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AN EXPLORATION OF A BODY SIZE ALLELE IN THE FIG WORM *CAENORHABDITIS INOPINATA*

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AN EXPLORATION OF A BODY SIZE ALLELE
IN THE FIG WORM *CAENORHABDITIS INOPINATA*

A THESIS APPROVED FOR THE

SCHOOL OF BIOLOGICAL SCIENCES

BY THE COMMITTEE CONSISTING OF

Dr. Gavin C. Woodruff, Chair
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ABSTRACT

As a model organism, the nematode *Caenorhabditis elegans* has been the subject of prolific biological research. Since its discovery, the genetic and phenotypic diversity of *Caenorhabditis inopinata*, the much larger sibling species of *C. elegans*, has been investigated. Comparative body size studies are ongoing between the two species. Because of the existing knowledge base on how body size in *C. elegans* has been studied, including its genetic landscape and life history traits, this prior research helps to inform on the ways the body size of *C. inopinata* may be studied. First discovered in Okinawa, Japan, wild-type *C. inopinata* lives in the interior of the fresh fig syconia *Ficus septica*. The genetic cause of the elongated appearance of *C. inopinata* has been studied. Postembryonic cell size expansion is primarily responsible for *C. inopinata's* large size, and gene expression patterns during the developmental stage from larval to adult have been described. As such, post embryonic growth is the period of interest in this body study study. In previous work, a forward genetic screen was utilized on wild type *C. inopinata* to create body size mutants in order to discover novel body size regulatory genes. Presented here is a study of a novel allele classified as *nok3* that presents with a shorter and wider body size in *C. inopinata*. This allele promotes a characterized as a dumpy phenotype (Dpy). This paper includes a series of genetic and observational studies of the cultivated mutant strain of *C. inopinata* made in an effort to fill in the gap in knowledge pertaining to this body size variant and adding to the existing knowledge base concerning body size regulation in *C. inopinata*. Through an initial genetic map, a region believed to harbor a causative gene for the Dpy mutation was determined. Gene editing using Crispr/Cas9 technology was performed on wild type *C. inopinata* in an attempt to identify a gene that controls the within species body size. A candidate gene known as Sp34_40141100 was targeted. Using Crispr/Cas9 technology, a deletion was generated which removed the entire open reading frame of the gene of interest creating the *nok6* allele. The *nok6* allele did not express the expected phenotype. Two other genes found in *C. inopinata* near the candidate region, were sequenced in the mutant strain. These genes are known orthologs to genes *dpy-13* and *col-34* that produce the dumpy phenotype in *C. elegans.* Sequencing revealed no differences in these genes compared to wild type. To further characterize the Dpy allele, a comparison of growth rates across lines of cultivated wild type and mutant *C. inopinata* was performed. Wild type females grew faster in length but not width compared to the mutant. An examination of individual fecundity over a series of 24 hour intervals after a single mating event found no significant difference in the total number of embryos laid between these strains. However, the timing of egg laying shows an oscillating patten within the Dpy strain which is not present within wild type. Dominance of *nok3* was determined to be recessive. These findings will aid in future efforts to discover more about the genetic, developmental, and evolutionary body size divergence of *C. inopinata* and add to the discussion of body size differences between these two closely related species.

Keywords: *Caenorhabditis*, *C. inopinata*, body size, Crispr/Cas9

INTRODUCTION

Body size diversity is well-documented across different animal species as well as within the same species (Woodward *et al.* 2005). Evolutionary and developmental biologists along with geneticists explore body size differences in order to gain a greater understanding of why this phenotype varies and to discover the causes of the variation. Body size is also researched because it may be used to evaluate other physical traits (Lindstedt and Boyce 1985). Indeed, in many taxa larger animals develop at a slower rate and larger females produce more or larger offspring (Blanckenhorn 2000). Body size of animal species is a life history trait that is well-suited for both genetic and comparative studies. The nematode *Caenorhabditis elegans* has been repeatedly used as a central model organism for biological research and genetic analysis because of their physiological attributes, including body size (Frezal *et al*. 2015; Corsi *et al*. 2015).

Wild-type *C. elegans* live in temperate and tropical regions around the world (Schulenburg and Félix. 2017). Adult worms are generally 1mm in length (Kuwabara and O'Neil 2001; Zhang *et al.* 2020). They can be cultured in large numbers on agar plates and cryo-preserved (Petersen *et al*. 2015). Their transparency permits studies of cellular processes. Additionally, isogenic and transgenic lines are easily produced because of their brief generation period and self-reproduction capabilities allowing for genetic manipulation and targeted mutagenesis (Petersen *et al*. 2015). The genome of C. *elegans* was the first animal organism to be completely sequenced (Kuwabara and O'Neil 2001). More specifically, the genes responsible for the nematode's body size have been the subject of multiple studies, including forward and reverse genetics to produce mutant strains for comparative studies (Schindelman *et al*. 2011; Corsi *et al*. 2015, Cho *et. al*. 2021). Standard body size mutations in *C. elegans* have been characterized as small, dumpy, and long phenotypes (Cho *et al.* 2021). The body shape variation of dumpy (Dpy) mutants is characterized by a thicker middle body and a shorter length often with an exaggerated reduction in body size (Cho *et al*. 2021). In adult *C. elegans*, more than 20 genes that promote a dumpy phenotype have been identified (Chandler and Chloe 2022). Many genes that encode cuticle collagen also produce dumpy worms when mutated (Kramer *et al*. 1990). *dpy-13* is a cuticle collagen gene that causes a dumpy phenotype when mutated in *C. elegans* (Von Mende 1988). *col-34* is another gene that encodes a

cuticle collagen (Mesbahi *et al*. 2020). The characterization of body size mutants in *C. elegans* has played a crucial role in discovering the underlying genetics for body size variants (Doitsidou *et al.* 2010; Savage-Dunn and Padgett 2017). While mutant strains can undergo genetic mapping and sequencing in order to identify causative genes, the gene editing technology Crispr/Cas9 allows for the creation of knockout mutations by using the Cas9 enzyme to cut DNA strands at specific points. This allows for precise editing of the genome which is useful in comparative genomic research (Boti *et al.* 2023). A variety of protocols for the use of Crispr/Cas9 in *C. elegans* have been developed and are aiding ongoing genetic research (Dickenson and Goldstein 2016).

With the discovery of the nematode *Caenorhabditis inopinata,* new opportunities are available to explore its divergent body size within the nematode family and specifically in relation to *C. elegans*, its closest related relative (Kanzaki *et al.* 2018; Woodruff *et al.* 2024). First discovered on Ishigaki Island, Okinawa, Japan in the fig syconia, and described by Kanzaki, *et al.* in 2018, *C. inopinata* is currently the closest living relative known to *C. elegans* as demonstrated by phylogenetic analysis (Kanzaki *et al.* 2018). *C. inopinata* and *C. elegans* differ from each other most notably in size and method of reproduction (Kanzaki *et al.* 2018). Physically, *C. inopinata* are typically larger in size than *C. elegans* (Kanzaki *et al.* 2018). Adult worms are approximately 60 percent longer (Woodruff *et al.* 2018). *C. elegans* are self-reproducing organisms and largely exist as hermaphrodites. Male individuals of the species do exist but occur in lower numbers (Brenner 1974). *C. inopinata* do not exist as hermaphrodites in the adult stage and are obligate outcrossers (Kanzaki *et al.* 2018). Both nematode species are found on or in fruit or plant matter and are bacterivores (Kanzaki *et al.* 2018). However, in nature *C. inopinata* live exclusively in fresh figs while *C. elegans* typically live in plant detritus (Kanzaki *et. al*. 2018). In laboratory conditions, both *C. elegans* and *C. inopinata* are grown on Nematode Growth Medium with peptone using OP50 *E. coli* as a food source (Brenner 1974; Dirksen *et al.* 2016). Generally, nematode populations are cultivated in incubators at 20ºC-25ºC (Dirksen *et al.* 2016). Both *C. elegans* and *C. inopinata* are easily cultivated in the laboratory using standard culturing protocols (Zhang, *et al.* 2017). Whole genome sequencing in *C. inopinata* has been completed (Kanzaki *et al*. 2018). In a previous study, it was shown that the body size difference between these two worm species largely occurs due to postembryonic events during the larval-to-adult transition (*Woodruff et al*. 2018).

