egl-32 Acts Through Sperm to Regulate Egg-laying in *C. elegans*

by
Marie McGovern

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

egl-32 ACTS THROUGH SPERM TO REGULATE EGG-LAYING IN _C.elegans_

by

Marie McGovern

Advisor: Dr. Cathy Savage-Dunn

A study of the egg-laying defective mutant, *egl-32,* in *Caenorhabditis elegans* has revealed that sperm have an active role in regulating egg-laying. Initially, our lab became interested in studying *egl*-32 because we believed it interacted directly with a TGF-β pathway that regulates dauer development. In studying this mutant we hoped we would gain a better understanding of the role of the TGF-β dauer pathway in egg-laying. We now believe that *egl*-32 and the TGF-β pathway only interact indirectly. However, studying the egg-laying defective mutant, *egl-32,* has lead to the novel finding that sperm have an influence on egg-laying. In an attempt to determine the cellular and anatomical basis for the *egl-32's* egg laying defect it was found that *egl-32* interacts with genes highly expressed in sperm. Here I present evidence that the cellular basis of the *egl*-32's egg-laying defective phenotype is due to a defect in sperm. I have investigated the possibility that sperm are playing an active role in egg-laying by performing simple mating experiments. These mating experiments have revealed the wild-type sperm can rescue the egg-laying defect of *egl-32* mutant animals. Also, introduction of mutant *egl-32* sperm into wild-type animals can induce an egg-laying defective phenotype. In an effort to determine the exact location of *egl-32* a candidate gene, *smn-1* was uncovered. A knockout of *smn-1* was obtained and characterized. The knockout is homozygous lethal. Heterozygous animals are egg-laying defective and respond similarly to *egl-32* in mating experiments. *smn-1* was also determined to interact with the same sperm proteins as *egl-32.*

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CHAPTER I: General Introduction

The Egg-laying System in *C. elegans*

The mutant I have characterized, *egl-32,* is an egg-laying defective mutant in *C. elegans.* Egg-laying defective mutants in *C. elegans* retain more eggs and lay fewer eggs than wild type animals. Egg-laying defective animals become bloated with eggs. The eggs eventually hatch inside the hermaphrodites, which become a 'bag of worms'. The Egg-laying defective phenotype can result from a variety of defects in the egg laying circuit.

Egg-laying requires the integration of various sensory cues. For example, eggs are laid in the presence of food. Once food is removed egg-laying ceases almost immediately (Trent et al., 1982). The vulval and uterine muscles, which are innervated by two types of neurons, are involved in egg laying. The neurons that innervate these sex muscles are the two Hermaphrodite Specific Neurons (HSNs) (Chalfie et al., 1985) and 6 VC motor neurons (White et al., 1986).

Three neurotransmitters have been shown to be important in egg laying: octopamine, serotonin, and acetylcholine (Horvitz et al. 1982; Trent et al. 1983). The small FMRFamide-related neuropeptide is also involved in egg-laying. A summary of the effects of these neurotransmitters is located in Table 1.1. Octopamine acts to inhibit egg-laying. Serotonin stimulates egg-laying. And, acetylcholine can act as a positive or negative regulator of egg-laying (Ramirez and Schafer, 1996). Pharmacological agents that affect the levels of these

Neurotransmitter	Serotonin	Octopamine	Acetylcholine	FMRFamide-related neuropeptide
Effects on Egg-laying	Stimulates egg-laying	Inhibits egg-laying	Can act as a positive or negative regulator of egg-laying	Stimulates egg-laying
Role in Egg-laying	Necessary for entry into the active phase	Unknown	Needed to maintain egg-laying while in the active phase	May act in parallel to serotonin for entry into the active phase

Table 1.1 – A summary of the neurotransmitters involved in egg-laying.

neurotransmitters also affect egg-laying in wild type animals. A summary of these pharmacological agents and their effects on egg-laying is found in Table 1.2. Phentolamine, an octopamine antagonist stimulates egg-laying (Horvitz et al., 1982). Drugs that potentiate cholinergic activity, such as aldicarb (an acetylcholinesterase inhibiter) and levamisole (an acetylcholine agonist), stimulate egg-laying. Mecamylamine, a cholinergic antagonist, inhibits egg-laying (Lewis et. al., 1980 a, b). Imipramine, a serotonin reuptake inhibitor, also stimulates egg-laying. Acetylcholine also stimulates muscle contraction in body wall muscles. Drugs that potentiate cholinergic activity also cause spastic paralysis.

Serotonin is produced by the HSNs as well as VC4 and VC5. Laser ablation of the HSNs results in animals that are egg-laying defective (Horvitz et al. 1982). Exogenous serotonin restores normal egg-laying in these animals. Imipramine has no effect on animals lacking HSNs, suggesting that the HSN is the endogenous source of serotonin. The HSNs are the only neurons of the egg laying circuit that have been shown to be required for normal egg laying. Laser ablation of both HSNs, but not the 6 VC neurons, results in failure to lay eggs.

Serotonin has also been shown to regulate the levels of the TGF-β dauer pathway ligand, *daf-7*, which is produced in the ASI neurons. A deletion mutation in *tph-1*, which encodes tryptophan hydroxylase, a key enzyme in serotonin biosynthesis, has a 6-fold reduction in *daf-7*::*gfp* expression (Sze et al. 2000) .

Pharmacological Agent	Imipramine	Levimosole	Aldicarb	Mecamylamine	Phentolamine
Neurotransmitter Effected	Serotonin re-uptake inhibitor	Acetylcholine agonist	Acetylcholine esterase inhibitor	Acetylcholine antagonist	Octopamine antagonist
Effects on egg-laying	Stimulates egg-laying	Stimulates egg-laying	Stimulates egg-laying	Inhibits egg-laying	Stimulates egg-laying

Table 1.2 – Summary of the pharmacological agents involved in egg-laying.

Acetylcholine is produced by the HSNs and all six of the VC motor neurons. Acetylcholine can act as a positive or negative regulator of egg laying (Ramirez and Schafer, 1996). Aldicarb and levamisole both initially stimulate egg laying in animals in M9 solution and cause spastic paralysis. Long-term exposure to levamisole leads to adaptation; the animals begin to move again and stop laying eggs in response to levamisole. Further evidence that acetylcholine can act as a positive or negative regulator of egg-laying comes from genetic studies. The *ace-2;ace-1* double mutants lack two of the three genes for acetylcholinesterase, which breaks down acetylcholine in the synapse. As a result these mutants have elevated acetylcholine levels and are egg-laying defective (Culotti et al., 1981). These mutants are sensitive to serotonin, but not levamisole. On the other hand, *cha-1* mutants have a decreased acetylcholine level due to a defect in choline acetyltransferase which in important in acetylcholine transmission (Alfonso et al. 1994). These mutants are egg-laying constitutive and levamisole hypersensitive (Ramirez and Schafer, 1996). A possible mechanism through which acetylcholine can be acting both as a positive and a negative regulator of egg-laying is through adaptation. It has been suggested that there is a signal transduction pathway that is responsible for this adaptation. Too much acetylcholine may result in adaptation and therefore an egg-laying defective phenotype, whereas not enough acetylcholine may result in a failure to activate the adaptation signal transduction pathway resulting in an egg-laying constitutive phenotype.

It was first thought that acetylcholine acted upstream of serotonin and the HSNs. This is because serotonin is a strong inducer of egg laying in both wild

type animals and animals that lack HSNs (Trent et al, 1983). Levamisole can strongly induce egg-laying in wild type animals, but not in animals that lack HSNs. However, it is more likely that these two neurotransmitters work in parallel. Mutants with low levels of acetylcholine, like *cha-1,* do not lay eggs in response to high levels of serotonin. Further evidence supporting the model that these two neurotransmitters act in parallel is that acetylcholine and serotonin are both released from the HSNs as well as VC4 and VC5. It is thought that serotonin is needed for entry into the active phase of egg-laying and that acetylcholine is necessary to maintain egg-laying while in the active phase (Weinshenker and Thomas, 1997).

The HSNs and the 6 VC motor neurons also contain one or more FMRFamide-related neuropeptides that may function as part of a hormonal pathway. These neuropeptides have also been shown to be important in controlling egg-laying.

The *flp-1* gene encodes a precursor of FMRFamide neuropeptides (Rosoff at al. 1992). Mutations in this gene have dramatically lengthened inactive egg-laying phases, but completely normal egg laying behavior while in the active phase. These mutants are also insensitive to food cues; they lay the same amount of eggs in the presences or absence of food. Serotonin stimulates *flp-1* mutants to lay eggs. However the rate and pattern of egg-laying is abnormal. Serotonin and the FLP-1 peptides may act in parallel to induce the switch from the inactive to the active phase (Nelson et al. 1998).

The *flp-1* mutants have a similar egg-laying defective phenotype as the TGF-β egg-laying defective/dauer-constitutive mutants such as *daf-1, daf-4, daf-7* and *daf-8*. Two of these, *daf-4* and *daf-8,* are also insensitive to food cues. The *flp-1* and dauer-constitutive genes may represent two hormonal pathways involved in relaying sensory cues to the egg laying circuit.

Defects in the HSNs or defects in the production of serotonin within the HSNs lead to an egg-laying defective phenotype (Trent et al.1983). Mutants with such defects will lay eggs if exogenous serotonin is provided. These mutants do not respond to imipramine, a serotonin reuptake inhibitor, as there is no serotonin present in the synapse.

The egg-laying defective phenotype can also result from defects in the vulval or uterine muscles (Trent et al. 1983). These mutants do not lay eggs in the presence of exogenous serotonin or imipramine, as they are unable to respond.

Other egg-laying defective mutants have been isolated that do not belong to either of the above-mentioned categories. Many dauer-constitutive/egg-laying defective mutants, including *daf-1, daf-7, daf-8* and *daf-14*, respond to both serotonin and imipramine (Trent et al. 1983). Other dauer-constitutive/egg-laying defective mutants, such as *daf-4,* have a mixed and variable response to serotonin and imipramine. Mutant *egl-32* animals also have a mixed and variable response to serotonin and imipramine.

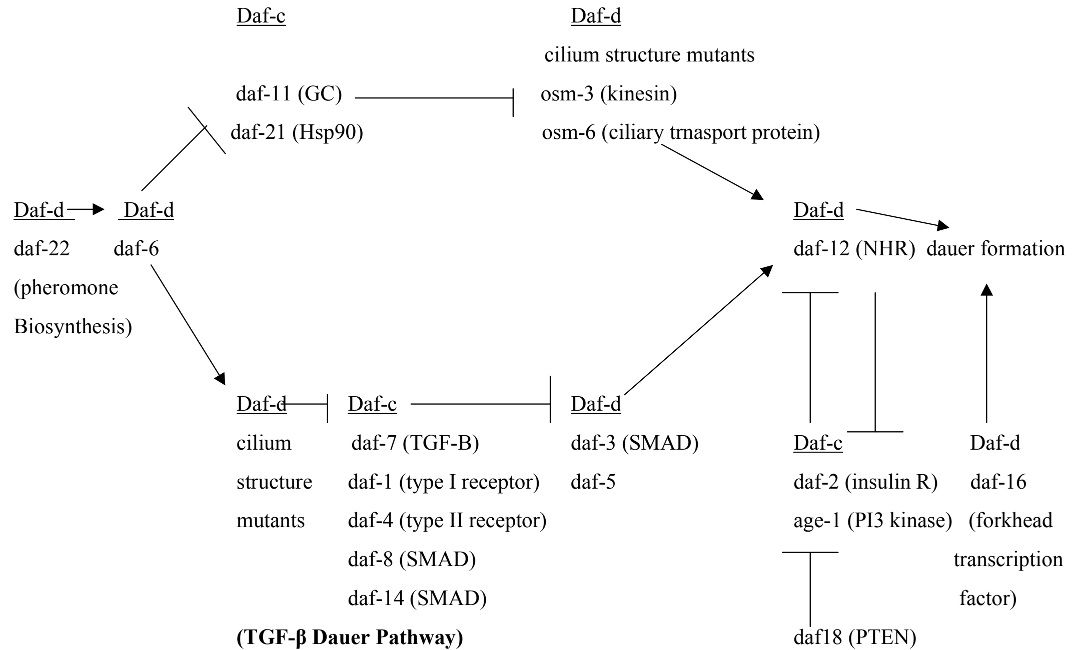
Egg-laying constitutive mutants have also been isolated. The egg-laying constitutive mutants in *C. elegans* retain fewer eggs than wild type animals. These animals also lay more eggs than wild type animals in the absence of food.

The TGF-β dauer Pathway in *C.elegans*

egl-32 is implicated in the transforming growth factor-β signal transduction pathway that regulates the dauer decision and egg-laying in *C.elegans*. The decision to form dauers is regulated by at least three signal transduction pathways: The transforming growth factor-β (TGF-β) pathway, an insulin-like pathway, and a cGMP pathway (Thomas, 1993) (Figure1.1). Only the TGF-β pathway regulates both the dauer decision and egg-laying (Trent et al. 1983). In the TGF-β pathway (Figure 1.2) the ligand, DAF-7 (Ren et al. 1996) is predicted to bind to the common type II receptor, DAF-4 (Estevez et al.,1993; Savage et al. 1996), and the type I receptor, DAF-1 (Georgi et al. 1990). Binding causes the constitutively active type II receptor to phosphorylate and active the type I receptor. The type I receptor is then predicted to phosphorylate and activate DAF-8 and DAF-14 Smads (Riddle & Albert, 1997; Inove & Thomas, 2000). The activated Smads antagonize the function of DAF-3 (Patterson et al. 1997) and DAF-5 (Riddle & Albert, 1997) (Figure 1.2). The DAF-7 ligand is expressed in a pair of chemosensory neurons called the ASI neurons (Ren et al.1996; Schackwitz et al. 1996) The ASI chemosensory neurons detect environmental conditions. In the first and second (L1 and L2) stages the ASI neurons are important in assessing

food availability, population density, and temperature to determine whether to proceed to the dauer larva or continue to adulthood (Ren et al. 1996; Schackwitz et al. 1996). Under favorable conditions, *daf-7* is expressed and the animal develops into an adult. Under unfavorable conditions, *daf-7* expression is repressed and dauer development occurs. Killing the ASI and ADF amphid chemosensory neurons with a laser microbeam has been shown to induce dauer development (Bargmann & Horvitz, 1991). Expression of *daf-7* is partially regulated by serotonin levels. Tryptophan hydroxylase, the key enzyme involved in serotonin biosynthesis, is encoded by *tph*-1. Mutants defective in TPH-1 have a 6 fold reduction in DAF-7::GFP expression, suggesting that serotonin may up-regulate *daf*-7 expression (Sze et al. 2000).

egl-32 had previously been implicated in the TGF-β dauer pathway because it was found that the egg-laying defective phenotype of *egl-32(n155)* is suppressed by mutations in *daf*-3 and *daf*-5 (Trent et all, 1983). Mutations in other members of the TGF-β pathway, upstream of *daf-3* and *daf-5,* are dauer-constitutive and also egg-laying defective. These mutants have both their dauer-constitutive and egg-laying defective phenotypes suppressed by mutations in *daf-3* and *daf-5.*



<u>Figure 1.1</u> – The three pathways regulating dauer formation in *C. elegans*. All three pathways converge on *daf-12*. Only mutants in TGF-β dauer pathway, which are suppressed by *daf-3* and *daf-5* are also egg-laying defective.

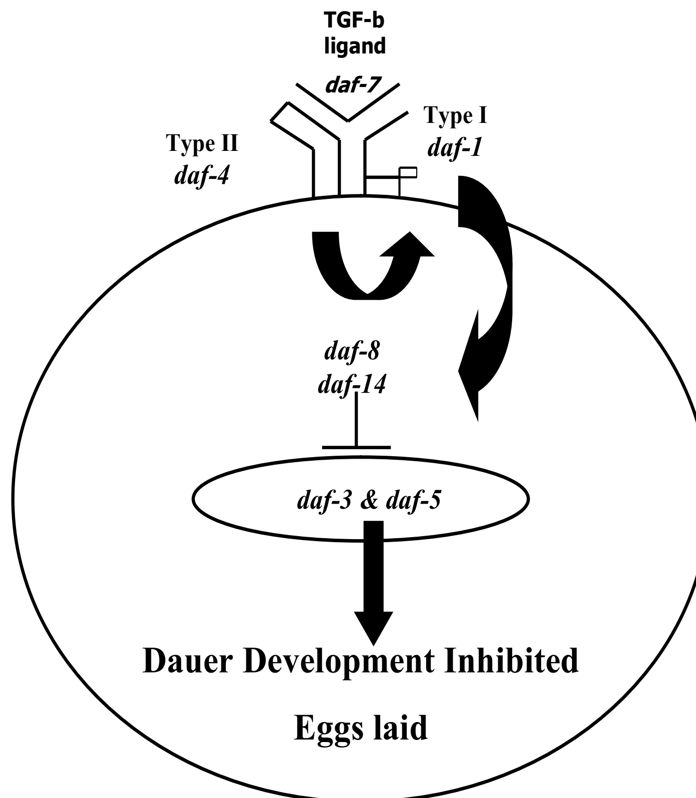


Figure 1.2 - The TGF-β dauer signal transduction pathways in *C. elegans.* This pathway regulates dauer development and egg-laying. The ligand, DAF-7 is produced when conditions are good. The ligand binds to the type I receptor, DAF-1, and the type II receptor, DAF-4. The constitutive kinase activity of DAF-4 phosphorylates and activates DAF-1, which then phosphorylates and actives the Smads, DAF8 and DAF-14, which function to suppress the Smad, DAF-3 and the ski/sno homologue DAF-5. As long as DAF_3 and DAF-5 are suppressed dauer development is inhibited in larvae and eggs are laid in adults

Major Sperm Proteins in *C.elegans*

I will show that EGL-32 acts through sperm to regulate egg-laying. Previously it has been shown that meiotic maturation and ovulation in *C. elegans* is regulated partially by sperm (Miller et al. 2001). In female mutants, oocytes fail to undergo meiotic maturation. Instead the oocytes arrest in diakinesis. Oocyte maturation is completed when sperm is introduced by mating. Sperm and oocytes are both thought to promote sheath cell contraction. Contraction of the six myoepithelial sheath cells that surround the proximal gonad in the hermaphrodite is responsible for expelling the oocyte from the gonad into the uterus at ovulation (Strome, 1986). In female mutants the sheath cells do not contract regularly until sperm are introduced. In males the sheath cells contact regularly, suggesting that sperm can directly stimulate sheath cell contraction even in the absence of oocytes. It has recently been shown that the major sperm proteins (MSPs) supply the signal for oocyte maturation and ovulation (Miller et al. 2001). When sperm extracts containing the MSPs were injected into *fog-2* females, which lack sperm due to a defect in germ-line sex determination, sheath cell contraction and oocyte maturation resumed. It has been shown that the signal for ovulation is conserved in the genus *Caenorhabditis.* Sperm from one species can promote ovulation in other species. In my work, I extend this finding to suggest that sperm also produce a signal that helps coordinate oocyte fertilization with egg laying, helping to insure that eggs are not laid too soon, or retained for too long.

The survival motor neuron gene

We have reason to believe that *egl*-32 and the *C.elegans* orthologue of the human survival motor neuron (smn) gene are allelic. In humans when the survival motor neuron (smn) gene is mutated the result is spinal muscular atrophy (sma)(Lefebvre, et.al. 1995). This is one of the leading genetic causes of infant mortality (Crawford & Pardo 1996). The survival motor neuron gene is ubiquitously expressed in human (Coovert et. al., 1997 & Lefebvre et. al., 1997) and *C. elegans* (Miguel-Aliaga et. al., 1997). Although *smn* is ubiquitously expressed in human cells, motor neurons seem to be especially susceptible to reduced levels of this gene (Coovert et. a., 1997). SMN is involved in snRNP biogenesis (Liu et. al., 1997 & Fisher et. al., 1997) and pre-mRNP splicing (Pellizzoni et. al., 1998). In *C. elegans* a knockout of the *smn-1* gene is homozygous lethal. Heterozygous animals are egg-laying defective at all temperatures.

Chapter II: Characterization of *egl-32(n155)*

Materials and Methods

Strains – The Bristol N2 strain is the wild-type strain. *egl-32(n155*) is a temperature sensitive egg laying mutant isolated by Trent et al (1983). Growth was on EZ worm plates.

Egg Retention Assay – L4 larvae were placed on plates with food and allowed to mature at 25°C overnight into young adults. The young adult worms were then placed on an agar pad on a glass slide in a drop of 10mM sodium azide. The worms were then viewed under a high power compound microscope (Zeiss Axioscope). The number of eggs retain in utero were counted. 36 N2 animals and 33 *egl-32* animals were used in this assay.

Egg-laying Assays - L4 larvae were placed on plates with food and allowed to mature at 25°C overnight to become young adults. The young adults were move to individual plates that either contained food or did not contain food. The worms were allowed to lay eggs at 25°C for 2 hours. The number of eggs (or hatched larvae, many times was the case with the egg-laying defective animals) present on the plate after 2 hours were then counted. The number of eggs laid in one hour was then calculated. 26 N2 animals and 26 *egl-32* animals were used in this assay.

