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A Developmental Exploration of the Role of Catp-6 gene in Model Organism C. elegans relating to Parkinson's Disease

Kaiser Kate Weafer
Bellarmine University, kkweafer@gmail.com

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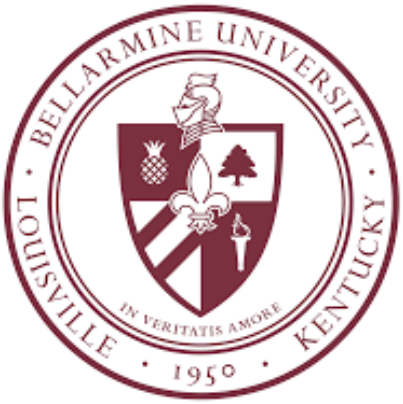
By: Kate Weafer

Bellarmine University



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ABSTRACT

Parkinson's disease (PD) is considered a common and complex neurogenerative disease characterized by the complete loss of dopaminergic neurons located in the substantia nigra pars compacta. With the incomplete understanding of this neurological disorder, Caenorhabditis elegans (*C. elegans*), a microscopic nematode, could serve as a model organism of study due to specific gene analogs, relating to PD, present in both *C. elegans* and humans. The gene of interest for this project is *catp-6* in *C. elegans* an ortholog of the human ATP132A gene. Mutations in the ATP132A gene correlates with an abnormal form of early onset PD called Kufor-Rakeb Syndrome, characterized by motor function loss. This project aims to utilize *C. elegans catp-6* knockout mutants to conduct studies focused on developmental explorations, and ultimately gender-related behavioral differences, with potential implications in PD.

INTRODUCTION

Parkinson's Disease (PD) Overview

Parkinson's disease (PD) is considered a common and complex neurogenerative disease, characterized by the complete loss of dopaminergic neurons located in the substantia nigra pars compacta (SNpc) (Kalia & Lang 2015). Dopaminergic neurons are responsible for a plethora of areas of brain functions, including motivation, working memory, and motor behavior. Furthermore, overall levels of dopamine, the primary neurotransmitters released by neurons in the SNpc has significant impact in physical and mental health (Chinta & Andersen 2005). The substantia nigra is a midbrain structure within the brainstem, that is involved in functions within the reward system and regulation of the motor movement. Specifically, the substantia nigra is divided into two areas, the pars reticulata (SNpr), comprising of gamma-aminobutyric acid-containing (GABAergic) neurons, and the pars compacta (SNpc), consisting of dopaminergic neurons, which is involved in PD (Sonne 2022). Figure 1 indicates the location of the substantia nigra within the human brain. In Parkinson's disease, the loss of those dopaminergic neurons in the SNpc results in deficiency of dopamine within the basal ganglia and ultimately leads to dysfunction in motor movements, including muscular rigidity, bradykinesia, rest tremor, and postural and gait impairment (Kalia & Lang 2015). Muscular rigidity refers to the inability of muscles to relax normally, while bradykinesia refers to the slowness of initiation of movement with reduction in contraction speed. Rest tremors refer to tremors individuals experienced at rest, while postural and gait impairment involves poor balance and instability in walking or standing (Kalia & Lang 2015). Further, Figure 2 expands on the pathophysiology of dysfunctions related to PD.