Previously, a forward mutagenesis screen was performed to discover novel body size mutations *in C. inopinata*.[1] Among these was *nok3*, an allele that promotes small and fat mutant phenotypes (*i.e*., dumpy phenotypes) in comparison to wild-type animals. Following extensive genetic mapping and whole-genome sequencing, a number of candidate genes harboring mutations in *nok3* homozygous animals were identified. These included the gene Sp34_40141100, which has an A-to-G transition in the *nok3* background that removes a stop codon and extends the protein sequence by 59 amino acids. This represented the most dramatic *nok3*-specific protein-coding mutation in the mapping interval. In addition, despite revealing no obvious protein-coding changes following short-read sequencing, two orthologs with known dumpy phenotype-promoting mutations in *C. elegans* were also present in the *nok3* mapping interval *cin-dpy*-13 (Von Mende 1988) and *cin-col-34* (Bird 1992, Riddle 1997).[2]

Here, this study compares wild type *C. inopinata* with *nok3* mutants in an attempt to identify genes that encode body size in *C. inopinata* and to characterize this allele. This study explores select aspects of the phenotypically dumpy mutant allele *nok3* in *C. inopinata*. First, the outcomes of using both Crispr/Cas9 technology to knockout a candidate gene and gene sequencing of additional candidate genes are reported. Next, a comparison of body growth and fecundity over time between wild type *C. inopinata* and its dumpy mutant strain to characterize the impact of the phenotype is presented. Dominance of the trait was determined. This research offers additional insight into the genetic profile of *C. inopinata* in an effort to aid in the discovery of novel body size regulatory genes.

MATERIALS AND METHODS

Strains and maintenance

The inbred line NKZ35 was cultivated from wild isolate *C. inopinata* from Ishigaki Island, Okinawa Prefecture, Japan (Woodruff et al. 2018). NZK35 and the mutant strain WOU11 used in this study were reared and maintained on 2% agar, peptone free, Nematode Growth Media (NGM)

[1] GC Woodruff (personal communication 2023-2024)
[2] GC Woodruff (personal communication 2023-2024)

plates seeded with OP50 *E. coli* at 25 °C (Brenner 1974). NKZ35 was maintained as the wild type control.

Targeted gene editing with Crispr/Cas9

Using Benchling software (Benchling 2023), sgRNA guides and a repair oligo were designed for the candidate gene sp34_40141100 (Hsu *et al*. 2013). These sgRNA guides were positioned flanking the gene of interest (Fig 1). To allow for post injection genotyping, three PCR primers were designed. One reverse primer was set within the locus to be excised to act as a wild type indicator. The other reverse primer was set down stream of both PAM sites to indicate if the deletion had been made (Fig 1). Both reverse primers were designed to work with the same forward primer placed upstream of the PAM sites.

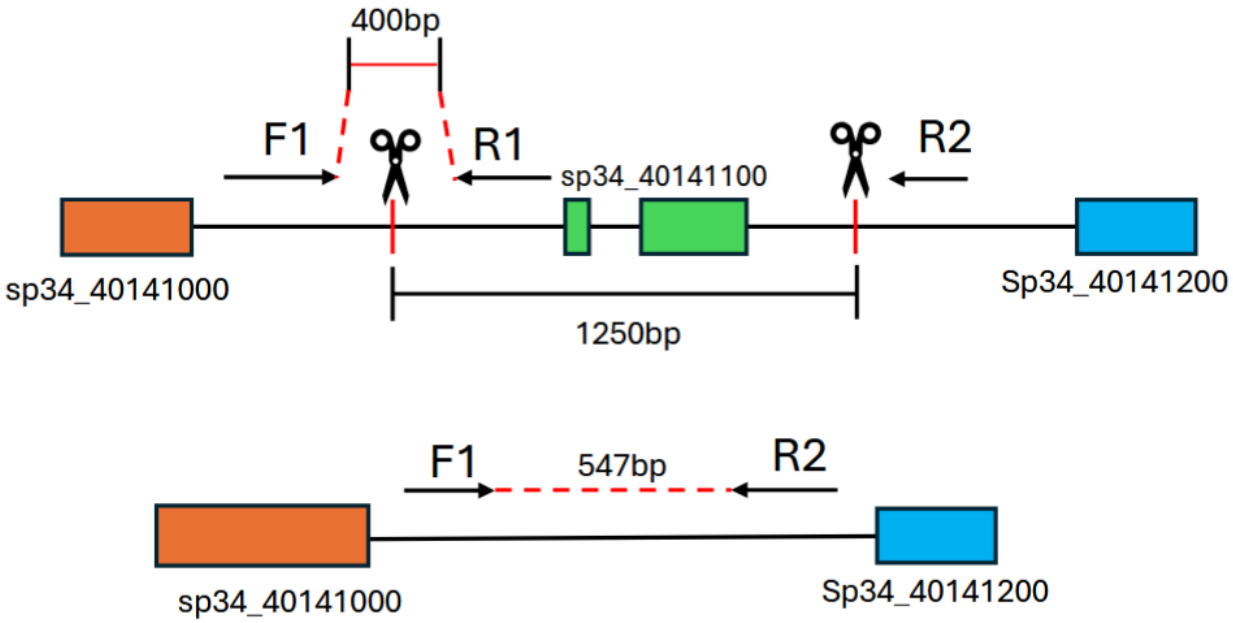


Figure 1. Diagram of the Crispr/Cas9 deletion framework and three primer PCR genotyping system. Unmarked genomic distances are relative.

Microinjections in *C. inopinata*

25 L4 female and male *C. inopinata* specimens from the strain NKZ35 were placed on an NGM plate and were allowed to grow for 48 hours. For candidate gene Sp34_40141100, an injection mixture was prepared consisting of IDT Alt-R S.p. Cas9 Nuclease V3, pure protein at a final concentration of 2µm, with two sgRNAs specific for the target gene (Table 1) at a final concentration of 1.65 pmol/µl along with the Repair oligo (Table 1) and the red fluorescent marker pZCS16 (*peft-3*::*wrmscarlet*) (Stevenson *et al.* 2020), both with a final concentration of 50ng/µl. This solution was brought to a total volume of 10µl with IDT duplex buffer. This mixture was injected into the distal arm of the gonad in the now reproductively active young adult female nematodes. A total of 68 nematodes underwent the microinjection process. Following the injection each female was placed on a NGM plate, with one male to ensure reproduction. Daily post injection screens for fluorescent F1 progeny were performed. All or nearly all F1s from fluorescent positive plates were then crossed individually with their siblings. Once the parental F1s had laid a sufficient mass of embryos, they were genotyped. Genotyping was performed via PCR amplification using a three primers system (Table 1, Fig.1). A PCR fragment indicating a wild-type individual was expected to be seen at 400bp. A 547bp PCR fragment was expected to be seen indicating the presence of the deletion. The standard single worm lysis protocol was used to extract the DNA (William *et al.* 1992). The PCR reaction mixture contained water, Onetaq 2X master mix polymerase and the three genotyping primers, each with a final concentration of 0.2 µM. The thermocycler program was optimized with temperature gradient. The PCR reaction was then performed (Table 2). All reaction products were visualized on a 1% agarose gel using SafeView dye. The process of inbreed crossing and genotyping was repeated four times until a homozygous population had been established. For each genotyping series, a sample of 32 animals were used. Body size measurements were performed under 5x, 10x or 20x magnification. To confirm the deletion a fragment spanning the open reading frame was generated with forward primer 1 and reverse primer 2. This fragment underwent Oxford Nanopore sequencing by Plasmidsaurus.

Table 1. Primer, repair oligo and sgRNA guide sequences for CriprCas9.

Type	Sequence
Sp34_40141100 Guide RNA 1	5’ ACAGAACAUGACCGAGCACG 3’
Sp34_40141100 Guide RNA 2	5’ GUGAAACGCACUAUACA 3’
Sp34_40141100 Repair Oligo	5’ATTTTTTGTTCGCCAAAAACAGAACATGACC GAGCGGGTGGCTGAAAATTACGTGCGCAAAAA AACGCTT 3’
Reverse Primer 1	5’ GCAAAGAAACTCGCCGAACT 3’
Reverse Primer 2	5’ TAGGGACAATTGAGGGACCG 3’
Forward Primer 1	5’ TCAAGAAGCACGCATAACATGA 3’

Table 2. PCR thermocycler program for sp34_40141100 genotyping. A 39x cycle was used during the extension.