Ovulation Rate Assay – L4 animals were placed on a plate and allowed to mature overnight at 25°C. The number of eggs retained by each adult was then counted

by placing the animals on an agar pad containing a small drop of food. The worms were then placed on individual plates and allowed to lay eggs for 5 hours at 25°C. The number of eggs retained after 5 hours was again counted. The number of eggs laid on the plate during the 5 hours was also counted. The ovulation rate was determined by adding the number of eggs retained and laid at the 5 hour time point and then subtracting number of eggs each animal originally retained. The number of ovulations per hour, per gonad arm was then calculated. 68 *him-5* animals (used as wild-type) and 100 *egl-32(n155);him-5* animals were used in this assay.

Food Cue Assay – L4 larvae were placed on a plate containing food and allowed to mature into young adults overnight at 25°C. The young adults were then either placed on plates containing food or lacking food. After 2 hours the number of eggs laid (or progeny present) was counted. 26 wild-type animals in the presence of food, 26 *egl-32(n155)* animals in the presence of food, 39 wild-type animals in the absence of food and 39 *egl-32(n155)* animals in the absence of food were assayed.

Temperature Shift Assay – Eggs were placed on a plate and allowed to hatch at either 15°C or 25°C. At various times after hatching (24, 30, 48 and 50 hours) the worms were shifted to a higher or a lower temperature. The worms that hatched at 25°C were shifted down to 15°C. The worms hatched at 15°C were shifted up to 25°C. The number of eggs retained in the young adults was then counted as

above. Ten animals were scored at each time point. Two hours of development at 15°C was considered equivalent to one hour of development at 25°C.

Brood Size Assay – L4 animals were placed on a plate and allowed to mature into young adults overnight at 25°C. The animals were then moved to a fresh plate and the number of eggs (or progeny) present on the plate was counted. This process was repeated each day for seven days. By the seventh day all the animals were dead. The total number of progeny was then calculated. 8 N2 and 8 *egl-32(n155)* animals were used in this assay.

Length Assay – To look at the length of L4 larvae L4 animals were collected. Pictures were taken and Sigma Scan Pro was used to calculate the length of the worms 6 N2 and 8 *egl-32* animals were used in this assay. To determine the length of young adult hermaphrodites, L4 larvae were allowed to mature overnight at 25°C. The young adults were then photographed and the length was determined using the same program. 10 N2 and 10 *egl-32(n155)* animals were used in this assay. For the male length assay, young males were collected and measured the same way as the hermaphrodites. 50 wild-type and 57 e*gl-32(n155)* males were used in this assay.

RESULTS

egl-32 Mutant Animals have Defects in Egg Retention, Egg-laying and Ovulation

In *C. elegans* a mutation in *egl-32* results in egg-laying defective animals. We used two assays to quantify egg-laying. For one assay, we counted the number of eggs (embryos) retained in the hermaphrodite uteri. L4 animals were allowed to mature for 24 hours at 25°C and then were examined to determine the number of eggs these young adults retained. Egg retention reflects both the rate of ovulation and the rate of egg-laying. For the second assay, we counted the number of eggs laid in a fixed time period. Again, L4 animals were allowed to mature for 24 hours at 25°C and then were placed on fresh plates for 2 hours. After 2 hours, the hermaphrodites were removed and the number of progeny on the plates counted. In the egg retention assay, mutant *egl-32* animals retain about twice as many eggs as wild type animals (Figure 2.1a). In the egg-laying assay, mutant *egl-32* animals were found to lay about half as many eggs as wild type animals (Figure 2.1b).

Since egg retention reflects both the rate of ovulation and the rate of egg-laying we determined the ovulation rate of *egl-32* animals (Figure 2.1c). Mutant *egl-32* animals ovulate slightly, but significantly, slower then wild-type animals. This suggests that *egl-32* animals have a defect in both ovulation rate and in the rate of egg laying. However, their egg-laying defect is more severe than their ovulation rate defect.

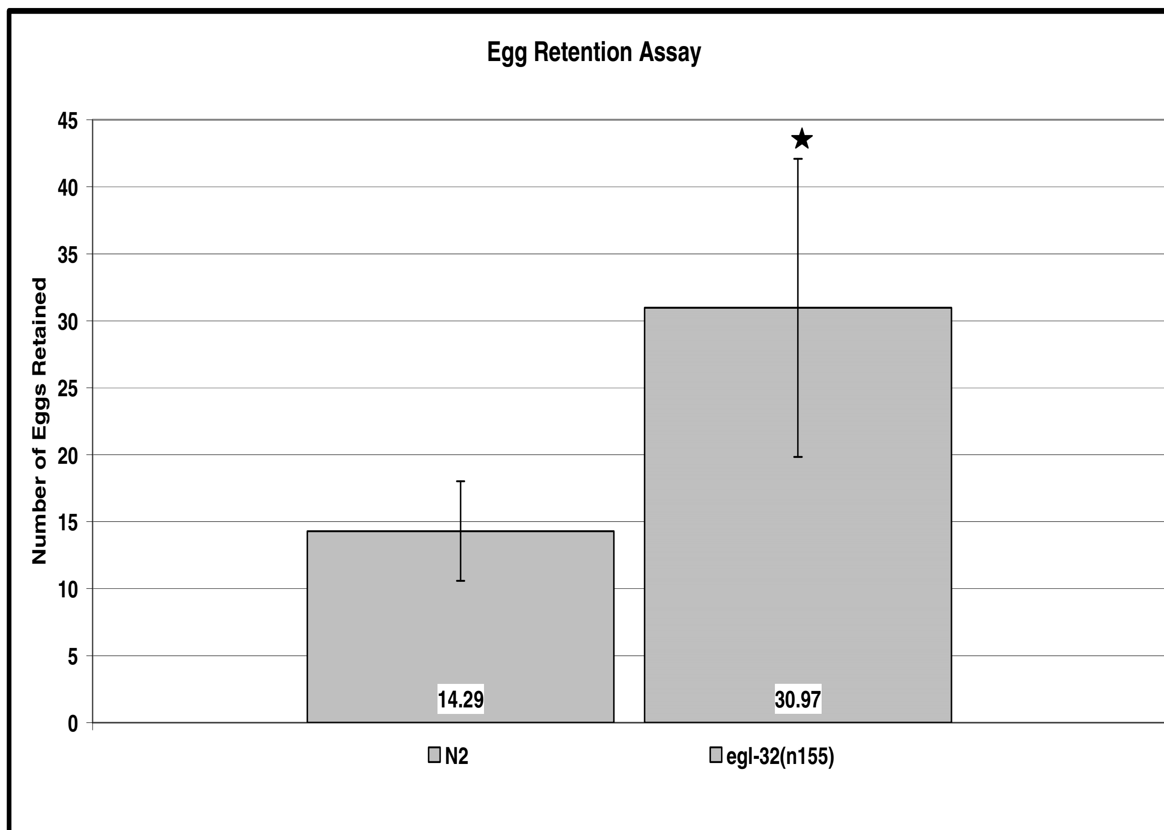


Figure 2.1a – *egl-32(n155)* animals retain about twice as many eggs as wild-type animals. * Statistically significant difference between N2 and *egl-32*. Student t-test value of 1.54582 X 10^{-12}. Bars indicate standard deviation.

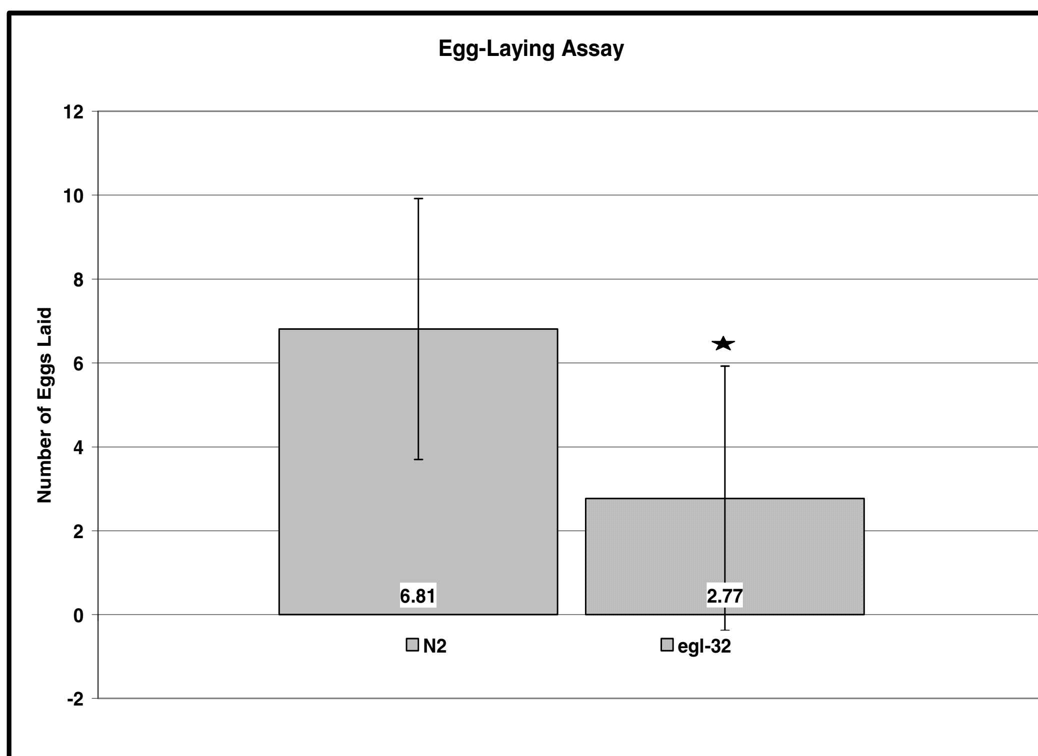
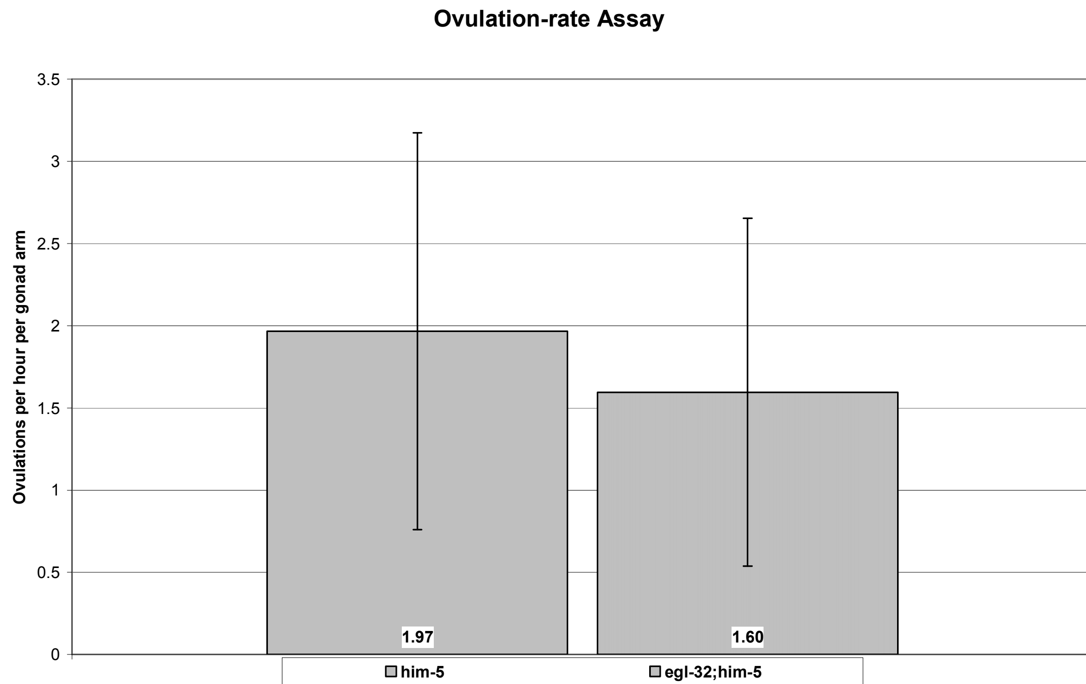


Figure 2.1b – e*gl-32(n155)* animals lay about half as many eggs in a 2 hour period as wild-type animals.* Statistically significant difference between N2 and *egl-32*. Student t-test value of 1.23 X 10^{-5}. Bars indicate standard deviation.



<u>Figure 2.1c</u> – *egl-32(n155)* animals ovulate at a slightly, but significantly slower rate than wild-type N2 animals.* Statistically significant difference between *him-5* and *egl-32;him-5*. Student t-test value of 0.042. Bars indicate standard deviation.

Mutant *egl-32* Animals Respond Normally to Food Cues

One of the important cues regulating the TGF-β dauer pathway is the presence or absence of food. In the absence of food young larvae will form dauers and older animals will begin to lay fewer eggs (Trent 1982). Since *egl-32* has been implicated in the TGF-β dauer pathway via suppression by *daf*-3 and *daf*-5, we were interested in determining if they were sensitive to food cues. In the absence of food wild-type animals begin to lay fewer eggs. This is also true of *egl-32* animals (Figure 2.1d) suggesting that the defect in *egl-32* is not at the level of chemosensation or sensory processing of food cues.

Mutant *egl-32* Animals have a Reduced Brood Size

We were interested in determining if the egg-laying defect of *egl-32* animals results in a decrease in the number of progeny produced during their lifetime. A reduction in the number of progeny could indicate germ-line defects including a reduction in the production of sperm or oocytes. The total number of progeny produced by both wild-type and *egl-32(n155)* animals was calculated over a one week period. During this time wild-type animals produced 3 times as many progeny as *egl-32* animals (Figure 2.2). This could be the result of a germline defect. However, it could also be due to a decrease in life span due to internal hatching of some of the progeny.

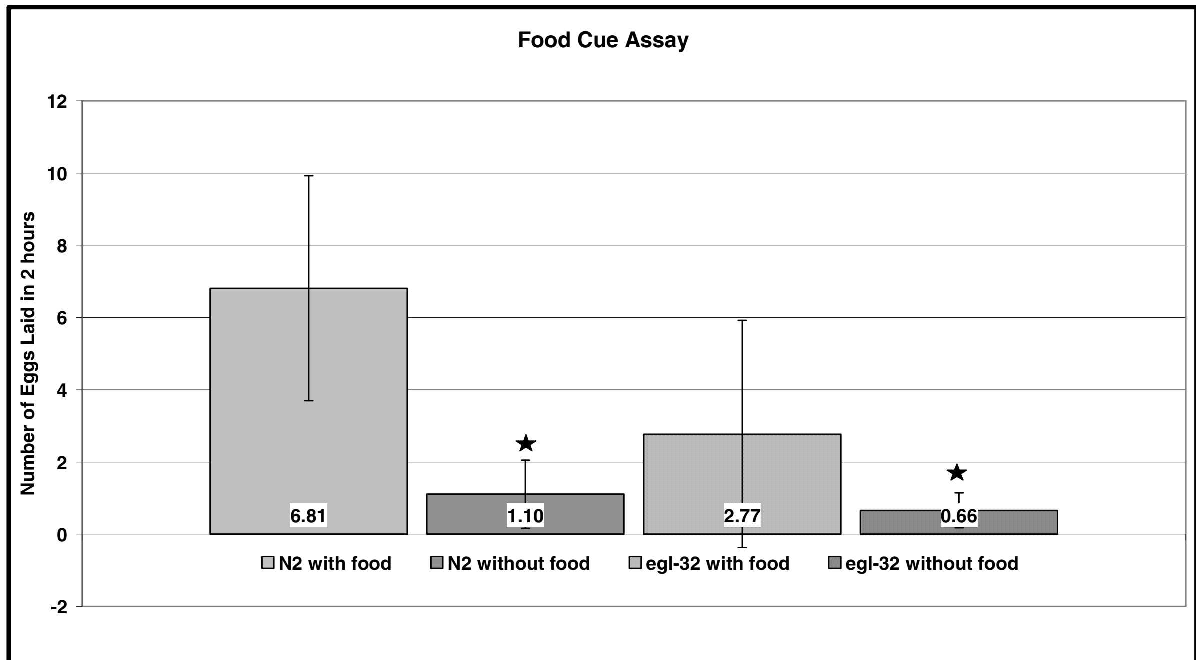


Figure 2.1d –*egl-32(n155)* animals respond normally to food cues. In the absence of food both wild-type and *egl-32n155)* animals lay fewer eggs than in the presence of food.* Statistically significant difference between N2 with food and without food, and *egl-32* with food and without food.. Student t-test for N2 with and with out food has a value of 3.16×10^{-16}. Student t-test value for *egl-32* animals with and without food has a value of 6.69×10^{-5}. Bars indicate standard deviation.

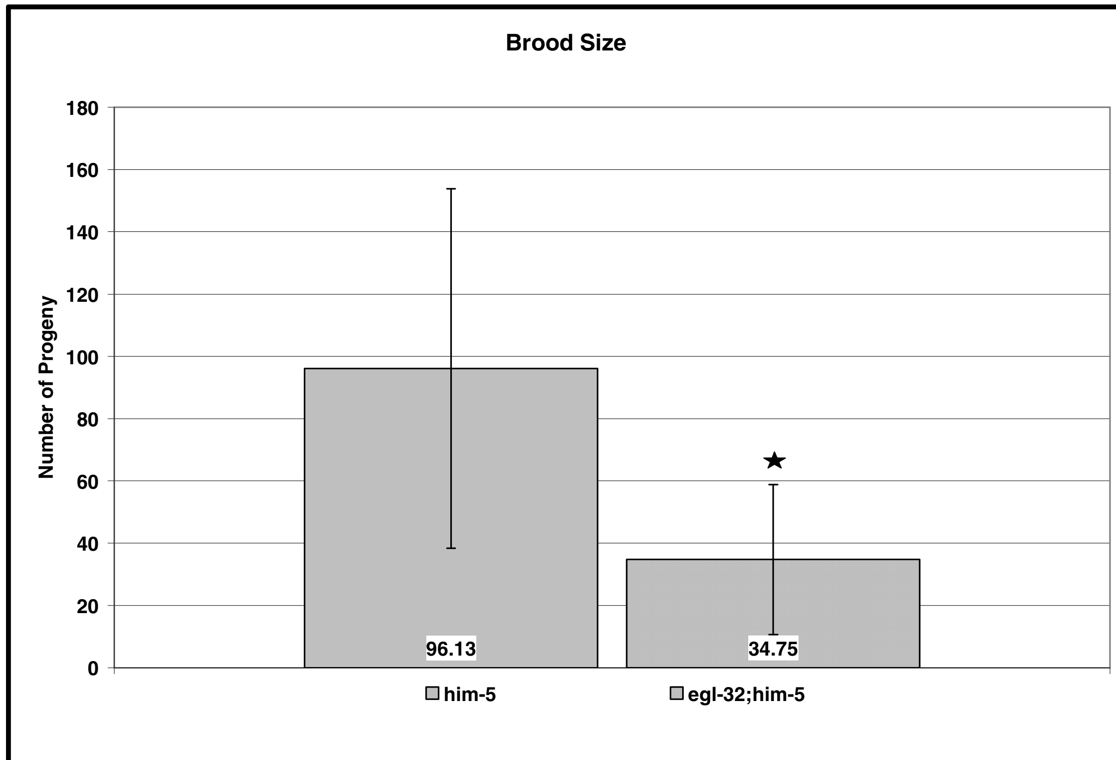


Figure 2.2 – Brood size assay. *egl-32(n155)* animals produce about half as many progeny during their life time then wild-type animals.* Statistically significant difference between *him-5* and *egl-32;him-5*.. Student t-test value of 8.06×10^{-3}. Bars indicate standard deviation.

The L4 stage is the Critical Period for *egl-32* Activity

The one existing allele of *egl-32(n155)* is temperature sensitive. The animals only display their egg-laying defect if they are grown at 25°C. Our technician, Ling Yu, performed a temperature shift assay to determine the critical period for *egl-32* activity. This turned out to be between 24 and 48 hours after egg collection at 25°C, which corresponds to the L4 stage of development (Figure 2.3). This was an interesting result because egg-laying does not occur at this stage. However, this is the time during which vulval morphogenesis occurs and the HSNs innervate their targets. Moreover, it is the one and only time during which hermaphrodites produce sperm (Ward & Carrel, 1979).

