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We recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science with a major in Biology .

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Behavioral characterization of *C. elegans gpa-16* mutant using temperature-sensitive and deletion alleles

by

TARIK MITCHELL

A THESIS

Submitted in partial fulfillment of the requirements

for the degree of Masters in Science

in the Biology Graduate Program of

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DEDICATION

I come from a large family that consists of educated men and women who share core values of integrity, faith, courage, and ambition. I'm a second generation college graduate and the first amongst my siblings. However the only reason that I was awarded the opportunity to attend graduate school or any type of school is because of the sacrifices that were made by others before me, specifically my two grandmothers, Mattie Mae Mitchell and Annie Pearl Green. Both coming from humble beginnings having to work instead of attend grade school paved the way for me to become the man that I am today. Everything that they endured was to ensure that generations to come did not have to endure the same hardship. It is therefore my honor to dedicate my thesis to them. The thesis, Behavioral characterization of *gpa-16* ts and *gpa-16* Δ mutants was done in dedication of Mattie Mae Mitchell and Annie Pearl Green.

ACKNOWLEDGEMENTS

This research project would not have been possible without the help of many people. First, I would like to thank my advisor Dr. Harbinder Singh Dhillon for his guidance, patience, and dedication. It is because of him that I can work as an open minded independent researcher. I would like to reserve a special thanks to my co advisor Dr. Theresa Szabo-Maas for the support and wisdom that she has consistently provided. I will like to convey special thanks to my committee members Dr. Robert MacBride, Dr. Thomas Mennella, Dr. Thomas Planchon and Dr. Theresa Szabo-Maas. Thanks to my fellow lab members Tiffany Cummings, Roderick King, Rochelle McDonald, Mahlet Mersha, and Diamond Moore for their assistance. I owe my deepest gratitude to Dr. Thomas Mennella for his help with my research, in addition to his vital role in my development as a graduate student, Dr. Amar Klar from NIH for his advice and knowledge on the entire project. The educational advancement alliance and HBCU-STEM fellowship program for funding me. Lastly, I want to thank my family and friends for their support and encouragement.

ABSTRACT

Caenorhabditis elegans is a nematode worm typically used in the laboratory as a model multicellular eukaryote with a comparatively simple nervous system. The complex body plans of multicellular organisms originate from a single fertilized cell. This requires that various symmetric and asymmetric cell divisions occur in order for organisms to develop into a functional form. It is known that formation of the anterior/posterior axis is generally defined by point of entry of the sperm, and the dorsal/ventral axis is shaped with the help of spindle positioning and specialized partitioning proteins. The specification of left-right laterality is required for the complete body plan including placement of visceral organs and brain laterality. We are interested in characterizing the developmental and behavioral effects exerted by a gene *gpa-16*, which codes for a Gα subunit. Two mutant strains *gpa-16* ts (or temperature sensitive) and *gpa-16* deletion have been characterized. The ts mutant has been previously shown to exhibit high embryonic lethality (~70%) with laterality reversal in about half of the survivors. Various behavioral assays were performed to characterize their behaviors. A chemotaxis based assay was used to characterize the mutant strains' associative learning, and habituation assays were used to characterize the mutant strains non-associative learning compared to the wild-type strain. Progeny viability was monitored at 15, 20, and 25 degrees Celsius, to compare temperature sensitivity between mutant and the wild-type strain. In summary, experimentation and tests have concluded that there is no significant difference between the behaviors of both *gpa-16* mutants, while both mutants have shown to be significantly different from wild-type N2 animals.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVATIONS

Δ	Deletion
1mm	One millimeter
7TM	Seven transmembrane
AB	Anterior blastomere
AP	Anterior posterior
BLAST	Basic Linear Alignment and Search Tool
C. elegans	*Caenorhabditis elegans*
cAMP	Cyclic adenosine monophospate
CEP	Cephalic neurons
CGC	Caenorhabditis Genetics Center
CRE	cAMP response element
CREB	cAMP response element-binding protein
DV	Dorsal ventral
E.coli	*Escherichia coli*
$G\alpha_s$	G-protein alpha stimulator
$G\alpha_i$	G-protein alpha inhibitory
GDP	Guanosine diphosphate
GNAI-1	G-prtoein O alpha-1
GPA-16	G-protein alpha -16
GPCR	G-protein coupled receptors
GTP	Guanosine triphosphate
IAA	Isoamyl alcohol
LR	Left right

LTD	Long term depression
LTP	Long term potentiation
MAP	Mitogen-activated protein
Mbp	Mega base pair
NaN3	Sodium azide
N2	Wild type
NGM	Nematode growth media
OP-50	*E.coli* strain
PAR	Psuedoautosomal region
PCR	Polymerase chain reaction
PDE	Posterior deirid neurons
PKA	Protein Kinase A
SEM	Standard error of mean
t.s.	Temperture sensitive

I. INTRODUCTION

C. elegans as a study model

Caenorhabditis elegans (*C. elegans*) has become a popular model organism for biological research since the initial proposal for using *C. elegans* as a multicellular lab model in 1974 by Sydney Brenner. This organism is a transparent, soil dwelling, nematode roundworm that can grow up to 1mm by feeding on bacteria. Its entire genome of 100.3Mbp was the first fully sequenced genome of a multicellular organism in 1998. It is estimated that *C. elegans* has approximately 19,735 protein coding open reading frames (Hillier et al., 2005). There are 959 somatic cells within the adult hermaphrodite; the developmental fate of each of these cells has been completely identified. *C. elegans* has a simple and compact nervous system, containing 302 nerve cells, which have all been identified as well as their neural connectivity (White et al., 1986).

Furthermore *C. elegans* has two sexes, hermaphrodite and male; despite hermaphrodites being the predominant sex form, males' compromise 0.05% of the population. Post self-fertilization, hermaphrodites lay eggs at ~32 cell stage; the hatched offspring goes through four cycles (L1-L4). The normal life cycle of *C. elegans* is approximately 14 days when grown at standard lab temperature of 20 °C. On an average, each hermaphrodite lays about 300 eggs.

In the laboratory, the organisms are easily maintained on solid agar in petri dishes or liquid media, with *E.coli* as a food source. When stored at -80°C in 15% glycerol base buffer, the worms can survive for long periods of time. These animals can spend years in this temperature/solution, and more importantly they can be revitalized when required.

This makes it convenient for researchers to maintain and share a large number of mutant strains.

In addition to the advantageous laboratory qualities mentioned above, mutant *C. elegans* strains can be easily obtained from the Caenorhabditis Genetics Center (CGC). These mutant strains are economical and efficient. Worm Base, an online database exclusively for *C. elegans* also affords extensive and reliable data about the *C. elegans* genome, neuroanatomy, and development. These resources are readily available for scientists to access and use for research (Mohri et al., 2005).

C. elegans development

Multicellular organisms go through a sequence of events in which they begin from a single cell to eventually become differentiated individuals. The initial cell divisions often give rise to several totipotent or multipotent cells. After subsequent cycles of cell division, the cells begin to develop specificity. Whereas each cell undergoes cellular differentiation, through processes that often result in asymmetrical cellular divisions. This may be in part due to various levels of concentration gradients of key morphogens (Levin and Palmer 2008, Armakolas et al., 2010). Morphogens are signaling molecules that travel away from their source to form a concentration gradient. The fate of each cell depends on the concentration of the morphogen signal; while the gradient predetermines the pattern of development (Tabata and Takei 2004).

Vertebrate embryos involve formation of three germ layers: ectoderm, mesoderm, and endoderm. These layers go on to form specific tissues and organs of the organism. Cells found in each layer become more specialized according to the system they will be a part of. Asymmetrical cell divisions during differentiation are required in order to ensure

proper formation of the anterior/posterior, dorsal/ventral axes as well as the left/right laterality of the organism. Asymmetrical cellular divisions early in development are proposed to be the source of specific human brain laterality (Klar, 2010). In all embryos for which there is evidence, LR polarity arises after anterior/posterior (AP) and dorsal/ventral (DV) polarities are fixed (reviewed by Wood, 1997).

In *C. elegans* the anterior-posterior axis is determined by the point of sperm entry at the one-cell stage, with entry actually marking the posterior end of the embryo (Goldstein and Hird, 1996). After the initial cleavage forming the AP axis (two-cell stage), a second cleavage takes place. Dorsal-ventral (D/V) polarity is established at the second cleavage, (Wood et, al. 1996). One spindle pole is found to be posterior to the other, which defines the dorsal side of the embryo this also, creates the DV axis (Zoines et al., 2010). The original AB (anterior blastomere) cell is elongated via restraints by the eggshell (Wood et al., 1996). The AB cell splits establishing the four-cell stage. This also marks the beginning of gastrulation in the emerging embryo.

Gastrulation is the period during embryogenesis when cells that are predetermined to produce internal tissues and organs move from the surface to the interior of the embryo (Rohrscheider and Nance 2009). During the third cleavage, the anterior and posterior AB cells (ABa and ABp) are rotated in the left-right direction to form the LR axis (at the six-cell stage). Thus, this cleavage is when L-R polarity becomes apparent, in which ABa and ABp divide in the L-R direction. Initially each spindle is formed in the plane of the L-R axis, orthogonally to both the A-P and D-V axes, but then skews in parallel just prior to cytokinesis so that one pole of each spindle assumes a position anterior to its opposite pole (Bergmann et al., 2003) .

It is therefore this twist which occurs with a consistent handedness (clockwise as viewed from the dorsal side) in the 6-cell embryo (Figure l) which essentially results in cells being on the left side, ABal and ABpl, which lie anterior to their sister cells on the right, ABar and ABpr, respectively (Sulston et al., 1983, Wood, 1991). The ensuing EMS and P, cell divisions, mainly A-P in direction, are also twisted as constrained by the positions of the AB-derived cells, so that the resulting and cell embryo are markedly L-R asymmetric (Wood et, al. 1996). This asymmetry continues throughout the cleavage stage while aiding the foundations for the body plan (Hamada et al 2002).

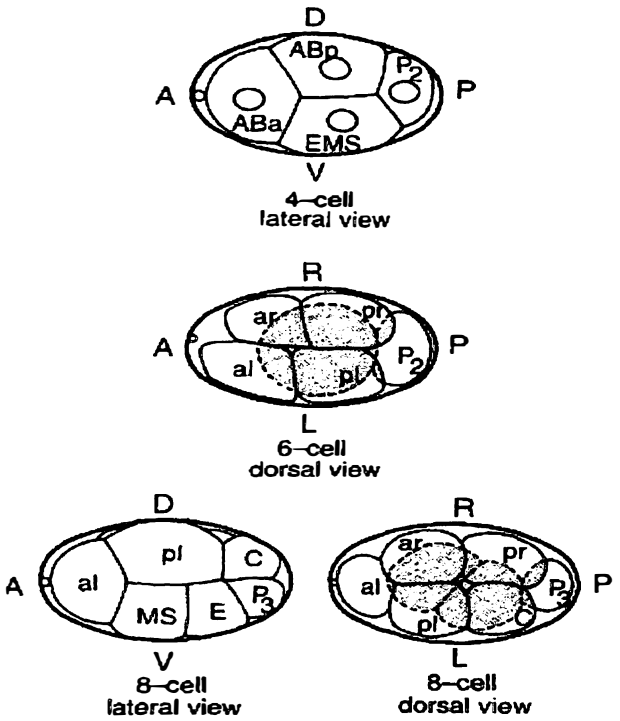

Figure 1. Early embryos, stages and views as indicated. A, anterior; P, posterior; D, dorsal; V, ventral, L, (animal's) left; R, (animal's) right. XC, excretory cell; CC's, coelomocytes; G, gonad; VNC, ventral nerve cord; V, vulva. (From Wood et al., 1996)

Cell polarity plays a crucial role in the determination of diverse cell types which eventually will contribute to the differentiation of cells (Zoines et al., 2010). There are three steps that occur during asymmetric cell division in *C. elegans*; first, a polarity cue must determine the cell's axis (fertilization); second, the cortical domains used to define cell polarity must be formed through polarity cues (PAR domains) Cortical domains defines the polarity of embryos by acting upstream of PAR proteins (Schenk et al., 2010); and third, polarization of the cytoplasm occurs through a downstream signal. PAR proteins are responsible for controlling cellular polarity and are involved in cell fate specification during gastrulation (Cowan and Hyman, 2004; Rohrschneider and Nance, 2009). They have a role in controlling the left-right (LR) axis orientation of cytoskeletal elements that are linked with the anterior-posterior (AP) and dorsal-ventral (DV) axes. PAR proteins are responsible for the transduction of signals downstream to molecules necessary for asymmetric cell division (Cowan and Hyman, 2004).

