probability density (Figure 2-1b). Thus, the worm's rate of movement, like the reversal probability, was apparently a stochastic process best modeled as a lognormal random variable. By estimating the logarithmic mean and standard deviation parameters from real data, we could thereby quantify the mean and variance in movement rate over time for a given nematode strain.

Temporal correlation between locomotion and egg-laying

Since egg-laying events were temporally clustered but spatially dispersed, we reasoned that egg-laying might be temporally coordinated with movement. Therefore, we investigated whether the patterns of reversals and velocity fluctuations changed during periods of active egg-laying. By synchronizing the time codes of the tracking computer (which recorded body position) and the videotape recorder (used to identify egg-laying events), we were able to examine the velocity pattern before and after each egg-laying event (Figure 2-2a and b). Surprisingly, we observed that in the 30 seconds immediately prior to egg-laying events, the animal's average velocity was significantly elevated, with the peak velocity occurring approximately fifteen seconds before the egg was laid. Direct observation of video recordings indicated that these velocity bursts corresponded to periods of uninterrupted forward movement at relatively high speeds. Not every egg-laying event was preceded by a velocity burst, and the magnitude of the velocity increase varied substantially from animal to animal. Thus, the increase in velocity did not appear to be an integral component of the egg-

laying motor program; rather, the initiation of the egg-laying motor program appeared to increase the probability of forward movement, or vice versa. Interestingly, the animal's velocity during the egg-laying event itself was essentially no different from the baseline locomotion rate. Thus, the spatial dispersal of egg-laying events appeared to result from a transient burst in locomotion velocity immediately prior to egg-laying.

Egg-laying, like locomotion, occurs in a specific temporal pattern. Specifically, egg-laying events are clustered in bursts, or active phases, which are separated by long inactive phases during which eggs are retained. Both the onset of the active phase and egg-laying within the active phase are aperiodic and model as Poisson processes with distinct rate constants. In principle, the velocity burst might correlate specifically with the onset of the active egg-laying phase; alternatively, it might precede all egg-laying events. To investigate this question, we compared velocities around the three different types of egg-laying events: the first egg laid in a cluster (i.e. a group of eggs each no more than two minutes apart), the middle eggs in cluster, and the last egg in a cluster (Figure 2-2c). We observed that all egg-laying events were preceded by significant increases in velocity, although the first egg in a cluster did exhibit the largest velocity peak. Nonetheless, the transient change in locomotor behavior appeared to correlate temporally with all egg-laying events and not merely the onset of the active egg-laying phase.

We also investigated whether egg-laying affected the likelihood of reversal events. To address this question we measured the frequency of reversal events during the time immediately before and after the occurrence of an egg-laying event. If egg-laying increased the probability of reversals, we would expect a peak in this trace around the time of egg-laying events; conversely, if egg-laying decreased the probability of reversals, this trace would be expected to show a dip. In fact, we observed a marked increase in reversal frequency from the

time of egg-laying until about 30 seconds subsequent to the egg-laying event. (Figure 2-2d). Thus, execution of the egg-laying motor program appeared to correlate with two changes in locomotive behavior: an increased propensity for forward locomotion before egg-laying and an increased propensity for reversals during and after an egg-laying event.

Coordination of egg-laying and locomotion requires the HSN motorneurons

What is the neural mechanism that coordinates egg-laying and locomotion? To address this question, we examined the effects of neurons that regulate egg-laying behavior on the control of locomotion. We first assayed the role of the HSNs, a pair of serotonergic motorneurons that have synaptic output to a number of brain interneurons and have also been shown to control the onset of the active phase of egg-laying. To assess the effects of the HSNs on locomotion, we recorded the movements of animals carrying a mutation in the gene *egl-1,* which causes the HSNs to undergo inappropriate cell death. When we analyzed the locomotive pattern of *egl-1* mutants, we observed that in contrast to wild-type animals, they did not display the velocity peak prior to egg-laying (Figure 2-3a). In addition, although the overall movement velocity of *egl-1* animals was normal, their reversal frequency was significantly higher than that observed for wild-type. Thus, the HSNs had two specific effects on locomotory behavior: they inhibited directional reversals and were specifically required for the velocity peak coordinating egg-laying and locomotion.

The HSNs contain multiple neurotransmitters, each of which could in principle be responsible for its effects on movement. One of these molecules, serotonin, appears to function specifically to facilitate the onset of the active egg-laying phase. To determine whether serotonin release by the HSNs could also be involved in promoting the velocity burst prior to egg-laying, we analyzed the egg-laying and velocity data for strains carrying loss-of-function mutations in three genes required for serotonin biosynthesis: *tph-1* (encoding

tryptophan hydroxylase), *bas-1* (encoding aromatic amino acid decarboxylase) and *cat-4* (a gene required for AA-decarboxylase activity). We observed that all three serotonin-deficient mutants lacked the velocity burst prior to egg-laying (Figure 2-3b). Moreover, all serotonin-deficient mutants showed both a significant decrease in locomotive velocity and a significant increase in overall reversal frequency. These results suggest that serotonin functions generally to promote forward locomotion and is specifically required for the velocity burst prior egg-laying events.