Mutant *egl-32* Animals have a Normal Length

Because other egg laying defective animals that are suppressed by *daf-3* and *daf-5* have lengths that vary from that of wild type animals we were curious to see if *egl-32* animals had a defect in body length. The *egl-32(n155)* mutant hermaphrodites were measured (Figure 2.4a). The *egl-32(n155)* hermaphrodites are 10% longer than wild type animals. L4 larvae were also measured and found to be the same length as wild-type L4 larvae (Figure 2.4b). In order to be sure that this increased length seen in the young adults was not a side effect of egg retention, we collected and measured *egl-32(n155)* males (Figure 2.4c). The *egl-32* males were found to be the

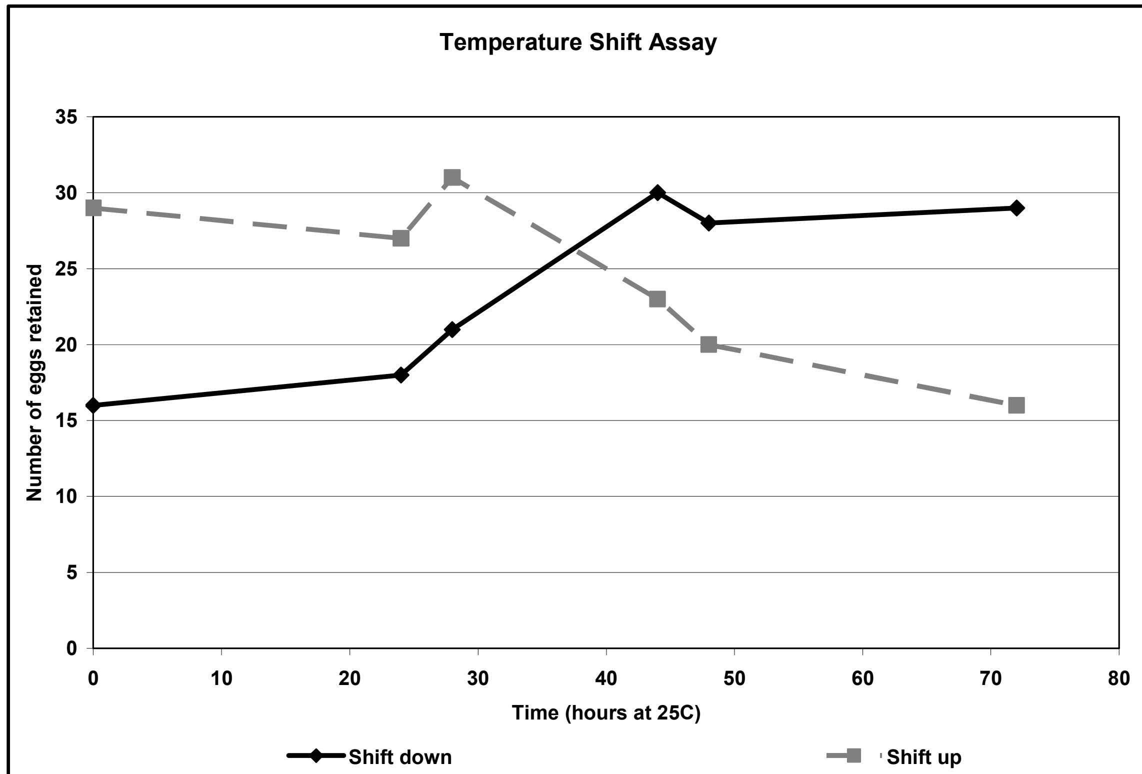


Figure 2. 3 – Temperature shift assay. Animals were either raised at 15˚C and shifted up to 25˚C at various time points, or they were raised at 25˚C and shifted down to 15˚C at various time point. The critical period for EGL-32 activity is the L4 stage of development. 10 animals were assayed at each time point. (work done by Ling Yu)

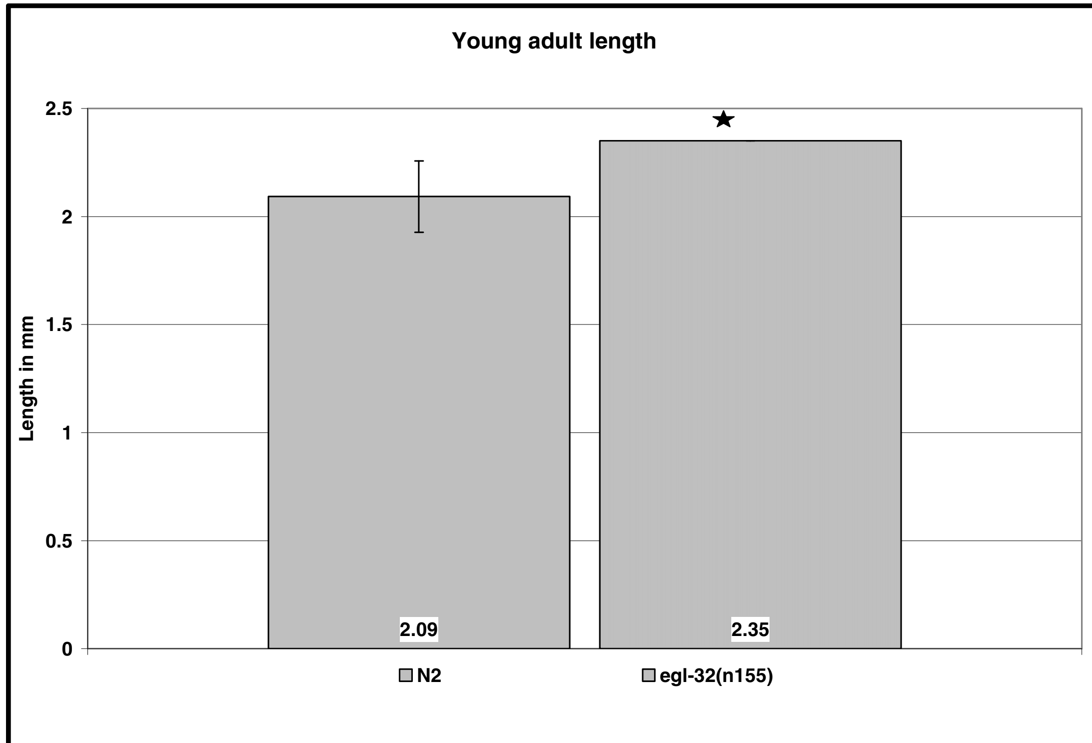


Figure 2.4a – Young adult *egl-32* animals are slightly longer then wild-type animals. 10 wild-type and *egl-32(n155)* animals were used in this assay.* Statistically significant difference between N2 and *egl-32*. Student t-test value of 2.6×10^{-5}. Bars indicate standard deviation.

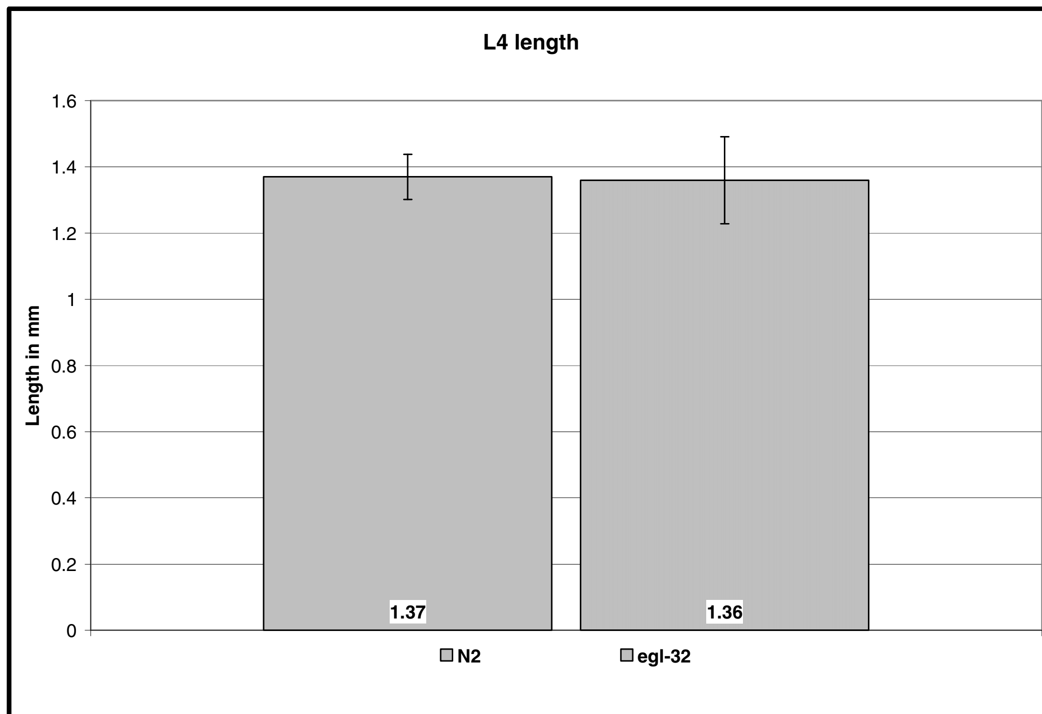


Figure 2.4b – Wild-type and mutant *egl-32(n155)* animals have no difference in length as L4 larvae. 6 wild-type and 8 *egl-32(n155)* animals were used in this assay. The difference is not statistically significant. Student t-test value of 0.35. Bars indicate standard deviation.

Figure 2.4c – Male *egl-32(n155)* animals are the same length as wild-type males. The difference is not statistically significant. Student t-test value of 0.429709. Bars indicate standard deviation.

same length as wild-type animals. Thus, the long length seen in the young adult hermaphrodites is most likely due to the increased number of eggs the animals are retaining.

Conclusion

Mutant *egl-32* animals retain about twice as many eggs, lay about half as many eggs and ovulate at a slightly, but significantly, slower rate than wild-type animals . This is not due to a defect in chemosensation or sensory processing of food cues as *egl-32* animals respond normally to food cues. Mutant *egl*-32 animals produce fewer offspring than wild-type animals. *egl-32(n155)* is a temperature-sensitive mutation. Temperature shift assays reveal that the L4 stage of development is the critical period for EGL-32 activity. This was an interesting finding because this is not when eggs are being laid. However, it is the one and only time that a hermaphrodite produces sperm. We have also shown that although *egl-32* hermaphrodites are approximately 10% longer than wild-type hermaphrodites, L4 larvae and *egl-32* males are not longer. Thus, the increase of length seen in the hermaphrodites is likely a side effect of their excessive egg retention.

Chapter III: Mapping and Genetic Interactions

Materials and Methods

Strains – The Bristol N2 strain is the wild-type strain. *egl-32(n155*) is a temperature sensitive egg laying mutant isolated by Trent et al (1983). A knockout of T08G11.2, *tm 336,* and knockouts of two of its homologues, *tm332* and *tm322,* were obtained from the knockout gene consortium. The HA strain and *dpy-5(e61);egl*-32(n155) double mutant were used for the SNP mapping. *smn-1(ok355),* a knockout of the *smn-1* gene, was characterized. Growth was on EZ worm plates.

Egg Retention Assay – L4 larvae were placed on plates with food and allowed to mature at 25°C overnight into young adults. The young adult worms were then placed on an agar pad on a glass slide in a drop of 10mM sodium azide. The worms were then viewed under a high power compound microscope (Zeiss Axioscope). The number of eggs retained in utero were counted. 39 N2 animals, 36 *egl-32* animals, 35 *tm336* animals, 67 *tm332* animals, 144 *tm322* animals and 86 *smn-1* heterozygous animals were used in this assay.

Egg-laying Assays - L4 larvae were placed on plates with food and allowed to mature at 25°C overnight to become young adults. The young adults were moved to individual plates containing food. The worms were allowed to lay eggs at 25°C for 5 hours. The number of eggs or hatched larvae present on the plate after 5

hours was then counted. The number of eggs laid in one hour was then calculated. 68 *him-5* animals and 25 *smn-1* heterozygous animals were used in this assay.

Ovulation Rate Assay – L4 animals were placed on a plate and allowed to mature overnight at 25°C. The number of eggs retained by each adult was then counted by placing the animals on an agar pad containing a small drop of OP 50. The worms were then placed on individual plates and allowed to lay eggs for 5 hours at 25°C. The number of eggs retained after 5 hours was again counted. The number of eggs laid on the plate during the 5 hours was also counted. The ovulation rate was determined by adding the number of eggs retained and laid at the 5 hour time point and then subtracting number of eggs each animal originally retained. The number of ovulations per hour, per gonad arm was then calculated. 68 *him-5* animals (used as wild-type), 53 *tm336* animals and 25 *smn-1* heterozygous animals were used in this assay.

Sequencing – The following primer pairs were generated;

egl32-9 5' -CCG GAT CCA CTG AGC ACA TTT- 3'

egl32-10 5' – CGC GTA CCG AAA CAC GCG CGC TGC GTC- 3'

egl32-13 5' - GCG GAT CCG CAA AGG TAA CGC TGC TCG G- 3'

egl32-17 5' –CTG TGC CAG TGA ATT CGT CG- 3'

egl32-19 5' –CGG CGA CAA GCG ATT CGA AC- 3'

smn1-4 5' –CGG GAT CCA AAC TTA AAC TCC GCC TCT- 3'

smn1-5 5' –GCG GAT CCG ATG GGT GGT GCG GCA GCA CG- 3'

These primers were used in a PCR reaction. The products of the PCR reactions

were sent to the DNA Sequencing Resource Center at Rockefeller University for sequencing. The sequences were then analyzed by performing a BLAST search of the *C.elegans* genome. The top hit was always from the expected area of the genome. The sequences were checked for any alterations in the nucleotide sequence between *egl-32,* or the three potential new alleles that were isolated, and the wild-type gene.

Screen for new allele – *dpy-5* worms were mutagenized using EMS following a standard protocol. The mutagenized worms were then crossed with *egl-32;him-5* animals. Non-dumpy, egg-laying defective animals were selected from the progeny. In the next generation dumpy, egg-laying defective animals were selected and used for sequencing and complementation testing.

Complementation test of new alleles – The three potential new alleles of *egl-32* were crossed with *egl-32;him-5* worms as follows; Double dpy-5;potential new alleles hermaphrodites were mated with *egl-32;him*-5 males . The next generation was screened to see if all the non-dumpy progeny were all egg-laying defective. 33 *egl-32* animals, 35 *tm336* animals and 36 double*, egl-32;tm336,* animals were used in this assay. .

SNP mapping – The following SNPs were chosen to map *egl-32*; T08G11, (T/C at position 17288), F36A2 (C/T at position 3370), and F16A11 (G/T at position 29146). The following primers were used;

Inner forward primer for F36A2 (for N2)– 5' CTA GGG TAA TCG TAC AGT ACT CCG AT-3'

Inner reverse primer for F36A2 (for HA) 5' –CTG TAG TGG TAC AAT GGT GGT AAGG-3'

Outer reverse primer for F36A2 5'-ATT CCG TTT GTC CAT AGT TTG TAG TC-3'

Outer forward primer for F36A2 5' –TCA GCA AAT TTT ATG GTT TTC TTT TT-3'

Inner forward primer for F16A11 (for HA) 5' –TCC CAC TGA CGA TAC TCT ATT TTT GAA CAT-3'

Inner reverse primer for F16A11 (for N2) 5' –GAG CAC TTT CTT TGC CAG CAA TAC TC- 3'

Outer reverse primer for F16A11 5' –ATT TAT CTT TGA TCC TGC ACG GTA CAT G- 3'

Outer forward primer for F16A11 5' –AAA TTT CCC GAT TTT GAA ATT TGG TAA A-3'

Inner forward primer for T08G11 (for HA) 5' –GCC AAA CTT GTC TCT AAA TGT TTT GGC- 3'

Inner reverse primer for T08G11 (for N2) 5' –GAA GTT TGG CTC TGA TGA TAG GAC CA- 3'

Outer reverse primer for T08G11 5' –AGA AAA CTT AGT GAC AGC GTC ATG TCA A- 3'

Outer forward primer for T08G11 5' –ATA TCT TCC TTG CCA GTA ACT GAA AGC A-3'

The primers were mixed as follows; For each SNP the forward and reverse primers were mixed in one tube with the N2 specific allele, in a separate tube the forward and reverse primers were mixed with the HA specific allele. PCR reactions were set up as follows; each reaction contained 16.5µl water, 2.5µl 10X PCR buffer, 0.5µl 50mM $MgCl_2$, 0.5µl 10mM dNTPs, 0.5µl taq polymerase, and 2.5µl of DNA. The PCR products were run on a 2% gel. Recombinants were generated as follows, *dpy-5;egl-32* animals were crossed with wild-type HA animals. F1 L4 larvae were placed on individual plates and allowed to mature and produce progeny. The F2 generation was examined for either egg-laying defective, non-dumpy animals, or dumpy non-egg-laying defective animals. These animals were placed on separate plates and allowed to self fertilize. Approximately 8-10 worms were place in 8µl of Lysis buffer and lysed to extract their DNA for use in PCR.

Background

Preliminary experiments on *egl-32* were performed by Ling Yu. *egl*-32 had previously been mapped to chromosome I at position 3.95. Ling attempted to narrow down the *egl-32* interval using chromosomal deficiencies in the region between *unc-29* and *mec-8* on LGI. Next, cosmids mapping to this interval were tested for the ability to rescue *egl-32(n155)*. One cosmid from this interval, C26G6, which overlaps the sequenced cosmid T08G11, rescued the *egl-32* phenotype. Among several subclones of this cosmid, one containing two predicted

ORFs T08G11.2 and T08G11.3, but not one containing only T08G11.3, also rescued *egl-32* (Figure 3.1). An RNAi was also performed using T08G11.2. Injection of dsRNA into adults gave no phenotypes in the offspring. However, injection of L4 animals with dsRNA from this gene results in a weak egg-laying defective phenotype.

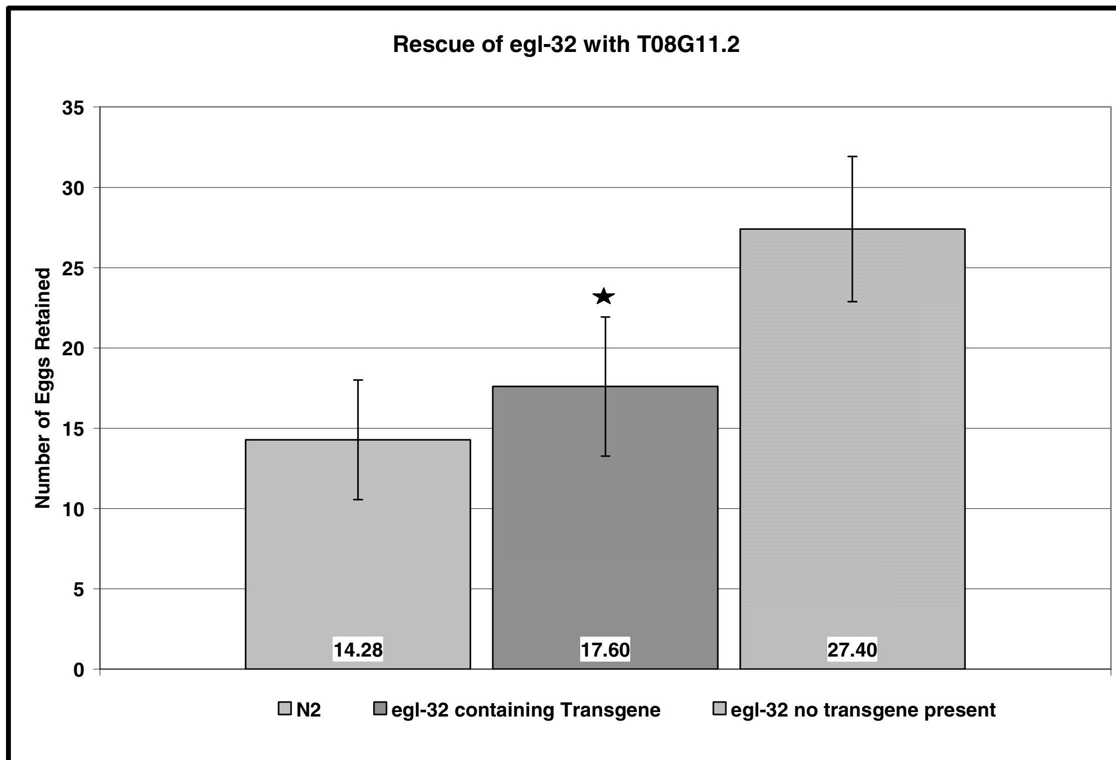


Figure 3.1 – Injection of the T08G11.2 rescues the egg-laying defective phenotype of *egl-32(n155).* The roller gene was co-injected as a marker gene. * Statistically significant difference between animals containing the transgene and those that do not contain it. Student t-test value of 5.17605×10^{-05}. Bars indicate standard deviation.

RESULTS

T08G11.2 Interacts with *egl-32*

Although the preliminary results suggest that *egl-32* is likely to correspond to the predicted ORF T08G11.2, sequencing of T08G11.2 from *egl-32(n155)* animals revealed no change to the genome. We extended our sequencing of this region from the previous ORF to the next ORF and still could not find a change to the genome. Furthermore, we performed a complementation test between *egl-32(n155)* and a knockout of T08G11.2 (*tm336)*. The two genes complement, providing further evidence that *egl-32* is not T08G11.2 (Figure 3.2). Collectively, these data suggest that T08G11.2 is not *egl-32* but that it may be an interacting locus.