Cell polarity in C. elegans embryos is defined by PAR proteins. The PAR domains establish the site of cell division by positioning the mitotic spindle. The PAR proteins control the segregation of fate determinants and position the spindle (Schenk et al., 2010). Initially, PAR proteins are found uniformly distributed throughout the cell cortex or in the cytoplasm (Tostevin and Howard, 2008, Zoines et al., 2010). PAR machinery has been found in a diverse group of animals, suggesting a conserved role in the mechanism for cell polarization (Goldstein and Macara, 2007). The localization of PAR proteins to their respective domains is required for the formation of the anterior and posterior axis during early embryogenesis (Nance et al., 2005). Fertilization, near the future posterior pole, provides the polarization necessary for the formation of the anterior

and posterior cortical domains, through the contraction of cortical actomyosin towards the anterior pole (Tostevin and Howard, 2008) (see Figure 2). The anterior domain (green) consists of PAR-3, PAR-6, and atypical protein kinase C (aPKC). The posterior domain (red) includes PAR-1 and PAR-2. The contraction of the anterior PARs allows PAR-2 to be free to move from the cytoplasm onto the cell cortex. The localization of PAR-2 onto the cell cortex continues to restrict the anterior PARs away from the posterior end of the zygote (Zoines et al 2010). The posterior domain is responsible for the segregation of cell fate determinants and the positioning of the mitotic spindle (Cowan and Hyman, 2004). PAR-2, more specifically, is found to determine mitotic spindle orientation (yellow), which ultimately defines the left-right axis (Bergmann et al 2003, Zoines et al 2010). The AP and DV axes must be predetermined before the LR axis can be defined (Levin and Palmer 2007 and Bergmann et al 2003). Lastly, PAR proteins are not only important in cellular polarity and mitotic spindle orientation, but also in positioning proteins and RNAs that have roles in cell fate distinctions between specific cell types, including those involved in gastrulation (Goldstein and Macara 2007).

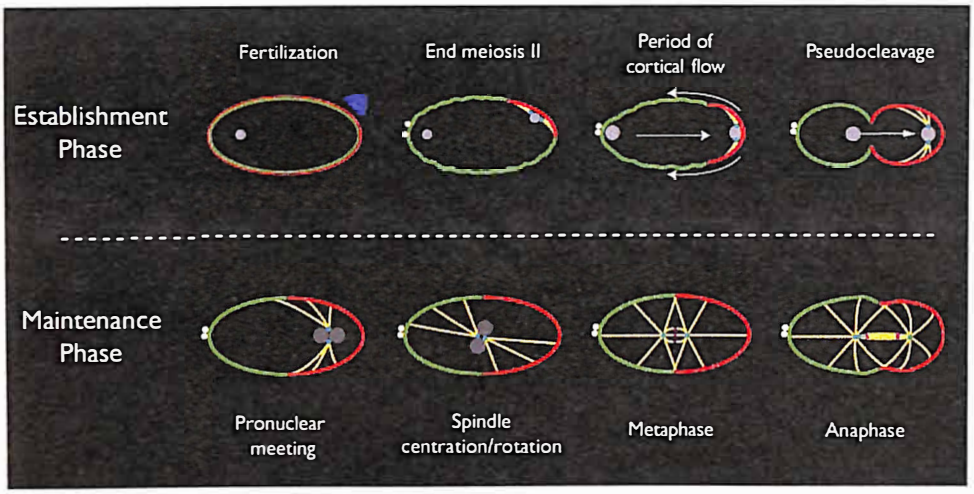

Figure 2. PAR Protein Distribution: Schematic view of the events that led up to the first asymmetric division in the *C. elegans* zygote. Nuclei are gray; Microtubules are yellow; Chromosomes are magenta; small filled cyan circles represent centrosomes; small filled white circles are polar bodies extruded during meiosis; red and green along the cell cortex represent cortical domains enriched in anterior PAR proteins (green = PAR-3, PAR-6, PKC-3) and posterior (red = PAR-1 and PAR-2) PAR proteins. (From Munro, 2006)

Learning and Memory

Many organisms are apparently born with some intrinsic information that is necessary for survival. Often times this information has already been programmed in their nervous system. Although in the case of *Homo sapiens*, we are born with inadequate information required for survival, and most of our novel learning is acquired through experience and observation. Learning is generally defined as the process of acquiring new knowledge or modifying the knowledge that has already been obtained (Kandel et al., 1991; Okano et al, 2000). In order to survive and reproduce, living organisms must not only possess the ability to adapt to their ever changing environment, but they must also store the information gathered for future recall. This process of storing and recalling information is known as memory (Bansal and Parle, 2010).

In non-associative learning, which is considered the simplest form of learning, the organism's response towards a harmless but repeated stimulus is tested (Kandel et al., 1991). Habituation and sensitization are viewed as non-associative learning. In the course of learning to habituate, the organism is exposed to a repeated stimulus. Exposure to this harmless stimulus results in reduced response towards the stimulus. This phenomenon explains why a person learns to ignore the smell of an innocuous odor after prolonged exposure to the odor. However, in the case of sensitization, the organism exhibits an increase of attention towards the stimulus. In the more complex associative

learning, the animal learns to associate one stimulus with another (Kandel et al., 1991). One of the earliest examples of associative learning was presented by Ivan Pavlov, in which a dog was trained to correlate the sound of a bell with food. Whenever the dog heard the sound of the bell, it would salivate in anticipation of food, even when the food was not presented (Pavlov, 1928).

Cellular and molecular aspects of habituation were studied by Eric Kandel using the marine sea slug *Aplysia californica* (Yan-You et al., 2005). Stimulus was delivered to the siphon resulting in the withdrawal of the siphon and the gills. After repeated exposure to this stimulus, the sea slug eventually stopped withdrawing its siphon and gills. This experiment indicated that recurring stimulation causes excitatory interneurons to yield weaker synaptic potentials, causing the motor neurons to drastically reduce their firing rates and eventually stop. This decrease in the synaptic potentials is caused by reduction in the number of transmitter vesicles released into the synapse (Kandel et al., 1991).

Once new information is acquired, memory is created as neural correlates that allow access to the information for future recall. Sequentially three levels of memory have been described. Short-term memory has a limited capacity for storing information, typically 7 plus or minus 2 items (Miller, 1955). It allows recall only after a few minutes and involves biochemical changes in key synapses (Kandel et al., 1991). Short-term memory and intermediate-term memory are often considered one and the same; they both involve long-term depression (LTD). Long-term depression is defined as a reduction in synaptic transmission induced by low frequency stimulation of the neurons (Bliss and Lomo, 1973). Long-term memory correlates with long term potentiation (LTP), which is

defined as an increase in communication between two nerve cells induced by high frequency stimulation of the neurons.

Long-term memory involves three processes; gene expression, the synthesis of new proteins and the growth of new synaptic connections (Kandel et al., 1991). In order for long-term memory to take place, protein kinase A (PKA) recruits mitogen-activated protein (MAP) kinase and enters the nucleus. Once in the nucleus, PKA phosphorylates and activates the transcription factor CREB-1. The activated CREB-1 then binds to the promoter element CRE (MAP kinase blocks the inhibitory actions of CREB-2). This leads to the initiation of two major events. First, the genes turned on as a result of CREB result in the production of the enzyme ubiquitin carboxyterminal hydroxylase. This ensures uninterrupted production of PKA, ensuring the continuity of the previously mentioned pathway. Secondly, the transcription factor C/EBP coding genes are turned on, causing the activation of genes needed for proteins associated with the growth of new synaptic neurons.

Previous studies have shown that appropriate acquisition and recall of learning and memory are strongly controlled by various neurotransmitters, in particular dopamine, glutamate, norepinephrine and serotonin (Chase and Koelle, 2007; Sawin et al, 2000). Experiments with *Aplysia californica* sensory neurons have shown that activation of serotonin receptors in post-synaptic neurons initiate a G-protein mediated signal transduction cascade that increases intracellular cAMP and Ca++ levels, which in turn initiate molecular cascades that can influence both short-term and long term synaptic changes. The changes in synaptic efficacy caused by cAMP are, in part, due to increased release of glutamate, an excitatory neurotransmitter that acts through an inhibitory Gα

subunit (Nicholls et al., 2006). The cellular correlate of memory in mammals is defined by long term potentiation, characterized by synaptic strengthening caused by high frequency stimulation of specific chemical synapses (Staubli and Lynch, 1987).

Previous studies with mammalian hippocampal neurons have confirmed the involvement of serotonin and glutamate in the cAMP mediated learning pathway. Mammalian studies have also revealed a role for another neurotransmitter, dopamine, which is required for memory stabilization possibly through a cytoplasmic polyadenylation element binding protein (Theis et al., 2003; reviewed in Barco et al., 2006).

Learning and memory in *C. elegans*

In *C. elegans,* behavior is primarily dictated by modifications in the environment. As a result, it has the aptitude to associate change in the environment with the existence or nonexistence of food. Its ability to learn through association makes *C. elegans* an ideal organism to study the molecular changes that take place during learning and memory.

C. elegans ability to learn can be tested through habituation (non-associative learning) and chemotaxis (associative learning). Habituation can be tested by observing a decrease of response by the nematode to repeated mechanical tap stimuli. Associative learning, such as classical conditioning and differential conditioning, can be tested by using assays in which worms are conditioned to specific chemicals that are paired with the presence or absence of a specific chemical, (this chemical can be harmless or harmful) and then assaying the worms for changes in chemotactic response towards the conditioned chemical (Saeki et al., 2001; Law et al., 2004).

Laterality reversal in C. *elegans gpa-16* mutants

The *C. elegans gpa-16* gene has been highly conserved throughout many organisms. Most notably in *D. rerio* (zebra-fish) and in *homo-sapiens* (see table 1). In zebra fish and in *homo-sapiens* the homologous *GNAI-1* showed a remarkable ~80% similarity when comparing the two protein sequences. Even more astonishing is that when comparing the zebra fish *GNAI-1* to the human homologue *GNAI-1*, there was a ~ 99% similarity among the protein sequences. This indicates that this gene has been highly conserved throughout evolution (Figure 3a-3c).

Sequence Alignment: *zebra-fish* GNAI-1 to *C. elegans* GPA-16

Score = 479 bits (1234), Expect = e-136, Method: Compositional matrix adjust.
Identities = 229/357 (64%), Length = 357, Positives = 277/357 (77%), Gaps = 3/357 (0%)

```
zebra-fish:1   MGCTLSTEDKAAVERSKMIDRNLRDDGEKAAREVKLLLLGAGESGKSTIVKQMKIIHEAG 60
               MGC +S ED+AA  RSK IDR L++DGE + R +KLLLLGAGESGKSTI+KQM+IIH+ G
C. elegans:1   MGCIMSQEDEAAKRRSKKIDRLLKEDGENSMRTIKLLLLGAGESGKSTILKQMRIIHDVG 60

zebra-fish:61  YSEEECKQYKAVVYSNTIQSIIAIIRAMGRLKIDFGXXXXXXXXXQLFVLAGSAEEGFMT 120
               Y+ EE K ++ VVY N I S+ AII AM +LKI F          +L + + + EE +
C. elegans:61  YTTEERKVFRGVVYGNIILSLNAIIHAMEQLKISFTTLDHESDARKLLMFSTTGEEDELP 120

zebra-fish:121 AELAGVIKRLWKDGGVQACFSRSREYQLNDSAAYYLNDLDRISQATYIPTQQDVLRTRVK 180
                EL  ++K +W D G+Q    RSREYQLNDSA YYL+ LDRI    YIPTQ D+LRTR+K
C. elegans:121 EELVVLMKSVWSDSGIQKALERSREYQLNDSAGYYLSQLDRICAPNYIPTQDDILRTRIK 180

zebra-fish:181 TTGIVETHFTFKDLHFKMFDVGGQRSERKKWIHCFEGVTAIIFCVALSDYDLVLAEDEEM 240
               TTGIVET F +KD  F +FDVGGQRSERKKWIHCFE VTA+IFCVALS+YD+VL ED +
C. elegans:181 TTGIVETQFVYKDRLFLVFDVGGQRSERKKWIHCFEDVTALIFCVALSEYDMVLVEDCQT 240

zebra-fish:241 NRMHESMKLFDSICNNKWFTDTSIILFLNKKDLFEEKIRKSTLTICYPEYAGSNTYEEAA 300
               NRM ES+KLFDSICNNKWF +TSIILFLNKKDLFEEKI +S LT C+PEY G+N YEEA+
C. elegans:241 NRMRESLKLFDSICNNKWFVETSIILFLNKKDLFEEKIVRSPLTHCFPEYTGANNYEEAS 300

zebra-fish:301 AYIQCQFEDLNKR---KDTKEIYTHFTCATDTKNVQFVFDAVTDVIIKNNLKDCGLF 354
               AYIQ QFED+NKR    + +EIYT FTCATDT N++FVFDAVTD+II++NL+ CGL+
C. elegans:301 AYIQQQFEDMNKRTTGEKNQEIYTQFTCATDTNNIRFVFDAVTDIIIRDNLRTCGLY 357
```