Stage	Temperature	Time
Denaturation	94° C	30 Seconds
Denaturation	94°C	30 Seconds
Annealing	55°C	60 Seconds
Extension	68°C	35 Seconds
Final Extension	68°C	5 Minutes
Hold	15°C	Indefinite

Sequencing of Nearby Candidate Genes

PCR primers were generated spanning the open reading frames of both *col-34* and *dpy-13* (Table 3). The standard bulk worm lysis protocol was used to extract the DNA (William *et al.* 1992). A PCR fragment for *col-34* was expected to be seen at 1412 bp. An 1800 bp PCR fragment was expected to be seen for *dpy-13*. The PCR reaction mixture contained water, Q5 High-Fidelity 2X Master Mix polymerase and the two primers, each with a final concentration of 0.5 µM. The thermocycler program was optimized with temperature gradient. The PCR reaction was then

performed (Table 4, Table 5). The fragments were purified to a final concentration of >15 ng/µl and underwent Oxford Nanopore sequencing by Plasmidsaurus.

Table 3. Primer Sequences.

Primer	Sequence
Col-34 Primer 1	5' ACCTGGGAGAATGAGCGAAT 3'
Col-34 Primer 2	5' AAACTGGCGACTTTCACACG 3'
Dpy-13 Primer 1	5' GTGGGGCTGAGAGATGCTAT 3'
Dpy-13 Primer 2	5' CATTCAGATGCCGCAGAAGA 3'

Table 4. Thermocycler protocol used for *col-34* amplification

Stage	Temperature	Time
Denaturation	98° C	30 Seconds
Denaturation	98°C	7 Seconds
Annealing	67°C	20 Seconds
Extension	72°C	35 Seconds
Final Extension	72°C	2 Minutes
Hold	15°C	Indefinite

Table 5. Thermocycler protocol used for *dpy-13* amplification

Stage	Temperature	Time
Denaturation	98° C	30 Seconds
Denaturation	98°C	7 Seconds
Annealing	68°C	20 Seconds
Extension	72°C	40 Seconds
Final Extension	72°C	2 Minutes
Hold	15°C	Indefinite

Body length and width measurements over time

NKZ35 and WOU11 were grown for three days until a sufficient number of gravid females and embryos were present. These populations were then synchronized by bleaching using the standard protocol (Stierngale 2006). The tubes were allowed to incubate for 48 hours at 25°C to induce an L1 arrest. After incubation, each tube was spun and the worm pellets were collected and plated to fresh NGM plates and allowed to grow for 24 hours. Following synchronization, 20 individuals were sampled from each plate every 24 hours and imaged. Individual worms were placed on 1% agarose pads with 0.25 mM levamisole and were photographed under magnification. Using ImageJ software, the body length and body width of each specimen were measured (Schneider *et al*. 2012). The "segmented line tool" in ImageJ was used to address line curvatures (Mörck and Pilon 2006). This was done until the populations were depleted.

Individual fecundity

20 L4 WOU11 females were picked along with 20 young males and were allowed to mate for 12 hours. This was done with the WT strain NKZ35 as well as a control. Mass-mating was done to account for nematode losses. The animals were monitored frequently to ensure that embryos were not missed due to early laying. Each gravid female was identified by a post mating mucous plug and/or the presence of embryos that were visible under magnification. The females were then plated individually before they began to lay. Each female was allowed to lay for ~24 hours at which point the female was transferred to a fresh NGM plate and the number of progeny was enumerated. Females were kept separate from adult males at all times after the initial mating event. This was repeated for each female until they were no longer laying. Females were determined to no longer be laying after two consecutive days without the presence of any progeny.

Mendelian segregation

20 L4 WOU11 females and 20 L4 NKZ35 males were crossed. The females were then picked to individual NGM plates and allowed to lay. The F1 heterozygotes were crossed with their siblings and a F2 generation was created. The F1 heterozygous parents were then measured using

ImageJ. Each plate of F2 progeny was counted and the phenotypes were visually categorized as Dpy or WT.

RESULTS

Successful application Crispr/Cas9 in *C. inopinata*

The use of Crispr/Cas9 technology has been established as a reliable method for targeted mutagenesis in *C. elegans* (Dickinson and Goldstein 2016, Dickinson *et al.* 2020). To determine the potential role that Sp_34-40141100 could play in body size regulation, it was targeted for deletion using Crispr/Cas9. A series of microinjections on young adult NKZ35 females were performed to that end over the course of tree trials. During the successful trial, two plates out of 25 with red fluorescent marker positive F1 progeny were detected post injection. From these red F1 populations a null mutant was identified via PCR genotyping (Fig. 2A). A homozygous line was subsequently established from it. This null mutant strain will now be known as WOU56 (Fig. 2B).

A deletion of Sp_34-40141100 did not yield a body size phenotype

To investigate whether the deletion of Sp_34-40141100 had any impact on the phenotype of WOU56, body length measurements were taken. NKZ35 and WOU56 adult females of similar age, were found to have average lengths of 1319.2 µm 1303.2 µm respectively. Males were likewise similar in size between strains, with a WT average length of 976.8 µm and 965.1 µm respectively. A Wilcoxon rank-sum test showed that when compared to NKZ35, this strain did not possess any statistically significant change in length resulting from the creation of the Sp34_40141100 null allele (*nok6*) for males (W = 206, p-value = 0.4604) or females (W = 270, p-value = 0.9243) (Fig. 2C). To further confirm the presence and the accuracy of the Sp_34-40141100 deletion, Oxford Nanopore sequencing was performed by Plasmidsaurus on a fragment spanning the 1250bp long locus (Fig. 3). The deletion was found to be exactly 1250bp long (Fig. 3).

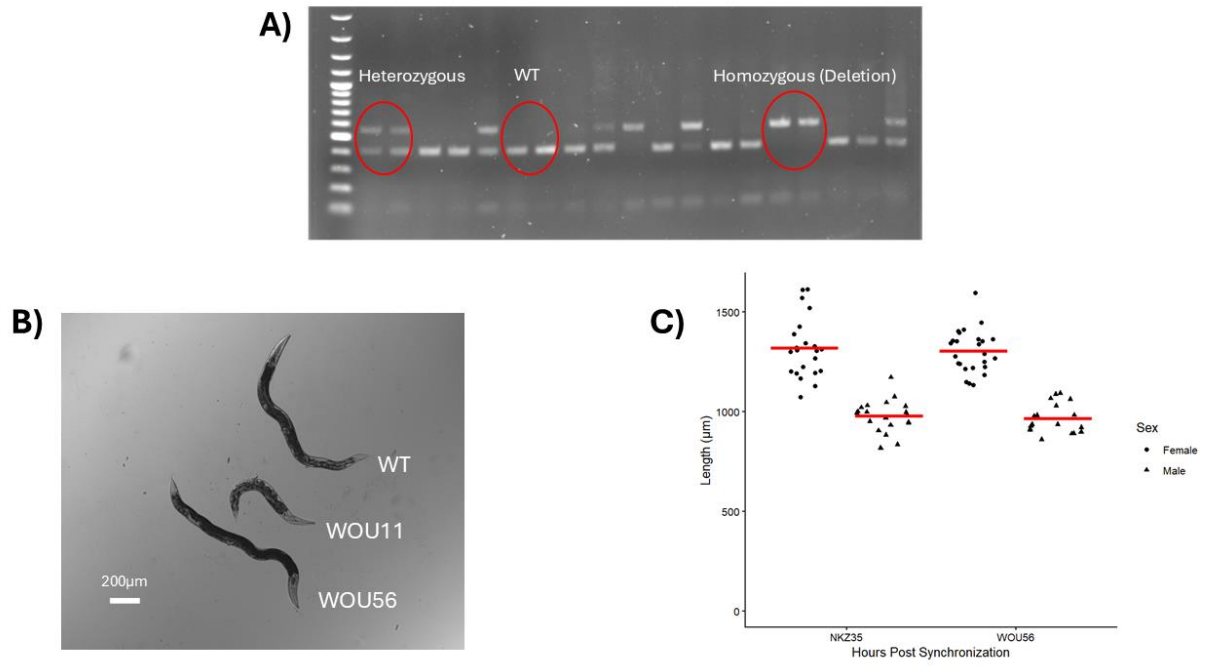


Figure 2. Cas9 mediated Sp_34-40141100 Null Mutant. (A) PCR genotyping of the adult red F1 progeny of *C. inopinata* showing deletion present at targeted gene. (B) Representative imaging of wild type *C. inopinata* WT, Dpy mutant WOU11, and the Cas9-induced mutant WOU56. (C) Comparison of body length measurements of WOU56 and WT show no statistically significant difference using a Wilcoxon rank-sum test.