T08G11.2 encodes a small novel protein of 282 amino acids. It contains an SH2-like domain. However, it lacks the critical arginine present in functional SH2 domains (Figure 3.3). A BLAST search revealed that T08G11.2 has 3 homologues in *C. elegans*. T08G11.2 and its three homologues have all been identified as sperm enriched transcripts (Kim 2000). T08G11.2 and its closest homologue, B0207.11, have about a 10,000-fold enrichment in sperm. A similar fold enrichment is found in the sperm specific proteins (*ssp-11*, *ssp-16*, *ssp-19*, *ssq-1*, *ssq-2*, *sss-1* and *sss-2*). To further characterize the biological roles of these sperm enriched proteins, we obtained knockouts from the *C.* elegans knockout consortium. These animals all retained far fewer eggs than wild-type animals

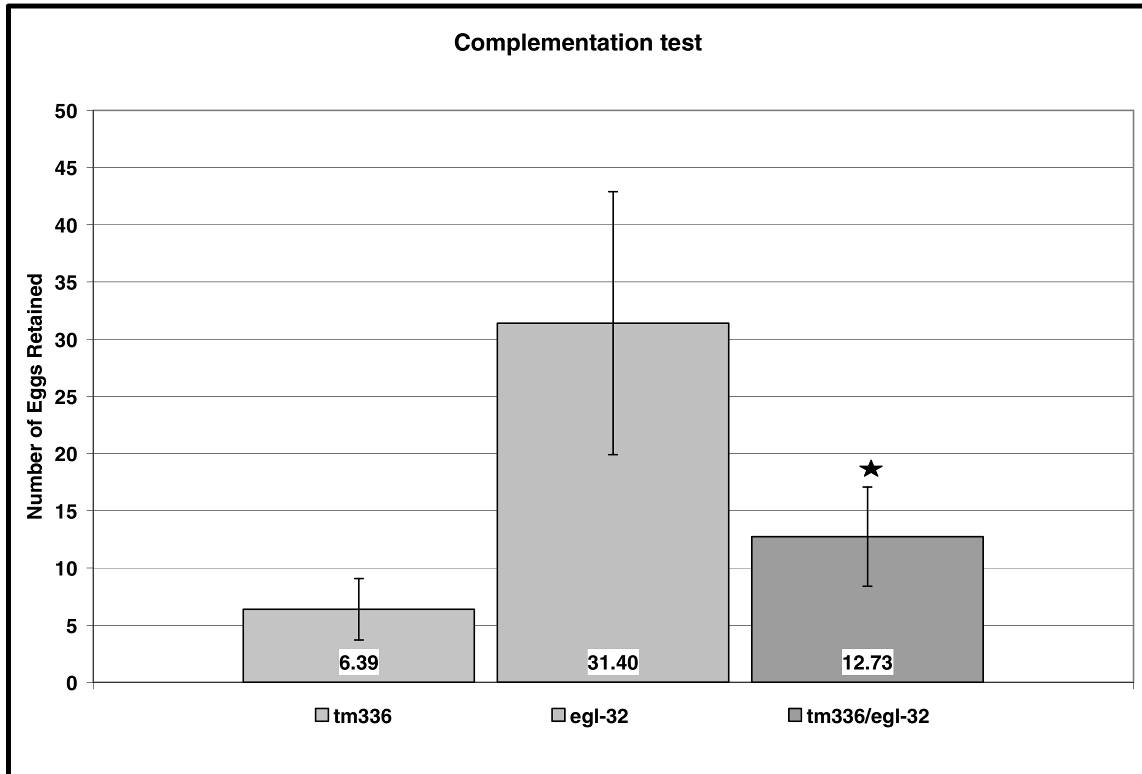


Figure 3.2 – *egl-32* and the knockout of T08G11.2 (*tm336)* complement each other. This suggests they are not the same gene. * Statistically significant. An ANOVA test was performed. The p-value comparing all groups is 7.47×10^{-27}. The double heterozygote retains significantly fewer eggs than *egl*-32 alone, p-value of 1.74×10^{-14}. The double heterozygote also contains significantly more eggs than the knockout of T08G11.2, *tm336,* alone, p-value of 8.78×10^{-11}. Bars indicate standard deviation.

MESDTSRTPYNSIYATLKNKKTDGEVVCCVHGRRCSYGKGNQDGKRDDNQYVNLPFLKNQDQIASKLTGKLNKLKI

AEEKENKIVPREKPEMMSLNTLVEIARAGEQPRKGRGDKEKRTKKAPVLVDDSQLYVMIGPPSEHPGEPPASEPTET

YKTNTIDESTFKGSEA<u>LSAYIGVVTLANAENHLTRRGEFALYHLFNPNGRLDTITDKLPLMIVYRTTTKKNRHYSIRTT</u>

<u>CDQQFFVDCGYPNVRKHYSLSHLVMFYKTSATC</u>EINPDDTSADSFSWWLE

<u>Figure 3.3</u> – We have cloned T08G11.2 from the *egl-32* mutant animals and determined its sequence. The predicted ORF encodes a small (282 aa) protein with a putative SH2-like domain (underlined). However, the important arginine found in functional SH-2 domains is missing. This protein product has been found to be enriched in sperm.

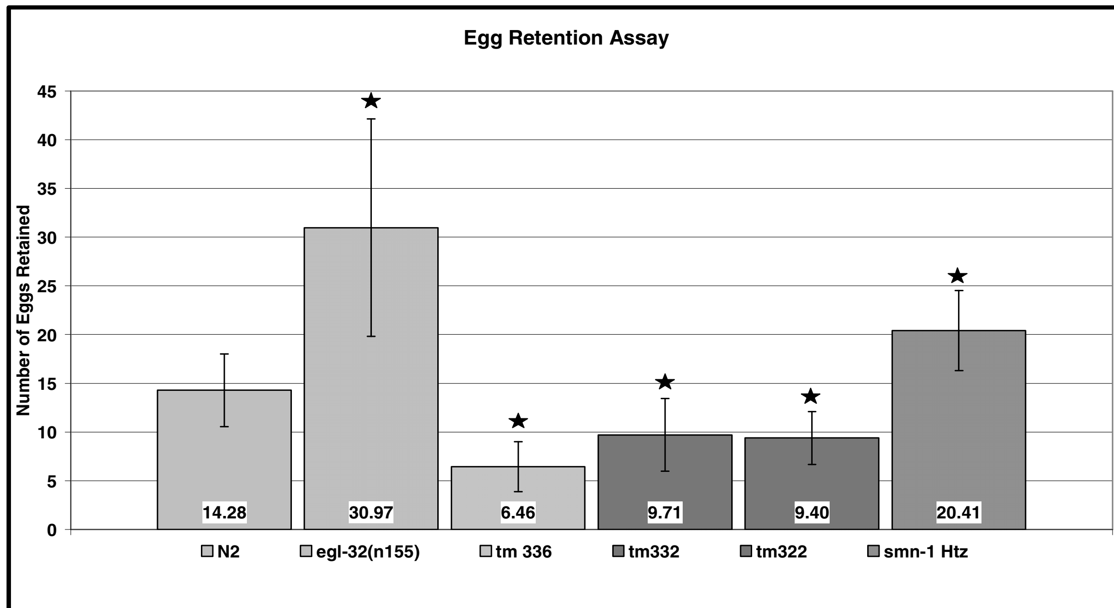


Figure 3.4a – A knockout of the gene associated with T08G11.2 (*tm336*) and two of its closest homologues, *tm332* and *tm322* retain far fewer eggs than wild type animals. Heterozygous animals containing a knockout of *smn-1* are egg-laying defective. Homozygous *smn-1* knockout is lethal. * Statistically significant difference. An ANOVA test was performed. The p-value comparing all groups is 1.5×10^{-102}. The p-value for *tm336* compared to N2 is 1.44×10^{-15}. The p-value for *tm332* compared to N2 is 3.66×10^{-8}. The p-value for *tm322* compared to N2 is 1.33×10^{-11}. The p-value for *smn-1* compared to N2 is 2.44×10^{-11}. Bars indicate standard deviation.

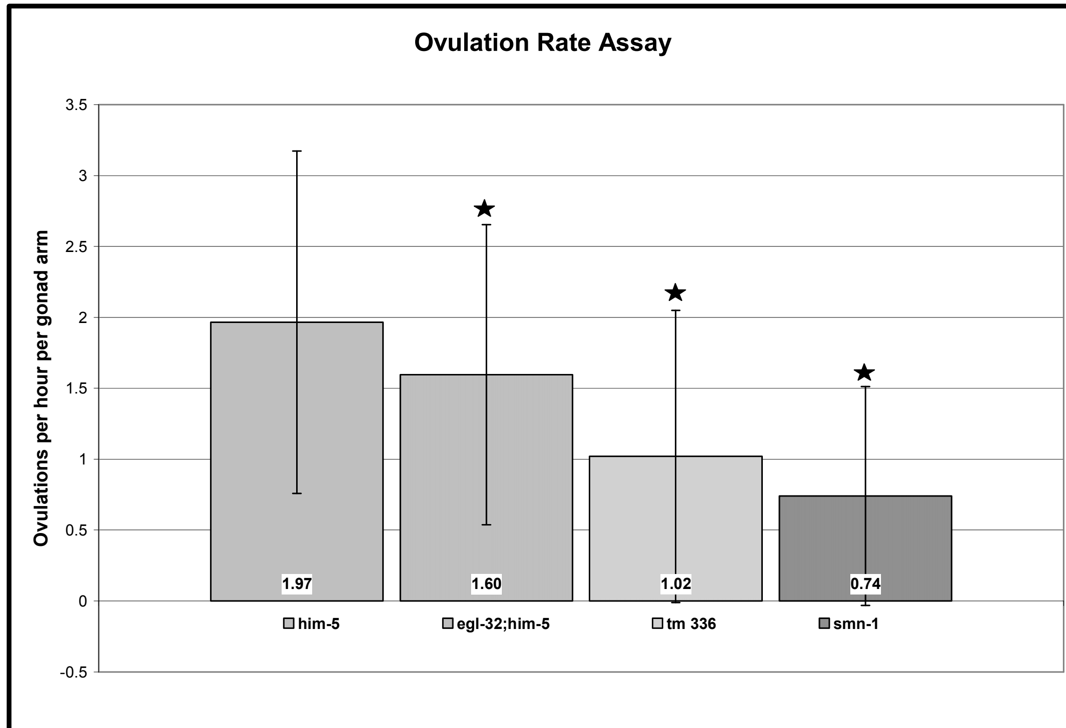


Figure 3.4b – Animals containing a knockout of T08G11.2, *tm336,* have a greatly reduced ovulation rate. This may be the reason they retain so few eggs. Animals heterozygous for a knockout of *smn-1* also have a greatly reduced ovulation rate. *egl-32;him-5* animals have a slight, but significant reduction in ovulation rates. *Statistically significant difference. An ANOVA test was performed. The p-value comparing all groups is $1.78 \times 10^{-7.}$ The p-value for *tm336* compared to *him-5* is 1.24×10^{-4}. The p-value for *smn*-1 compared *to him-5* is 8.22×10^{-6}. The p-value for *egl-32;him-5* compared to *him-5* is 3.72×10^{-2}. Bars indicate standard deviation.

(Figure 3.4a). However, upon careful inspection, we noticed that older embryos were often seen still retained within the hermaphrodites. Normally eggs are laid by the time the embryo reaches the comma stage of development. This suggests that T08G11.2 is egg-laying defective. We thought perhaps they were not retaining a large number of eggs due to a reduced ovulation rate. We found this to be true for the knockout of T08G11.2 (*tm336)* (Figure 3.4b). *tm336* animals have a greatly reduced ovulation rate. This suggests that T08G11.2 has both an egg-laying defective phenotype and an ovulation rate defect. It is possible that these animals have a defect in MSP distribution or production. This possibility is being investigated at this time. MSP's are known to control meiotic maturation and ovulation rates in *C.elegans.* Our results suggest that they may also have a role in regulating egg-laying.

egl-32* May Be *smn-1

SNP mapping was performed to help narrow down the interval in which *egl-32* may be located (Table 3.1). The original reported position for *egl*-32 was on LG1 at position 3.95. Our deficiency mapping suggested it was located at position 3.40. Preliminary SNP mapping suggest that *egl-32* is closer to its original reported position (Figure 3.5). The data suggest that *egl-32* may be on the other side of *mec*-8. An inspection of candidate genes in this area revealed *smn-1*. This gene has been reported to have a 12 base pair deletion in the 5' UTR immediately before the TATAA box, 141 base pairs upstream of ATG in *egl-32(n155)* mutant animals (Miguel-Aliaga et. al., 1999).

SNP	Strain difference N2/HA	Genomic Position on LG1	# of recombinants right of SNP	Location of *dpy-5*	Suggested location of *egl-32*
F36A2	C/T	3.14	6/34	0.0	3.7
T08G11	T/C	3.35	2/17	0.0	3.7
F16A11	G/T	3.76	8/27	0.0	4.8

Number of recombinants counted between *dpy-5* and *egl-32* = 46/1273 = 3.61 cM

Table 3.1- SNP mapping suggests that *egl-32* is located near 3.7 cM on chromosome LG1. We investigated candidate genes in this region.

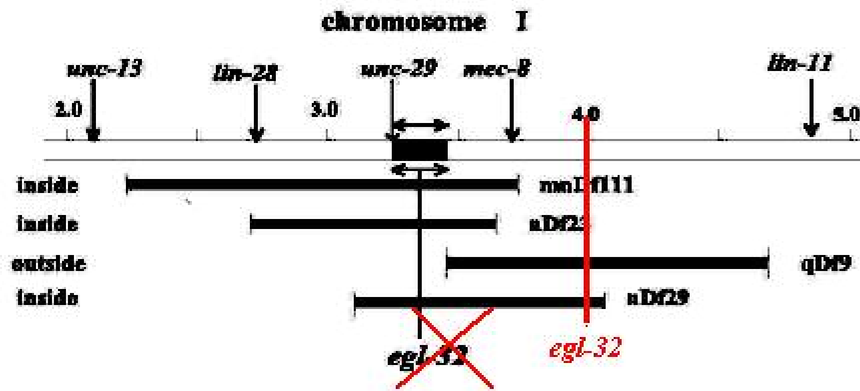


Figure 3.5 – SNP mapping indicates that *egl-32* is located on the other side of *mec-8* closer to its original reported position.

When the survival motor neuron (*smn*) gene is mutated in humans the result is spinal muscular atrophy (Lefebvre et al., 1995). This is one of the leading genetic cause of infant mortality (Crawford & Pardo, 1996). The survival motor neuron gene is ubiquitously expressed in human (Coovert et. al., 1997 & Lefebvre et. al., 1997) and *C. elegans* (Miguel-Aliaga et. al., 1997). Although *smn* is ubiquitously expressed in human cells, motor neurons seem to be especially susceptible to reduced levels of this gene (Coovert et. a., 1997). SMN is involved in snRNP biogenesis (Liu et. al., 1997 & Fisher et. al., 1997) and pre-mRNA splicing (Pellizzoni et. al., 1998).

***smn-1* Functions in Egg-Laying**

Since a knockout of *smn*-1 is available, we have asked whether this gene functions in egg-laying. In fact, the knockout of *smn-1* is homozygous lethal but heterozygous animals are egg-laying defective at all temperatures. Heterozygous *smn-1*/+ KO animals retain more eggs than wild type animals (Figure 3.4a). We have tested whether the *smn-1* heterozygous egg-laying defect shares properties with that of *egl-32(n155)*. We have found that, similar to *egl*-32, *smn*-1 heterozygous animals lay significantly fewer eggs than wild-type animals (Figure3.5). We have found that the number of eggs retained by *smn*-1 heterozygous animals is lower than that of *egl-32* animals. However the ovulation rate of *smn-1* heterozygous knockout animals is even more greatly reduced as compared to *egl*-32 animals (Figure3.4b).

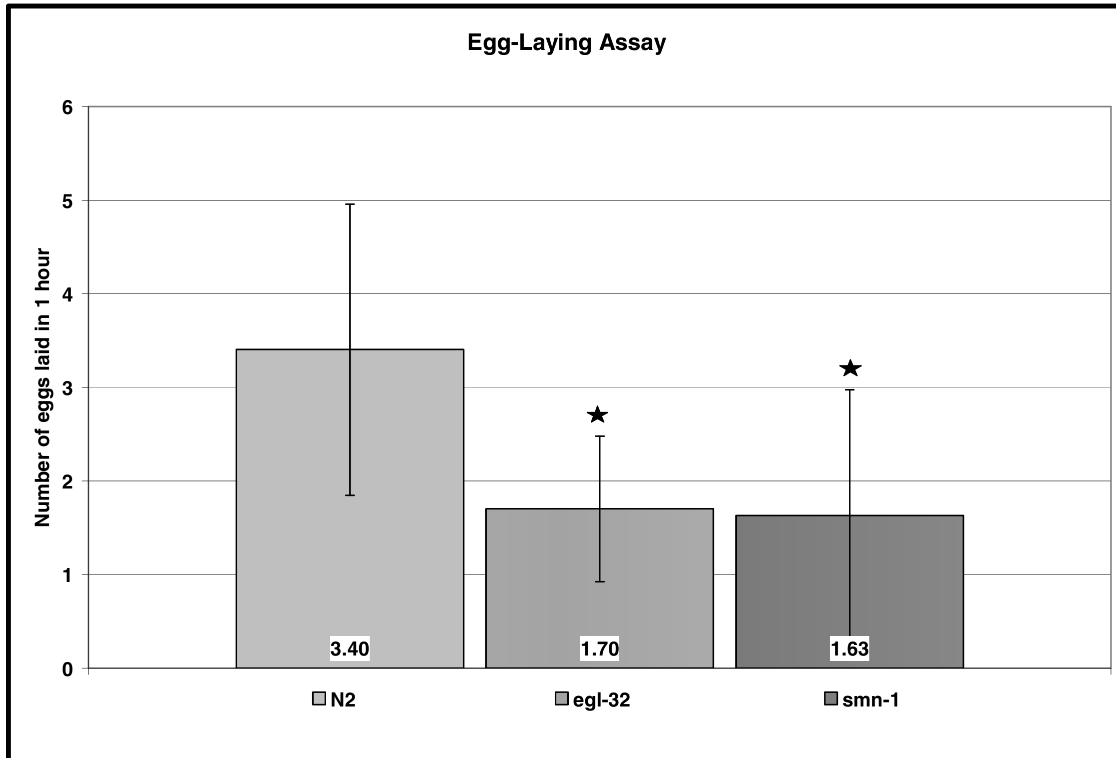


Figure 3.6 –Both *egl-32(n155)* animals and heterozygous *smn*-1 animals lay fewer eggs than wild-type animals.* Statistically significant difference. An ANVOA test was performed. The p-value between all groups is 1.49×10^{-6}. The p-value for N2 vs. *egl-32* is 7.72×10^{-6}. The p-value for N2 vs. *smn-1* is 6.95×10^{-5}. Bars indicate standard deviation.