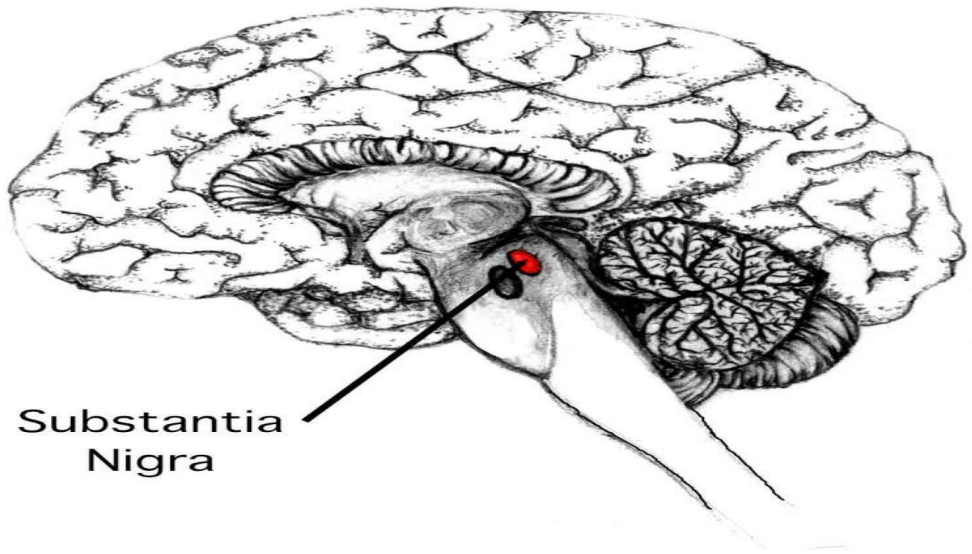

Figure 1. Location of the substantia nigra of the human brain (Griffiths 2022)

In the U.S., approximately 90,000 people are diagnosed with PD annually with an estimation 1.2 million new people being affected by 2030. Further, there is a drastic 50% increase of PD diagnoses from formally projected rates of 60,000 annual diagnoses. Additionally, the incidence rates of PD are greater in specific geographic regions, specifically Southern California, Central Pennsylvania, Southeastern Texas, and certain areas of northeastern and midwestern U.S. that is considered the "Rust Belt", which is characterized by industrial manufacturing. Further, one of the primary risk factors for PD is age, so the increase of PD incidence is directly correlated with growth of the aging population growth. Estimates of incidence additionally indicate that PD occurs more frequently in males than females at all ages (*Prevalence & Incidence* 2024).

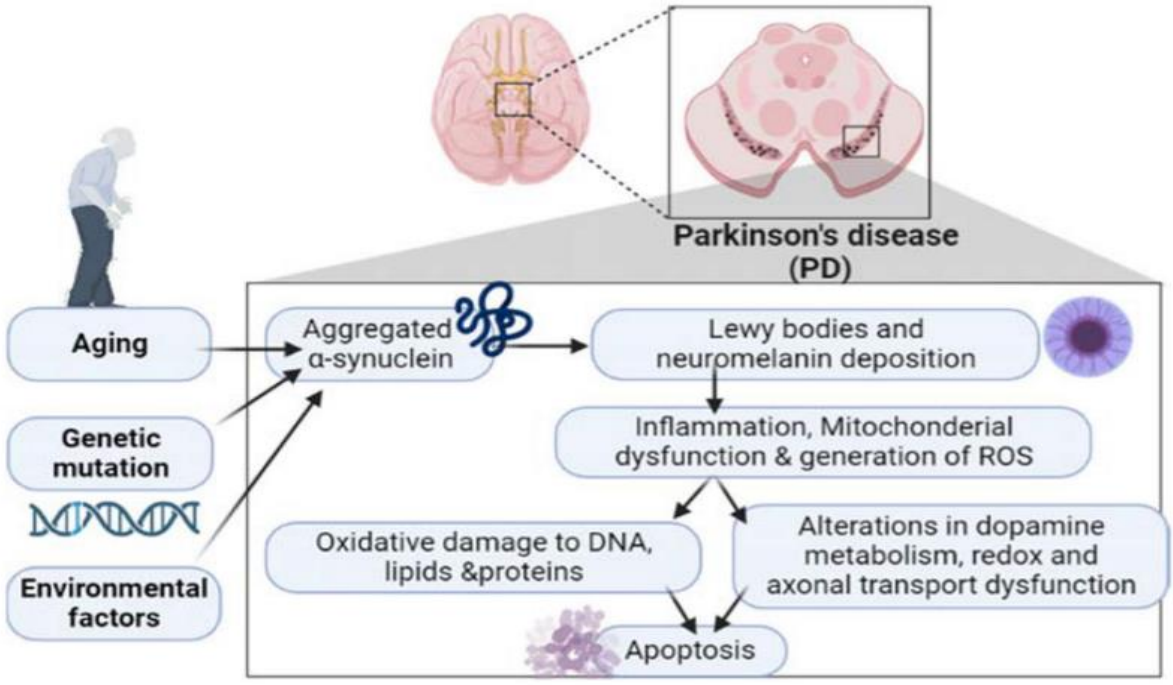

Figure 2. Schematic representation of the pathophysiology of Parkinson's disease: Factors such as age, genetics, and the environment contribute to the aggregation (formation to clusters) of α-synuclein, leading to formation of Lewy bodies, which are abnormal protein aggregates in the brain. The formation of Lewy bodies leads to inflammation, mitochondrial dysfunction, and production of reactive oxygen species (ROS). Further, these dysfunctions lead to oxidative damage to DNA, lipids, and proteins as well as contributing to dysfunctions in dopamine metabolism and axonal transport. Ultimately, these lead to death of dopaminergic neurons and clinical manifestations associated with Parkinson's disease (Al-Gareeb et. al 2023).