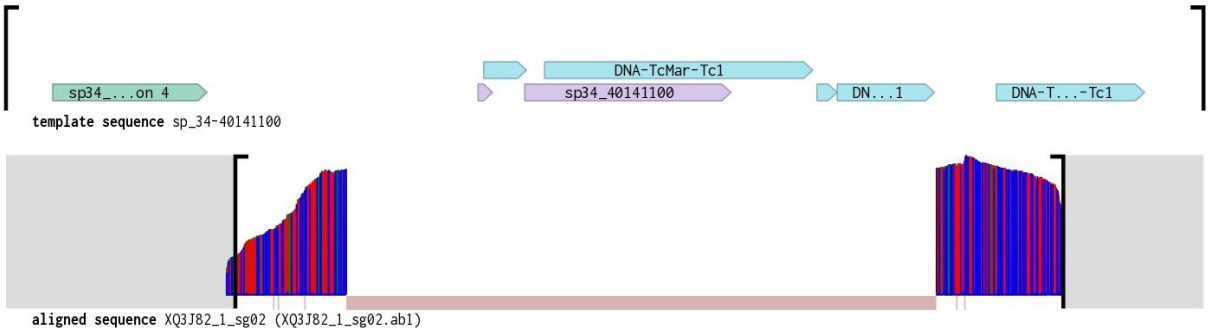


Figure 3. Sequence alignment of the candidate gene Sp34_40141100 in WOU56. A Cas9 mediated deletion is visible, spanning the open reading frame of Sp34_40141100.

Local Dpy associated genes do not harbor nonsynonymous or deleterious mutations

Within *C. elegans* there are many genes which are known to exhibit a dumpy phenotype when perturbed (Chandler and Chloe 2022). In the *nok3* mapping interval, two genes orthologous to *ce-col-34* and *ce-dpy-13* were identified in *C. inopinata*. In order to determine whether these orthologs possess mutations that may be underlying the Dpy phenotype in the mutant line, fragments spanning these genes were amplified by PCR and re-sequenced with Oxford Nanopore sequencing performed by Plasmidsaurus. Subsequently, neither was found to carry any notable sequence changes when compared to the reference genome (Fig. 4, Fig. 5).

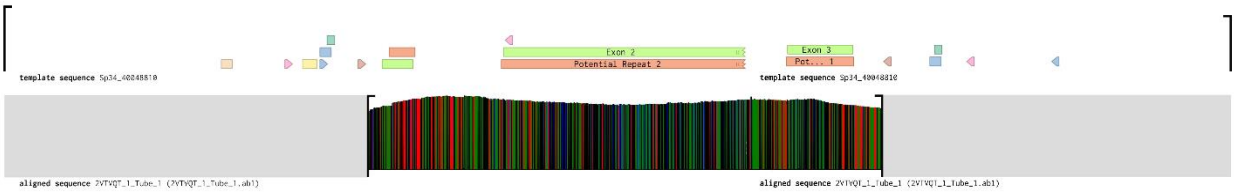


Figure 4. Sequence alignment of the candidate gene dpy-13 in WOU11 shows no change compared to the wild type reference genome.

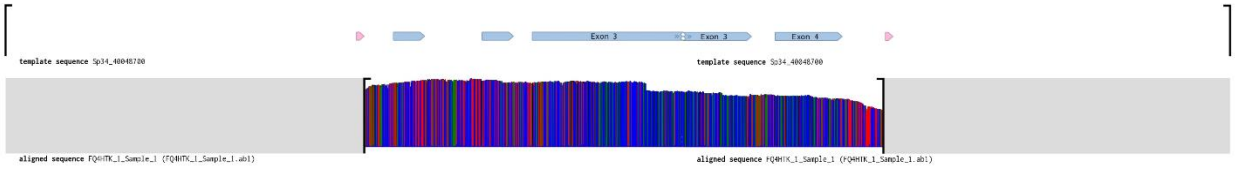


Figure 5. Sequence Alignment of the candidate gene *col-34*-13 in WOU11 shows no change in comparison to the wild type reference genome.

***nok3* homozygotes display body length differences in both length and width over time**

Many body size mutations in *C. elegans* do not impact every developmental stage (Soete *et al*. 2007). To access when in development the Dpy mutation impacts body size, organism size was measured over time. Body growth of *C. inopinata* is known to be slow growing (Soete *et al.* 2007). To explore if the *nok3* allele has an effect on the rate of body size growth and the rough

timing of when the Dpy phenotype appears, a body size assay was conducted. Over the course of 96 hours the length and width of both NKZ35 and WOU11 were measured. Both strains maintained a similar length and width for the first 24 hours (Fig. 6). WOU11 begins to diverge between 48 and 72 hours as both strains approach adulthood (Fig.6, Fig. 7).

It was observed that WOU11 females are both wider and shorter than NKZ35 females past the 24-hour point (Fig. 6, Fig. 7, Fig. 10). An ANOVA did show a significant difference between WT and mutant female length and width ($F = 16.2$, p-value = 1.4×10^{-4}) ($F = 96.6$, p-value = 1.2×10^{-17}). Interestingly, male WOU11 and male NKZ35 seem to maintain similar lengths as they grow at each interval (Fig. 6, Fig. 11). An ANOVA showed no significant difference between male length of either strain (p-value = 0.75). However, WOU11 males are significantly wider than NKZ35 at each time ($F = 53.6$, p-value = 3.17×10^{-8}) (Fig. 7). The rate of body length growth in NKZ35 females is higher than WOU11 (Fig. 8). This is contrasted by WOU11 females having a higher rate of change in its width than NKZ35 (Fig. 9).

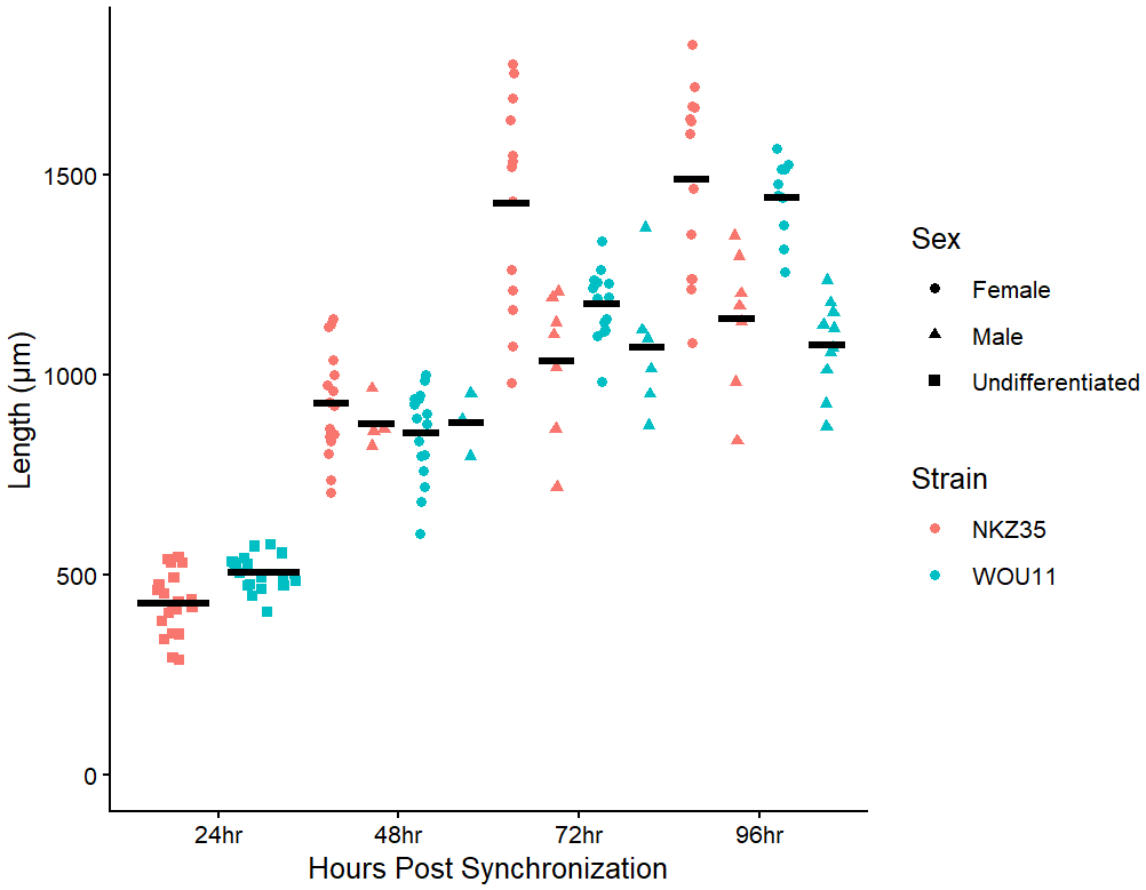


Figure 6. Body length measurements of NKZ35 and WOU11 over 24 hour intervals for 4 days shows female WOU11 are shorter than female NKZ35 but the male WOU11 are not shorter than their wild type counterpart. Both a Two and a Three way ANOVA were performed and provided support for this trend. The variance of length of WOU11 is lower at every time point than NKZ35.