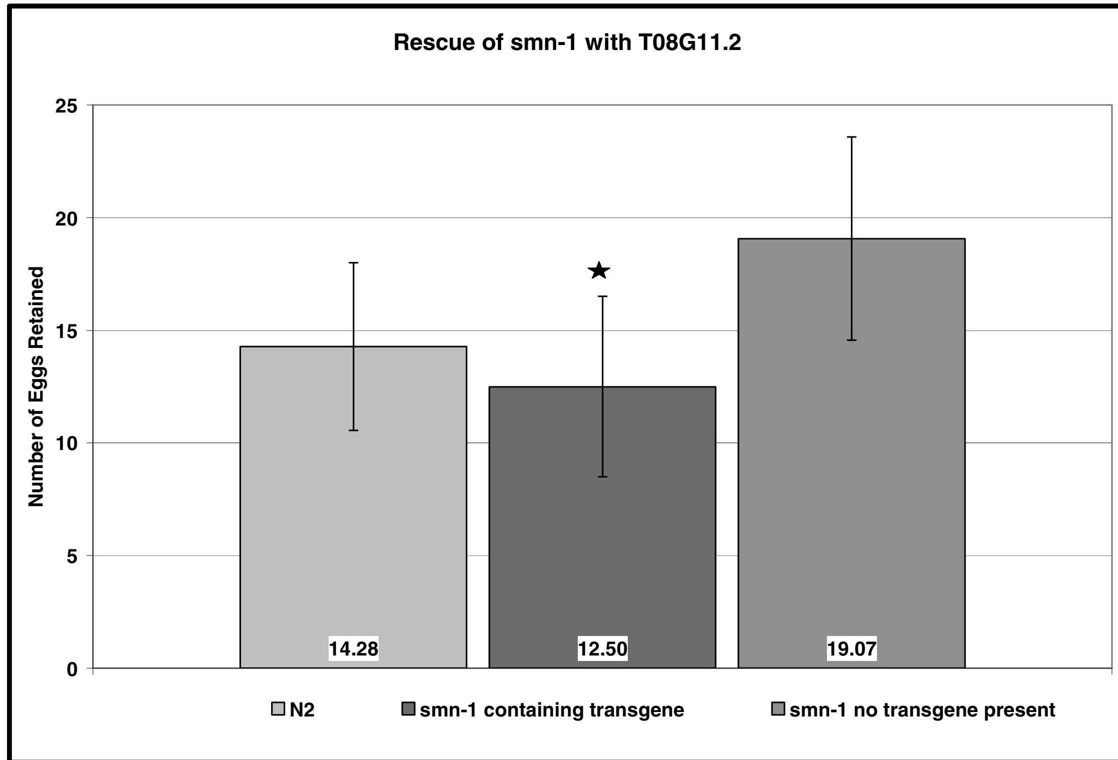


Figure 3.7 – Injection of T08G11.2 rescues the egg-laying defect seen in *smn-1* heterozygous knockout animals. This suggests that T08G11.2 and *smn-1* interact. * Statistically significant difference between those that contain the transgene and those that do not. Student t-test value of 4.72135×10^{-8}. Bars indicate standard deviation.

smn-1 is also similar to *egl-32* in that injection of T08G11.2 into *smn-1* heterozygous knockout animals also rescues their egg-laying defective phenotype (Figure 3.6). This suggests that T08G11.2 and *smn*-1 are interacting loci. A deficiency spanning the *smn-1* locus, *qDf9*, was used in Ling's deficiency mapping of *egl-32(n155)*. However, since heterozygous animals containing a knockout of the *smn-1* gene are egg-laying defective, it is unlikely that this gene was absent in the deficiency. We have attempted to clone *smn-1* for use in rescue experiments. To date, we have been unsuccessful. We have also attempted to directly inject the PCR product of the wild-type *smn-1* gene into *egl-32* animals to test for rescue. However, it has been reported that too much, or too little of this gene product can have deleterious effects. At higher concentrations we could not isolate a line. This was most likely because those containing the transgene died. At lower concentrations we were able to isolate a line. However, these worms could not be accurately scored for egg-laying defects since they had multiple other defects. Animals containing the transgene were severely blistered.

Screen for new alleles

egl-32 was originally isolated in 1983. Since that time no new alleles of *egl*-32 have been found. A mutagenesis was performed in order to obtain new alleles. Animals mutant for a linked gene, d*py-5,* were mutagenized with EMS using a standard protocol. The mutagenized worms were then crossed with *egl-32;him-5* animals, and non-dumpy, egg-laying defective animals were selected for from the progeny. In the next generation dumpy, egg-laying defective animals were selected and used for sequencing and complementation testing. Upon

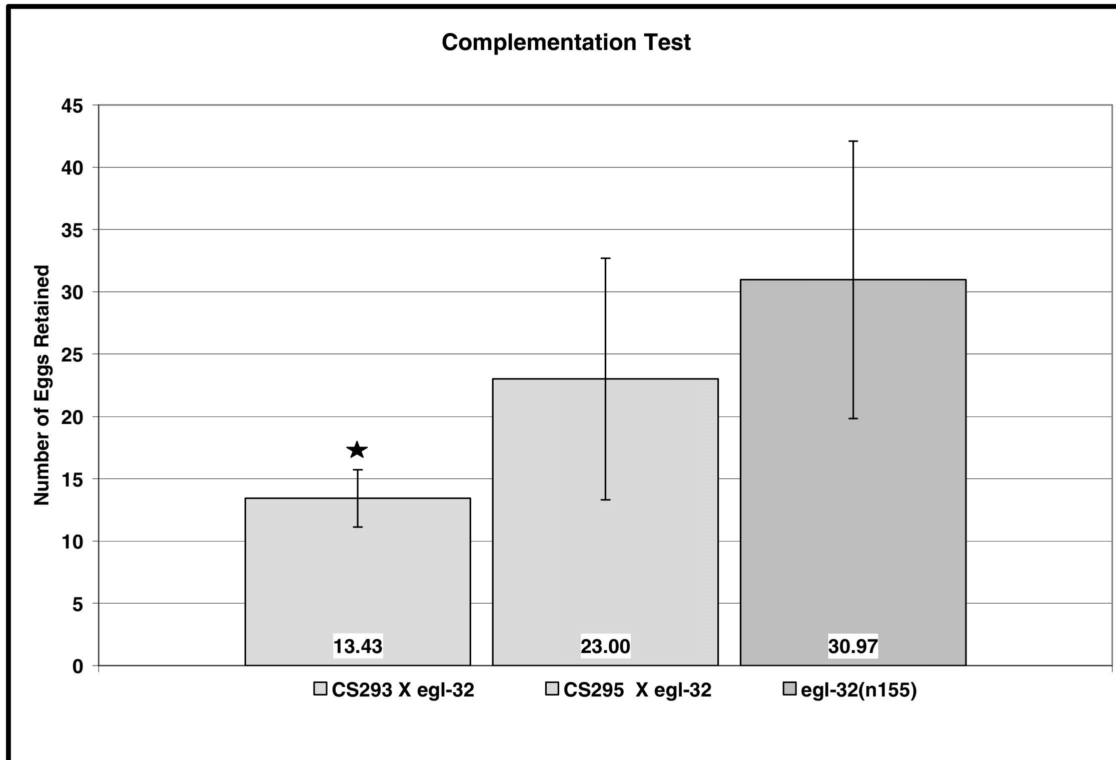


Figure 3.8 – Complementation test for *egl-32* and new alleles. CS293 complements but CS295 does not. * Statistically significant difference between the number of eggs retained by *egl-32* and *egl-32/*CS293. An ANOVA test was performed. The p-value comparing all groups is 6.06×10^{-4}. The p-value is between *egl-32* and CS293 is 2.05×10^{-4}. The p-value between *egl*-32 and CS295 is 0.201. Bars indicate standard deviation.

sequencing, all three potential new alleles were also found to contain no change to the genome at the T08G11.2 locus or in *smn-1.* A complementation test between *egl-32* and the potential new alleles was performed (Figure3.7). CS293 complements *egl-32.* However, CS295 failed to complement. Since it shows no change to the genome at either T08G11.2 or *smn-1* there are two possibilities: it may contain a mutation in one of the close homologues, or it may be an allele of *egl-32* and which may be neither T08G11.2 nor *smn-1.* The third allele did not freeze properly and was lost.

Conclusions

Although the egg-laying defective phenotype of *egl-32* is rescued by injection of T08G11.2 they are not the same locus, but are interacting loci. Finer mapping revealed a candidate gene, *smn-1* that may be an allele of *egl-32*. *smn-1* behaves similar to *egl-32* in many ways. A knockout of *smn-1* is homozygous lethal and heterozygotes are egg-laying defective. *smn-1's* egg-laying defective phenotypes is also rescued by injection of T08G11.2. However *smn-1,* unlike *egl-32,* has a greatly reduced ovulation rate.

The *egl*-32 interacting locus, T08G11.2, has three homologues in *C.elegans.* T08G11.2 and its three homologues are all sperm enriched transcripts. We obtained a knockout of T08G11.2 and two of its closest homologues. All retain far fewer eggs than wild-type animals. However, they all retain late stage embryos suggesting that they are egg-laying defective. The low egg retention reflects a greatly reduced ovulation rate detected in these animals.

e*gl-32* and *smn-1* may be alleles of each other that interact with sperm enriched transcripts that regulate both ovulation rates and egg-laying in *C.elegans.*

Chapter IV: Sperm Regulate Egg-Laying in *C.elegans*

Materials and Methods

C. elegans stocks – The following worm stocks used in this study were obtained from the Caenorhabditis Genetics Center (CGC):

egl-32(n155), him-5(e1490), smn-1(ok355), fog-2(q71), egl-33(n151)

The following double mutant was generated in our lab;

egl-32(n155);him-5(e1490)

It was generated as follows: *him-5(e1490)* males were allowed to mate with *egl-32(n155)* hermaphrodites. In the next generation wild-type animals were selected and placed on individual plates. Egg-laying defective progeny were selected and placed on separate plates. These plates were screened in the next generation for the presence of a high frequency of males.

Worms were raised at 25°C and grown on EZ worm plates.

The *spe-38;him-5* animals were obtained from Andrew Singson.

Mating Experiments – The hermaphrodites, or female *fog-2* animals, used in the mating experiments were selected as L4 larvae were allowed to mature overnight at 25°C. The males used in the mating experiments were stained with SYTO-17 as follows:

Worms were washed off plates with M9 solution and transferred to a 1.5 ml microcentrifuge tube. The worms were pelleted by microcentrifugation. The supernatant was removed. The dye was prepared by taking 3µl of 5mM stock solution into 1497µl M9 to make a 10µM solution (occasionally a solution as

concentrated as 100µM was used. This was done only when the dye was old and appeared to be losing its ability to fluoresce strongly). 500µl of dye was added to the worm pellet. 250µl of dyed worms were transferred to two clean dry plates. The plates were wrapped in aluminum foil and places at 25°C for a minimum of 2 hours. To remove the dye solution the worms were pelleted in a fresh 1.5 ml microcentrifuge tube. The supernatant was removed. The worms were resuspended in a few drops of M9 and transferred to a fresh plate for recovery. Once the plates were dry, stained males were picked to plates containing the young adult hermaphrodites or females and allowed to mate for a minimum of 2 hours.

The hermaphrodites were then transferred to an agar pad on a microscope slide and placed in a drop of 10mM sodium azide. The number of eggs retained in the uterus was then counted under a high power compound microscope. 89 unmated *egl-32;him-5* animals, 60 *egl-32;him-5* animals in which a mating was not detected, 77 *egl-32;him-5* animals in which a mating with *him-5* males was detected, 10 *egl-32;him-5* animals in which a mating with *spe-38;him-5* males was detected, 71 *him-5* unmated animals, 80 *him-5* animals in which a mating with *egl-32;him-5* animals was not detected, 38 *him-5* animals in which a mating with *egl-32;him-5* animals was detected,76 *smn-1* heterozygous animals that were not mated, 53 *smn-1* heterozygous animals in which a mating with *him-5* males was not detected, 41 *smn-1* heterozygous animals in which a mating was detected, 59 *egl-33* animals in which a mating was not detected, 29 *egl-33* animals in which a mating with *him-5* males was detected, 29 *fog-2* females mated with *him-5*

males, and 38 *fog-2* females mated with *egl-32;him-5* males were used in this assay.

Background

Egg-laying in *C.elegans* is a carefully monitored process. Egg-laying is biphasic, alternating between an active phase when eggs are laid, and an inactive phase (Waggoner et al. 1998). The hormone serotonin is necessary to initiate the active phase of egg-laying. Serotonin is produced by the hermaphrodite specific neurons (HSNs) (Trent et al., 1983). Hermaphrodites integrate many internal and external cues in deciding whether or not to enter the active phase. One well known external cue is the presence or absence of food (Trent, 1982). In the presence of food the worms enter the active phase more frequently, resulting in more eggs being laid and fewer being retained. In the absence of food, egg-laying nearly ceases and animals begin to bloat with eggs. We have uncovered an internal cue, the presence of sperm, which also stimulates egg-laying.
C.elegans animals are either hermaphrodites or males. Although the worms are hermaphroditic they do not produce sperm and oocytes at the same time. Sperm are exclusively produced during the fourth larval stage (Ward & Carrel, 1979). The sperm are stored in the spermathecae. When the worms undergo their final molt to become adults they switch to producing exclusively oocytes and will do so for the remainder of their life. As the oocytes mature they are ovulated and pass though the spermathecae where they come in contact with sperm. The oocytes are fertilized, pass into the uterus, and are eventually laid. Unlike humans,

almost all the sperm in the hermaphrodite are used to fertilize oocytes (Ward & Carrel, 1979). The number of sperm limits the number of potential offspring a self-fertilizing hermaphrodite is capable of producing to approximately 300 (Hodgkin, 1983). If a hermaphrodite is allowed to mate with a male the number of cross progeny can increase to approximately 1,400 (Hodgkin, 1986). Males produce exclusively sperm for their entire lives. If a male is allowed to mate with a hermaphrodite his sperm will crawl (they are amoeboid not flagellated) into the spermathecae where they will be preferentially used to fertilize the oocytes (Ward & Carrel, 1979).

Given the active role that sperm play in oocyte maturation and ovulation, we have asked whether they also regulate egg-laying. The Major Sperm Proteins (MSPs) are necessary for oocyte maturation and ovulation (Miller et al., 2001). In mutant female animals the worms produce only oocytes. The oocytes in these female animals will not mature and ovulation occurs at a very low level. But, when MSPs are introduced the oocytes begin to mature and the sheath cells surrounding the ovary begin to contract at a faster rate resulting in more frequent ovulations. Our studies of the egg-laying defective mutant, *egl-32,* have led to the identification of an influence of sperm on egg-laying. The *egl-32* mutants were previously shown to have mixed and variable responses to serotonin and imipramine (a serotonin re-uptake inhibitor) (Trent et al., 1983). These pharmacological results did not allow the identification of a cellular or anatomical basis for the *egl-32* egg laying defect. To address this issue we have been further characterizing *egl-32* and have found that it interacts with genes expressed in

sperm. We have evidence that the cellular basis of the *egl*-32 egg-laying phenotype is a defect in sperm.

RESULTS

egl-32 and *smn-1* Interact with T08G11.2

In transformation experiments, we identified a cosmid, C26G6, which could suppress the *egl-32* phenotype. Subclones of this cosmid containing the predicted open reading frame (ORF) T08G11.2 can also rescue *egl*-32 (Figure 3.1c). For reasons described in Chapter 3 we believe that *egl-32* does not encode T08G11.2, but rather that they are interacting loci.

A BLAST search revealed that T08G11.2 has 3 homologues in *C. elegans*. T08G11.2 and its three homologues have all been identified as sperm enriched transcripts (Kim, 2000). T08G11.2 encodes a small novel protein of 282 amino acids. It contains an SH2-like domain. However, it lacks the critical arginine present in functional SH2 .

To characterize further the biological roles of these sperm enriched proteins, we obtained knockouts from the *C.elegans* knockout consortium. Although these animals all retained far fewer eggs than wild-type animals we have found that they are indeed egg-laying defective (Figure 3.5a). *tm336* animals retain late stage embryos and have a greatly reduced ovulation rate (Figure 3.5b)

Because *egl-32* maps close to *smn-1,* and *egl-32* mutant animals contain a 12 base pair deletion in the promoter region of *smn-1,* we have reason to believe that they are alleles of the same gene. We have found that heterozygous animals

containing a knockout of *smn-1* behave similarly to *egl-32* animals. Both are egg-laying defective (Figures 2.1a & 3.6a) and have reduced ovulation rates (Figures 2.1c & 3.6c). Also, injection of T08G11.2 into both *egl*-32 and *smn-1* heterozygous animals rescues their egg-laying defective phenotype (Figures 3.1c & 3.7) suggesting that *smn-1*, similar to *egl-32*, interacts with the sperm enriched transcript of T08G11.2.

Sperm can Modulate Egg-laying Rates

We became interested in the possibility that sperm may be playing an active role in egg-laying when we discovered that the interacting gene T08G11.2 and its three homologues are all highly expressed in sperm. This interest was elevated by the temperature shift assay, revealing that the L4 stage of development, the stage when hermaphrodites are making sperm, is the critical period for *egl-32* activity (Figure 2.3). We investigated the possibility that sperm are playing an active role in egg-laying by performing simple mating experiments. Young adult hermaphrodites were allowed to mate with males stained with a vital dye for a minimum of 2 hours. Hermaphrodites were then examined for the presence or absence of fluorescent sperm as an indicator of the success or failure of mating. If the *egl*-32 egg-laying defect is due to a defect in the sperm, then we might expect that the introduction of wild-type sperm could correct this defect. Consistent with this hypothesis, the experiments revealed that the introduction of

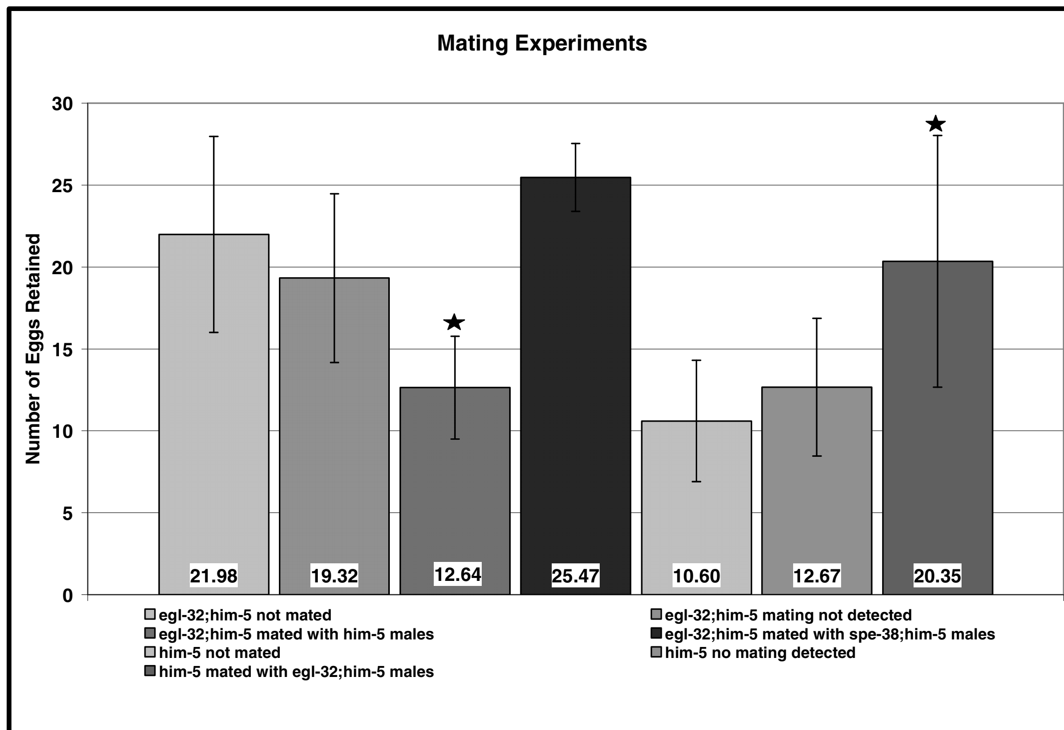


Figure 4.1 - Mating experiments. When mutant *egl-32* animals are provided with wild-type (*him-5*) sperm via mating they begin to retain a wild-type number of eggs. When wild-type, *him-5* animals are provided with mutant *egl-32* sperm via mating they begin to retain more eggs. Fertilization is required for the rescue of *egl-32* by wild-type sperm. When *spe-38;him-5* males are mated with *egl-32;him-5* hermaphrodites they do not begin to retain fewer eggs as they did when *him-5* sperm was introduced via mating. * Statistically significant difference between mated and unmated animals. An ANOVA test was performed. The p-value comparing all groups is 1.40×10^{-60}. The p-value between *egl-32* not mated and mated with *him-5* males is 2.02×10^{-24}; The p-value between *him-5* not mated and those mated with *egl-32;him-5* males is 1.73×10^{-20}. Bars indicate standard deviation.

wild-type sperm, via mating, into *egl-32* mutant animals rescued the egg-laying defect (Figure 4.1). Furthermore, the introduction of mutant *egl-32* sperm into wild-type animals induced an egg-laying defective phenotype (Figure 4.1). The presence of males in itself did not lead to this change in egg-laying behavior, since control animals on the same plates without detectable stained sperm were not affected significantly. This experiment indicates that sperm and/or seminal fluid from an *egl-32* male are sufficient to cause an egg-laying phenotype, even in the presence of wild-type hermaphrodite sperm. Since mutant *egl-32* hermaphrodites, in the absence of seminal fluid, are egg-laying defective, the more likely focus of the defect is the sperm.

To be sure that the egg-laying defect was due solely to the presence of the sperm and /or seminal fluid we were introducing into the animals, without interference from endogenous hermaphroditic sperm we performed mating experiments using female, *fog-2* animals. These animals produce no sperm of their own. Consistent with our previous mating experiments, *fog-2* animals mated with *egl-32;him-5* males retained significantly more eggs than *fog-2* females mated with *him-5* males (Figure 4.2).

Similarly, when wild-type sperm was introduced into heterozygous *smn-1* knockout animals via mating, they began to retain fewer eggs (Figure 4.3). The introduction of sperm cannot rescue every egg-laying defect. We tested *egl-33* mutant animals by introducing wild-type sperm into *egl-33* hermaphrodites. The animals containing stained sperm did not retain fewer eggs than those not mated with wild-type males (Figure 4.3).

Because the L4 stage is the critical period for EGL-32 activity we were interested in determining if *egl*-32 acts during spermatogenesis. Since this is a temperature sensitive mutation and since males, unlike hermaphrodites, make sperm their entire lives we wanted to determine if males raised at a permissive temperature (20°C) were capable of inducing an egg-laying defect in *fog-2* females. We mated *him-5* males and *egl-32;him-5* males that were raised at 20°C with *fog-2* females. We found no difference in the number of eggs retained in these two different matings (Figure 4.4). So, *egl-32* mutant males must be raised at 25°C in order to be capable of inducing an egg-laying defect in mated females.