Figure 3a. One-to-one sequence alignment between *C. elegans GPA-16* protein sequence and the zebra-fish homologue GNAI-1 protein sequence. The sequence from *C. elegans* shares an approximate 80% similarity to the zebra-fish homologue GNAI-1.

```
Sequence Alignment: Homo-sapien GNAI-1 to C. elegans GPA-16

Score = 499 bits (1286), Expect = e-142, Method: Compositional matrix adjust
Identities = 237/357 (66%), Length = 357, Positives = 283/357 (79%), Gaps =
3/357 (0%)

H. sapiens:1    MGCTLSAEDKAAVERSKMIDRNLREDGEKAAREVKLLLLGAGESGKSTIVKQMKIIHEAG 60
                MGC +S ED+AA  RSK IDR L+EDGE + R +KLLLLGAGESGKSTI+KQM+IIH+ G
C. elegans:1    MGCIMSQEDEAAKRRSKKIDRLLKEDGENSMRTIKLLLLGAGESGKSTILKQMRIIHDVG 60

H. sapiens:61   YSEEECKQYKAVVYSNTIQSIIAIIRAMGRLKIDFGDSARADDARQLFVLAGAAEEGFMT 120
                Y+ EE K ++ VVY N I S+ AII AM +LKI F       DAR+L + +   EE  +
C. elegans:61   YTTEERKVFRGVVYGNIILSLNAIIHAMEQLKISFTTLDHESDARKLLMFSTTGEEDELP 120

H. sapiens:121  AELAGVIKRLWKDSGVQACFNRSREYQLNDSAAYYLNDLDRIAQPNYIPTQQDVLRTRVK 180
                 EL  ++K +W DSG+Q    RSREYQLNDSA YYL+ LDRI  PNYIPTQ D+LRTR+K
C. elegans:121  EELVVLMKSVWSDSGIQKALERSREYQLNDSAGYYLSQLDRICAPNYIPTQDDILRTRIK 180

H. sapiens:181  TTGIVETHFTFKDLHFKMFDVGGQRSERKKWIHCFEGVTAIIFCVALSDYDLVLAEDEEM 240
                TTGIVET F +KD  F +FDVGGQRSERKKWIHCFE VTA+IFCVALS+YD+VL ED +
C. elegans:181  TTGIVETQFVYKDRLFLVFDVGGQRSERKKWIHCFEDVTALIFCVALSEYDMVLVEDCQT 240

H. sapiens:241  NRMHESMKLFDSICNNKWFTDTSIILFLNKKDLFEEKIKKSPLTICYPEYAGSNTYEEAA 300
                NRM ES+KLFDSICNNKWF +TSIILFLNKKDLFEEKI +SPLT C+PEY G+N YEEA+
C. elegans:241  NRMRESLKLFDSICNNKWFVETSIILFLNKKDLFEEKIVRSPLTHCFPEYTGANNYEEAS 300

H. sapiens:301  AYIQCQFEDLNKR---KDTKEIYTHFTCATDTKNVQFVFDAVTDVIIKNNLKDCGLF 354
                AYIQ QFED+NKR   +  +EIYT FTCATDT N++FVFDAVTD+II++NL+ CGL+
C. elegans:301  AYIQQQFEDMNKRTTGEKNQEIYTQFTCATDTNNIRFVFDAVTDIIIRDNLRTCGLY 357
```