Along with other factors, such as age and genetics, biological sex has significant influence in PD diagnosis and experience. As stated previously, PD diagnosis is greater than males than females with clear sex differences in symptomology; however, women experience quicker progression of the disease and have a higher mortality rate compared to men. In female individuals, motor symptoms occur later with the primary evident symptoms being reduced rigidity and tremors as well as postural instabilities. Additionally, non-motor symptoms such as depression, fatigue, and excessive sweating are more prevalent in female individuals as well dysphagia, the difficulty in swallowing. In males, the freezing of gait, which initially presents as the lack of ability to walk intentionally at the initial stages and is one of the most disabling motor symptoms of PD. Another prominent symptom more likely found in male individuals is camptocormia, which is severe forward bending of the trunk while standing or walking. Further, affected male individuals are more likely to experience REM sleep behavior disorder (RBD) than female individuals. Additionally, with RBD, women generally experience less aggressive and harmful RBD than men. Overall, there are distinct sex differences in clinical manifestations in PD; however, the exact cause behind them is relatively unknown. Because of this, it is important to further explore these differences to aid in the understanding of the complexity of Parkinson's disease and to further determine how to better the quality of life for these individuals living with PD and those around them (Cerri et al 2019).

The exact etiology of Parkinson's disease remains unknown and is not well understood; because of this, PD is considered to be an idiopathic disease influenced by a multitude of factors, such as a combination of environmental and genetic influence (Kalia & Lang 2015; Cerrri et. al 2019). Environmental factors associated PD include previous head injuries, beta-blocker use, and pesticide exposure (Kalia & Lang 2015). In addition to this, an important environmental factor

associated with PD is chronic stress. For example, a possible stress factor could be work conditions. PD cases are prominent in men with stress based on high career and job demands, particularly men with high education. Correspondingly, women with low education, but high career and job demands, experience a greater risk in PD diagnosis (Cerri et. al 2019). In addition to chronic stress, reduced physical activity is a significant factor in PD risk. Specifically, increased exercise activity in midlife is associated with lower PD risk as well as lower degree of complications and better prognosis (Cerri et. al 2019). Genetic factors suggest the influence of the first identified gene, SNCA, whose mutations correlate with autosomal dominant Parkinsonism, which additionally encodes for the protein α-synuclein (Kalia & Lang 2015). Specifically, alterations in this gene cause missense mutations, causing multiplications of the gene locus and substitutions (Kalia & Lang 2015). Other genes associated with Parkinson's disease include: *LRRK2, VPS35, EIF4G1, DNAJC13, and CHCHD.* The *LRRK2* gene encodes the leucine-rich repeat kinase 2, which is involved in a plethora of cellular processes, such as protein synthesis and neurite growth, along with a potential involvement with the innate immune system. Mutations in this gene have been determined to be one of the most frequent causes of genetic PD. The *VPS35, EIF4G1, DNAJC13,* and *CHCHD* genes are correlated with the dominantly inherited Parkinson's disease. First, the *VPS35* gene encodes for vacuolar protein sorting 35 (VP35), which is associated with endosomes, Golgi apparatus, and lysosomes. With the *EIF4G1* gene, there is little known of its relation to PD, so further research needs to be done. *DNAJC13* encodes for receptor-mediated endocytosis 8 (REM-8), which is a chaperone protein, and mutations in this gene correlate to PD, particularly with individuals of Dutch German-Russian Mennonite ancestry. Lastly, *CHCHD* encodes for *coiled-coil-helix-coiled-coil-helix*

domain containing 2 and considered to be a mitochondrial protein. However, further studies need also to be conducted to determine its influence on Parkinson's disease.