Fertilization is required for *egl-32* activity.

We were interested in determining if fertilization is required for *egl-32's* activity. Alternatively, *egl-32* may function in the seminal fluid. To determine if fertilization is required we mated *egl-32;him-5* hermaphrodites with *spe-38;him-5* males. These animals produce structurally normal looking sperm. The sperm can crawl into the spermathecae. However, they are incapable of binding to and fertilizing an oocyte (Chatterjee et al., 2005). In these mating experiments, we found that *egl-32;him-5* animals did not retain fewer eggs after these matings. This suggests that fertilization is required for *egl-32's* activity (Figure 4.1).

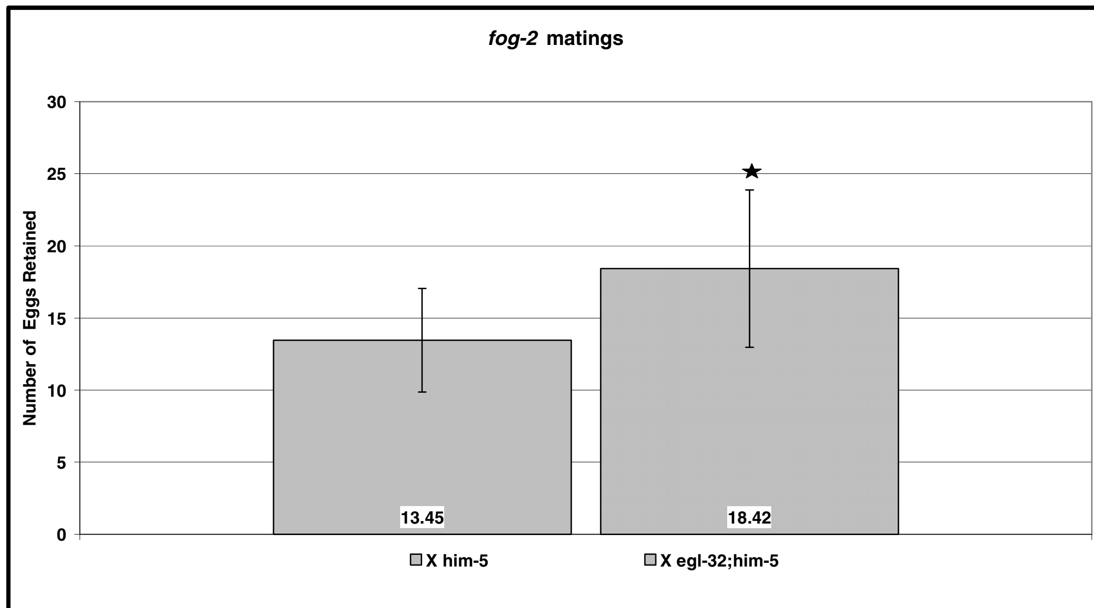


Figure 4.2 – When *fog-2* female animals are mated with *egl-32;him-5* males they retain significantly more eggs than when they are mated with *him-5* males. * Statistically significant difference between *fog-2* females mated with *him-5* males and those mated with *egl-32; him-5* males. The Student t-test value is 3.4288 X 10^{-5}. Bars indicate standard deviation.

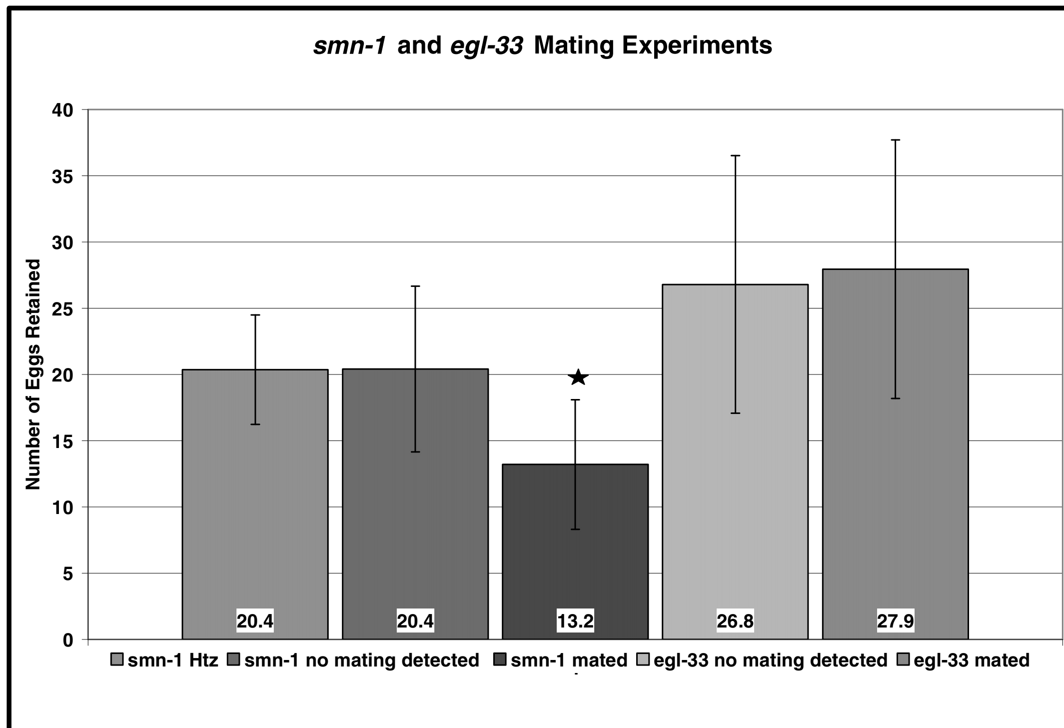


Figure 4.3 – *smn-1* animals heterozygous for a knockout of this gene behave similar to *egl-32* mutant animals in mating experiments. *smn-1* hermaphrodites retain fewer eggs when wild-type (*him-5*) sperm is introduced via mating. This is not true of all egg-laying defective mutants. Control e*gl-33* hermaphrodites did not begin to retain fewer eggs when wild-type sperm was introduced. * Statistically significant difference. An ANOVA test was performed. The p-value between all groups is 5.59×10^{-24}. The p-value between *smn-1* heterozygous animals not mated and those that were mated with *him-5* males is 3.25×10^{-15}. The p-value between *egl-33* mated and not mated is 0.603. Bars indicate standard deviation.

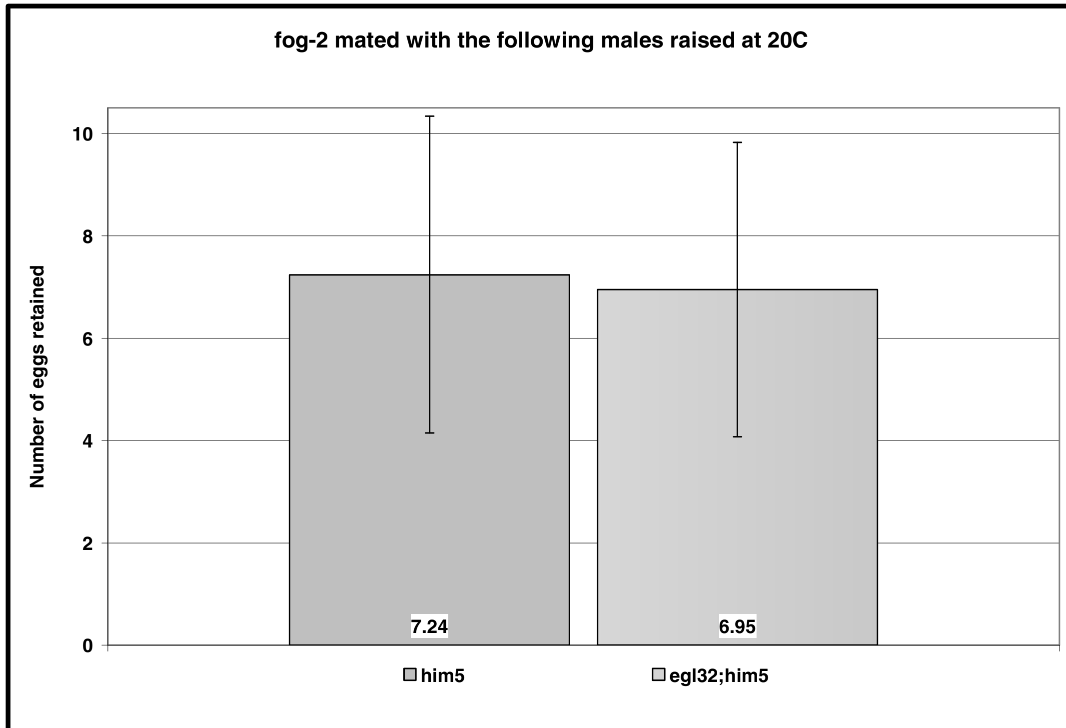


Figure 4.4 – When *egl-32* males are raised at 20°C they are no longer able to induce an egg-laying defective phenotype in *fog-2* females. Bars indicate standard deviation.

Conclusions

egl-32 and *smn-1*, which may be the same gene, both interact with the sperm enriched transcript, T08G11.2. When wild-type sperm is introduced into either *egl-32* or *smn-1* heterozygous animals, they begin to retain fewer eggs. When mutant *egl-32* sperm is introduced into wild-type animals, via mating, they begin to retain more eggs. We have shown that this is due to the presence of the introduced sperm, and not the endogenous hermaphroditic sperm by repeating these experiments with mutant *fog-2* females. Female *fog-2* animals retained significantly more eggs when they were mated with *egl-32* males than when they were mated with wild-type males. However, when *fog*-2 females were mated with males raised at the permissive temperature of 20°C there was no difference in the number of eggs retained. We have also shown that fertilization is required for *egl-32* to affect egg-laying. When *spe-38;him-5* males containing sperm that act similar to wild-type sperm in all ways except they are incapable of fusing with the oocyte to fertilize it, were mated with *egl-32* hermaphrodites they did not begin to retain fewer eggs.

Chapter V: Interactions with the TGF-β Signal Transduction Pathway

Materials and Methods

Strains – The Bristol N2 strain is the wild-type strain. *egl-32(n155*) is a temperature sensitive egg laying mutant isolated by Trent et al (1983). *egl-32(n155)*;*daf-3(e1376)* and *egl-32(n155); daf*-5(e1386) mutant animals constructed by Rafal Tokarz, were used to confirm the suppression of *egl-32(n155).*d*af-5 (*e1386);*him-5*(e1490) animals were crossed with *egl-32*(n155)*;him-5*(e1490) animals in order to generate *egl-32;daf-5;him-5* animals. Growth was on EZ worm plates.

Mating Experiments - The hermaphrodites used in these mating experiments were selected as L4 larvae and were allowed to mature overnight at 25°C. The males used in these mating experiments were stained with SYTO-17 dye as follows; Worms were washed off plates with M9 solution and transferred to a 1.5 ml microcentrifuge tube. The worms were pelleted by microcentrifugation. The supernatant was removed. The dye was prepared by taking 3µl of 5mM stock solution into 1497µl M9 to make a 10µM solution (occasionally a solution as concentrated as 100µM was used. This was done only when the dye was old and appeared to be losing its ability to fluoresce strongly). 500µl of dye was added to the worm pellet. 250µl of dyed worms were transferred to two clean dry plates. The plates were wrapped in aluminum foil and places at 25°C for a minimum of 2 hours. To remove the dye solution the worms were pelleted in a fresh 1.5 ml

microcentrifuge tube. The supernatant was removed. The worms were resuspended in a few drops of M9 and transferred to a fresh plate for recovery. Once the plates were dry, stained males were picked to plates containing the young adult hermaphrodites or females and allowed to mate for a minimum of 2 hours.

The hermaphrodites were then transferred to an agar pad on a microscope slide and placed in a drop of 10mM sodium azide. The number of eggs retained in the uterus was then counted under a high power compound microscope. 71 unmated *him-5* hermaphrodites, 38 *him-5* hermaphrodites in which a mating with *egl-32;him-5* males was detected, 50 *him-5* hermaphrodites in which a mating with *egl-32;daf-5;him-5* males was detected, 14 *him-5* hermaphrodites in which a mating with *daf-5;him-5* males was detected, 53 unmated *egl-32;him-5* hermaphrodites, 77 *egl-32;him-5* hermaphrodites in which a mating with *him-5* males was detected, 44 *egl-32;him-5* hermaphrodites in which a mating with *egl-32;daf-5;him-5* males was detected, 30 *egl-32;him-5* hermaphrodites in which a mating with *daf-5;him-5* males was detected, 39 *egl-32;him-5* hermaphrodites in which a mating with *egl-32;him-5* males was detected, 54 unmated *egl-32;daf-5;him-5* hermaphrodites, 54 *egl-32;daf-5;him-5* hermaphrodites in which a mating with *him-5* males was detected, 18 *egl-32;daf-5;him-5* hermaphrodites in which a mating with *egl-32;him-5* males was detected, 17 *egl-32;daf-5;him-5* hermaphrodites in which a mating with *daf-5;him-5* males was detected, 18 *egl-32;daf-5;him-5* hermaphrodites in which a mating with *egl-32;daf-5;him-5* males was detected, 38 *daf-5;him-5* unmated hermaphrodites, 29 *daf-5;him-5*

hermaphrodites in which a mating with *him-5* males was detected, 25 *daf-5;him-5* hermaphrodites in which a mating with *egl-32;him-5* males was detected, 12 *daf-5;him-5* hermaphrodites in which a mating with *egl-32;daf-5;him-5* males was detected and 20 *daf-5;him-5* hermaphrodites in which a mating with *daf-5;him-5* males was detected were used in this assay.

Egg retention assays – The number of eggs retained by young adult animals were counted as follows; L4 larvae were placed on a plate with food and allowed to mature overnight at 25°C. The worms were then placed in a drop of 25 mM sodium azide on an agar pad on a microscope slide. The number of eggs retained by each animal was then counted using a high powered light microscope. 32 *egl-32;daf-3*, 28 *egl-32;daf-5,* 75 wild-type, 106 *egl-32;him-5,* 38 *daf-5* ;*him-5,* 40 *daf-3;him-5,* and 19 *daf-7*animals were used in this assay.

Egg-laying Assays - L4 larvae were placed on plates with food and allowed to mature at 25°C overnight to become young adults. The young adults were moved to individual plates containing food. The worms were allowed to lay eggs at 25°C for 5 hours. The number of eggs or hatched larvae present on the plate after 5 hours was then counted. The number of eggs laid in one hour was then calculated. 68 *him-5* animals, 100 *egl-32;him-5,* 30 *daf-5;him-5,* 37 *daf-3;him-5,* and 18 *daf-7*animals were used in this assay.

Ovulation Rate Assay – L4 animals were placed on a plate and allowed to mature overnight at 25°C. The number of eggs retained by each adult was then counted by placing the animals on an agar pad containing a small drop of OP 50. The worms were then placed on individual plates and allowed to lay eggs for 5 hours at 25°C. The number of eggs retained after 5 hours was again counted. The number of eggs laid on the plate during the 5 hours was also counted. The ovulation rate was determined by adding the number of eggs retained and laid at the 5 hour time point and then subtracting number of eggs each animal originally retained. The number of ovulations per hour was then calculated. 68 *him-5*, 100 *egl-32;him-5,* 30 *daf-5;him-5,* 36 *daf-3;him-5,* and 18 *daf-7* animals were used in this assay.

RESULTS

daf*-5 Suppresses *egl-32* More Strongly than *daf-3

egl-32 had previously been implicated in the TGF-β dauer pathway because it was found that the egg-laying defective phenotype of *egl-32(n155)* is suppressed by mutations in *daf*-3 and *daf*-5 (Trent et al, 1983). Recall that the ligand in the TGF-β dauer pathway, DAF-7, is produced when conditions are good (i.e. there is plenty of food available, the animals are not overcrowded, and the temperature is not too high) (Ren et al., 1996). When DAF-7 is produced it causes the association of the type I receptor, DAF-1 (Georgi et. al., 1990), and the type II receptor, DAF-4 (Savage et al., 1996 & Estevez et al., 1993). Association of the two receptors allows the constitutively active kinase activity of DAF-4 to phosphorylate and activates DAF-1. When DAF-1 is activated it phosphorylates and activates the Smads, DAF-8 (Riddle & Albert, 1997) and DAF-14 (Inoue & Thomas, 2000).When DAF-8 and DAF-14 are active they function to suppress DAF-3 (Patterson et al. 1997), which is a Smad, and DAF-5 (Riddle & Albert, 1997), a Sno and Ski homologue. As long as DAF-3 and DAF-5 are suppressed larvae do not become dauers and adults lay eggs at a regular rate. Mutants in all of the components upstream of *daf-3* and *daf-5* in this pathway are dauer constitutive and egg-laying defective. We and others (Daniels et al., 2000) have found that *daf*-5 suppresses *egl-32* more strongly than *daf*-3 (Figure 5.1). It had previously been found that *egl*-4, another egg-laying defective mutant implicated in the TGF-β pathway, is more strongly suppressed by *daf*-3 than *daf-5* (Daniels et al., 2000).

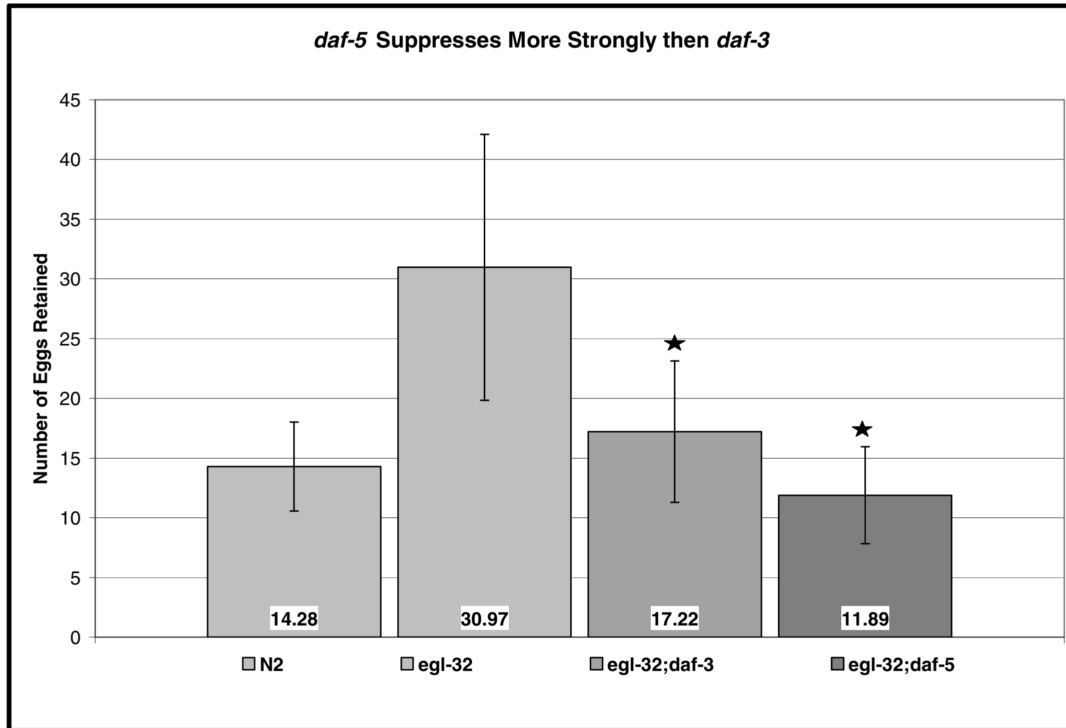


Figure 5.1 – Both *daf-3* and *daf-5* suppress *egl-32's* egg-laying defective phenotype. However, *daf-5* suppresses more strongly than *daf-3.* *Statistically significant difference between *egl-32* and either *egl-32;daf-3* or *egl-32;daf-5*. An ANOVA test was performed. The p-value comparing all groups is 5.13 x $^{10-}$21. The p- value for *egl-32;daf-3* vs. *egl-32* is 5.07 x 10^{-8}. The p-value for *egl-32;daf-5* vs. *egl-32* is 5.62 x 10^{-12}. Bars indicate standard deviation.

Both findings are interesting because it is thought that *daf-3* and *daf*-5 function together. However, Gene Expression Profiles performed by Reinke et al. show differential timing for expression of *daf-3* and *daf-5* (Reinke et al., 2000).

The TGF-β Pathway acts via sperm or spermatogenesis to regulate egg-laying.

Because *egl-32* interacts with sperm proteins to regulate egg-laying and since it is implicated in the TGF-β dauer pathway, which also regulates egg-laying, we were interested in finding out if the TGF-β pathway functions in the sperm, the soma, or both. We performed mating experiments similar to those done to determine that *egl-32*'s action is in the sperm. In this case we used a variety of different males and hermaphrodites to see where the *daf-5* mutation had to be to suppress *egl-32*.