Specific interneurons are necessary for the velocity peak prior to egg-laying

How might serotonin released from the HSNs stimulate locomotion? Although the HSNs make neuromuscular junctions only with the vulval muscles, they also make synaptic connections with several interneurons in the head. Among the most prominent synaptic outputs of the HSNs are to a pair of interneurons called AVFs. AVF's synaptic output is primarily directed to AVB, a so-called command interneuron implicated in promoting forward locomotion, and to AVJ, which also directs its synaptic output to AVB as well as the other forward command interneuron PVC. Thus, a reasonable hypothesis to explain the HSN's effects on locomotion was that the HSNs might modulate the activity of AVF and AVJ, which in turn could activate the forward command interneurons and promote forward movement.

To test this hypothesis, we examined the effects of ablating each of these neurons on the animal’s locomotion and egg-laying behavior. When we assayed the effect of ablating the AVFs, we observed an effect similar to that caused by a serotonin-deficient mutation: the velocity burst prior to egg-laying was reduced if not eliminated, and the average velocity overall was significantly reduced (Figure 2-4a and b). In contrast, the effect of AVF ablation on egg-laying was quite distinct from that of HSN ablation or serotonin deficiency. Whereas HSN-ablations and serotonin deficient mutations slowed the rate of egg-laying and increased

the duration of the inactive egg-laying phase, AVF ablations did not (data not shown). These results suggested that AVF mediated the effects of the HSNs on locomotion, but was unconnected to its effects on egg-laying.

We also investigated the roles of the command interneurons themselves, in particular AVB, in the temporal coupling of egg-laying and locomotion. We analyzed the locomotion behavior of two strains in which specific command interneurons undergo inappropriate cell death due to misexpression of the ICE protease under a cell-type-specific promoter. In one strain, all the command interneurons (i.e. AVA, AVB, AVC, and PVC) were absent; in the other AVA, AVC and PVC were killed but AVB was spared. Consistent with previously published analyses of these strains, we observed that both showed abnormal locomotion patterns: their average velocity was significantly reduced, and the intervals between reversals was significantly increased. However, perhaps surprisingly, both strains exhibited a significant velocity burst prior to egg-laying (Figure 2-4c). Thus, these data indicate that the coordination of egg-laying and movement does not require the command interneurons, and may involve direct modulation of motorneurons by AVF or other decision-making interneurons.

DISCUSSION

Stochastic modeling of locomotor events

Locomotion involves some of the most intricate and complex motor patterns displayed by *C. elegans* and represents a critical aspect of the worm's behavioral repertoire; yet, the long-term temporal patterns of locomotion in *C. elegans* are not well understood. We have shown here that two key aspects of nematode locomotion, translational velocity and reversal frequency, can be closely modeled as stochastic processes with lognormal probability

densities. Both translational velocity and directional reversals reflect the activity of two antagonistic, mutually exclusive locomotory programs leading to forward or backward movement respectively. Intervals of backward movement tend to be relatively brief; thus, 10-second intervals of high average velocity generally correspond to periods of uninterrupted forward movement. Likewise, continuous forward movement leads to longer inter-reversal intervals. The stochastic nature of both these features of locomotive behavior implies that the fluctuations between forward and backward movement are arrhythmic and involve random fluctuation in behavioral states.

Both velocity changes and directional reversals have been shown to play a key role in the responses of nematodes to sensory stimuli. For example, an animal's velocity has been shown to decrease significantly when the animal enters a lawn of its bacterial food source. Likewise, a nonlocalized mechanical stimulus (i.e. "tap") causes an animal to change its direction of movement, either from forward to backward or vice versa, while a localized touch to the head or tail causes the animal to change its direction of movement away from the stimulus. Moreover, changes in the occurrence of long periods of uninterrupted forward movement (i.e. "runs") and bouts of frequent reversals (i.e. "pirouettes") have been shown to mediate navigation in a chemoattractive gradient. The behavioral models described make it possible to quantitatively describe the temporal patterns of velocity fluctuations and reversals in terms of two specific parameters of a lognormal random variable: the logarithmic mean and logarithmic variance. By estimating these parameters from behavioral mutants or animals carrying specific neuronal ablations, it is possible to gain insight into how individual genes and neurons influence the timing of these locomotory events.