Caenorhabditis elegans as a Model Organism

Caenorhabditis elegans (*C. elegans*), a microscopic nematode, has been continuously applied to be an organism of interest for research due to its well-defined nervous system (Sengupta & Samuel 2009) Further, this organism is easily manageable with minimal requirements for growth and nutrition, while producing a large number of offspring in a relatively short amount of time. To better visualize the organism, Figure 3 indicates the life cycle of *C. elegans.* Moreover, *C. elegans* can be utilized as a model organism for particular bodily structure, such as neural circuits and behavior, emphasizing its complexity (Sengupta & Samuel 2009). This nematode has anatomically been mapped out to the cellular level including the 58 motor neurons present in the ventral cord with approximately 302 neurons overall (Sengupta & Samuel 2009); Zhen & Samuel 2015). This organism is not anatomically segmented; however, the adult motor circuit consists of a distinguished pattern, specifically six repeating units of about 12 muscle cells and 12 motor neurons present along the adult body of *C. elegans* (Zhen & Samuel 2015).

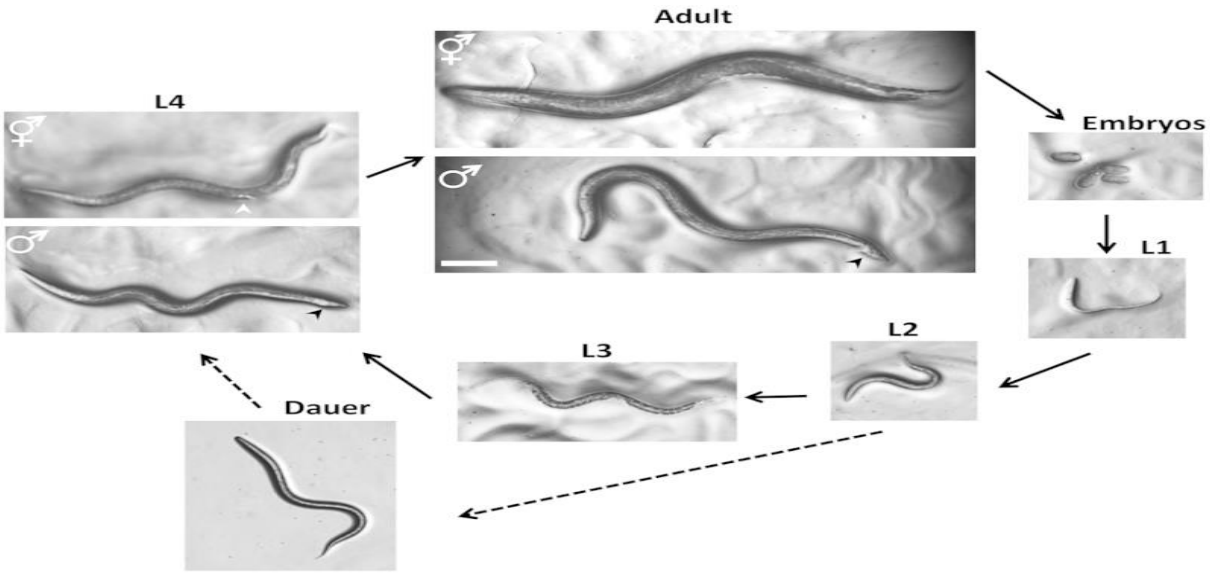

Figure 3. This figure indicates the four larval stages of *C. elegans* for the hermaphrodite and male. The L4 stage shows the first easily apparent distinction between the sexes in which hermaphrodites are characterized by a narrowed tail and a half clear circle midway down the side of the body, which is the developing vulval tissue, and can easily distinguished in a stereomicroscope. In contrast, the males have a wider-finned tail compared to the hermaphrodites (Corsi 1970).