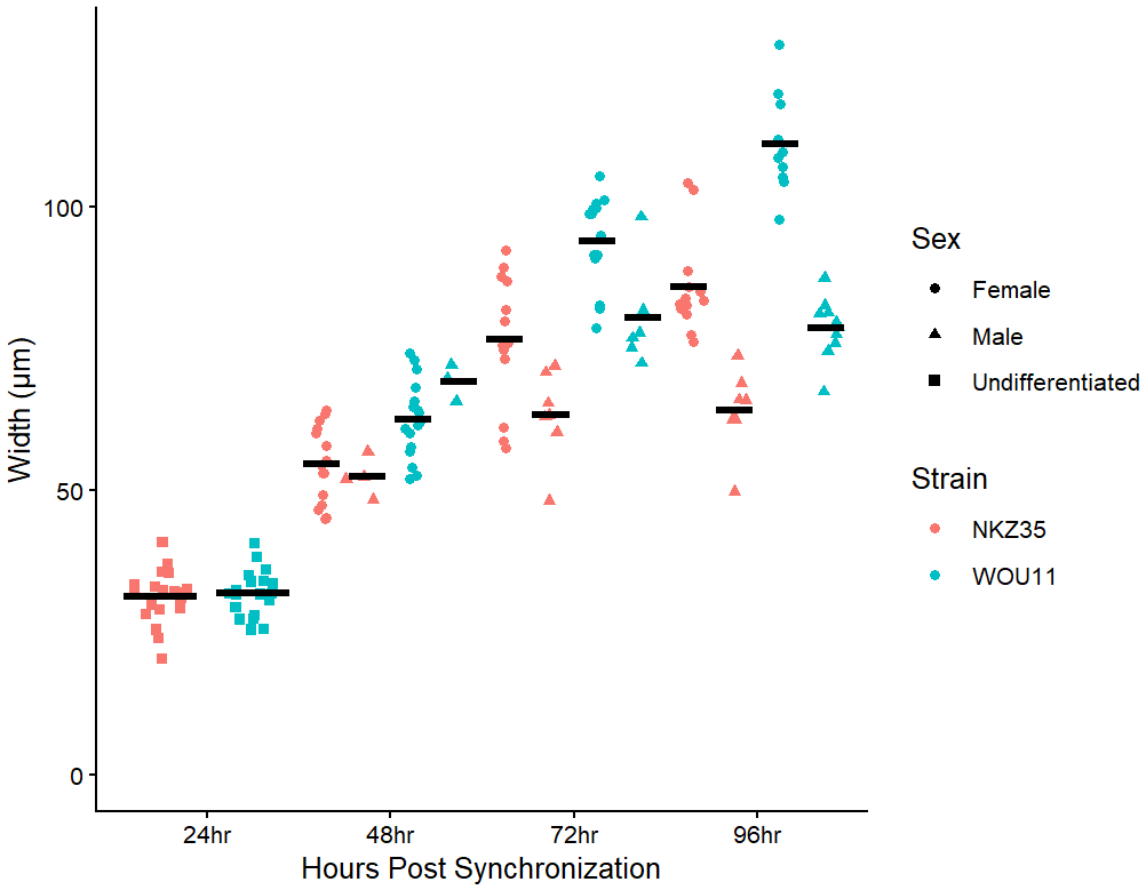


Figure 7. Body width measurements of NKZ35 and WOU11 over 24 hour intervals for 4 days shows female WOU11 are wider than female NKZ35 and male WOU11 are wider than their wild type counterpart. Both a Two and a Three way ANOVA were performed and provided support for this trend.

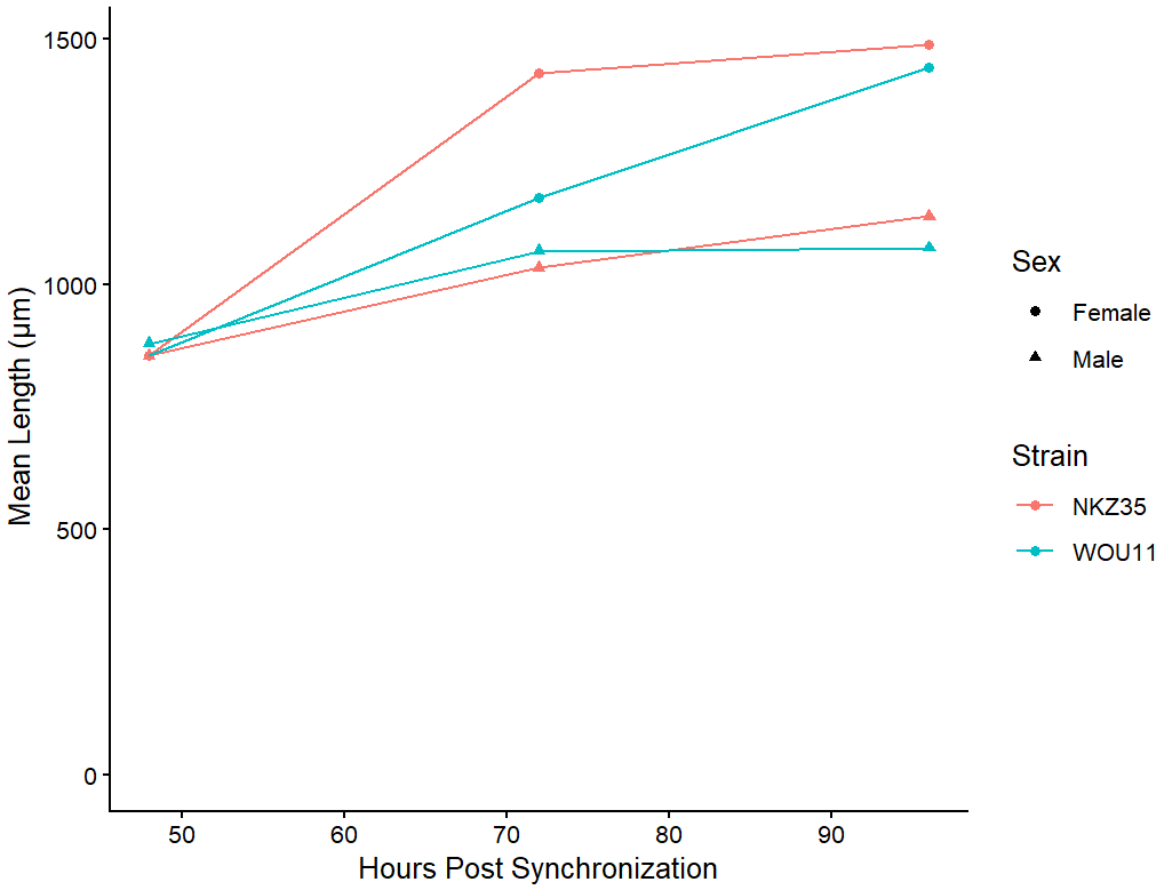


Figure 8. The rate of length growth is higher in wild type females than mutant females at every interval based upon mean length measurements. The rate of length growth remains similar across wild type males and mutant males at every interval based upon mean length measurements.