Figure 3b. A one-to-one sequence alignment between *C. elegans GPA-16* protein sequence and the *homo-sapien* homologue GNAI-1 protein sequence. The sequence from *C. elegans* shares an approximate 80% similarity to its *homo-sapien* homologue GNAI-1.

```
Sequence Alignment: Zebra-fish GNAI-1 to Homo-Sapien GNAI-1

Score = 1787, E-value = 0, Method: Compositional matrix adjust.
Identities = (96%), Match length = 354, Positives = 283/357 (98%), Query length
= 354

Zebra-fish:1    MGCTLSTEDKAAVERSKMIDRNLRDDGEKAAREVKLLLLGAGESGKSTIVKQMKIIHEAG 60
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H. Sapiens:1    MGCTLSAEDKAAVERSKMIDRNLREDGEKAAREVKLLLLGAGESGKSTIVKQMKIIHEAG 60

Zebra-fish:61   YSEEECKQYKAVVYSNTIQSIIAIIRAMGRLKIDFGD+ARADDARQLFVLAG+AEEGFMT 120
                YSEEECKQYKAVVYSNTIQSIIAIIRAMGRLKIDFGDAARADDARQLFVLAGSAEEGFMT
H. Sapiens:61   YSEEECKQYKAVVYSNTIQSIIAIIRAMGRLKIDFGDSARADDARQLFVLAGAAEEGFMT 120
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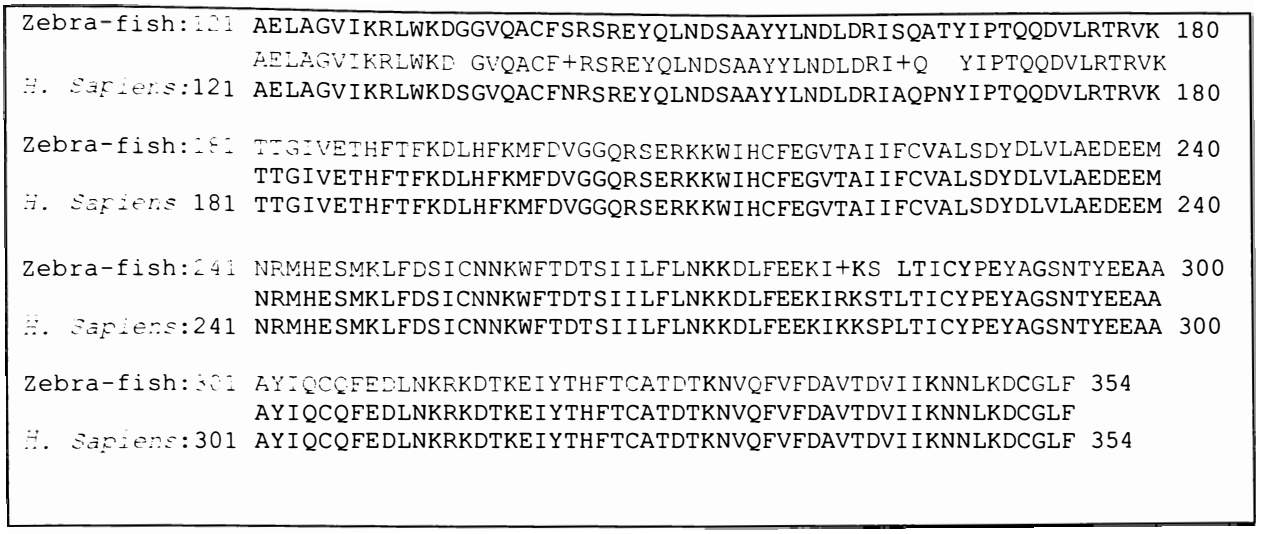

Figure 3c. A one-to-one sequence alignment between *zebra-fish GNAI-1* protein sequence and its *homo-sapien* homologue GNAI-1 protein sequence. These sequences share nearly 100% similarity. Both are homologues to *C. elegans* GPA-16.

Studies in *C. elegans* have shown that a strong loss-of-function mutation in a gene initially called *spn-1* affects early spindle positioning and results in inexact choice of handedness (Wood et, al. 1996). This mutation interrelates genetically with mutations in three par genes that are known to play key roles in localized cortical components. Furthermore, the *spn-1* gene encoded the Gα protein GPA-16. Because of this, the originally named *spn-1* has since been referred to as *gpa-16*. Furthermore, *gpa-16* has previously been described to be required for centrosomal association of a Gβ protein (Wood et, al. 1996).

Hypothesis

We hypothesized that laterality reversal in worms is likely to have neural consequences that can be tracked using specific behavioral assays. Our experimental approach included behavioral analysis, viability testing, and an attempt to achieve genetically back-crossed animals. Our results showed that both *gpa-16 ts* and *gpa-16*Δ mutants show statistically identical deficiencies in learning, memory and viability.

II. MATERIALS AND METHODS

Strains

All *C. elegans* were obtained through the *Caenorhabditis* Genetic Center, University of Minnesota; Minneapolis, MN. Worms were grown in nematode growth media (NGM) plates at 20°C (some at 25°C and 15°C) as described in (Hope, 1999). The *E. coli* strain OP50 was used as a food source. N2; wild type Bristol isolate; *gpa-16 {it143 I; him-5e1490 V, strain BW1890};* and *gpa-16 {ok2349 I, strain RB1816}* were the strains used throughout the study.

Nematode growth media plates (NGM plates)

C. elegans are maintained in the laboratory on Nematode Growth Medium (NGM) agar which has been sterilized and poured into petri plates (Brenner, 1974). The methodology for NGM that was used is as followed. [Mix 1.5g NaCl, 8.5g agar, and 2.5g peptone in a 2 liter Erlenmeyer flask. Add 500 ml H2O]. Cool flask in 55°C water bath for 15 min or allow the flask to cool down at room temperature. [Add .5 ml 1 M $CaCl_2$, .5 ml 5 mg/ml cholesterol in ethanol, .5 ml 1 M $MgSO_4$ and 12.5 ml 1 M KPO_4 buffer].

Preparation of bacterial food source

E. coli OP50 is a uracil auxotroph whose growth is limited on NGM plates. A limited bacterial lawn is necessary because it allows easier observation and better mating of the worms. A starter culture of *E. coli* OP50 can be obtained from the CGC or can be recovered from worm plates. Use the starter culture to isolate single colonies on a streak plate of a rich medium such as LB agar [10g Bacto-tryptone, 5g Bacto-yeast, 5g NaCL, 15g agar, H_2O to 1 litre, pH 7.5] (Byerly et al., 1976). Using a single colony from the

streak plate, aseptically inoculate a rich broth, such as L Broth [10g Bacto-tryptone, 5g Bacto-yeast, 5g NaCl, H_2O to 1 litre, pH to 7.0 using 1 M NaOH. Allow inoculated cultures to grow overnight at 37°C. The *E. coli* OP50 solution after being inoculated overnight is then ready for use in seeding NGM plates. The *E. coli* OP50 streak plate (the original source from which the *E. coli* was taken) and liquid culture should be stored at 4°C to ensure its usefulness for several months to come.

Transferring worms onto NGM plates

Several methods are used for transferring *C. elegans* from one petri plate to another. One method that was used is to pick single animals with a worm picker while using a dissecting microscope. A worm picker can be made by mounting a 1-inch piece of 32 gauge platinum wire into either the tip of a Pasture pipet or in a bacteriological loop holder. Platinum wire heats and cools quickly and can be flamed often (between transfers) to avoid contaminating the worms, this is done before and after picking. The end of the wire, used for picking up worms, can be flattened slightly with a coin/penny and then filed to remove sharp edges; sharp points can poke holes in the worms and kill them or make holes in the agar. To pick a worm identified under the dissecting microscope, slowly lower the tip of the wire and gently swipe the tip at the side of the worm and lift up. Another method is to get a blob of *E. coli* OP50 on the end of the wire before gently touching it to the top of the chosen worm. The worm will stick to the bacteria. Several animals at a time can be picked by this method. To put a picked worm on a fresh plate, slowly lower the tip of the worm picker, gently touch the surface of the agar, and hold it there to allow the worm to crawl off of the picker. A more convenient method of transferring worms is sub culturing or "chunking", wherein a sterilized scalpel

or spatula is used to move a chunk of agar from an old plate to a fresh plate by simply cutting the desired region on the agar. More often than not there will usually be hundreds of worms in the piece of agar. The worms will crawl out of the chunk and spread out onto the bacterial lawn of the new plate. This method works well for transferring worms. Especially for instances where the worms have crawled into the agar or are difficult to pick individually.

Synchronizing worm populations

Prior to every experiment all animals that were tested were synchronized. Synchronization ensures that all animals were at the same stage during testing and more importantly the removal of any and all possible contamination. Obtaining synchronous cultures of C. elegans included the following. Freshly prepare 50ml of hypochlorite solution. M9 Buffer [3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 litre. Sterilize by autoclaving]. Methods: Pipette 1ml of M9 buffer unto the NGM plate that is being synchronized. Aseptically transfer the axenized eggs to 10ml tube whereas the adults will release their eggs. Allow the 10ml tube to settle for 5 minutes. Aspirate most of the liquid from the 10ml tube and transfer 10ml of hypochlorite solution from 50ml sterile tube that was freshly prepared. Spin the 10ml tube that now contains the eggs and hypochlorite solution for at least 2 min at 1150 x g to pellet the worms. Once spinning is complete aspirate the hypochlorite solution, and add 10ml of M9 buffer to initiate the washing of the worms. Spin for at least 2 min at 1150 x g to pellet the worms. Repeat this 3-5 times until the pellet is transparent. Upon visualizing a clear pellet, aspirate most of the liquid and pipette the pellet on to a seeded NGM plate. This plate should be stored at the permissible temperature (16˚C - 25˚C).

Behavioral Assays

For each experiment, well fed, three-day old synchronized young adult worms were used. Special attention was given to make sure that the worms were not over crowded, starved, or contaminated.

Habituation assays: In preparation for the assay both non seeded and seeded Normal Growth Media (NGM) plates prepared fresh the night before the assay and left overnight at room temperature. Approximately 10 worms were transferred to the new NGM plates. Using an eyelash hair, the worm was gently tapped on its anterior portion, better termed the head of the worm. In response to this stimulus, the worms typically regress and move backwards. The number of times the animal moves backward until it no longer responds to the stimulus was counted (hence, at this point the worm has habituated).

Chemotaxis based conditioning assays: chemotaxis plates were prepared the night before the assay and kept at room temperature for 1 hour before use as described by (Bargmann et al, 1991). Animals were collected with M9 buffer and washed two more times before the assay. For conditioning, the animals were exposed to 3 µl of isoamyl alcohol for 90 minutes. In order to immobilize the animals 2µl of 1M sodium azide (NaN_3) was placed on the trap/control and gradient points 10 minutes before the start of the assay and served as an anesthetic. Worms were placed at the starting point equidistant to both the trap and gradient points. 1/100 isoamyl alcohol diluted in 100% ethanol was placed on the gradient while 100% ethanol was placed on the trap point. Plates were left undisturbed for one hour and then kept at - 10°C for 10 minutes after which the number of worms in each plate section was counted. Chemotaxis index was calculated by