Influence of egg-laying behavioral states on locomotion

Interestingly, we observed that both directional reversals and velocity fluctuations were temporally correlated with egg-laying behavior. Specifically, egg-laying events tended to be preceded by an increase in locomotor velocity and followed by an increase in reversal frequency. Previous studies of the timing of egg-laying events indicated that animals fluctuate between inactive and active egg-laying phases; eggs are retained during the inactive phase and laid in bursts during the active phase. The results described here indicate that the active egg-laying phase also involves changes in locomotor behavior, such that animals have an increased propensity for directional changes and rapid body movement between clustered egg-laying events. The primary consequence of these changes in locomotor pattern is that egg-laying events are spatially dispersed despite being temporally clustered. It should be noted that these behaviors are not mechanistically linked, and both egg-laying and locomotion can occur normally in the absence of temporal coordination with the other behavior. Moreover, the temporal link between egg-laying and locomotive behavior is not deterministic; the extent and duration of the velocity burst varies widely from egg-laying event to egg-laying event, and some events are not preceded by a velocity burst at all. Thus, egg-laying appears to influence locomotion stochastically by modifying the probability of executing behavioral events associated with forward locomotion.

What is the biological purpose for this coordination of egg-laying and movement? Clearly, there is no mechanical requirement for increased motility during egg-laying, since the processes can be uncoupled (e.g. in an AVF-ablated animal) without impairing normal egg-laying behavior. Moreover, an opposite correlation between egg-laying and movement has been reported for other nematode species; some species lay eggs only during periods of inactive locomotion. This variation in the nature of the correlation between egg-laying and movement may reflect different mating or foraging strategies utilized by different nematode

species or subspecies. Recently, it was demonstrated that wild subspecies of *C. elegans* exhibit one of two distinct foraging strategies; "social" strains cluster together on food, whereas "solitary" strains disperse across a food source. An increase in locomotion during the active egg-laying phase could perhaps facilitate a solitary foraging strategy by scattering an animal's progeny across the food source.

Effects of interneurons and motorneurons on behavioral decision-making

By analyzing the locomotion patterns of animals carrying cell-specific neuronal ablations, we identified specific roles for several classes of neurons in coordinating egg-laying and movement. Together, these data provide an outline of the neural circuitry that controls locomotory behavioral decision-making and mediates the temporal coordination of egg-laying with movement. Specific roles in these processes were identified most strikingly for two classes of neurons: the AVF decision-making interneurons and the HSN egg-laying motorneurons.

The AVF neurons appear to play a specialized role in controlling the onset of reversals and velocity fluctuations. Ablation of AVF caused a marked decrease in the mean translational velocity and increased the frequency of reversals. Moreover, these ablations caused a nearly complete elimination of both the velocity burst prior to egg-laying and the reversal burst following egg-laying. Thus, the AVFs can probably best be described as decision-making interneurons, since they do not affect the ability to generate the reversals or velocity fluctuations but strongly influence the frequency and timing of these events. The AVFs direct most of their synaptic output to AVB, a forward command interneuron thought to be a key component of the central pattern generator for forward locomotion. Therefore, it is reasonable to hypothesize that AVF's ability to increase the likelihood of sustained forward movement is mediated at least in part through modulation of AVB. However, since animals

lacking all the command interneurons still exhibit the velocity burst, AVF may also promote forward movement through an AVB-independent neural mechanism. Since the AVFs direct some synaptic output to the VB motorneurons, which have been implicated in directly generating the forward locomotion pattern, it is possible that the velocity burst could also involve direct modulation of the VBs by AVF.

Another class of neurons that were required to coordinate egg-laying and movement were the HSNs, a pair of egg-laying motorneurons. Although the HSNs are important for promoting egg-laying, they are not essential; HSN-ablated animals still lay eggs, and these egg-laying events are still clustered in active phases. However, animals carrying an ablation of the HSNs completely failed to undergo the changes in both velocity and reversal frequency that normally accompany periods of active egg-laying, indicating that these neurons were essential for coupling the temporal patterns of egg-laying and movement. Yet in contrast to the AVF interneurons, the HSNs had little effect on the frequency of reversals or the pattern of velocity fluctuations except during periods of active egg-laying. Thus, the HSNs appear to specifically affect locomotory behavior during the active egg-laying phase, probably through modulation of decision-making neurons such as AVF. Since mutants deficient in serotonin, a neuromodulator released from the HSNs, also showed an uncoupling of egg-laying and locomotor patterns, it appears likely that the modulation of locomotory interneurons by the HSNs probably involves serotonergic neurotransmission.

Interestingly, serotonin deficiency, unlike HSN ablation, had a significant effect on the pattern of locomotion during inactive egg-laying periods. In fact, the locomotion phenotype of *tph-1* and *cat-4* mutants was remarkably similar to that of the AVF-ablated animals. These observations suggest that serotonin may function more generally as a modulator of AVF activity, and that other neurons in addition to HSN may promote increased

locomotor activity through serotonin release. The evidence that serotonin is a modulator that promotes locomotor activity was unexpected, as exogenous serotonin has been shown previously to inhibit movement. The inhibitory action of serotonin on movement appears to involve direct inhibition of neurotransmitter release from ventral cord motorneurons. Thus, the seemingly paradoxical actions of serotonin as both an activator and an inhibitor of locomotion can be explained most simply as reflecting two distinct cellular targets for serotonin within the neural circuitry (i.e., the AVF interneurons and the ventral cord motorneurons, respectively). The application of quantitative analytical techniques such as those described here should make it possible to dissect apart the distinct features of locomotory behavior affected by serotonin and other modulators, and hence identify the specific neural mechanisms that underlie these distinct modulatory actions.