The entirety of its genome has been sequenced, conveying its genetic makeup of six chromosomes and about 20,000 genes (Cooper & Raamsdonk 2018). This could provide incredible insights to further explore certain disorders, such as Parkinson's disease, at a molecular level (Cooper & Raamsdonk 2018). Understanding these diseases include classifying biomarkers, which can be utilized for developing potential treatments. However, there is an emphasis in the incomplete understanding of this particular neurological disorder. Because of this further exploration is needed, and *C. elegans* can serve as a model organism of study due to specific gene analogs present in both *C. elegans* and humans. Some of these gene analogs

include *PRKN/pdr-1*, *PINK1/pink-1,* and *ATP13A2/catp-6* . Mutations in *PRKN/pdr-1* is involved in early age onset of PD, and additionally, *pdr-1* mutation in *C. elegans* consist of the aggregation of abnormal mitochondria and oxidative phosphorylation deficiencies. *PINK1/pink-1* is also involved in early onset form of PD and is considered autosomal recessive. *Pink-1* mutant worms are also involved in oxidative phosphorylation deficiencies and morphology alterations. Lastly, *ATP13A2/catp-6* is the gene of focus and will be expanded later on this paper. Overall, deletions of these analogs in *C. elegans* have led to dysfunctions including dopamine neuronal loss and movement deficiency. These manifestations can be used to further understand the mechanisms and pathogenesis of Parkinson's disease, which could possibly provide insight to therapeutic treatments (Cooper & Raamsdonk 2018).

Him-5/Sex Differences

As stated previously, biological sex plays a significant role in Parkinson's disease, especially in prevalence and symptomology. Because of this, more exploration is needed to better understand the disease. This can be further explored by looking at male and hermaphrodite, which are the two natural sexes of *C. elegans*. Specifically, hermaphrodites are considered females somatically, and they are able to reproduce by mating with males or through self-fertilization (Zarkower 2006). Further, self-progeny are mostly hermaphrodites, and approximately 1/500 are males. However, greater proportions of males can be increased by *him* mutations, the *him* gene name is an abbreviation for high incidence of males. With this particular project, *him-5* worms were used to cross with *catp-6* mutant *C. elegans* in order to increase the number of male *catp-6* worms in a given generation. This will allow explorations of sex differences and ultimately determine its effects as it relates to PD.

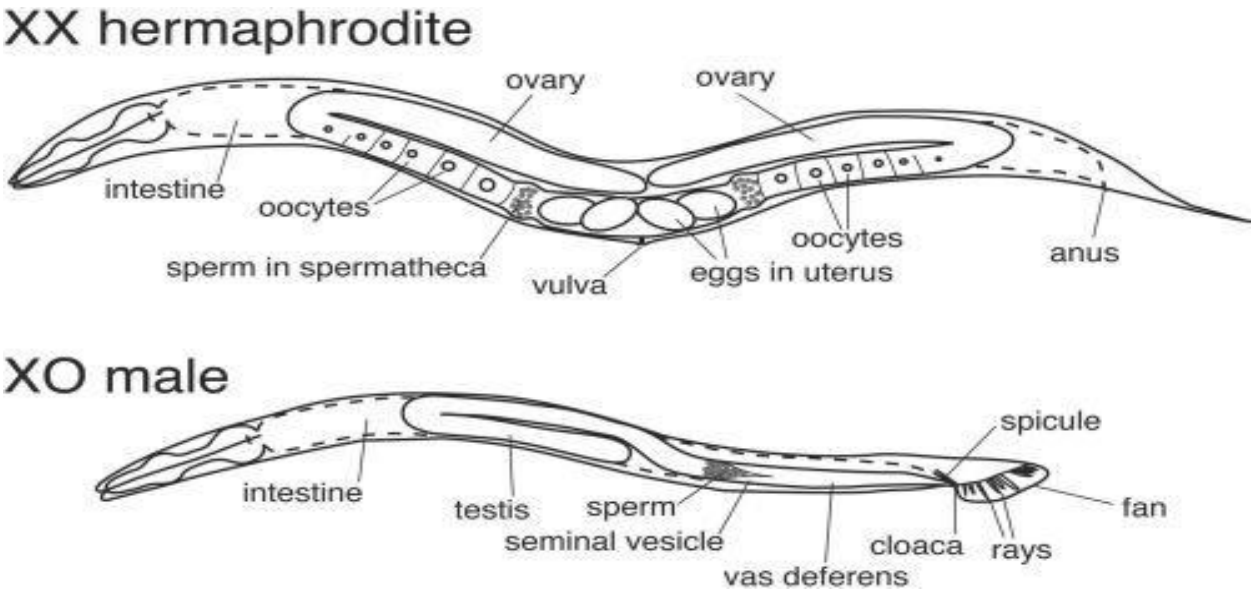

Figure 4. This figure illustrates the physical structural differences between the hermaphrodite and male *C. elegans*. There are distinct differences between the body sizes, and in particular, in tail morphology and the gonads (Zarkower 2006).