subtracting the number of worms at the trap point from the number of worms found at the gradient point and dividing it by the total number of worms found on the plate. Worms located at the starting point were excluded from counting, as these worms were dead or damaged while processing the samples and were therefore immobile.

Generating Males

Five well-fed L4 worms transferred to NGM plates seeded with OP50. They were then heat shocked at 31.5° C for 6-7 hours. After 2-3 days, three males were picked and transferred to a plate with at least two hermaphrodites.

Single Worm PCR

DNA from a single worm was extracted using proteinase K. 1 µl 20mg/ml proteinase case was dissolved with 95 µl 1x PCR buffer. Each worm was lysed in 5µl proteinaseK-buffer solution. After freezing and thawing for five minutes, worms were lysed in the PCR machine using the following setting:

65˚C for 60-90 min

95˚C for 15 min

Once DNA was extracted, PCR was used to amplify 350bp of wild-type N2. The following *dop-2* primers were used as a control to:

Fwd: 5'- AAC GAT TCC TTG CGA TTC TG- 3'

Rev: 5'- AAA GGA CTT CAC TGC ACG AC-3'

The following settings were used for PCR:

1. 94°C, 4 min

2. 94°C, 30 sec

3. 58°C, 30 sec

4. 72°C, 1 min

5. Steps 2, 3 and 4 repeated for 30 cycles.

6. 72°C, 7 min

After freezing and thawing for five minutes, worms were lysed in the PCR machine using the following setting:

65˚C for 60-90 min

95˚C for 15 min

Once DNA was extracted, PCR was used in an attempt to amplify 3kb of wild-type N2 and *gpa-16* Δ. The following *gpa-16* Δprimers were used:

Outer Left Sequence: 5'-AGCGCAATGGGGTGTATTAT- 3'.

Outer Right Sequence: 5'-CGAATCGGACCAACACTCT- 3'.

Inner Left Sequence: 5'-AGCGAAACGAAGATCCAAGA- 3'

Inner Right Sequence: 5'-ATTCGTGATCGAGTGTGGTG- 3'.

The following settings were used for PCR (gradient testing was used as well):

1. 94°C, 4 min

2. 94°C, 30 sec

3. 58°C, 30 sec

4. 72°C, 1 min

5. Steps 2, 3 and 4 repeated for 30 cycles.

6. 72°C, 7 min

PCR products were confirmed by gel electrophoresis.

Sequence analysis

The Basic Linear Alignment and Search Tool (BLAST) was used for sequence comparison. The DNASTAR Laser Gene seqbuilder software was used to analyze deletion sequences.

Statistical analysis

Sigma plot (Statistical software) was used for behavioral assays; statistical analysis was carried out using Two Way Analysis of Variance.

III. RESULTS

Comprehensive phenotypic and behavioral analysis of *gpa-16 t.s* and *gpa-16* deletion mutants

The *gpa-16 {it143 I; him-5e1490 V, strain BW1890}; and gpa-16 {ok2349 I, strain RB1816}* mutant strains were examined for visual and phenotypic abnormalities. Compared to wild type *N2* worms, these strains were found to be normal in terms of body size, shape, movement and locomotion.

Behavioral characterization of the mutant strains was carried out using a series of behavioral assays, specifically non associative learning; habituation, and associative learning, chemotaxis. Since the *gpa-16 ts* animal has been classified as a temperature sensitive mutant, we were curious to investigate if the learning and memory of this animal would vary depending on the temperature. Therefore, experiments were done under permissive and non-permissive temperatures (15 and 20 °Cs) that were known to yield a sufficient amount of progeny, which would cause the temperature sensitive mutant to mimic wild-type mutant phenotypes.

Non-associative learning results

The first habituation experiment was conducted on animals that were grown under 15 °C conditions; the animals' ability to no longer respond to a repeated harmless stimulus was tested using the habituation assay as stated previously. Surprisingly when compared to the wild-type N2, both *gpa-16 t.s.* and *gpa-16*Δ mutants exhibited significantly slower habituation rates (with $p<0.001$) when tested after being raised at 15 °C conditions, Most interestingly the two mutants did not show a significant difference when compared to each other (Figure 4).

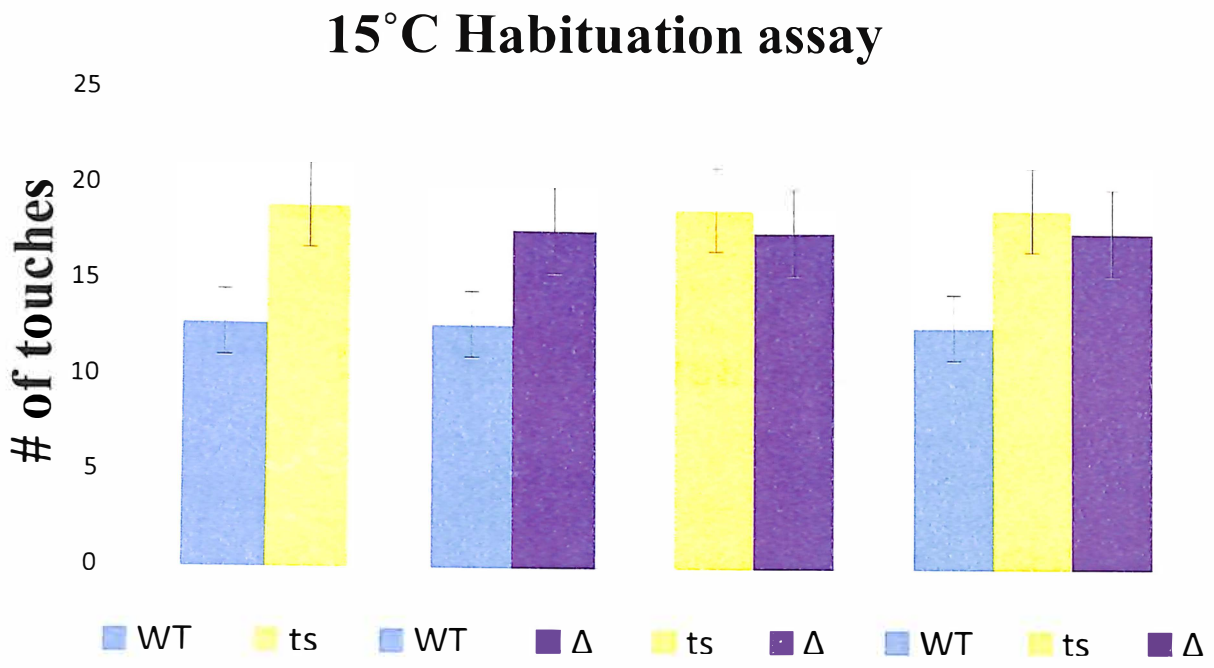

Figure 4 15°C Habituation assay for wild-type and mutants' *gpa-16* ts and *gpa-16* Δ. Stimulus was given every 5 seconds until habituation occurs. After testing animals that were grown at 15°C, mutants with a point mutation in the *gpa-16* gene (referred to as ts) along with mutants with a deletion in the *gpa-16* gene (referred to as Δ) both displayed abnormal habituation rates in comparison to wild-type (referred to as WT) animals. In addition the two mutants did not show any significant difference in the amount of touches it took them to habituate. These results indicate that both mutants learn slower than WT and that they both learn the same after being grown at 15°C. (Colors: teal= wild-type N2 worms, yellow= *gpa-16 ts*, purple= *gpa-16*Δ) N=44 for each strain. (p value < 0.001, two way analysis variance)

The second habituation experiment was conducted on animals that were grown under 20 °C conditions; the amount of touches it took the animals to ignore the repeated harmless stimulus was tested again, using the habituation assay as previously mentioned. No

difference from the 15 degree results, when compared to the wild-type N2, both *gpa-16 t.s.* and *gpa-16*Δ mutants exhibited significantly slower habituation rates (with $p<0.001$) when grown in 20 °C conditions, the two mutants did not show a significant difference when compared to each other (Figure 5).

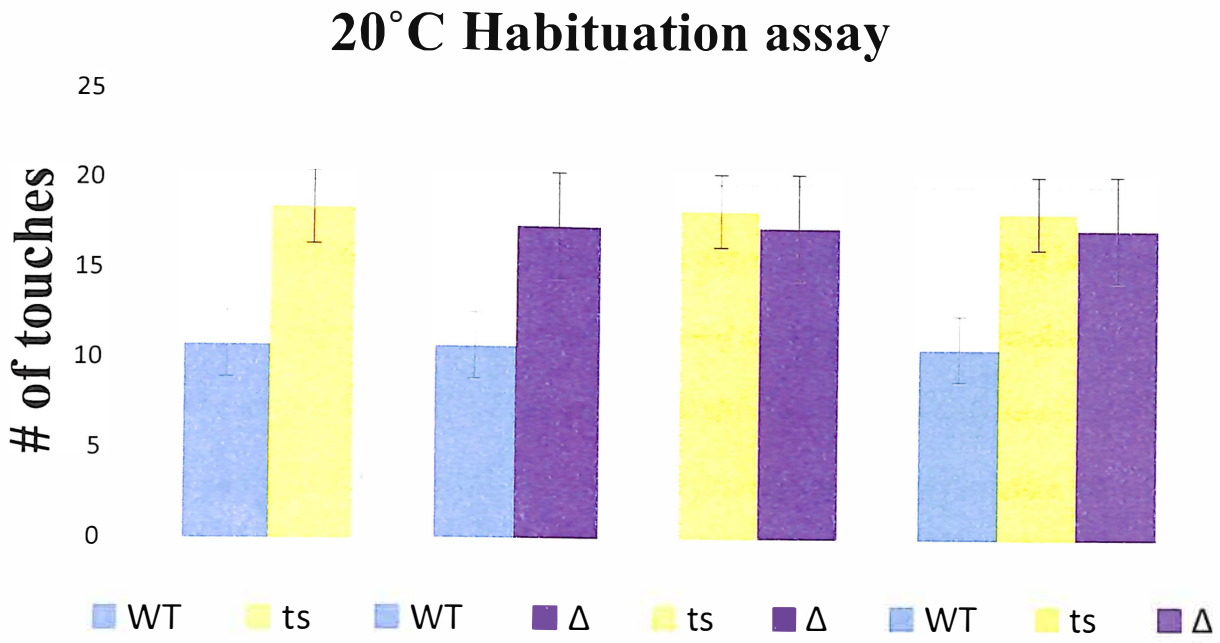

Figure 5 20°C Habituation assay for wild-type and mutants' *gpa-16* ts and *gpa-16* Δ. Stimulus was given every 5 seconds animals habituated. After testing animals that were grown at 15°C, both *gpa-16 ts* and *gpa-16* Δ mutants displayed abnormal habituation rates in comparison to wild-type animals. The two mutants did not show a significant difference in their habituation rates. These results suggest that both mutants learn slower than WT and that both mutants learn the same after being grown at 20°C. (Colors: teal= wild-type N2 worms, yellow= *gpa-16 ts*, purple= *gpa-16*Δ) N=44 for each strain. (p value < 0.001, two way analysis variance)

Associative learning results

After the non-associative results indicated that both mutants behave the same, and that neither of them behaved as wild-type further behavioral characterization became a necessity. In theory, when using a temperature sensitive mutant under permissible temperatures, the mutant should resemble wild-type. However the non-associative results do not indicate that. Therefore a more complex method of testing the animals' behavior was done using chemotaxis based associative learning assays. There were separate chemotaxis experiments that were performed on animals that were grown at 15 and 20 °C. Chemotaxis usually consists of an organism being exposed to a chemical that is either harmful or desired. The experiments conducted here used a chemical that resembled the smell of food. However the chemical was not food and the worms could only smell the chemical. Without being pre-exposed to this chemical (naïve) one would postulate that the animals would be more attracted towards it, with the expectation of finding food. However after being conditioned with this chemical and then exposed to it, one would postulate that the animals would become less attracted to this chemical, and that this chemical would be related to starvation. During the chemotaxis experiments the chemo-attractant isoamyl alcohol was paired with starvation. In conditioned chemotaxis, worms that are usually attracted to a particular compound tend to avoid it after experiencing starvation in presence of that compound (Colbart and Bergmann, 1995). First, we challenged a group of naive worms that were raised in 15 °C conditions. These worms were challenged with 1:100 isoamyl alcohol (no conditioning). The worms (all three strains) showed strong attraction towards isoamyl alcohol after being raised at 15 °C (Figure 6). As previously stated, the smell of isoamyl alcohol is known to resemble the

smell of their food. When the conditioned wild-type N2, *gpa-16 ts* and *gpa-16*Δ mutants were conditioned with isoamyl alcohol, they all exhibited reduced avoidance (Figure 6). However when comparing wild-type to *gpa-16 ts* and *gpa-16*Δ mutants the wild-type showed a significant difference in the amount of reduced avoidance when compared to the two mutant strains. When comparing *gpa-16 ts* and *gpa-16*Δ mutants, there was no significant difference in their amount of reduced avoidance (Figure 6).

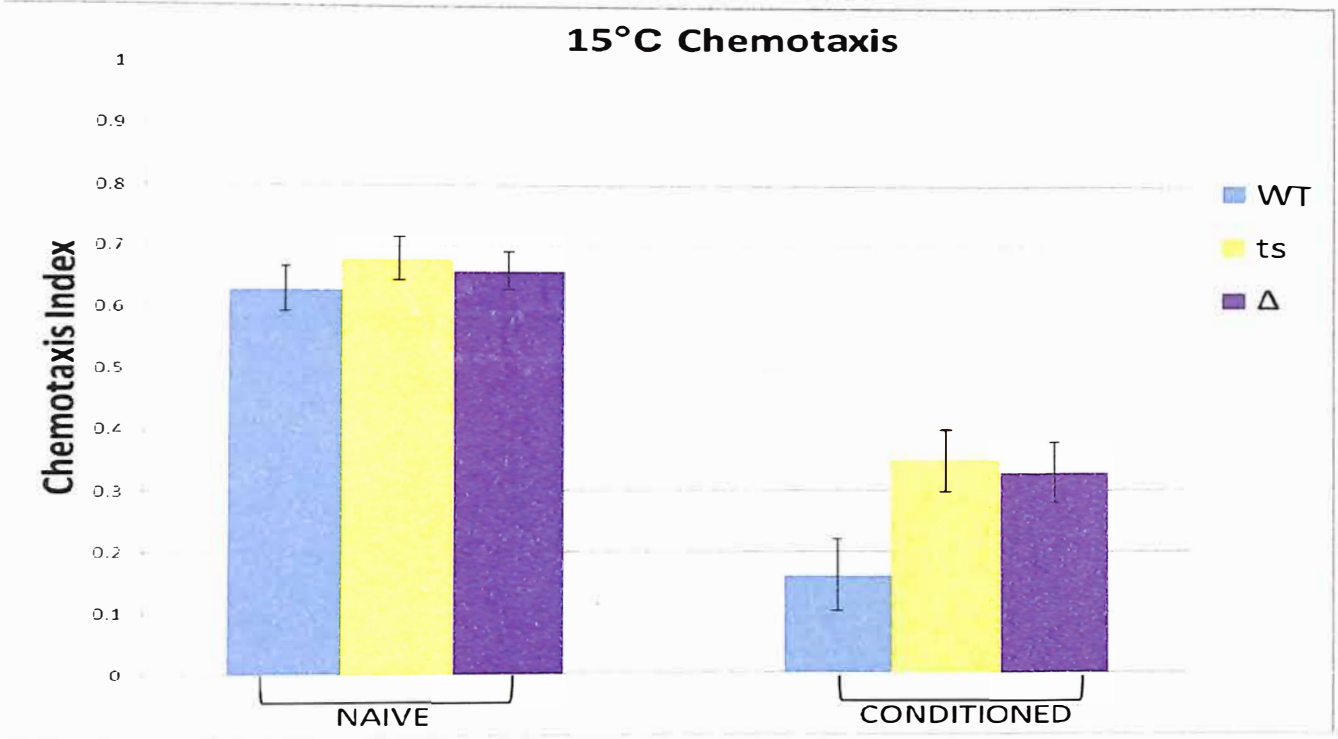