If *daf-5(-)* is functioning in the soma to suppress *egl-32(-),* then we would expect that any time the mutant allele of *daf-5* is present in the soma it will suppress *egl-32.* This suppression would include preventing *egl-32* sperm from inducing an egg-laying defect in both *daf-5* (-) and *egl-32;daf-5* (-) hermaphrodites. A summary of these expected results is in Table 4.1.

If *daf-5(-)* is functioning in either the sperm, or during spermatogenesis then we would expect that anytime the mutant allele of *daf-5* is present in the sperm it will be able to rescue *egl-32's* egg-laying defect. This also means that

Soma	Wild-type sperm	*egl-32* sperm	*egl-32;daf-5* sperm	*daf-5* sperm
Wild-type	Wild-Type	Egg-laying Defective	Egg-laying defective	Wild-Type
egl-32	Wild-type	Egg-laying defective	Egg-laying defective	Wild-Type
egl-32;daf-5	Wild-Type	Wild-Type	Wild-Type	Wild-Type
daf-5	Wild-Type	Wild-Type	Wild-Type	Wild-Type

Table 5.1 - This table shows the predicted results if the TGF-β pathway acts in the soma of the hermaphrodite to suppress *egl-32*.

Soma	Wild-type sperm	*egl-32* sperm	*egl-32;daf-5* sperm	*daf-5* sperm
Wild-type	Wild-Type	Egg-laying Defective	Wild-Type	Wild-Type
egl-32	Wild-Type	Egg-laying defective	Wild-Type	Wild-Type
egl-32;daf-5	Wild-Type	Egg-laying defective	Wild-Type	Wild-Type
daf-5	Wild-Type	Egg-laying defective	Wild-Type	Wild-Type

Table 5.2 This table shows the predicted results if the TGF-β pathway is functioning in sperm or in the male soma during spermatogenesis.

Soma	Wild-type sperm	*egl-32* sperm	*egl-32;daf-5* sperm	*daf-5* sperm
Wild-type	Wild-Type	Egg-laying Defective	Egg-laying defective	Wild-Type
egl-32	Wild-Type	Egg-laying defective	Egg-laying defective	Wild-type
egl-32;daf-5	Wild-Type	Egg-laying defective	Wild-Type	Wild-Type
daf-5	Wild-Type	Egg-laying defective	Wild-Type	Wild-Type

Table 5.3 – This table shows the predicted results if the TGF-β pathway is functioning in both the sperm and the soma.

sperm from *egl-32;daf-5* (-) double mutants would be unable to induce an egg-laying defect .A summary of these expected results are shown in Table 4.2.

If *daf-5(-)* is needed in both the soma and the sperm in order for it to suppress *egl-32,* then we would expect that *egl-32;daf-5* (-) sperm would be able to induce an egg-laying defect in a wild-type hermaphrodite, but not in a *daf-5(-)* hermaphrodite. A summary of the expected results is in Table 4.3.

We found that when wild-type (*him-5*) hermaphrodites were mated with *egl-32;him-5(-)* males, as previously shown, the number of eggs retained increases from 13 to 25 (Figure 5.2). However, when *egl-32;daf-5;him-5* (-) sperm was introduced into wild-type animals they showed no increase in the number of eggs retained. There are two possible explanations for this; *daf*-5(-) may be acting in the sperm to suppress *egl-32's* ability to induce an egg-laying defect. Alternatively, *daf-5* (-) may be acting in the soma to suppress *egl*-32 during spermatogenesis. We introduced *daf-5;him-5* (-) sperm into wild-type animals and also saw no change to the number of eggs being retained. We mated *him-5* males with *him-5* hermaphrodites to determine if the simple act of mating could alter egg retention rates. We found that mating had no effect on egg-retention.

When *egl-3(-);him-5* hermaphrodites were mated with wild-type (*him-5*) males, as previously shown the number of eggs retained was greatly reduced, from 30 to 12 (Figure 5.3). When either *daf-5(-);him-5*) or *egl-32(-); daf-5(-);him-5.*

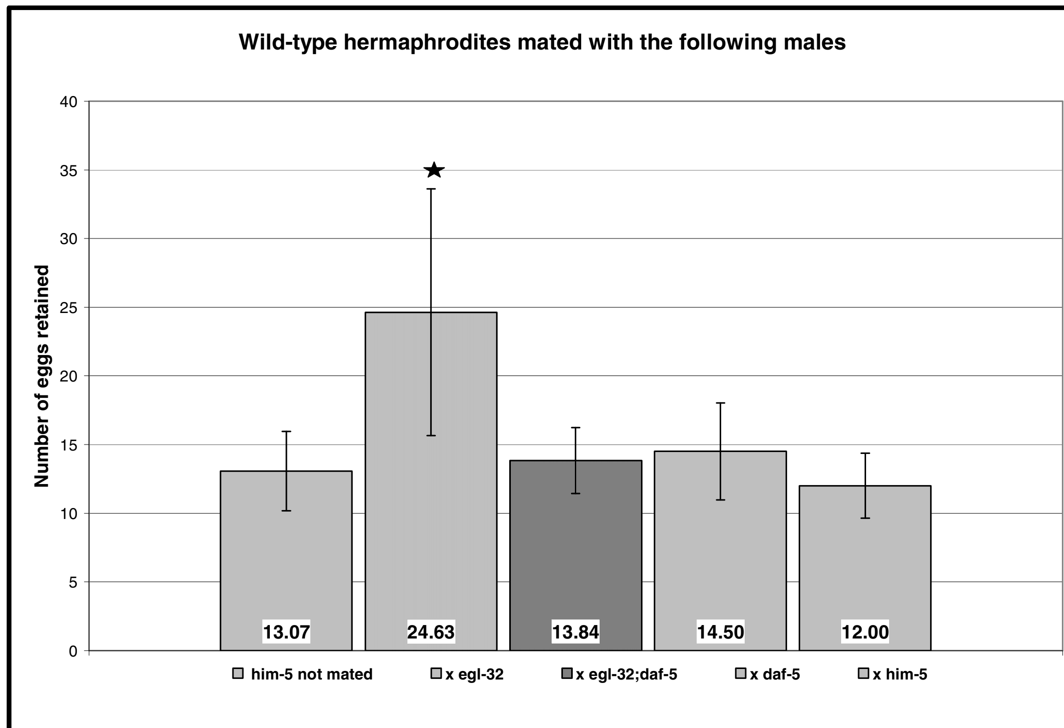


Figure 5.2 – When wild-type (*him-5*) hermaphrodites are mated with *egl-32* males they retain significantly more eggs. This increase in the number of eggs retained is not seen when wild-type hermaphrodites mated with *egl-32;daf-5;him-5, daf-5;him-5* or wild-type males. * Statistically significant difference. An ANOVA test was performed. The p-value comparing all groups is 4.72×10^{-25}. The p-value for *him-5* hermaphrodite's mated with *egl-32;him-5* males vs. *him-5* males that have not been mated is 6.24×10^{-17}. The p-value comparing *him-5* not mated to those mated with *egl-32;daf-5* males is 0.125. The p-value comparing *him-* not mated with *him-5* mated with *daf-5* males is 0.107. The p-value comparing *him-5* not mated to those mated with *him-5* males is 0.247. Bars indicate standard deviation.

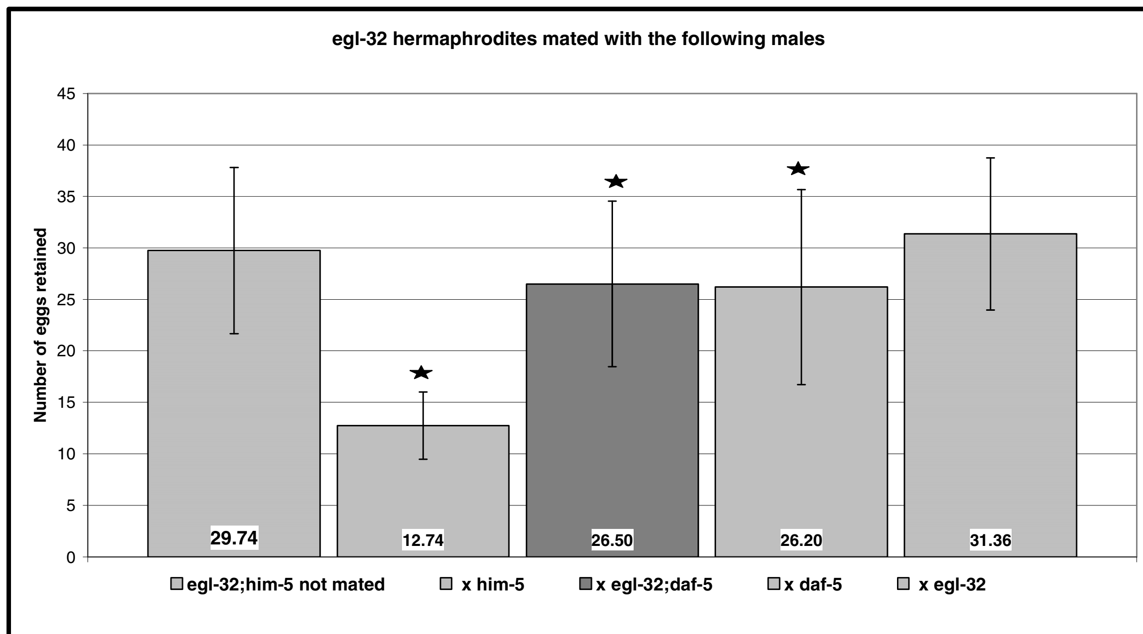
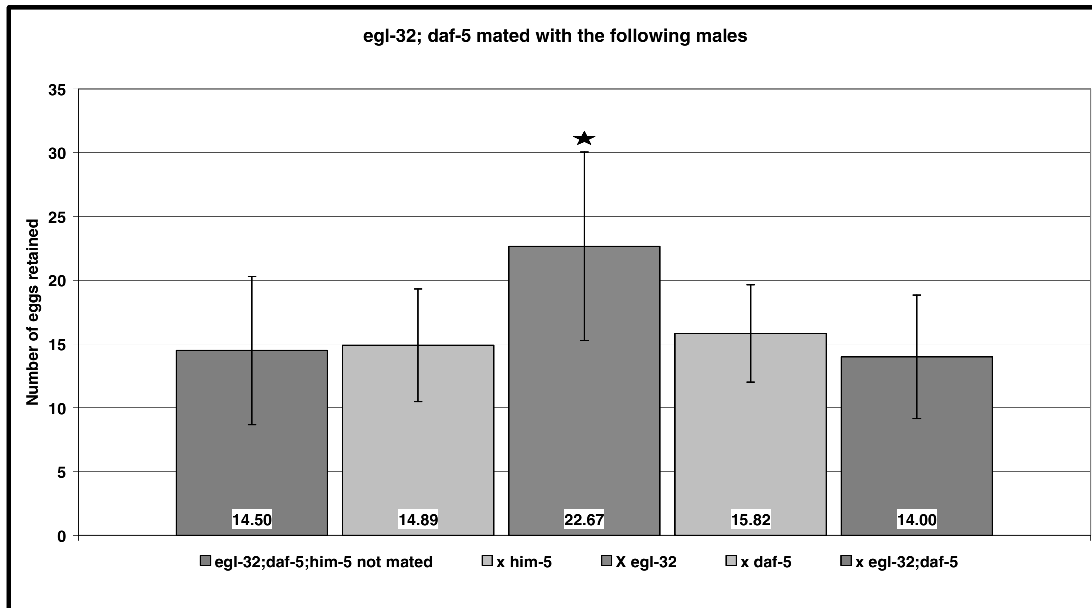


Figure 5.3 When *egl-32;him-5* animals are mated with wild-type males they retain significantly fewer eggs. There is also a slight, but significant, difference in the number of eggs retained when *egl-32;him-5* hermaphrodites are mated with either *egl-32;daf-5;him-5* or *daf-5;him-5* males. When *egl-32;him-5* hermaphrodites are mated with *egl-32;him-5* males there was no change in the number of eggs retained. *Statistically significant difference. An ANOVA test was performed. The p-value comparing all groups is 1.02×10^{-39}. The p-value for *egl-32;him-5* hermaphrodites mated with wild-type males vs.*egl-32;him-5* animals that have not been mated is 8.62×10^{-34}. The p-value for *egl-32;him-5* hermaphrodites mated with *egl-32;daf-5;him-5* males vs. *egl-32;him-5* animals that were not mated is 3.74×10^{-3}. The p-value for *egl-32;him-5* hermaphrodites mated with *daf-5;him-5* males vs. *egl-32;him-5* animals that have never been mated is 8.85×10^{-3}. The p-value comparing *egl-32;him-5* not mated to those mated with *egl-32;him-5* males is 0.326. Bars indicate standard deviation.

sperm was introduced into *egl-32(-);him-5* hermaphrodites they remained egg-laying defective. However, there was a slightly, but significant reduction in the number of eggs retained. This slight rescue suggests that *daf-5* (-) may act in the sperm to suppress *egl-32(-).* The slight reduction in the number of eggs retained may reflect an inadequate ability of *daf-5 (-)* sperm to out compete endogenous hermaphrodite sperm.

When *egl-32(-);daf-5(-);him-5* hermaphrodites were mated with *egl-32(-);him-5* males the number of eggs retained increased (Figure 5.4). This suggests that *daf-5* (-)is not suppressing *egl-32(-)* in the soma. When *egl-32(-);daf-5(-);him-5* hermaphrodites were mated with either wild-type, *daf-5(-)him-5*, or *egl-32(-);daf-5(-);him-5* males they did not show a significant change in the number of eggs they retained.

When *daf-5;(-)him-5* hermaphrodites were mated with *egl-32(-);him-5* males there was a significant increase in the number of eggs retained (Figure 5.5). Again, this suggests that *daf-5* (-) is not able to suppress *egl-32's* ability to induce an egg-laying defect when present only in the soma. When *daf-5(-);him-5* hermaphrodites were mated with either *him-5, egl-32(-);daf-5(-);him-5* or *daf-5(-);him-5* males, there was no significant change to the number of eggs retained. All the above data are summarized in Table 4.4.



<u>Figure 5.4</u> – When *egl-32;daf-5;him-5* hermaphrodites are mated with *egl-32;him-5* males they begin to retain significantly more eggs. When *egl-32;daf-5;him-5* hermaphrodites are mated with *him-5; daf-5;him-5, or egl-32daf-5;him-5* males they show no change in the number of eggs being retained. *Statistically significant difference. An ANOVA test was performed. The p-value comparing all groups is 9.76×10^{-7}. The p-value for the number of eggs retained by *egl-32;daf-5;him-5* hermaphrodites mated with *egl-32;him-5* males vs. *egl-32;daf-5;him-5* animals that were not mated is 8.03×10^{-6}. The p-value between *egl-32;daf-5;him-5* animals that are not mated compared to those mated with *him-5* males is 0.696. The p-value between *egl-32;daf-5;him-5* not mated and those mated with *daf-5;him-5* males is 0.382. The p-value between *egl-32;daf-5; him-5* animals not mated compared to those mated with *egl-32;daf-5;him-5* males is 0.743. Bars indicate standard deviation.

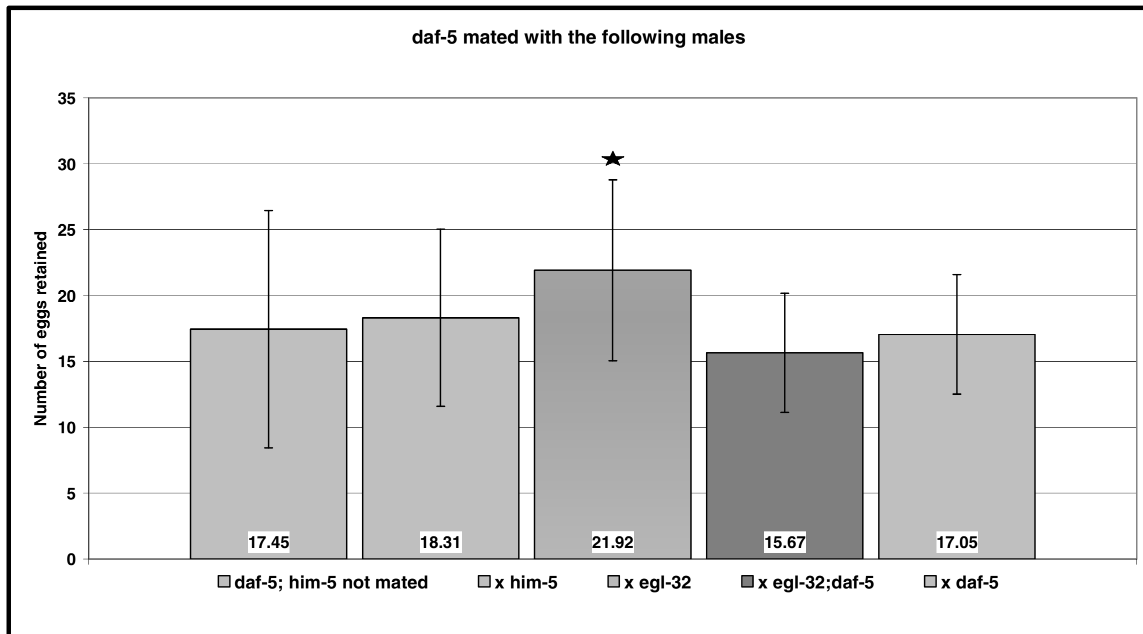


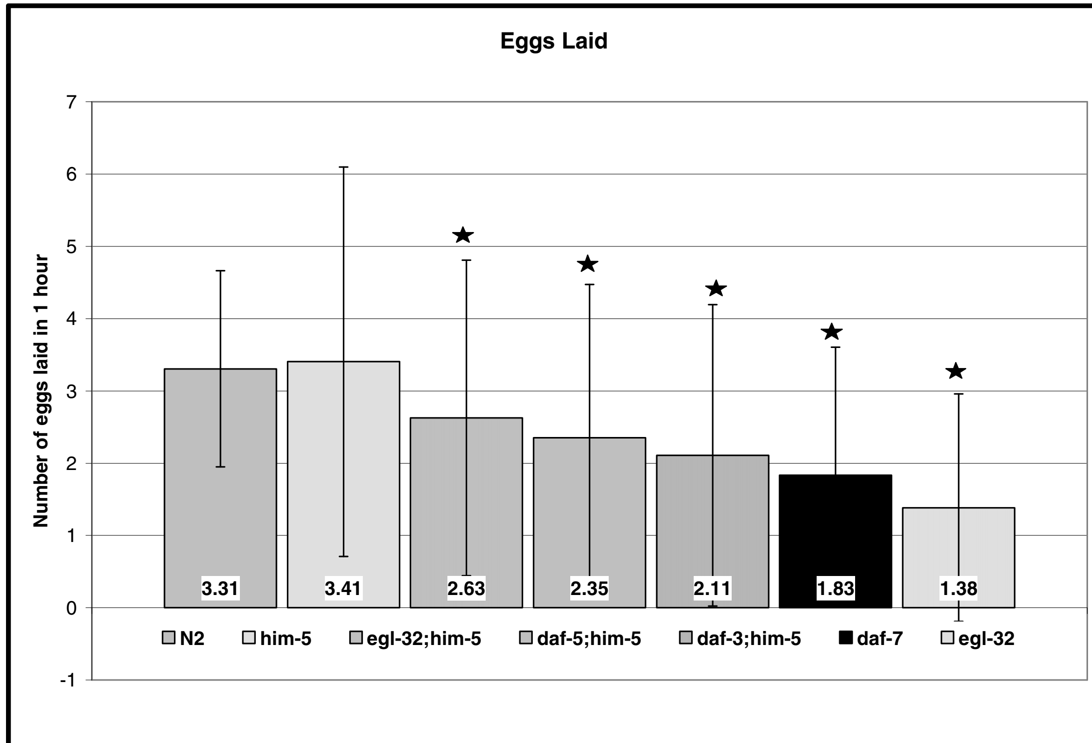
Figure 5.5 – When *daf-5;him-5* hermaphrodites are mated with *egl-32;him-5* they retain significantly more eggs. When *daf-5;him-5* hermaphrodites are mated with *him-5, egl-32;daf-5;him-5* or*daf-5;him-5* males they do not show a change in the number of eggs retained. *Statistically significant difference. An ANOVA test was performed. The p-value comparing all groups is 0.060 The p-value for the number of eggs retained by *daf-5;him-5* hermaphrodites mated with *egl-32;him-5* males vs. *daf-5;him-5* animals that were not mated is 3.89×10^{-2}. The p-value comparing *daf-5* ;him-5 animals that were not mated to those mated with *him-5* males is 0.667. The p-value comparing *daf-5;him-5* animals that were not mated to those mated with *egl-32;daf-5;him-5* males is 0.515. The p-value between *daf-5;him-5* animals that were not mated compared to those mated with *daf-5;him-5* males is 0.854. Bars indicate standard deviation.