Gene of Interest: catp-6

For this project, the gene of interest is in *C. elegans catp-6*,, the human gene ATP132A analog used to study PD. In humans, mutations in this results in an abnormal form of early onset PD called Kufor-Rakeb Syndrome, characterized by motor function loss. Normal gene function is associated with a lysosomal ATPase transporter, and its dysfunction leads to protein misfolding, oxidative stress, and aggregation because of lysosomal deficiencies (Cooper & Raamsdonk 2018). *catp-6* is expressed in several tissues of the body, including head and tail neurons, all body muscles, pharyngeal cells, coelomocytes, lateral hypodermis, spermatheca, vulval muscles, and gonadal sheath cells (Lambie et. al 2013). In *C. elegans, catp-6* mutations lead to deficits stated earlier, including dopamine neuronal loss and decreased movement. This gene may also be correlated with the accumulation of α-synuclein, which is known to relate to

PD (Cooper & Raamsdonk 2018). Additionally, mitochondrial dysfunction has been known to be associated with neurodegenerative diseases, such as PD; specifically, mutation of *catp-6* illustrated enhance neuronal loss due to loss of α-synuclein, which is known to be correlated with mitochondrial deficits (Cooper & Raamsdonk 2018).

Purpose of the Study

The goal of this thesis project is to further explore the *catp-6* (Strain: RB2510; Allele: ok3473) gene, which is present in *C. elegans*, as it relates to Parkinson's disease. The effects of this knockout gene will be observed in multiple settings, determining the manifestations of physiological dysfunctions. Additionally, sex differences of *C. elegans* will be taken into consideration when observing physiological or behavioral changes to further understanding its effects on different genders. With this project, a deeper understanding of potential contributors Parkinson's disease could be gained through the observations and data gathered. Data gathered could be used on a more applicable basis, which is significant considering the increasing prevalence of PD.

METHODS

Cultivation and Maintenance of C. elegans (catp-6 mutants)

catp-6 (Strain: RB2510; Allele: ok3473) mutant *C. elegans* were obtained from University of Minnesota (Minneapolis, MN) and were maintained on Nematode Growth Medium (NGM) agar plates. Further, they were regularly stored at 15 °C. However, other temperatures were used (20 °C and 22°C) because they accelerate the growth phases of *C. elegans*, meaning the worms' growth stages process through in a shorter amount of time. There are several

methods for transferring *C. elegans* from one agar plate to another. The methods used for this project were “chunking” and utilizes a worm pick. “Chunking” consists of removing a portion of agar from one plate to another, and typically, this portion will have many worms on the agar chunk that is transferred. The second method involves using a worm pick to transfer worms from one plate to the other. This worm pick is utilized to pick up specific ages or sexes of worms using a stereomicroscope and transfer them to a different plate.The transfer frequency of these worms was normally once a week, picking two worms for each transfer plate.

Developmental Observations at Different Temperatures

The typical maintenance of *catp-6* worms did not align with wild-type worms, so developmental observations were performed in order to find the optimal conditions for growth in these mutant worms. All worms were picked at L4 stages. The following conditions were used to find the optimal temperature and number of worms per plate to discover the ideal conditions for growth:

Temperature (°C)	**Number of *catp-6* Mutant Worms**
15	5
15	10
15	20
20	5
20	10
20	20
22	5
22	10
22	20

Figure 5. This table consists of the 3 different temperatures: 15°C, 20°C, 22°C and the 3 different number of worms per temperature: 5, 10, 20 used to find which conditions would be best for *catp-6* mutant growth.