Figure 6 15 °C Associative learning chemotaxis assay for young adult hermaphrodites of *C. elegans gpa-16 ts* and *gpa-16*Δ mutants. Worms were collected, washed and assayed for isoamyl alcohol chemotaxis on chemotaxis plates after being raised at 15 °C. Bars on the left in the naïve bracket represent naïve, unconditioned worms while bars on the right in the conditioned bracket represent worms conditioned with isoamyl alcohol for 90 minutes before the assay. After testing animals that were grown at 15°C, both mutants displayed abnormal chemotaxis learning behavior in comparison to wild type animals and showed no significant difference in their 15 °C chemotaxis learning ability. The x axis

indicates the average chemotaxis index toward isoamyl alcohol. (Colors: teal= wild-type N2 worms, yellow= *gpa-16 ts*, purple= *gpa-16Δ)*. $P < 0.001$

Next, experiments were done using a group of naive worms that were raised in 20 °C conditions. These worms were also challenged with 1:100 isoamyl alcohol (no conditioning). The worms also showed a strong attraction towards isoamyl alcohol after being raised at 20 °C (Figure 7). When the conditioned wild-type N2, *gpa-16 ts* and *gpa-16Δ* mutants were conditioned with isoamyl alcohol, they all exhibited reduced avoidance (Figure 7). However when comparing wild-type to *gpa-16 ts* and *gpa-16Δ* mutants, the wild-type worms showed a significant difference in the amount of reduced avoidance when compared to the two mutant strains. When comparing *gpa-16 ts* and *gpa-16Δ* mutants, there was no significant difference in their amount of reduced avoidance (Figure 7).

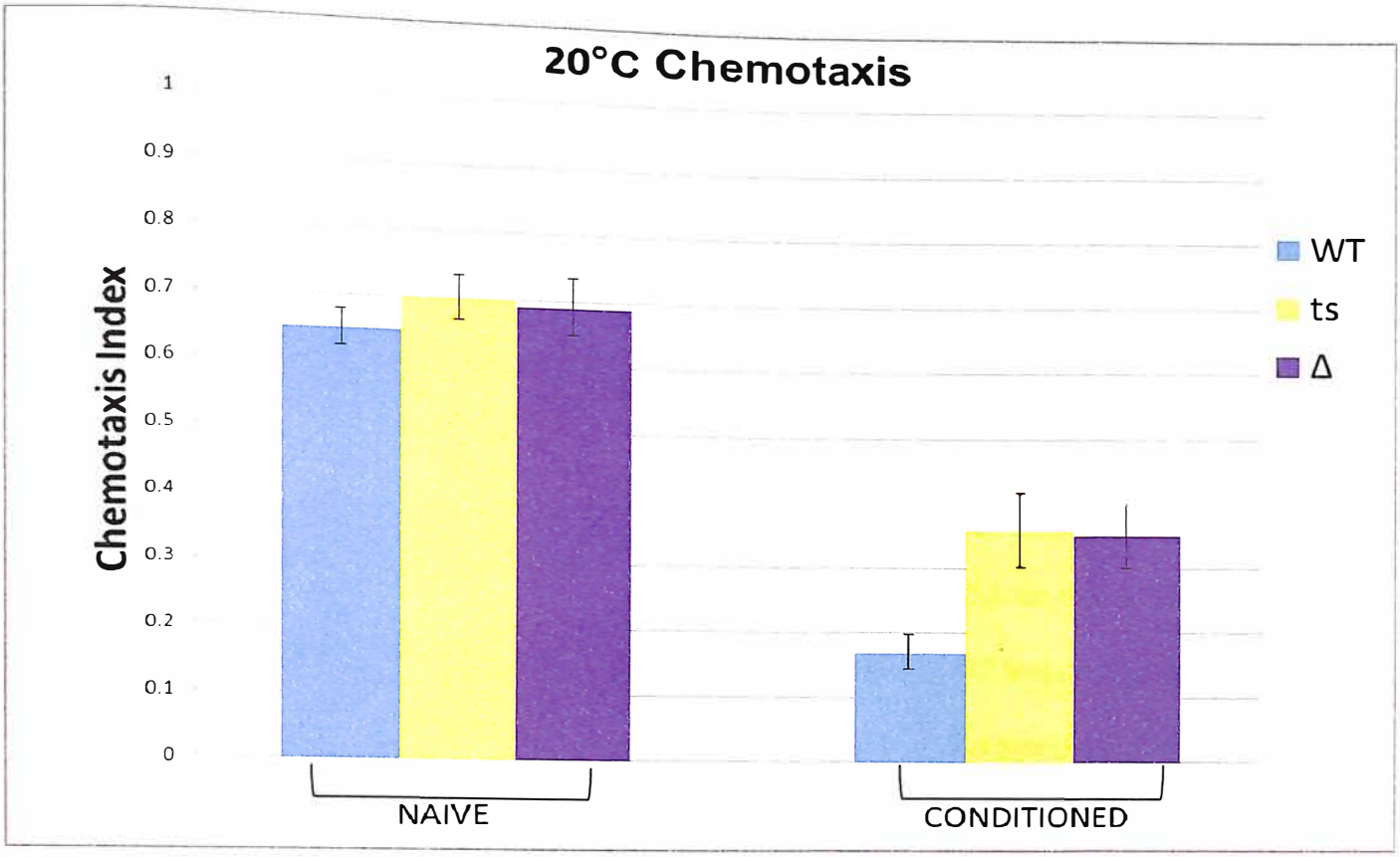

Figure 7 20 °C Associative learning chemotaxis assay for young adult hermaphrodites of *C. elegans gpa-16 ts* and *gpa-16*Δ mutants. Worms were collected, washed and assayed for isoamyl alcohol chemotaxis on chemotaxis plates after being raised at 20 °C. The naïve bracket represents naïve, unconditioned worms. While the conditioned bracket represent worms conditioned with isoamyl alcohol for 90 minutes prior the assay. After testing animals that were raised at 20°C, both *gpa-16* mutants displayed abnormal chemotaxis learning behavior in comparison to wild type animals and showed no significant difference in their 20 °C chemotaxis learning ability. The x axis indicates the average chemotaxis index toward isoamyl alcohol. (Colors: teal= wild-type N2 worms, yellow= *gpa-16 ts*, purple= *gpa-16*Δ*).* $P < 0.001$

After the interpretation of our results from the behavioral assays we were interested in finding out if the learning discrepancies of the different strains were significantly different when compared to the habituation/chemotaxis behavioral data from different temperatures (15 and 20°C behavioral data comparison). In short, I wanted to find out the

outcome when comparing two animals of the same strain but from two different temperatures. This would tell me if the animals learned differently in opposing temperatures. I also wanted to know if the results would be different if two different strains from two different temperatures were compared. This would tell whether or not the animals learned differently depending on the temperature. For example, when analyzing two animals that are the same strain but different temperatures I would compare the data from *gpa-16 ts* 15 °C Habituation and Chemotaxis to the data from *gpa-16 ts* 20 °C Habituation and Chemotaxis. If I were comparing two different strains that were grown in different temperatures I would compare *gpa-16 ts* 15 °C Habituation and Chemotaxis to wild-type N2, 20 °C Habituation and Chemotaxis. I compared the results from strains that were tested after been raised under 15 °C conditions to the results of animals that were tested after being raised under 20 °C conditions (figures 8 -9).

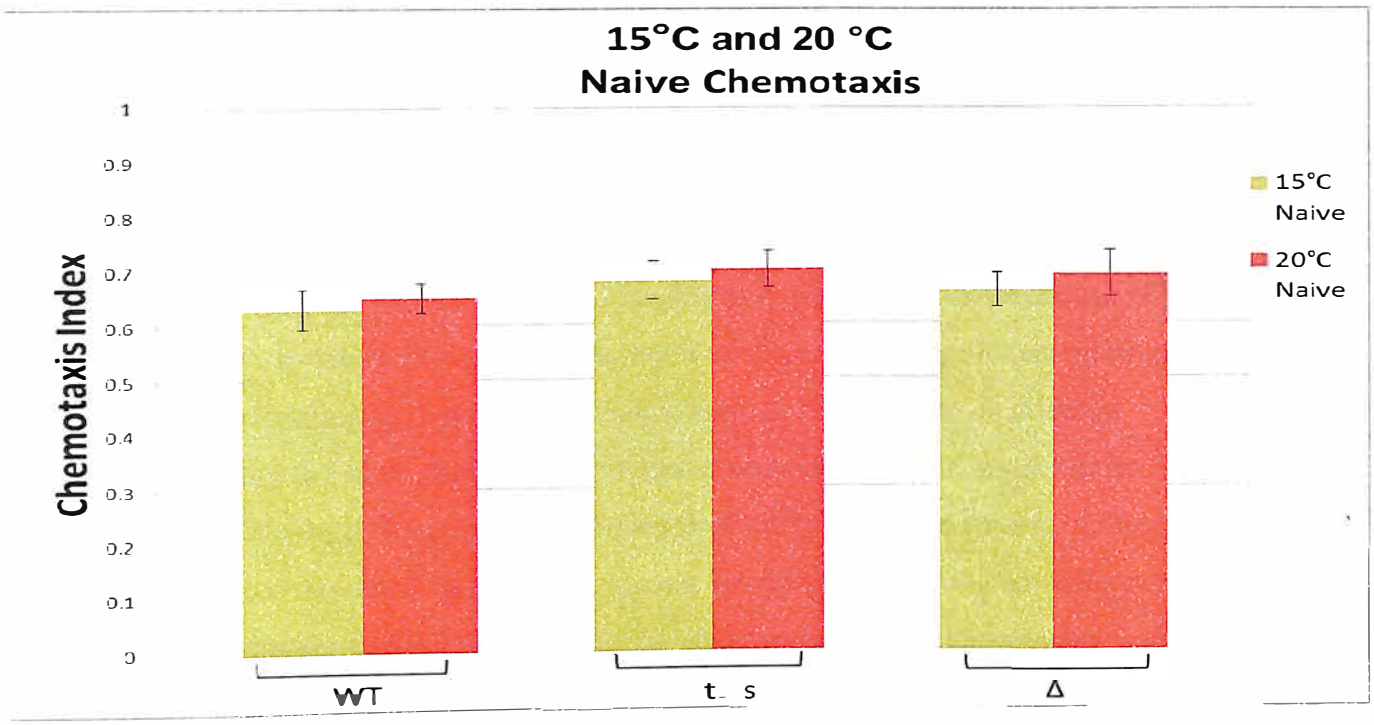

Figure 8a .The comparison of 15 and 20 °C Naïve chemotaxis. Using two-way analysis variance I was able to conclude that there was no significant difference in the associative learning amongst the naïve group of animals tested, regardless of the temperatures they had been grown at. Colors: green= 15 °C, red= 20 °C. (WT=wild-type N2, ts= *gpa-16* ts, Δ= *gpa-16*Δ) $P < 0.001$

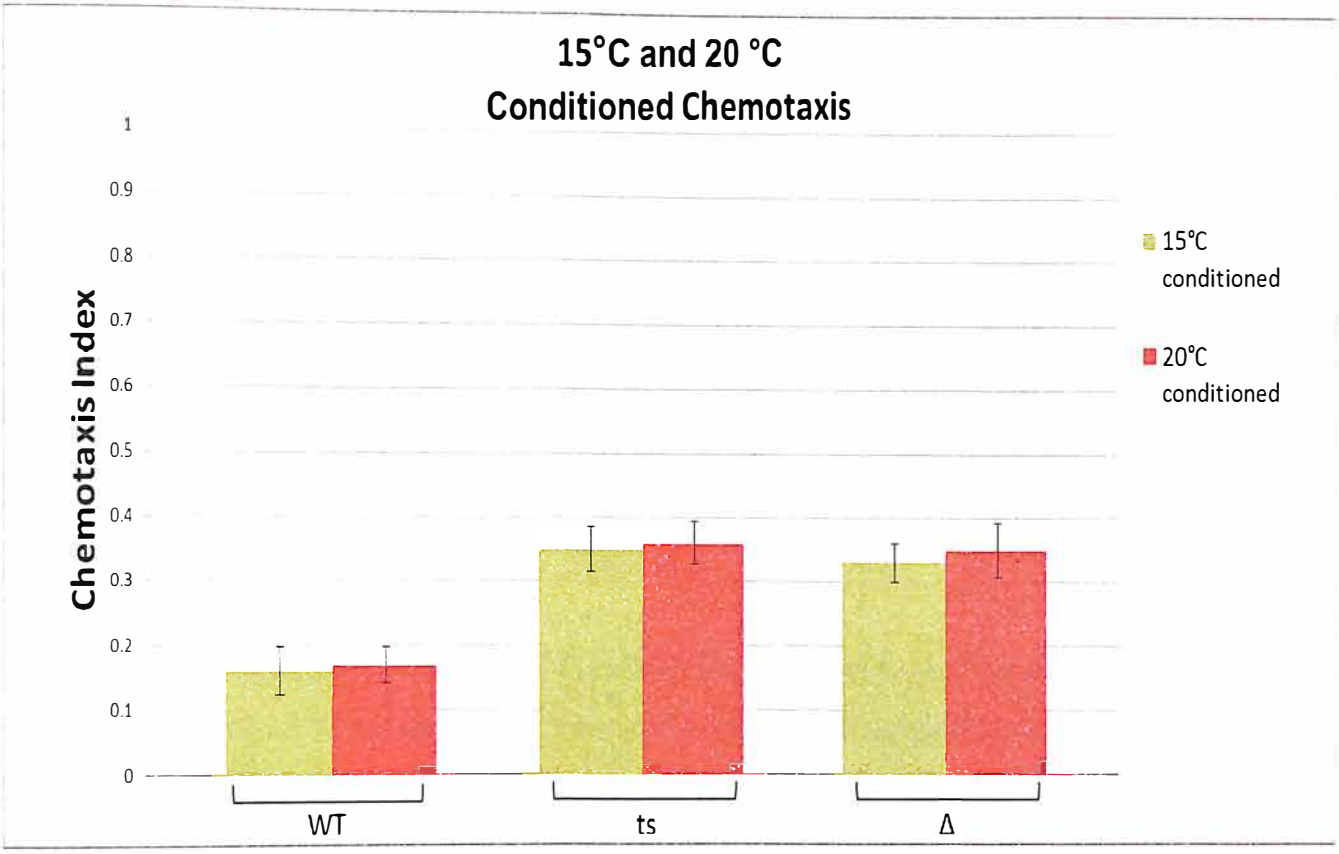

Figure 8b .The comparison of 15 and 20 °C conditioned chemotaxis. After the use of two-way analysis variance I was able to conclude that there was no significant difference in the associative learning as it pertains to strains learning slower/faster in different temperatures. When the same strains were compared amongst different temperatures, there was no significant difference. The comparing of different strains amongst different temperatures was consistent with my initial results. Resulting in a significant difference between the two mutant strains in comparison to wild-type and while showing no significant difference when comparing the two mutant strains to each other. Colors: green= 15 °C, red= 20 °C. (WT=wild-type N2, ts= *gpa-16* ts, Δ= *gpa-16*Δ) $P < 0.001$

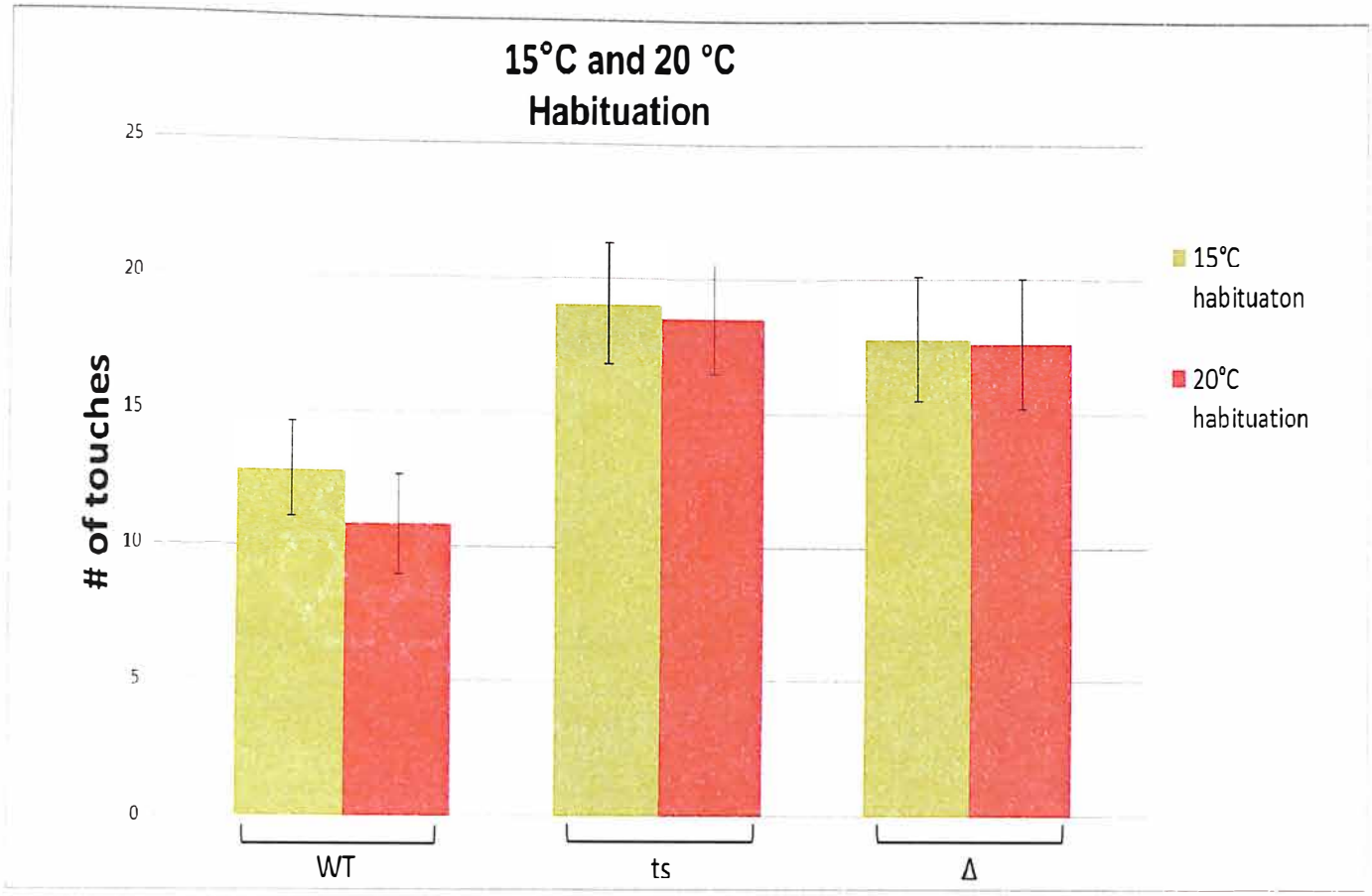

Figure 9 The comparison of 15 and 20 °C habituation was done to analyze the role of identical genes and different genes that were grown at different temperatures. I used two-way analysis variance to interpret my previously collected results. I was able to conclude that there was no significant difference in the non-associative learning as it pertains to strains learning slower/faster in different temperatures. When the same strains were compared amongst different temperatures, there was no significant difference, to what my previous results indicated. The comparing of different strains amongst different temperatures was consistent with my initial results. Resulting in a significant difference between the two mutant strains when compared to wild-type, while showing no significant difference when comparing the two mutant strains to each other. Colors: green= 15 °C, red= 20 °C. (WT=wild-type N2, ts= *gpa-16* ts, Δ= *gpa-16*Δ) $P < 0.001$

Using two-way analysis variance we concluded that there was no significant difference in the learning and memory of the animals tested, regardless of the temperatures they had been grown at. More specifically the comparison of habituation and chemotaxis data amongst different temperatures provided a level of understanding on the role temperatures can have on the specific animals that we worked with, in particular the *gpa-16* mutants.

Viability results

In addition to learning and memory testing, we were interested in viability testing as well. The *gpa-16 ts* mutant has been fundamentally described and based on its previous description there are two main characteristics in which these animals display. The first is that at higher temperatures (25 °C) they display reversed laterality of internal organs and secondly, they also display a high lethal rate (occurring within the 4-6 cell stage) at higher temperatures (25 °C) (figure 10). So far all experiments revealed that both mutants behave significantly different from wild-type, while not exhibiting a significant difference when compared to each other. To ensure the most accurate interpretation for the characterization of the mutants, it was suggested that I analyze the viability of both the *gpa-16 ts* and *gpa-16*Δ mutants. Animals were raised at permissible and non-permissible temperatures (15, 20, and 25 °C). Previous studies have indicated that at 15 and 20 °C *gpa-16 ts* behaves similar to wild-type and that at 25 °C *gpa-16 ts* no longer functions as wild-type, thus making this mutant temperature sensitive.

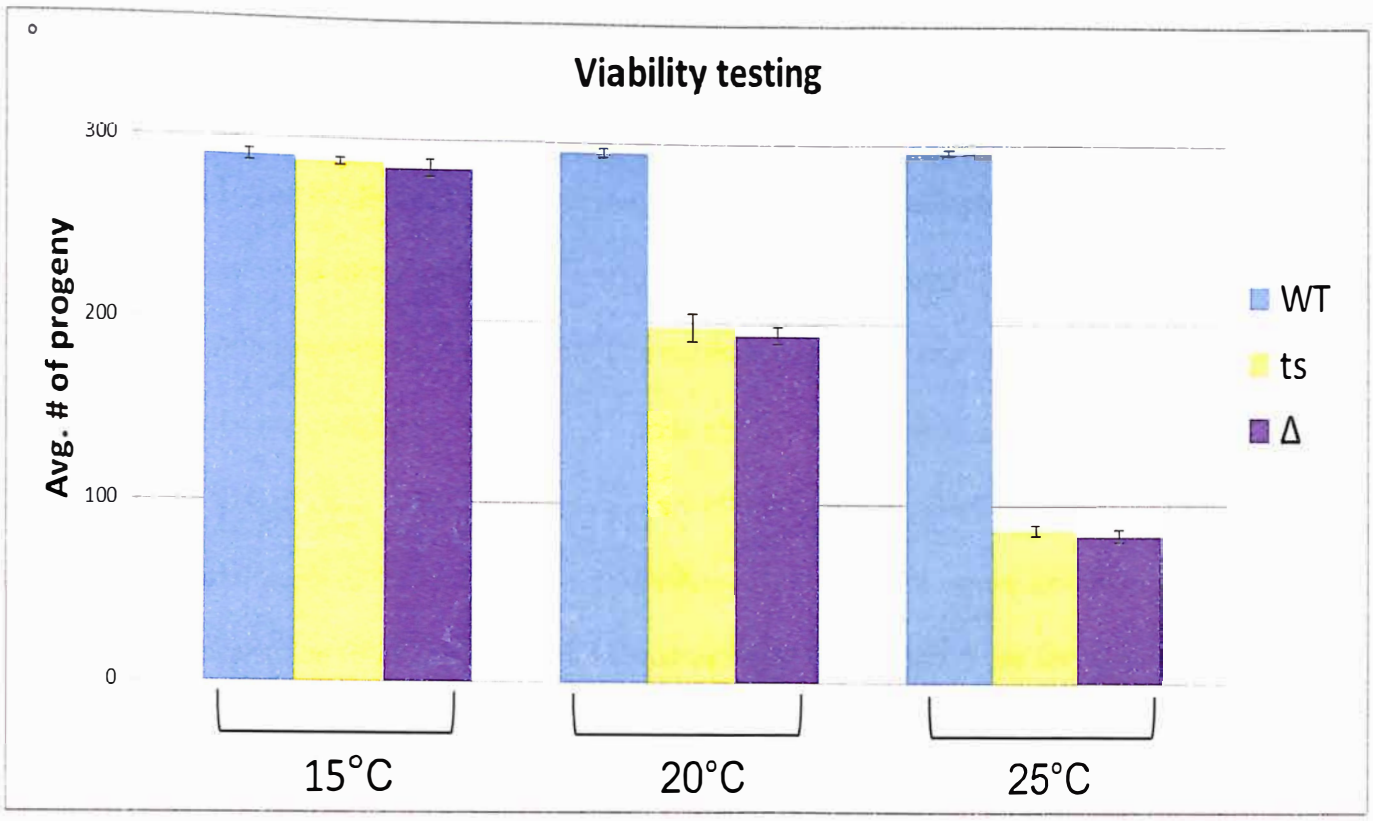

Figure 10 The viability for each strain was achieved by counting the number of progeny from each strain. Animals were raised at permissible and non-permissible temperatures (15, 20, and 25 °C). The 15 °C bracket represents animals that were grown at 15 °C. While the 20 and 25 °C brackets represent worms that were raised at either 20 or 25 °C. After testing animals that were raised at 15°C, there was not a significant difference in the amount of progeny amongst any strain. After testing animals that were raised at 20°C, there was a significant difference in the amount of progeny produced when comparing both mutants to wild-type. However, when comparing both mutant strains to each other there was no significant difference in the amount of progeny that had been produced. This was also true for animals that were raised at 25 °C. (Colors: teal= wild-type N2 worms, yellow= *gpa-16 ts*, purple= *gpa-16Δ).* $P < 0.001$

PCR Results

It was suggested that I conduct back crossing experiments using male *gpa-16* Δ worms and wild-type N2 hermaphrodite animals. The purpose of back crossing is to ensure that the genome of the mutant is identical to that of wild-type with the exception of the targeted mutation within the mutant. The methodology that I would use to ensure that the cross was executed properly is polymerase chain reaction most commonly referred to as PCR. Prior to starting the crosses we attempted to standardize PCR. Standard protocol procedures and gradient procedures were used. We were unable to confirm the crosses due to PCR failure. We construed that the primers were the reason for this failure. Primers that were previously standardized were used as a control to aid in our efforts to conduct PCR.

IV. DISCUSSION AND CONCLUSION