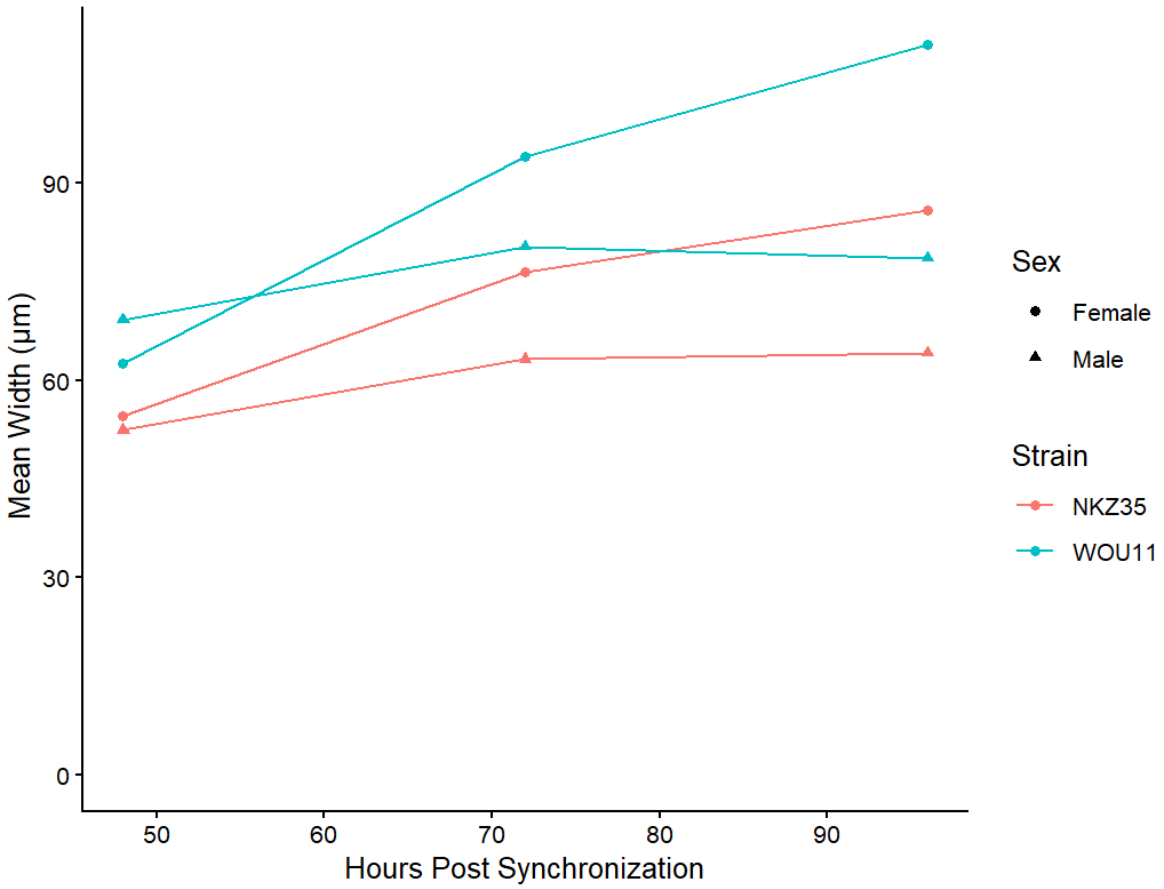
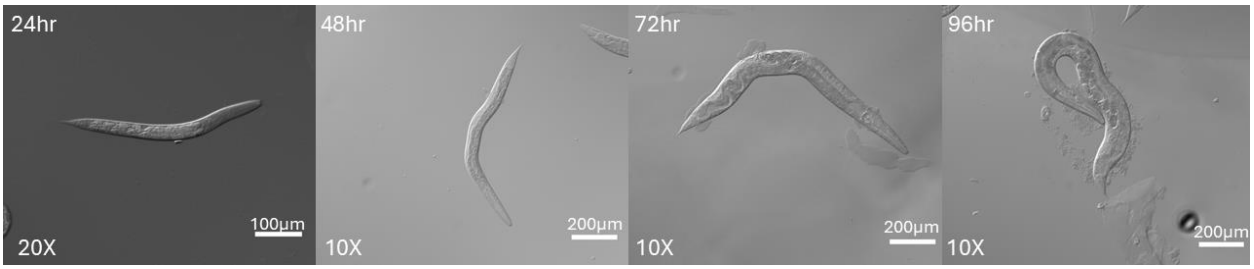


Figure 9. The rate of width growth is higher in mutant females when compared to wild type males and females at every interval based upon mean width measurements. Mutant males are consistently wider than wild type males as well.

A) Dpy



B) WT

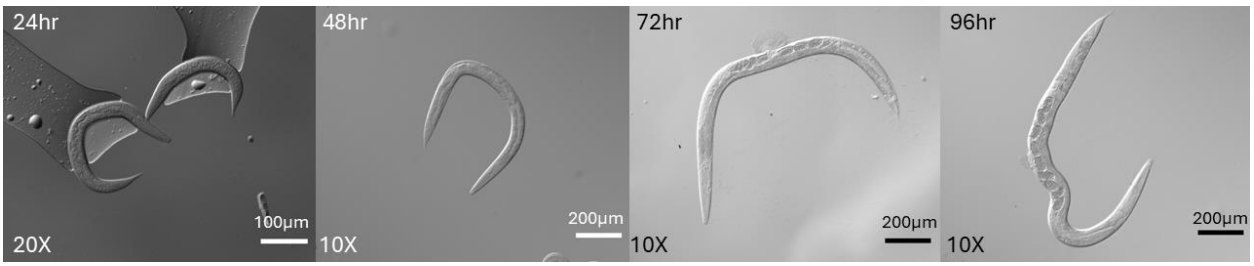


Figure 10. (A) Representative images of Dpy females at each time interval. (B) Representative images of WT females at each time interval.

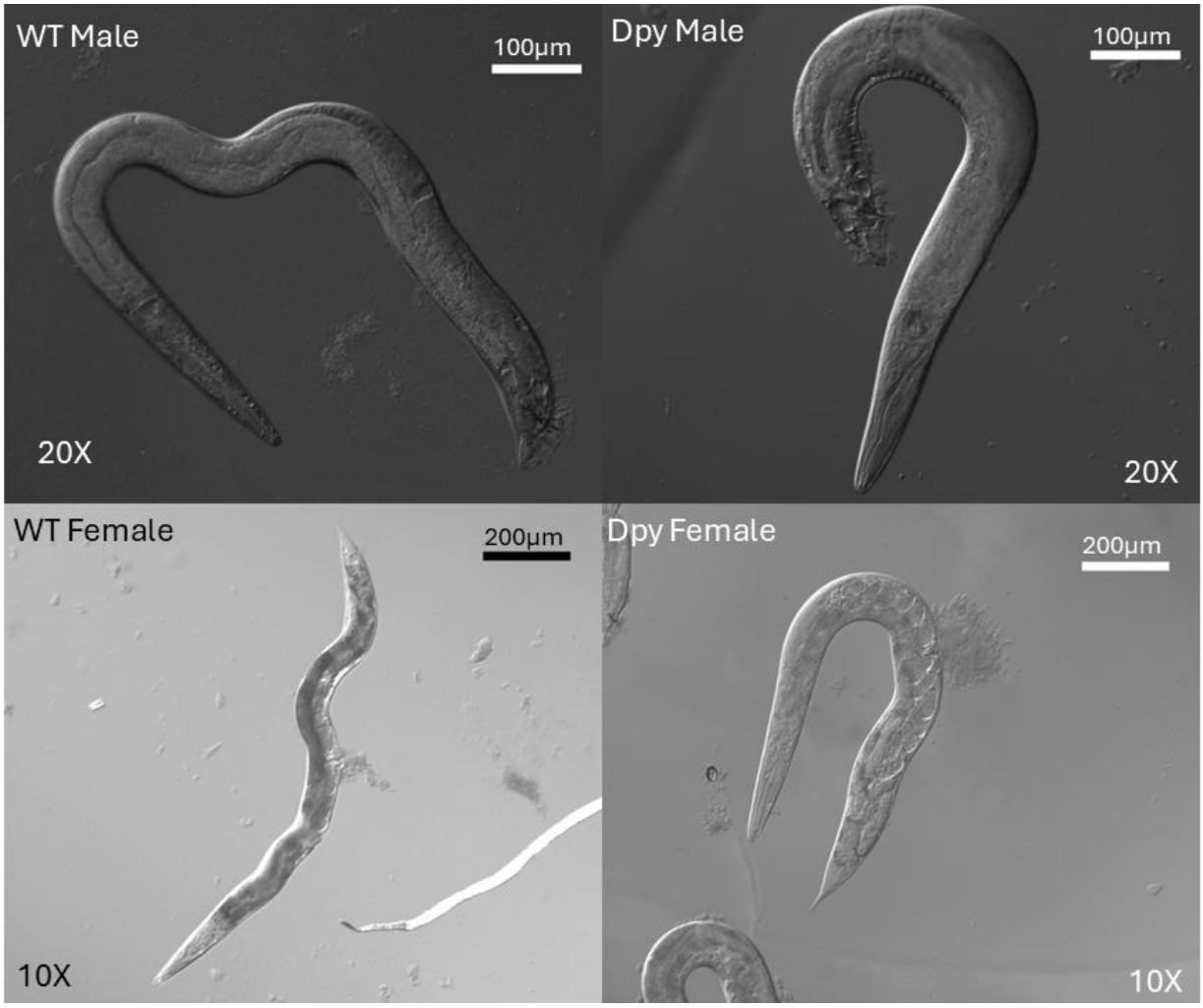


Figure 11. A visual comparison of adult wild type male and female *C. inopinata* with Dpy mutants.