Synchronization of catp-6 mutant *C. elegans*

The purpose of the synchronization of *catp-6* mutant *C. elegans* ensures that the worm population are the same age in order to perform behavioral assays. The following steps showcase synchronization steps (Kudumala et. al 2019)

1. Gravid adults were collected from at least 2 plates by utilizing deionized water from a squirt bottle, and this water was dispense on the plate. The plates were gently swirled, and worms were collect into a 15 mL conical tube using a disposable plastic pipette.
2. The conical tube was spun down at 140 x g for 2 minutes in order to pellet the worms. After, the supernatant was gently removed using a disposable plastic pipette, taking care to not disturb the pellet.
3. The worms were resuspended and washed by filling the tube to 10 mL with deionized water. The tube was then centrifuged at 140 x g for 2 minutes. The supernatant was removed, and this same step was performed two more times or until the water/supernatant appeared clear.
4. 5 mL of freshly made sodium hypochlorite/NaOH solution was added to the worm pellet and was vortexed to be thoroughly mixed. The tube was incubated on a rocker for about 4-8 minutes
5. About a drop of solution (~30 μL) was placed on a glass microscope slide and was used to check for worm lysis. When approximately 70% of the worms are lysed and embryos were released, the tube was filled with egg buffer up to 10 mL. This tube was immediately centrifuged for 1 min at at 140 x g to pellet.

6. The supernatant was removed and the pellet was washed 3 more times by filling the tube with egg buffer or M9 each time. The tube was spun down at 140 x g for 1 minute per wash, and the pellet should turn white at the end of the washes.
7. After the final wash, 30% sucrose solution was used to separate the embryos from the dead carcasses. 5 mL deionized water was added to the pellet, and 5 mL 60% sucrose was then added. The tube was thoroughly mixed and was centrifuged at 160 x g for 6 minutes.
8. A glass Pasteur pipette was used to transfer the embryos floating at the top of the tube. The amount removed was about 4 mL. Any remaining sucrose was removed by washing the embryos 3 times with deionized water by centrifuging at 140 x g for 3 minutes, removing the supernatant and resuspending the pellet (by filling the tube) each time.
9. The washes were repeated with 1X M9 buffer. After the final wash, the pellet was resuspended in 10 mL M9. The tube was then placed on the shaker overnight in order to hatch into L1 larvae. The worms will remain at this stage until food is given.
10. The L1 larvae were washed 3 times with deionized water by centrifuging at 140 x g for 2 minutes the tube. The larvae were then placed in 1 mL water.
11. A small drop was pipetted from the tube and was plate on NMG plates. Then, the plates were left half-open until drop dried out and was covered and incubated upside down in 20 °C until ready for use.

Behavioral Assays to Assess Locomotor Function of catp-6 C. elegans

- *Radial Locomotion Assay for Thrashing Rate*

To observe motility or movement in *C. elegans*, the ‘thrashing assay’ could be used, which consists of placing the organism in liquid and determining its frequency of thrashing

movement or body bends (Currey & Liachko 2021). This technique is well-established, making it reliable:

1. Flip labeled NGM assay plates upside down
2. Make small dot at the center of the upside-down plate
3. Transfer worms to the designated plates
4. Set a timer for 30 minutes and put the lid back on the plate and set aside
5. After 30 minutes, remove lid and place plate upside down
6. Use different colored pen from the center point and put a small dot at the location of each worm. Count and record how many worms did not move from the center
7. Measure the distance from the center point to the final location markings for each worm, and record distance using a ruler. Using the distance, thrashing rate can be determined.

RESULTS

Observations on Developmental Abnormalities in catp-6 mutants

The following tables illustrate the observations of *catp-6* mutants at different temperatures and different number of worms placed on plates. From the observations, it seems that 22 °C with 10 or 20 worms have the greatest number of worms compared to other plates at different temperatures. These numbers may also be due to the higher temperatures, leading to faster growth rates of these worms; therefore, there will be more worms present at these temperatures. Further, a particular abnormal observation in the plates was the worm bagging. In typical *C. elegans,* the worms lay embryos, and they hatch outside the parental body. In “worm bagging”, the embryos remain internal and hatch inside the parental body (Mosser et. al 2011). In

the plates with worm bagging, this phenomenon was observed, and progeny were seen inside the parental body with attempts to eat their way out. Additionally, observations indicated that worm bagging occurred quicker among higher temperatures (20 °C & 22 °C). This may be due to the higher temperatures accelerating worm growth rates.

For the synchronization of *catp-6 C. elegans*, multiple attempts were performed using various protocols. The final attempt using the protocol described above was ultimately successful. Additionally, behavioral assays, such as radial locomotion, were not performed due to those time constraints as well.