The *gpa-16* gene plays a central role in the development of *C. elegans*. A well-studied temperature sensitive allele has been previously described to display laterality reversal in half of the surviving progeny under non-permissive conditions. However, no other abnormality or atypical phenotype has been previously reported in these animals.

We hypothesized that laterality reversal in worms is likely to have neural consequences that can be tracked using specific behavioral assays. Our experimental approach included behavioral analysis, viability testing, and an attempt to achieve genetically back-crossed animals. Our findings suggest that *gpa-16* has a role in learning and memory as well. Furthermore, our results also show that both *gpa-16 ts* and *gpa-16*Δ mutants show statically identical deficiencies in learning, memory and viability.

In order to test our hypothesis, we conducted a series of behavioral assays including non-associative learning (habituation assays), associative learning (chemotaxis-based) and viability testing. Prior to all behavioral testing animals were grown at two different temperatures (15 and 20 °C). This was done to analyze if different temperatures affected the learning and memory of the mutant strains. Our behavioral assays showed that *gpa-16 ts* and *gpa-16*Δ share similar behavioral abnormalities.

This was a clear indication that *gpa-16 ts* did not resemble the behavior of other typical temperature sensitive mutants. Typically, temperature sensitive mutants mimic wild-type under permissible temperatures. However, our results yielded that both mutants displayed similar behavioral deficits at both permissive and non-permissive temperatures.

Behavioral analysis of *gpa-16 ts* and *gpa-16Δ* mutants

We were curious to find out if the learning and memory of *gpa-16 ts* and *gpa-16Δ* would vary depending on the temperature. Therefore experiments were conducted that emphasized on animals that were grown under permissive 15 °C and non-permissive 20 °C temperatures. The first experiment conducted was 15°C habituation. Whereas animals were grown under 15 °C conditions and the animals' ability to no longer respond to a repeated harmless stimulus was tested using the habituation assay as stated previously. Surprisingly when compared to the wild-type N2 animals, both *gpa-16 t.s.* and *gpa-16Δ* mutants exhibited significantly slower habituation rates. Most interestingly the two mutants did not show a significant difference when compared to each other.

The second habituation experiment was conducted on animals that were grown under 20 °C conditions; Abnormalities in response towards a single touch stimulus were observed during 20 °C habituation as well. Nearly identical to the 15 degree results, when compared to the wild-type N2 worms, both *gpa-16 t.s.* and *gpa-16Δ* mutants exhibited significantly slower habituation rates when grown in 20 °C conditions. Notably the two mutants did not show a significant difference when compared to each other.

After now having 15 and 20 °C habituation results we decided to compare the two results. We wanted to know the outcome when comparing two animals of the same strain but from two different temperatures. This would determine if the animals learned differently in opposing temperatures. We also wanted to know if the results would be different if two different strains from two different temperatures were compared. This would tell whether or not the animals learned differently depending on the temperature. Using two-way analysis variance to interpret my previously collected results we

concluded that there was no significant difference in the non-associative learning as it pertains to strains learning slower/faster in different temperatures. When the same strains were compared amongst different temperatures, there was no significant difference, to what my previous results indicated. The comparing of different strains amongst different temperatures was consistent with my initial results as well.

A chemotaxis assay was used to detect abnormalities in the animals' ability to sense and respond to chemical stimulus. Generally, *C. elegans* move away from chemicals that smell harmful and towards chemical compounds that resemble the smell of food. Iso-amyl alcohol (water soluble), is one of the chemical that is widely used for conducting chemotaxis assays. Chemoattractants are detected by the neurons ADF, ASE, ASG, ASI, ASJ and ASK located in the amphid organs which are exposed to the environment (Bergmann et al., 2006). Studies have shown that abnormalities with ASE neurons reduce but do not eradicate chemotaxis behavior in *C. elegans*. However ablation of the ASE, ASI, ADF and ASG neurons abolishes the chemotaxis effect completely (Bergmann et al., 1991). This suggests that all of the previous mentioned neurons do not play the same role in sensing chemicals. Although it is believed to play a minor role, the *gpa-16* gene is highly likely to be expressed in the ASI, ASJ and ASK neurons (Bargmann et al., 2006).

Both of our chemotaxis assays (15 and 20 °C) showed that both *gpa-16 ts* and *gpa-16*Δ mutants were defective in chemoreception when compared to N2, while showing no difference from each other. After being conditioned with iso-amyl alcohol in the absence of food, the mutants failed to move as far away from the iso-amyl alcohol as wild-type. This could indicate that certain neurons could play a bigger role than

previously thought. We question whether or not neurons are being rearranged as well, similar to how the internal organs of the mutants become reversed.

After now having 15 and 20 °C chemotaxis results we decided to compare the two results. Identical to our aim in habituation, we wanted to know the outcome when comparing two animals of the same strain but from two different temperatures and I also wanted to know if the results would be different if two different strains from two different temperatures were compared. This would tell whether or not the animals learned differently depending on the temperature.

After the use of two-way analysis variance we concluded that there was no significant difference in the associative learning as it pertains to strains learning slower/faster in different temperatures. When the same strains were compared amongst different temperatures, there was no significant difference. The comparing of different strains amongst different temperatures was consistent with my initial results. Resulting in a significant difference between the two mutant strains in comparison to wild-type and while showing no significant difference when comparing the two mutant strains to each other.

A vital experiment that was conducted was the viability testing. Animals were raised at permissible and non-permissible temperatures (15, 20, and 25 °C). After testing animals that were raised at 15°C, there was not a significant difference in the amount of progeny amongst any strain. After testing animals that were raised at 20°C, there was a significant difference in the amount of progeny produced when comparing both mutants to wild-type. However, when comparing both mutant strains to each other there was no

significant difference in the amount of progeny that had been produced. This was also true for animals that were raised at 25 °C. This experiment was the one experiment that included the trademark of the previously described temperature sensitive mutant. Our results indicating no significant difference between the two mutants while both mutants display significant difference when compared to wild-type, indicates a lack of temperature sensitivity by the previously described *gpa-16* temperature sensitive mutant.

Back-crosses and western blot analysis were advised for the deletion mutant. However after not being able to successfully standardize PCR due to the *gpa-16*Δ primers not working properly, crosses were unsuccessful. Western blot analysis was not feasible because of the inability to obtain the 1° antibody. This item was not sold commercially.

Our behavioral results indicate the possibility of rearranged neuronal circuitry in the *gpa-16* mutants. It is premature to speculate a mechanism, but reversal of internal organs as a result of the mutation could possibly have an effect on neuronal connectivities due to anatomic constraints. This could further explain the behavior aberrations. Additionally, our results suggest that perhaps the previously described *gpa-16 ts* mutant is not temperature sensitive. Thus if it were, it would behave similar to wild-type under permissible temperatures as oppose to the deletion mutant.

After interpreting our results we became curious as to why the *gpa-16 ts* mutant behaved as a deletion mutant under permissive temperatures. We propose that there are two main possibilities as to why the *gpa-16 ts* mutant displays statistically identical behavior to the *gpa-16* deletion. We have also proposed specific experiments that will enable us to investigate the two possibilities.