METHODS

Strains and Genetic Methods

Routine culturing of *C. elegans* was performed as described (Brenner 1974). The chromosomal locations of the genes studied in these experiments are as follows: LGII, *tph-1*; LGIII, *bas-1*; LGV, *cat-4, egl-1*.

For ablation experiments, we used the following strains:

NC197 (genotype: dpy-20(e1282)IV; wdIs4[dpy-20(+)unc-4::GFP]; kindly provided by David Miller), which carries an integrated unc-4::GFP fusion;

akIs11, which uses the nmr-1 promoter to express ICE protease in a subset of command interneurons, and kyIs36, which uses the glr-1 promoter to express ICE protease in a subset of command interneurons (both kindly provided by Villu Maricq).

Analysis of locomotion behavior

Locomotion behavior of individual animals on solid media (NGM agar) was recorded at room temperature (22°) for 4-8 hr as described (Waggoner *et al*. 1998) using an automated tracking system. For each recording, a six column matrix was saved by the tracker computer: hour, minute, second, millisecond, x-position of the worm, y-position of the worm. The velocity of the animal was calculated at one second intervals over the entire recording at a resolution of 10 seconds. We first measured the animal's turning angle throughout the course of a behavioral recording. The animal's direction of movement was determined by sampling the positional data at intervals of constant distance, converting the data into a polygonal path composed of discrete line segments of constant size. We then computed turning angles by determining the change in direction of bearing between adjacent segments. Analysis of the distribution of turning angles in wild-type animals revealed two distinct peaks. One peak, centered at 0 degrees, corresponded to animals continuing in approximately the same direction. The other peak, centered at 180 degrees, corresponded to a switch between forward and backward locomotion. Defining a reversal event as a turning angle greater than 90 degrees, we then calculated the intervals between reversal events.

Analysis of egg-laying behavior

The time of egg-laying events and the intervals between them were determined from analysis of videotapes obtained using the automated tracking system. Quantitative analysis of the egg-laying pattern using this interval data was performed as described (Zhou *et al*. 1997).

Ablation of Neurons

To ablate the AVF neurons, we used the strain NC197 (genotype: dpy-20(e1282)IV;wdIs4[dpy-20(+)unc-4::GFP]; kindly provided by David Miller), which carries

an integrated unc-4::GFP fusion. Both the SAB and AVF neurons express unc-4::GFP, and in the larvae SABVL and SABVR are difficult to distinguish from the AVFL and AVFR. We therefore used a two-step laser ablation procedure to determine the effect of AVF ablation. In early first-stage larvae, the SABVL and SABVR were ablated. We allowed these worms to recover overnight before ablating the AVFs the following day. On the third day, cell killing of all four neurons was verified by scoring for the absence of GFP-expressing neurons in the head region. SAB-only ablated worms were used as a control.

To ablate the ASH neurons, we first identified these neurons by filling with the lipophilic dye diI. Early-first stage larvae were placed in a solution of m9 and diI for one hour. The diI was absorbed by the neurons which have processes extending to the amphids, ASH being one of them. The ASHs were identified based on position, and confirmation of the ablation occurred the following day using the same dye-filling procedure.

The results presented in this chapter are being revised for publication in a paper entitled "Serotonin modulates locomotory behavior and coordinates egg-laying and movement in *Caenorhabditis elegans*" (Hardaker, L.A., Singer, E., Kerr, R., Zhou, G, and Schafer, W. R.) Laura Anne Hardaker was the primary investigator and author of this paper.