Variance in the reproductive schedule despite unchanged total fecundity

To investigate if *nok3* impacts the fecundity of *C. inopinata,* the number of offspring was measured. Over the course of 144 hours, 20 individual NKZ35 and WOU11 females that had previously mated once with males of their respective strain were plated and the fecundity of both was monitored. It was found that the total amount of laid embryos did not significantly differ between NKZ35 and WOU11, with an average total count of 27.7 and 31.70 F1 progeny respectively (Fig. 12). A Wilcoxon rank-sum test showed no significant difference (W = 152.5, p-value = 0.2028). However, it was observed that WOU11 laid embryos in a less continuous manner than NKZ35. WOU11 females were found to lay embryos approximately every 48 hours. This is

in contrast to the NKZ35 females that were observed to have laid every 24 hours (Fig. 13). In both trial groups, the number of F1 progeny decreased with each plating event as the females were not repeatedly mated.

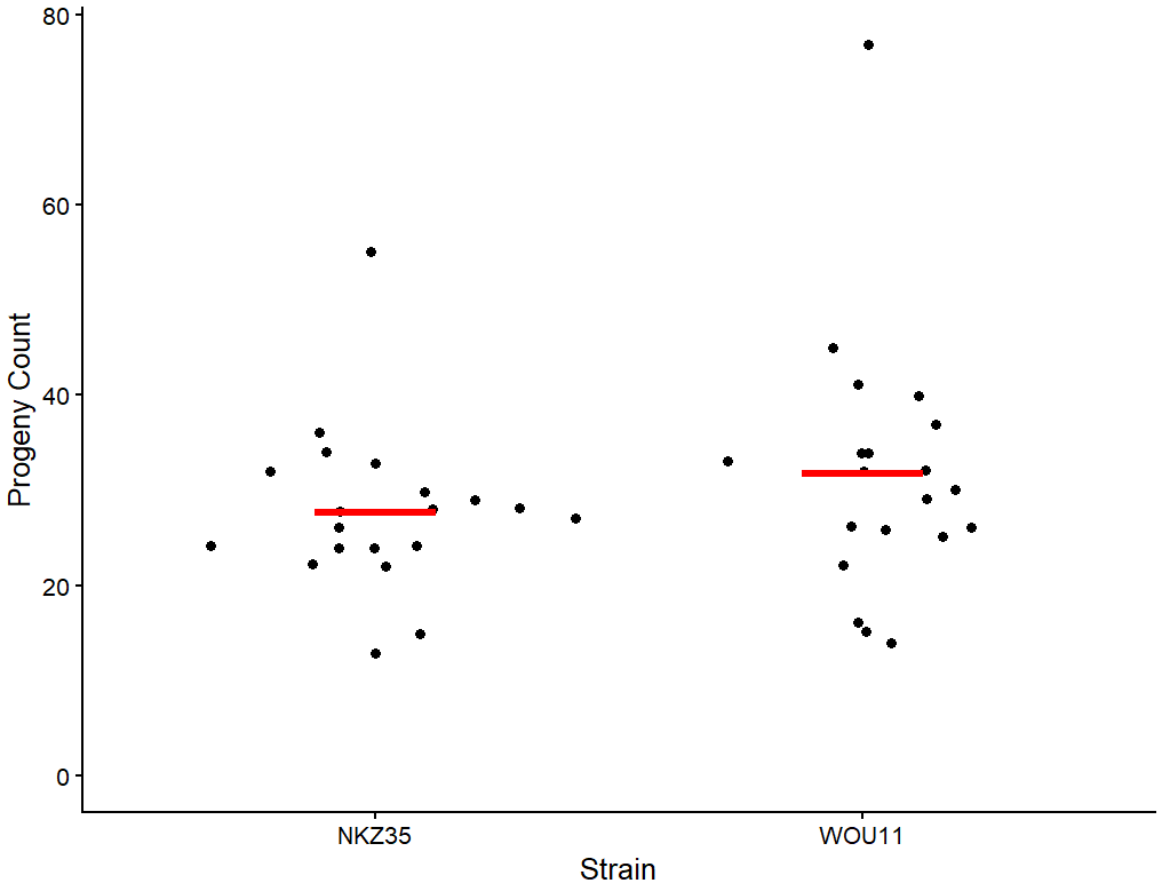


Figure 12. Total progeny after a single mating event in NKZ35 and WOU11 shows no statistically significant differences using a Wilcoxon rank-sum test.

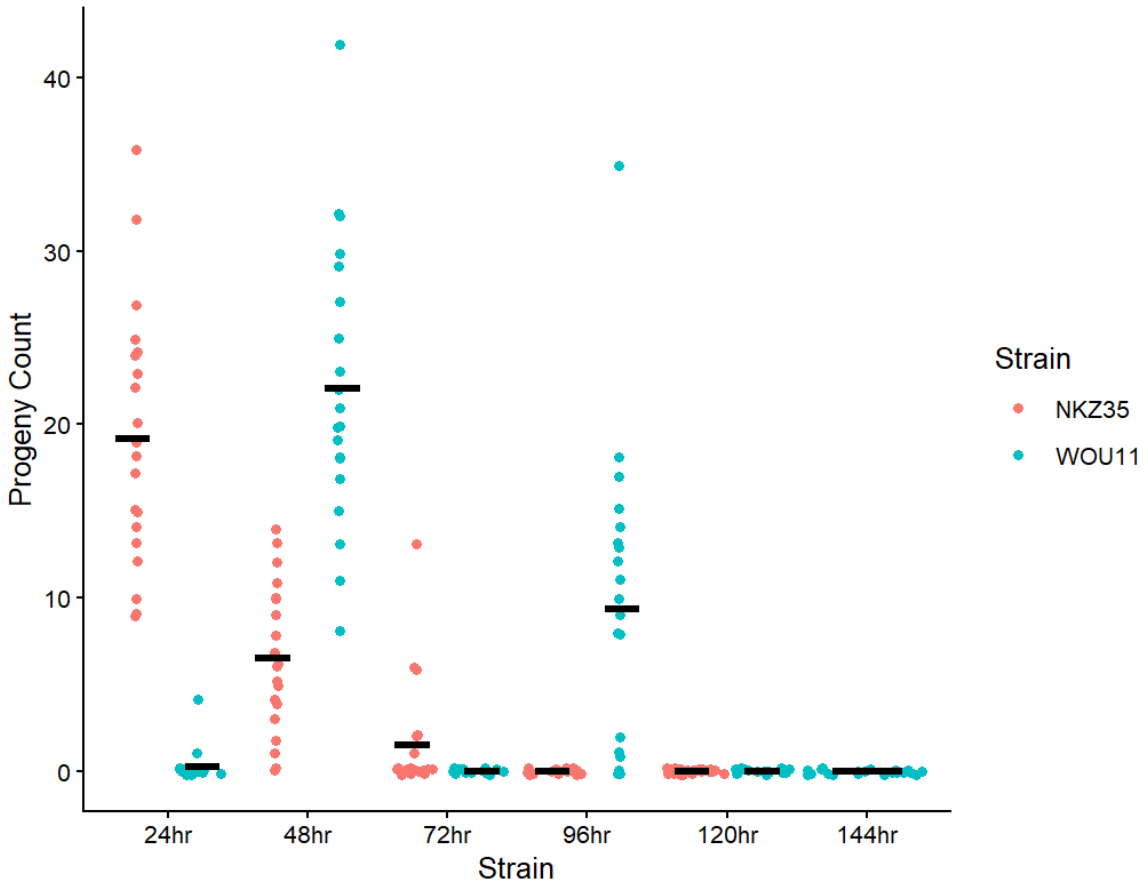


Figure 13. Time distribution of egg laying after a single mating event in NKZ35 and WOU11 shows discontinuous patterns in WOU11.