The first probable reason for the nearly identical behavior amongst the temperature sensitive and deletion mutant in permissive temperature 15 °C is the possibility of the ts mutant having a null allele. The ts mutant having a null allele would result in an absence of product or protein production. Essentially this would cause the ts animal to become identical to the deletion mutant considering that both will not produce a product. If this were true, this would explain the similarities in their behavior. To test if the ts mutant has a null allele at permissive temperature 15 °C, it will be imperative to sequence the genomic DNA sequence of *gpa-16* from the ts mutant. It will also be useful to screen for the presence of the protein through western blot analysis. Testing for the presence of protein production will clearly tell whether a protein is being produced. It would also be essential to ensure that the ts animals are grown and raised under permissive temperature 15 °C upon testing.

Although our *gpa-16 ts* mutants were grown and raised at permissive temperature 15 °C, they were not actually tested at 15 °C. They were tested at room temperature which is approximately 20-22 °C. Therefore, the second possible reasoning the ts mutants behave as the deletion mutant under permissive temperature 15 °C might be due to the actual testing temperature. It is possible that testing the ts animals under non-permissive temperature of ~20 °C was sufficient to cause destabilization in the G alpha protein structure. However, based on previous reports this mutation has been documented to occur exclusively during early embryogenesis and under non permissive 20 and 25 °C temperatures (Wood et al., 1996). Nevertheless, to uncover the role (if any) of room temperature testing on the ts mutants, we would suggest not only raising and growing the ts animals at the permissive 15 °C temperature but also to actually test the ts animals at

the permissive 15 °C temperature. It would be ideal if the testing could take place in a room that is consistently 15 °C. Using a temperature plate might also be feasible.

Lastly, we also hypothesized that the reversal of the ts mutant internal organs could possibly lead to neuronal consequences. Additionally, we noticed behavior aberrations in animals that were known to have internal organ reversal. Despite being unable to successfully distinguish left handed worms from right handed worms, based on previous reports we think that there were a significant portion of ts animals that had lateralization reversal. Though it is premature to provide specific mechanisms supporting our findings or to confirm if our hypothesis is exclusively true we have postulated a supporting idea in case it was true. We postulate that in addition to internal organs being reversed, that maybe specific neurons are being reversed as well, including neuronal circuits that effect learning and memory. For example, our lab has previously shown us that *gpa-14* expressed in dopaminergic neurons have a role in dopamine release during learning and memory. Let's hypothetically imagine that *gpa-14* neurons are always in a specific location within the pre-synaptic cell and when a certain amount of dopamine is released into the synaptic cleft and activating specific molecules in the post-synaptic cell. This pathway advances downstream which eventually yields the learning and memory phenotype. Our proposition is that perhaps when the internal organ reversal occurs, dopaminergic neurons (including those expressing *gpa-14*) are reversed as well, or may simply be either atypically placed or may form atypically connections. This in turn affects the dopaminergic learning and memory circuit and thereby we observe deficits. Therefore animals with reversed neurons that have role in learning and memory such as *gpa-14* will learn slower than those without reversal. We know that our *gpa-16* mutants

have a reversal of internal organs, but it is not known if there is a reversal in learning and memory related neurons or neuronal circuits. If there was neuronal or neuronal circuit reversal, however subtle, we would anticipate a correlation between the learning and memory aberrations found in the *gpa-16* ts mutants. Although doubtful we have not ruled out the possibility of mislabeling the mutant strains for one another or for wild type. This is common in many laboratories, therefore we cannot rule this out.

V. FUTURE DIRECTION

Previous studies have revealed that a mutation in the *gpa-16* gene, which codes for a G_{α} subunit of a worm G-protein, causes 70% lethality which occurs in the embryonic 4-6 cell stage. Out of the portion of animals that survive 50% have either left or right lateralization (Bergmann et al., 2003). This is interesting considering that 100% of wild type worms have right lateralization. For future studies, we are focused on two things: a) The determinations if lack of lateralization (or symmetry) plays a role in killing the 70% of embryos that do not survive. More specifically we're intrigued to find out if there's a specific or random lateralization pattern that is occurring, which could possibly be responsible for the lethality. Therefore video-microscopy has been standardized in our lab. This was done by me and a fellow graduate student, Tiffany Cummings. Video-microscopy will aid in examining embryos at specific stages. b) Examining the learning, memory, and behavior of the mutants that survive which are known to have 50% L/R lateralization and being able to distinguish if left or right handed animals learn differently. More specifically we want to gain a greater understanding to how learning, memory, and behavior of worms are affected by L/R lateralization. The transparency of the *C. elegans* model is highly beneficial in determining the worms' anatomy. Thus it is the worms' anatomy that denotes L/R lateralization.

The first approach is to study the L/R lateralization in adult worms and to become comfortable and efficient with distinguishing L/R lateralization whereas the body plan of adults will be more developed which will allow for more recognizable markers. In addition, a cross between the *gpa- 16* mutants with GFP worms that are specific for L/R florescence allows for the utilization of the GFP expression as a marker for L/R

determination. Lastly, after the mutants and the GFP-expressing worms have reproduced, the dead embryos will be examined under the confocal microscope. It is expected that they will have L/R GFP expression that will ultimately determine whether or not lateralization is specific or random.

VI. APPENDIX A

This appendix section outlines experiments that were carried during the course of this project but were not considered in the main body of the thesis. For example, a significant portion of time was spent in trying to distinguish L/R handedness in adult worms. We tried to accomplish this by manually picking the worms and rotating them ~90°, we also analyzed the worms under various microscopes with high magnification in hope to identify L/R handedness; additionally we took bright field images and tried to rotate those as well. After numerous trial and errors we decided to focus on other aspects of the project. One of the methods we tried was exploiting worms expressing *gfp* in the intestines or gonads. Despite our limited success I think that this may still be the best way to distinguish L/R handedness in *C. elegans* (Figure 11).

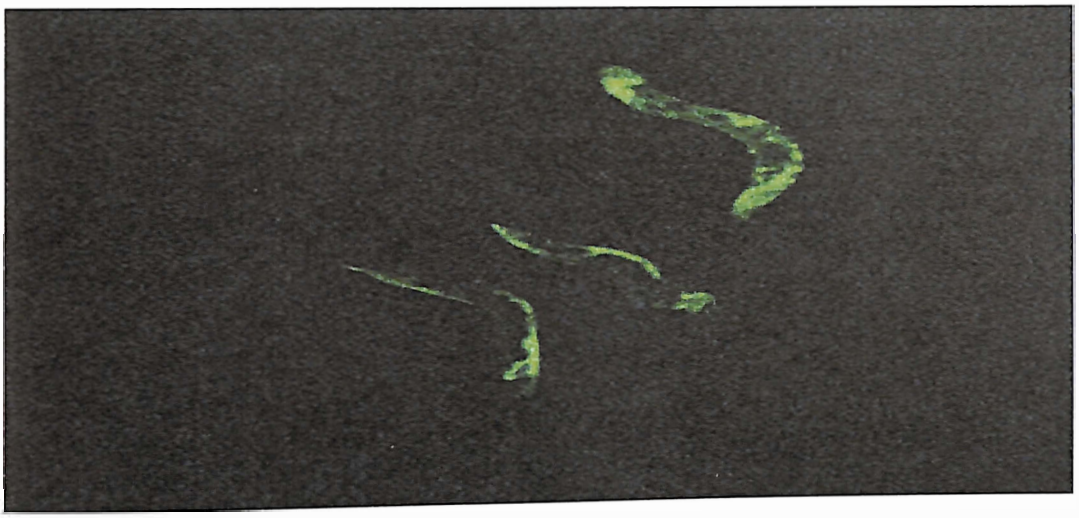

Figure 11. GFP expression on the L/R, A/P, and V/D of the worm. In the future GFP strain(s) can be crossed with *gpa-16* mutants.

Both the anterior gonand and the intestine are used as markers for L/R handed worms. In all wild-type animals the anterior gonand sits on the right side of dorsal lying *C. elegans*

making them right handed and their intestine are directly opposite also denoting them as right handed. The strains that we tested showed excessive auto-fluorescence as well as background fluorescence and were not usable for our purpose. In addition there was some ambiguity in that all animals displayed dumpy phenotype which was not expected, and was brought to notice of the lab from which the strain was obtained.

Aside from being able to distinguish L/R handedness in worms we wanted to backcross our *gpa-16* deletion mutant with WT animals. This would ensure that the molecular background of *gpa-16* Δmutants were the same as WT, with the exception of *gpa-16*. However after not being able to successfully standardize PCR due to *gpa-16*Δ primers not working properly, the crosses were unsuccessful.

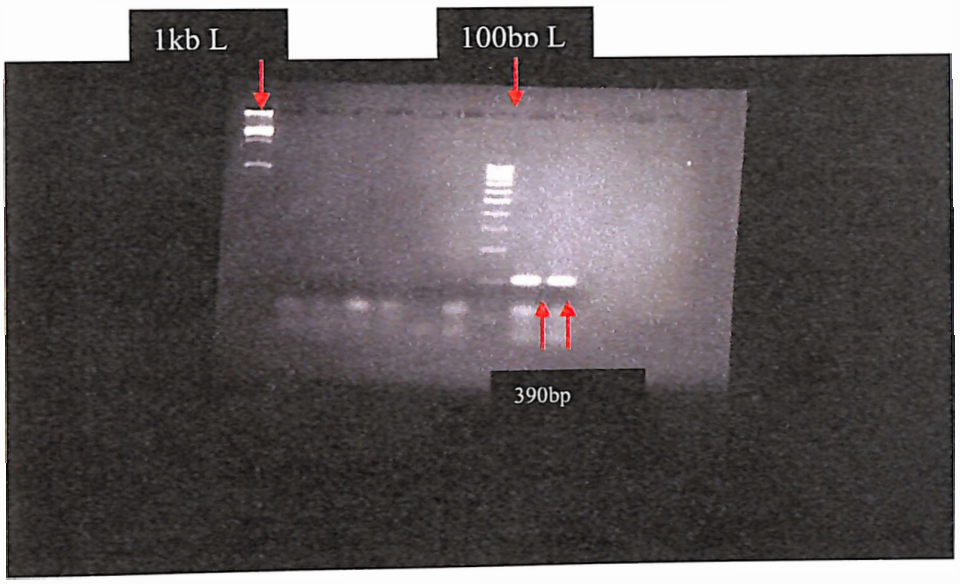

Figure 12 Agarose gel electrophoresis confirmation of *dop-2* primers working (lanes 9 and 10), while in lanes 2-7 *gpa-16*Δ primers showed no amplification from PCR.

With feasible *gpa-16*Δ primers we would anticipate amplification within the 3kb region. As a control we used *dop-2* primers. These primers were known to work and express in the 390bp region. Additionally they were prior used by multiple researchers within our lab. By following the exact same protocol with the only difference being the primers, we were able to conclude that our *gpa-16*Δ primers were not working properly.

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