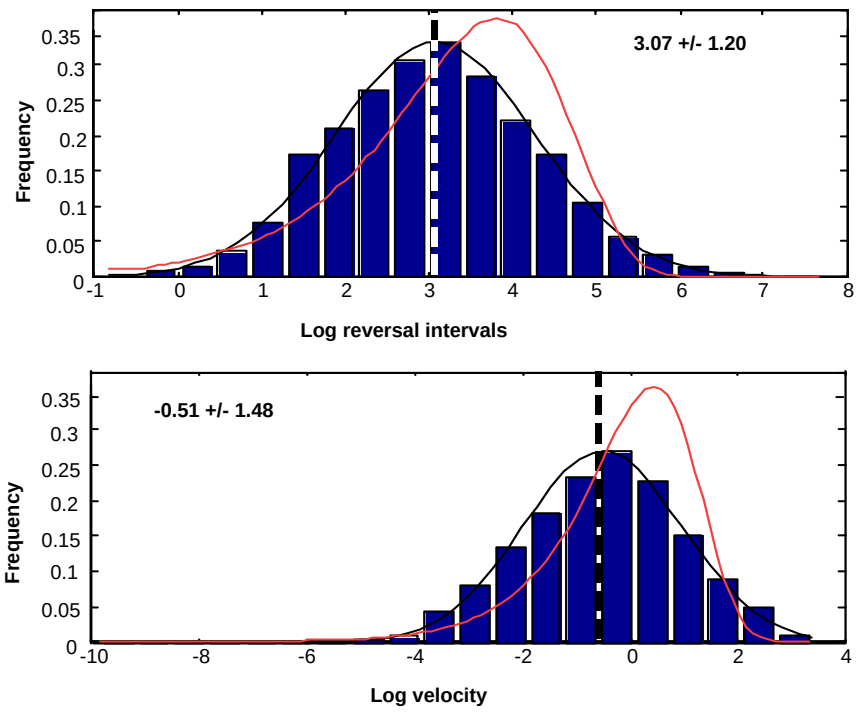


Figure 2-1. Temporal pattern of reversal frequency and velocity
a) Shown is the histogram of the observed natural log of the reversal interval times (in log sec), with the relative frequency on the y-axis. The graph shows a gaussian distribution between reversals, in which the distribution of reversal intervals indicate a temporal pattern that could be modeled as a random process with a log normal probability density. A lognormal random variable has two parameters: the logarithmic mean and logarithmic variance (3.07 corresponds to 21.5 seconds, and 1.20 corresponds to 3.3 seconds). Shown are a histogram of 7305 reversal intervals by wild-type *C. elegans* over ten recordings. b) Shown is the histogram of the observed values of log velocities (in log sec). Again, the distribution is a lognormal probability density, with log mean of -0.51 and log variance 1.48. Shown is the distribution of 366,383 velocities by wild-type *C. elegans* over ten recordings. The dark line indicates the ideal gaussian distribution based on ML analysis of the real data. The lighter line indicates the ideal distribution if the reversal intervals modeled as a poisson process.

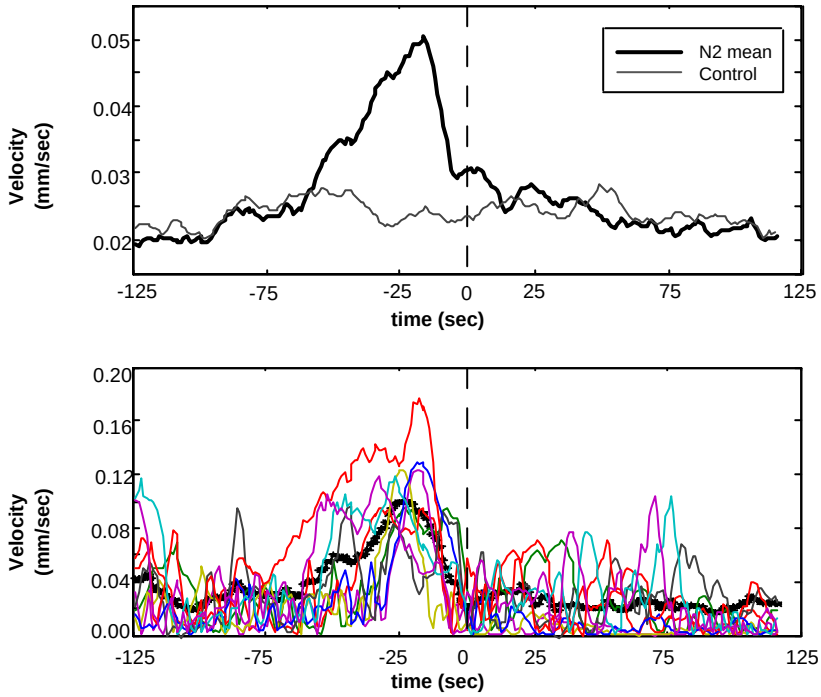


Figure 2-2a. Mean velocity pattern surrounding egg-laying events
Shown is a plot of the mean wild-type velocity surrounding egg-laying events (dotted vertical line at t=0), determined by synchronizing the time codes of the tracking computer (which records body position) to the videotape recorder (used to identify egg-laying events), and using ML analysis. The highest peak occurs at fifteen seconds before egg-laying. Shown is the mean data of 10 worms, 61 hours, 186 egg-laying events. Shown also is a plot of control data, the velocities around a list of random times over the length of the recordings.

Figure 2-2b. Individual data set of velocity pattern around each egg-laying event
Shown is a plot of every velocity trace around the egg-laying events of one recording, with the mean velocity in bold.