Collectively these data suggest that *daf-5* functions to suppress *egl-32* in the sperm. However, these data do not exclude the possibility that the TGF-β pathway is functioning in the somatic gonad during spermatogenesis.

The TGF-β Pathway Functions in both Egg-laying and Ovulation

We wanted to determine whether the suppression by *daf*-5 (-) was mediated by a change in egg-laying rates, ovulation rates, or both. We also wanted to characterize the function of *daf*-7 in regulating these events. Therefore, we determined the egg retention, the ovulation and the egg-laying rates of *daf-3, daf-5,* and *daf-7* mutant animals. We found that all three have a significantly reduced egg-laying and ovulation rate as compared to wild-type (*him-5*) animals (Figure 5.6 and 5.7). It is surprising to us that *daf-7* animals which are egg-laying defective (Figure 5.8) also have a reduced ovulation rate. However, *daf-7's* egg-laying rates, egg retention rates, and ovulation rates are all similar to those seen in *egl-32* (Figures 5.6, 5.7 and 5.8). Similarly, it was surprising to also find a decrease in the ovulation and egg-laying rates in *daf-3* and *daf-5* animals since these animals have no egg retention defect at all (Figure 5.8). These data show that the TGF-β pathway influences both egg-laying and ovulation.

Interestingly, in spite of the varied ovulation and egg-laying rates seen in these different mutant backgrounds all had ovulation rates that were very similar to their egg-laying rates (Figure 5.9). This suggests that ovulation and egg-laying



<u>Figure 5.6</u> – *egl-32;him-5, daf-5;him-5, daf-3;him-5* and *daf-7* animals all lay fewer eggs than wild-type animals. *Statistically significant difference. An ANOVA test was performed. The p-value comparing all groups is 5.31×10^{-4}. The p- value for the number of eggs laid in *him-5* vs. *egl-32;him-5* is 3.07×10^{-2}. The p-value for *him-5* vs.*daf-5;him-5* is 1.02×10^{-2}. The p-value for *him-5* vs. *daf-3;him-5* is 1.25×10^{-2}. The p-value for N2 vs. *daf-7* is 3.21×10^{-3}. Bars indicate standard deviation.

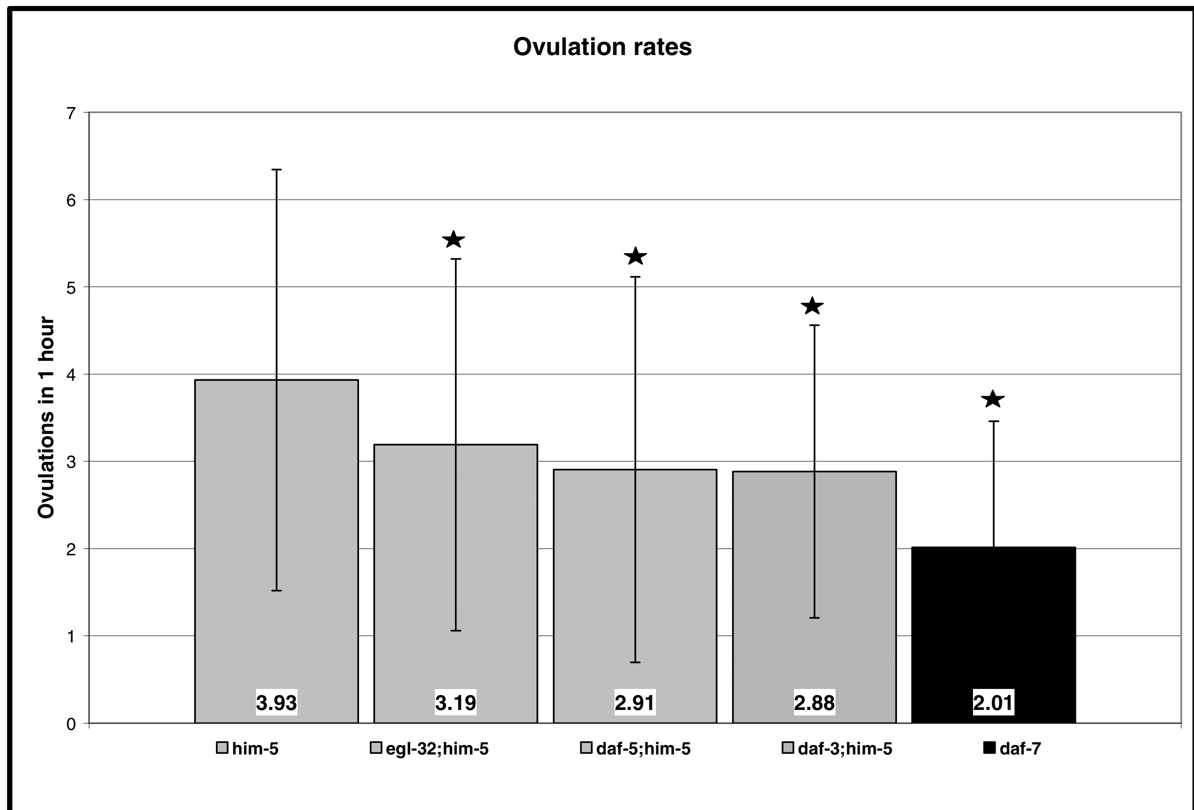


Figure 5.7 – *egl-32;him-5, daf-5;him-5, daf-3;him-5* and *daf-7* animals all have reduced ovulation rates compared to wild-type (*him-5*) animals. *Statistically significant difference. An ANOVA test was performed. The p-value comparing all groups is 5.46×10^{-3}. The p-value for wild-type vs. *egl-32;him-5* is 3.72×10^{-2}. The p-value for *him-5* vs. *daf-5;him-5* is 4.96×10^{-2}. The p-value for *him-5* vs. *daf-3;him-5* is 2.20×10^{-2}. The p-value for *him-5* vs. *daf-7* is 1.83×10^{-3}. Bars indicate standard deviation.

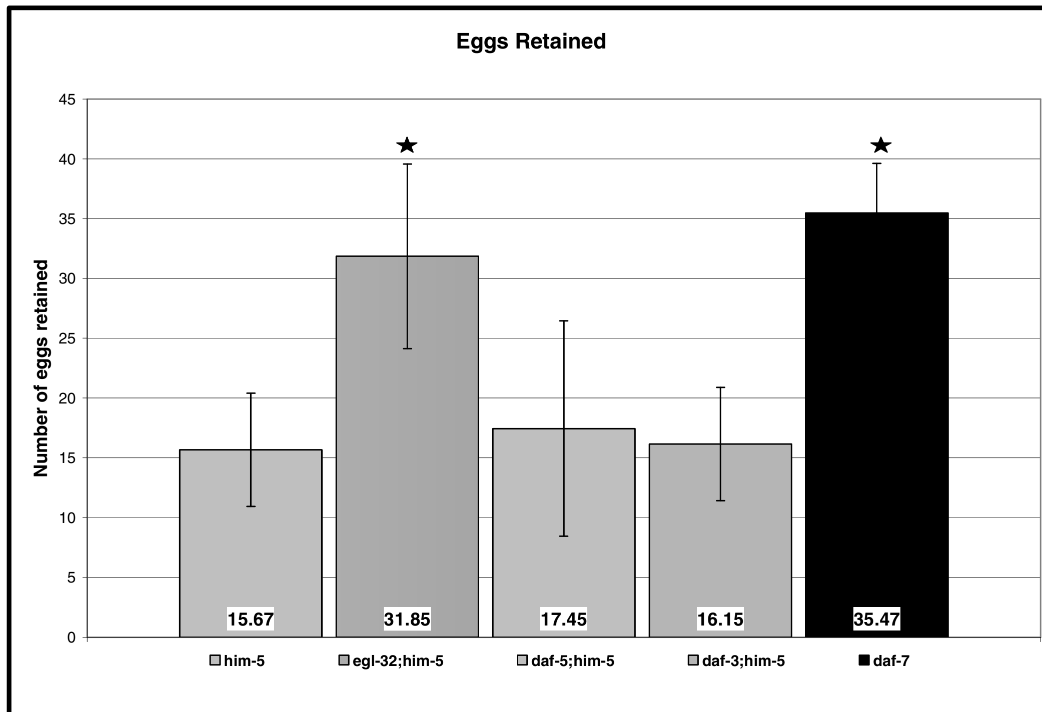


Figure 5.8 – Although *daf-3* and *daf-5* have reduced ovulation and egg-laying rates they do not retain more eggs than wild-type animals. However, *egl-32* and *daf-7* which also have reduced ovulation and egg-laying rates do retain significantly more eggs than wild-type animals. *Statistically significant difference. An ANOVA test was performed. The p-value comparing all groups is 2.57×10^{-53}. The p-value for wild-type (*him-5*) vs. *egl-32;him-5* is 1.14×10^{-36}. The p- value for wild-type vs. *daf-7* is 1.43×10^{-29}. The p-value between *himn-5* and *daf-5;him-5* is 0.170. The p-value between *him-5* and *daf-3;him-5* is 0.603.Bars indicate standard deviation.

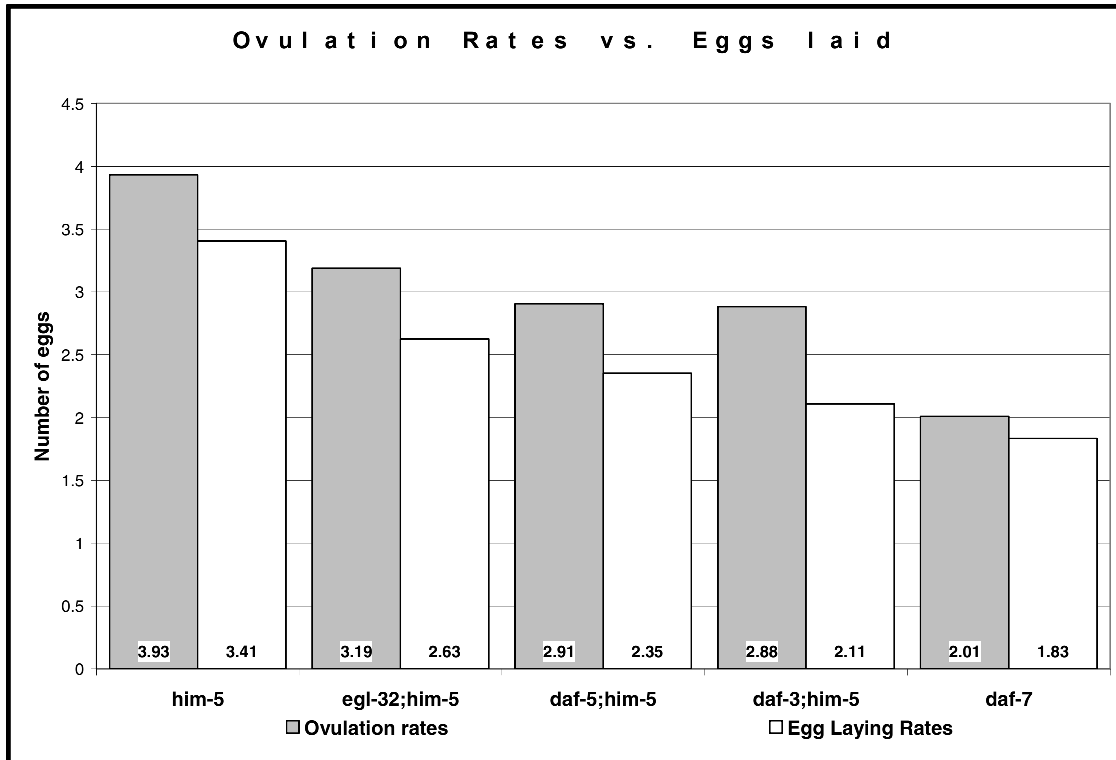


Figure 5.9 – Wild-type (*him-5*), *egl-32;him-5, daf-5;him-5, daf-3;him-5* and *daf-7* animals all have ovulation rates that are very close to, but slightly greater than the number of eggs they lay.

Soma	Wild-type sperm	*egl-32* sperm	*egl-32;daf-5* sperm	*daf-5* sperm
Wild-type	Wild-Type	Egg-laying defective	Wild-Type	Wild-Type
egl-32	Wild-Type	Egg-laying defective	Wild-Type	Wild-type
egl-32;daf-5	Wild-Type	Egg-laying defective	Wild-Type	Wild-Type
daf-5	Wild-Type	Egg-laying defective	Wild-type	Wild-Type

Table 5.4 – Summary of the mating experiments. e*gl-32* sperm is able to induce an egg-laying defective phenotype when introduced into any of the hermaphrodites used in this experiment. Wild-type and *daf-5* sperm are able to rescue the egg-laying defect when introduced into *egl-32* hermaphrodites. Sperm from *egl-32;daf-5* males are incapable of inducing an egg-laying defective phenotype in wild-type, or *daf-5* animals. However, *egl-32;daf-5* sperm are also incapable of rescuing *egl-32's* egg-laying defective phenotype. This table exactly matches Table 4.2 which contains the predicted results for the TGF-β pathway functioning in the sperm or during spermatogenesis.

rates may be tightly linked. In all cases the ovulation rates were slightly greater than that of the egg-laying rates. Over time this would lead to a build up of eggs in the uterus. This, in fact, is what is seen in older worms. However, these results do not explain why *egl-32* and *daf-7* animals become egg-laying defective. They may have many more ovulations as very young adults and as the uterus fills with eggs they are eventually stimulated to lay eggs at a normal rate. To determine if *egl-32* and *daf-7* mutants have highest ovulation rates as very young adults the ovulation rates should be determined at various time points after L4 instead of waiting for 24 hours. These should be compared to the ovulation rates at similar time points for that of wild-type, *daf-3* and *daf-5* animals.

Conclusions

egl-32 interacts with the TGF-β dauer pathway. It is suppressed by both *daf-3* and *daf-5*. However, we have shown that *daf-5* suppresses more strongly than *daf-3.* This is an interesting result since it is thought that *daf-3* and *daf-5* function together to promote dauer formation and prevent egg-laying under harsh conditions.

We were able to use the knowledge that *egl-32* functions in sperm to determine if the TGF-β pathway is functioning in the sperm, the soma, or both. Our mating experiments suggest that *daf-5* is suppressing *egl-32* in the sperm. However, we can not rule out at this time the possibility that *daf-5* is functioning to suppress *egl-32* in the soma during spermatogenesis.

We have shown that the TGF-β pathway has a role in both egg-laying and ovulation. d*af-3, daf-5* and *daf-7* mutants were all shown to have a reduction in both egg-laying rates and ovulation rates. This is interesting since of these three, only *daf-7* is egg-laying defective. We looked to see if there was a difference between the egg-laying and ovulation rates that could account for this. For example, if *daf-7* animals laid far fewer eggs than they ovulated we would expect them to bloat with eggs. However, this is not the case. In all cases we found that the ovulation rates slightly exceeded the egg-laying rates, even in wild-type animals. This could account for the fact that older animals normally become bloated with eggs.

Chapter V1: General Conclusions

egl-32 Acts through Sperm to Regulate Egg-laying

We became interested in the possibility that *egl-32* may be acting through sperm to regulate egg-laying when we discovered that the critical period for EGL-32 activity was during the L4 stage of development. This coincides with the one and only time that a hermaphrodite makes sperm. Our interest was heightened when we discovered that *egl-32* interacts with T08G11.2, which is known to be highly expressed in sperm. We performed simple mating experiments that revealed that *egl-32* does act through sperm to regulate egg-laying. When wild-type sperm are introduced, via mating, into *egl-32* hermaphrodites they begin to retain a wild-type number of eggs. When *egl-32* sperm are introduced into wild-type hermaphrodites, they begin to retain more eggs. This suggests that there is something about the mutant *egl-32* sperm that is able to affect the wild-type hermaphrodite. Recall that male sperm will out-compete hermaphrodite sperm. We have shown that fertilization is required for the rescue of *egl-32* by endogenous male sperm. It is likely that a signal is released from sperm upon fertilization that induces egg-laying. This signal may be either missing, or incapable of being released from mutant *egl-32* sperm.

Thus, we conclude that *egl-32* activity is required for a sperm-derived signal that promotes egg laying and acts at or after fertilization. Consistent with this model, *tm336* mutants, in which a gene expressed in sperm is deleted, have defects in both ovulation and egg laying. The defects in ovulation and egg laying

are at least partially separable genetically as seen in the *egl-32(n155)* mutant, which has only a slightly reduced ovulation rate. One possibility is that these defects share a common cause, but require different threshhold levels of signaling activity.

A role for seminal fluid in regulating female oocyte maturation and ovulation, among other behaviors, has been well established in *Drosophila ,* (Wolfner, 2002). In particular, the hormone Acp70A (sex peptide) stimulates oogenesis and Acp26Aa (ovulin) stimulates ovulation. Similarly, factors in mammalian seminal fluid, such as TGFβ and prostaglandin, promote changes in the female to facilitate embryo survival and implantation (Robertson, 2005). The rapid evolution of male seminal fluid proteins suggests a role in male-female sexual conflict, in which the male benefits most by increasing the quantity of offspring, while the female benefits more by selecting high quality mates (Chapman, 2001 & Clark and Swanson, 2005). In *C. elegans*, although competition between hermaphrodite and male sperm occurs (Ward & Carrel, 1979), it might be expected that the hermaphrodite's sperm would play a limited role in regulating its own reproductive behaviors due to the lack of sexual conflict. This, apparently, is not the case. Instead, hermaphrodite sperm actively signal to promote oocyte maturation and ovulation via the MSPs (Miller et. al., 2001), and, as shown here, to promote egg laying via an *egl-32*-dependent activity. Sperm signaling in the hermaphrodite may exist to ensure prudent investment of resources. In the absence of sperm, ovulation would be an energetically wasteful behavior. Similarly, a sperm-derived signal may exist to

coordinate the rate of fertilization to the rate of egg laying. As long as sperm are present the rate of egg laying is close to the rate of ovulation resulting in a fairly constant number of eggs present in the uterus. Older, wild-type worms normally bloat with eggs. These older hermaphrodites may become defective in egg laying because they no longer contain the internal cue present in sperm to promote egg laying.

egl-32 and _smn-1_ may be Allelic

egl-32 and *smn-1* may be alleles of the same gene. SNP mapping places *egl-32* in the same region of LG1 as *smn-1*. Also, both *egl*-32 and *smn-1* have been shown to interact with the sperm enriched transcript, T08G11.2. A knockout of *smn-1* results in a more severe defect than that seen in *egl*-32 but they share many of the same characteristics. Homozygous *smn-1* animals die at or before the L4 stage of development. Heterozygous *smn*-1 animals are egg-laying defective. Similar to *egl*-32 they retain more eggs, lay fewer eggs and have a decreased ovulation rate when compared to wild-type animals. When wild-type sperm is introduced into either *egl-32* or *smn-1* heterozygous animals, they begin to retain fewer eggs.

smn-1 is known to be involved in spliceosomal assembly and SnRNP biogenesis. It is possible that *egl-32* interacts with T08G11.2 and its homologues, as well as other unknown transcripts, by recognizing, and properly splicing them. In the complete absence of *egl-32/smn-1*, such as in the knockout, all the transcripts normally recognized and spliced fail to do so and are degraded. This

results in lethality. However, when the level of *egl-32/smn-1* are reduced, as in the heterozygous knockout or in *egl-32(n155,)* enough transcripts are properly spliced to allow the animals to survive. However, certain protein products produced by these animals will be reduced. This may include the sperm enriched transcripts of T08G11.2 and its homologues. When these sperm proteins are reduced or absent, the result is an egg-laying defect.

The Role of TGF-β dauer Pathway in Egg-laying and Ovulation

We performed a number of mating experiments to show the TGF-β dauer pathway functions in either the sperm or in the soma during spermatogenesis. We were able to show this using the knowledge that *egl-32's* egg-laying defect is suppressed by *daf-5*. We asked whether *daf-5* needed to be present in the sperm, the soma, or both in order for it to suppress *egl-32.* These mating experiments revealed that *daf-5* must be present in the sperm to suppress *egl-32.* This does not rule out the possibility that *daf-5* is functioning in the soma during spermatogenesis to bring about this suppression.

We have also shown that TGF-β pathway has a role in both egg-laying and ovulation. Mutant d*af-3, daf-5* and *daf-7* animals were all shown to have a reduction in both egg-laying rates and ovulation rates. This is interesting since of these three, only *daf-7* is egg-laying defective. We thought perhaps there may be a greater difference between the egg-laying and ovulation rates in *daf-7* animals that could account for this. If *daf-7* animals lay far fewer eggs than they ovulate we would expect them to bloat with eggs. However, this is not the case. In all

cases we found that the ovulation rates slightly exceeded the egg-laying rates, even in wild-type animals. This could account for the fact that older animals normally become bloated with eggs. It is possible that in very young adult *daf-7* animals the difference between egg-laying and ovulation in greater. This difference may even out as the worms being to bloat with eggs. This possibility could be investigated by looking at much younger adults and determining if their egg-laying and ovulation rates.

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