Observations on Different Conditions: Day 1			
	Temperature (°C)		
Number of *catp-6* Mutant Worms	**15**	**20**	**22**
5	adults, no abnormal observation	adults, no abnormal observation	adults, no abnormal observation
10	adults, no abnormal observation	adults, no abnormal observation	adults, no abnormal observation
20	adults, no abnormal observation	adults, no abnormal observation	adults, no abnormal observation

Table 1. Day 1 of Observations

Observations on Different Conditions: Day 2			
	Temperature (°C)		
Number of *catp-6* Mutant Worms	**15**	**20**	**22**
5	Adults, embryos present, no visible progeny	Adults, bagging	Adults, bagging
10	Adults, embryos	Adults, bagging	Adults, bagging

	present, no visible progeny		
20	Adults, embryos present, no visible progeny	Adults, bagging	Adults, bagging

Table 2. Day 2 of Observations

Observations on Different Conditions: Day 3			
	Temperature (°C)		
Number of *catp-6* Mutant Worms	**15**	**20**	**22**
5	Adults, embryos present, no visible progeny, slow motor movement	Adults, embryos present, no visible progeny, slow motor movement	Adults, significant visible progeny present, slow motor movement
10	Adults, embryos present, no visible progeny	Adults, visible progeny present, slow motor movement	Adults, significant visible progeny present, slow motor movement
20	Adults, embryos present, visible progeny present, slow motor movement	Adults, visible progeny present, slow motor movement	Adults, significant visible progeny present, slow motor movement

Table 3. Day 3 of Observations

Observations on Different Conditions: Day 4			
	Temperature (°C)		
Number of *catp-6* Mutant Worms	**15**	**20**	**22**

5	Adults, visible progeny present, slow motor movement	Adults, significant visible progeny present, slow motor movement	Adults, significant visible progeny present, slow motor movement
10	Adults, visible progeny present, slow motor movement	Adults, significant visible progeny present, slow motor movement	Adults, significant visible progeny present, slow motor movement
20	Adults, visible progeny present, slow motor movement	Adults, significant visible progeny present, slow motor movement	Adults, significant visible progeny present, slow motor movement

Table 4. Day 4 of Observations

Observations on Different Conditions: Day 5			
	Temperature (°C)		
Number of *catp-6* Mutant Worms	**15**	**20**	**22**
5	visible progeny present, slow motor movement, plate was not full of worms	visible progeny present, slow motor movement, number of progeny equivalent to plate 10 at 15 °C	visible progeny present, slow motor movement, number of progeny equivalent to plates at 15 °C
10	visible progeny present, slow motor movement, plate was not full of worms but more than plate above	visible progeny present, slow motor movement, number of progeny equivalent to plate 20 at 20°C	visible progeny present, slow motor movement, number of progeny greater than plates at 20 °C
20	visible progeny present, slow	Mold growth, visible progeny present, slow	visible progeny present, slow motor movement,

	motor movement, plate was not full of worms but more than plate above	motor movement, number of progeny greater than plate 20 at 15 °C	number of progeny greater than to plates at 20 °C

Table 5. Day 5 of Observations

DISCUSSION

Parkinson's disease is a neurogenerative disease that is characterized in by the death of dopaminergic neurons present in the substantia nigra pars compacta in the brain. This disease has led to a variety of symptoms, both motor and cognitive. Further, there are distinct sex differences in symptomology, but reasoning remains unknown as well as the etiology of the disease. In this project, *C. elegans* were utilized as the model organism of study to further explore Parkinson's disease, with a particular focus on the *catp-6* gene in *C. elegans*. The initial project was to further explore the sex differences that is correlated with Parkinson's disease that were previously mentioned; however due to sequenced complications, such as crossing not being successful after concerted efforts and not having enough offspring was the time frame they were supposed to along with the maintenance of these mutant worms differing from wildtype worms, there was shift a focus of the project to developmental observations and ultimately find the optimal conditions for growth in order to perform behavioral assays. With this reprioritization, developmental observations were performed, and optimal growth conditions of the mutant worms were found. Certain observations, such as worm bagging are typical characterizations of the *catp-6* mutant worms. These observations ultimately give insight for future maintenance of these worms, and hopefully can be utilized to further explore this mutation through behavioral assays.

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