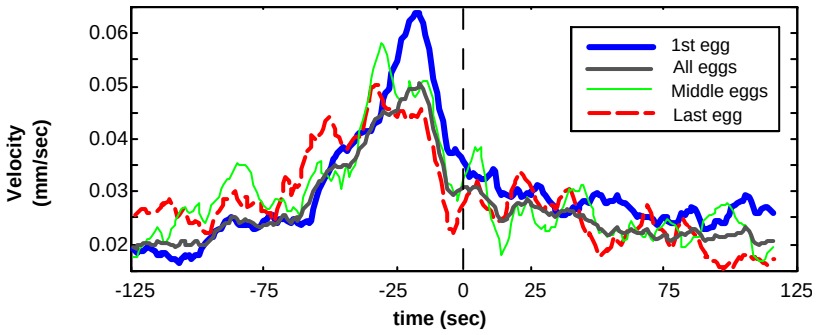


Figure 2-2c. Velocity pattern surrounding egg-laying events
Shown are the plots of the velocities around the three different egg-laying events: the first egg laid in a cluster (i.e. a group of eggs each no more than two minutes apart), the middle eggs in a cluster, and the last egg in a cluster. (The mean is also shown for comparison). All egg-laying events were preceded by statistically significant increases in velocity ($p<0.001$), and the velocity burst of the first eggs was statistically significant from the velocity burst of the last eggs ($p<0.05$), according to the Mann-Whitney rank sum test.

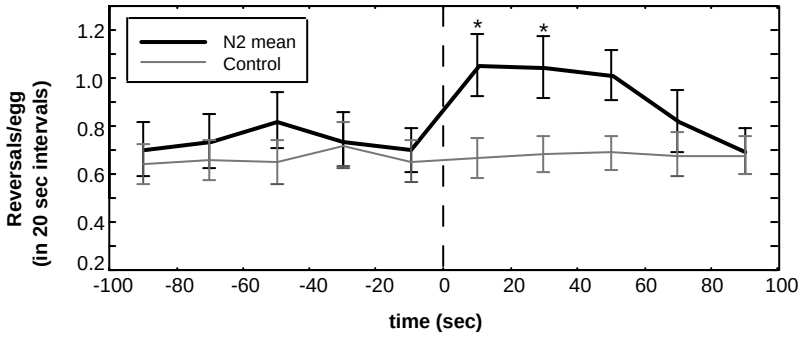


Figure 2-2d. Reversal frequency surrounding egg-laying events
Shown is a plot of the mean reversal frequency surrounding egg-laying events (dotted vertical line) in wild-type *C. elegans*, over the course of 10 recordings, 61 hours, and 186 egg-laying events. After egg-laying occurs, there is a statistically significant increase ($p<0.05$) in the frequency of reversals, according to the Mann-Whitney rank sum test. Shown also is a plot of control data, the reversal frequencies around a random list of imaginary egg-laying times over the length of the recordings. This plot does not show any significant differences from the mean reversal frequency.

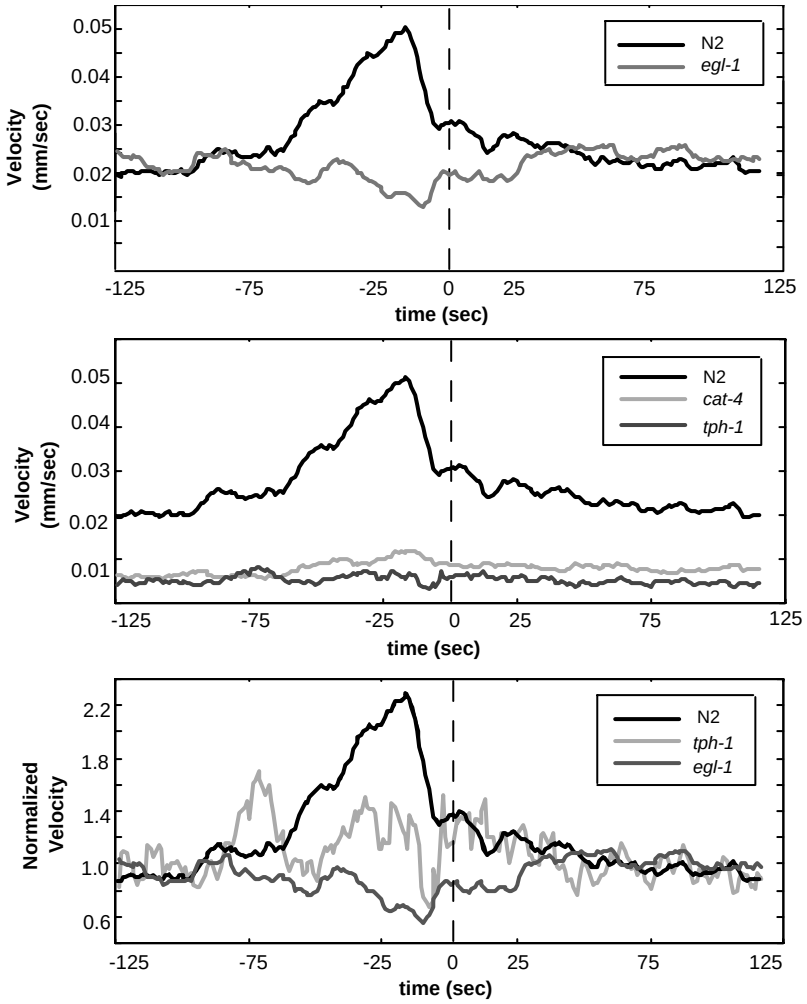


Figure 2-3a. Effect of *egl-1* mutation on the velocity burst
Shown are the plots of mean velocity surrounding egg-laying events for wild-type (10 recordings, 61 hours, 186 egg events) and *egl-1* mutants (8 recordings, 61 hours, 63 egg events). Although the mean velocity is similar in wild-type *egl-1* mutants, *egl-1* mutants show no significant increase in velocity prior to egg-laying.

Figure 2-3b. Effect of serotonin deficient mutants *tph-1* and *cat-4* on the velocity burst
Shown are the plots of mean velocity surrounding egg-laying events for wild-type, *cat-4* mutants (10 recordings, 55 hours, 139 egg events), and *tph-1* mutants (6 recordings, 27 hours, 56 egg events). The mean velocity of these mutants is significantly reduced, and there is a statistically significant difference between the velocity of wild-type and the mutants.

Figure 2-3c. Normalized effect of *tph-1* and *egl-1* on the velocity burst
Shown are the plots of the normalized velocity around egg-laying events, where normalized velocity equals velocity divided by the mean velocity of the period without the velocity burst, to get a mean normalized velocity of approximately 1.0 for each strain. Here, it is possible to visualize that the velocity burst in the *tph-1* or *egl-1* mutants is dramatically reduced, although there is more noise in the *tph-1* mutants.

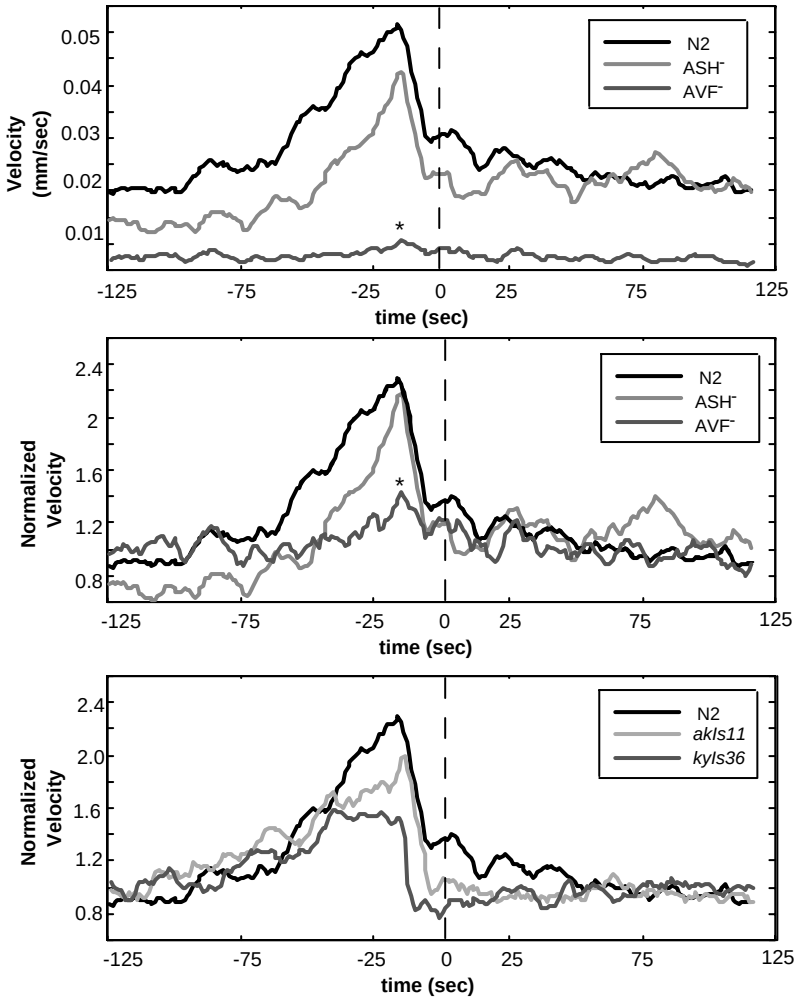


Figure 2-4a. Effect of decision interneuron ablation on the velocity burst
Shown are the plots of mean velocity surrounding egg-laying events for wild-type, ASH-ablated animals (5 recordings, 34 hours, 85 egg events) and AVF-ablated animals (7 recordings, 42 hours, 174 egg events). Although ASH-ablated animals appear to have a smaller peak, they also have a lower mean velocity; when the velocities are normalized (Figure 2.4b), there is no statistically significant difference in the velocity peak. However, this is not the case for AVF-ablated animals. In these mutants, both the mean velocity and the velocity peak are significantly reduced (* $p<0.01$). This can be visualized in this figure and in Figure 2.4b.

Figure 2.4b. Normalized effect of decision interneuron ablation on the velocity burst
Described above.

Figure 2.4c. Effect of killing command interneurons on the velocity burst
Shown are the plots of normalized velocity surrounding the egg-laying events for wild-type, *akIs11* mutants (6 recordings, 52 hours, 173 egg events), and *kyIs36* mutants (6 recordings, 52 hours, 112 egg events). Although the average velocity was significantly reduced for both strains (data not shown), both also exhibit a significant velocity burst prior to egg-laying.

Strain (worms,hr,eggs)	Mean log (velocity)	Standard Deviation	Mean log (rev interval)	Standard Deviation
N2 (10,61,186)	**-0.51**	1.48	**3.07**	1.2
egl-1 (7,61,63)	**-0.40**	1.49	**3.13**	1.13
tph-1 (6,27,56)	**-1.41**	1.45	**3.22**	1.69
kyIs36 (6,52,112)	**-1.36**	1.43	**3.86**	1.80
akIs11 (6,52,173)	**-1.17**	1.44	**3.62**	1.60

Table 2-1. Reversal frequency and velocity parameters of mutant animals

CHAPTER IV CONCLUSION

In this thesis, I have described specific mechanisms through which the decision to execute a given motor program is influenced by sensory information as well as the activity of other pathways, using *Caenorhabditis elegans* egg-laying behavior as my model. I have shown evidence of how the peptides encoded by the *flp-1* gene may be involved in relaying food sensory cues to the egg-laying circuitry by a hormonal mechanism, and I have demonstrated that the egg-laying circuitry back-modulates the brain to affect locomotion (Figure 3-1). There is potential for future studies to analyze these results further. For instance, the exact cellular and molecular pathway through which the FLP-1 peptides function has yet to be discovered, and the most downstream effectors of the velocity burst prior to egg-laying has not been elucidated. Discovering these answers will yield an even clearer picture of how behavior is modulated by cellular and molecular mechanisms.

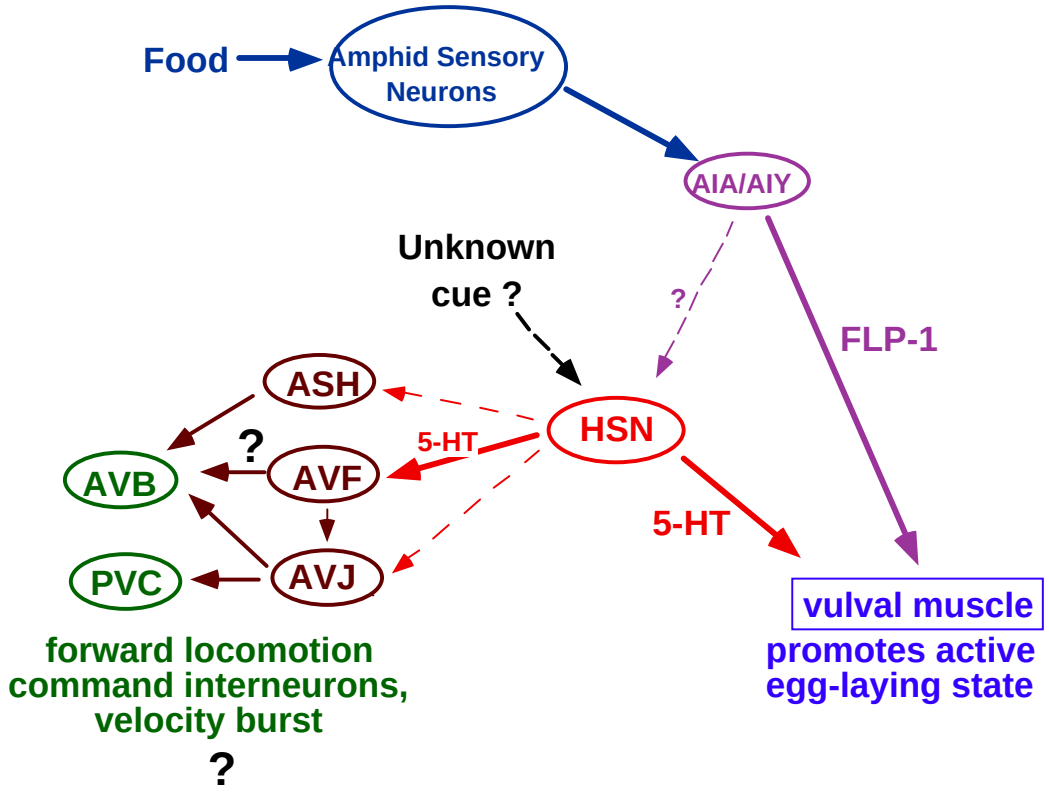


Figure 3-1. Model of neural and molecular circuitry involving food sensory cues, egg-laying, and locomotion. Upon food presence, the amphid chemosensory neurons send a signal to the AIA/AIY interneurons, which release FLP-1 peptides. These neuropeptides then facilitate modulation of egg-laying by serotonin. Serotonin not only affects the egg-laying muscles, but also plays a role in the burst of velocity prior to egg-laying, perhaps by being released from the HSNs and signaling the AVFs, both of which are necessary for the velocity burst. How the decision interneurons (AVFs) affect the velocity burst has yet to be determined. Although the forward locomotion command interneurons AVB and PVC are needed for normal velocity, they do not seem to be responsible for the velocity burst.

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