The *nok3* allele is recessive

The extent of an allele's dominance can reveal mechanistic insights into a gene's function (Hawley and Walker 2003). To investigate the dominance of the *nok3* allele, heterozygous females were generated. These individuals were measured and compared to females from the wild type NKZ35 strain. It was seen that the average length of the heterozygotes did not differ significantly from the expected wild type length. Wild type NKZ35 and the heterozygous females had an average length of 1375 µm and 1272.7 µm respectively. A Wilcoxon rank-sum test does not show a significant difference (W = 463, p-value = 0.09) (Fig. 14). When comparing the widths, a similar trend is seen (Fig. 15). NKZ35 with an average width of 82.98 µm and the heterozygotes with 81.01 µm (W = 268, p-value = 0.097). To further explore the segregation pattern of the *nok3* allele the ratio of Dpy/WT F2 progeny was calculated. This revealed an overall Dpy:WT ratio of 28.1%

with an expectation of a Dpy:WT ratio of 25% (Fig.16). A Chi-square goodness-of-fit test showed no significant difference (X-squared = 5.6, df = 1, p-value = 0.18). Taken together, this data confirms that the *nok3* allele is recessive.

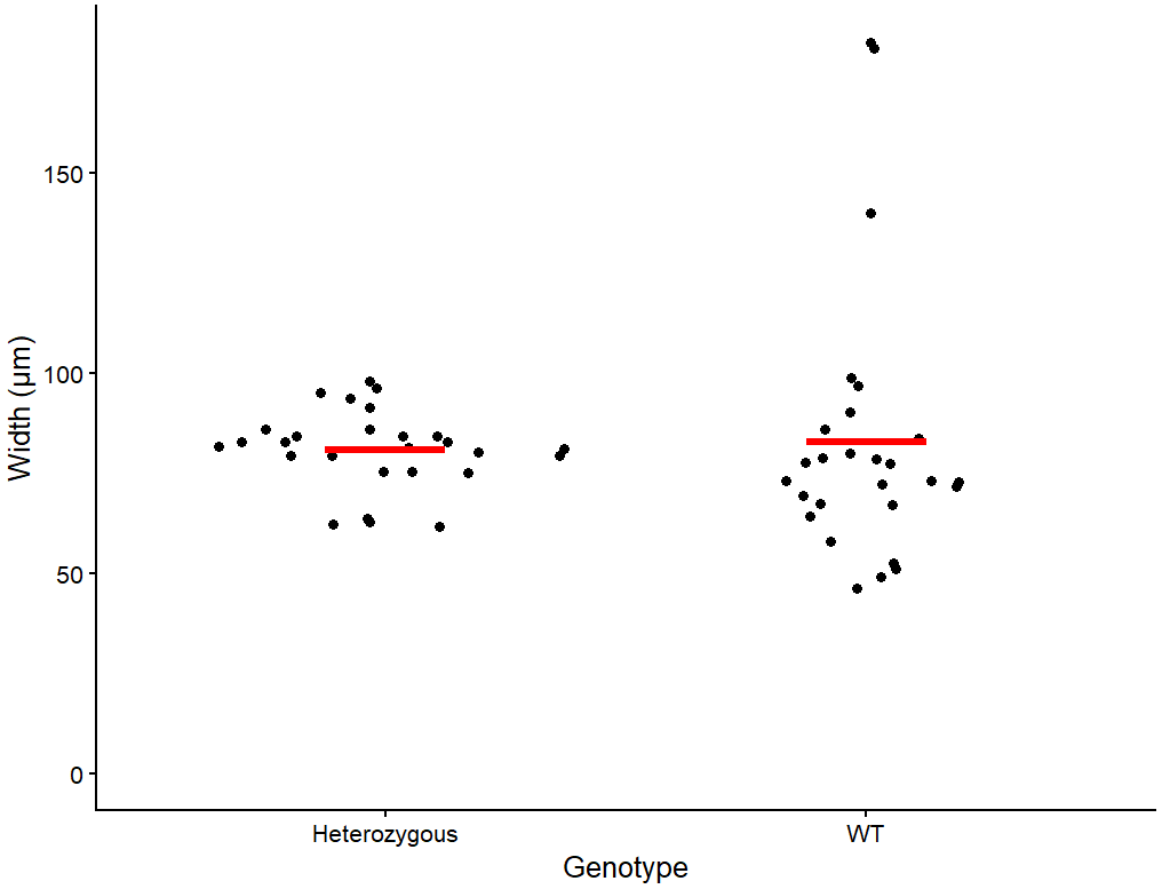


Figure 14. Heterozygous female body width comparison with wild type *C. inopinata* shows no statistically significant difference using a Wilcoxon rank-sum test.

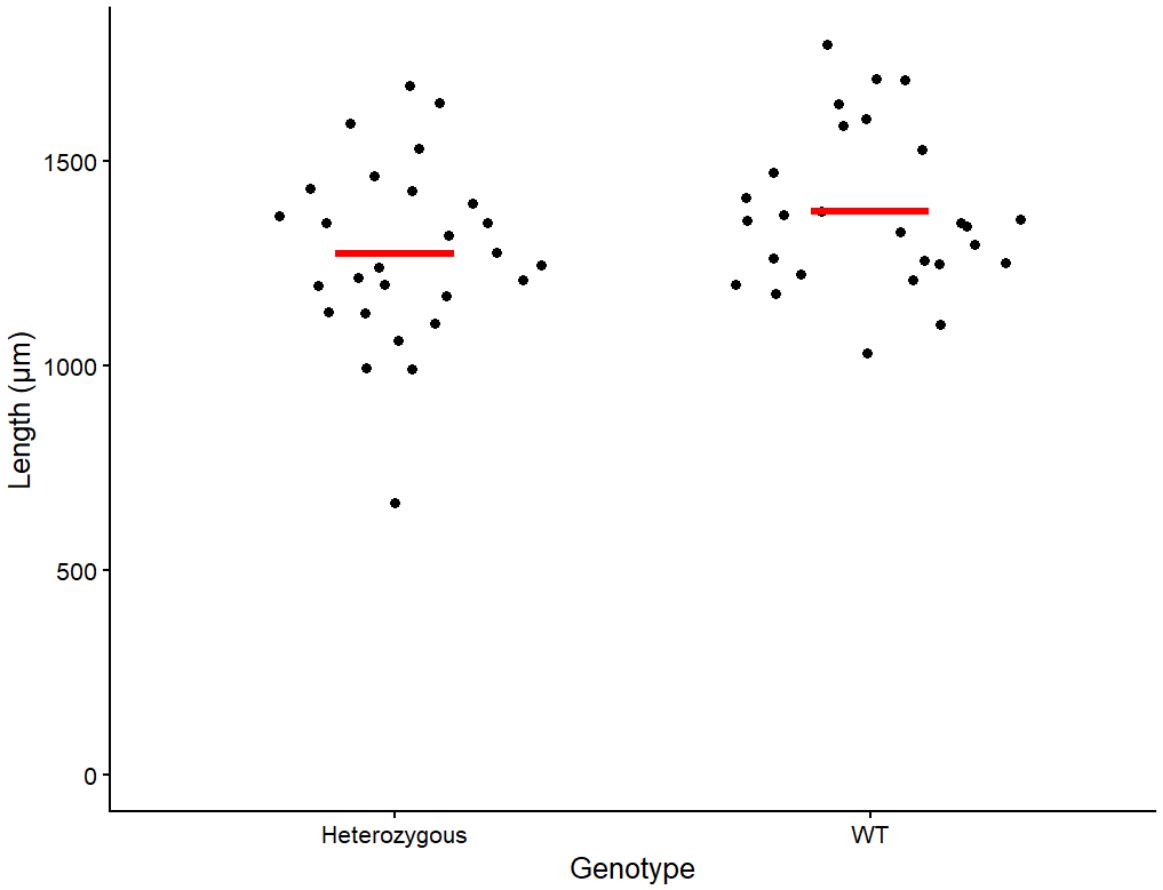


Figure 15. Heterozygous female body length comparison with wild type *C. inopinata* shows no statistically significant difference using a Wilcoxon rank-sum test.

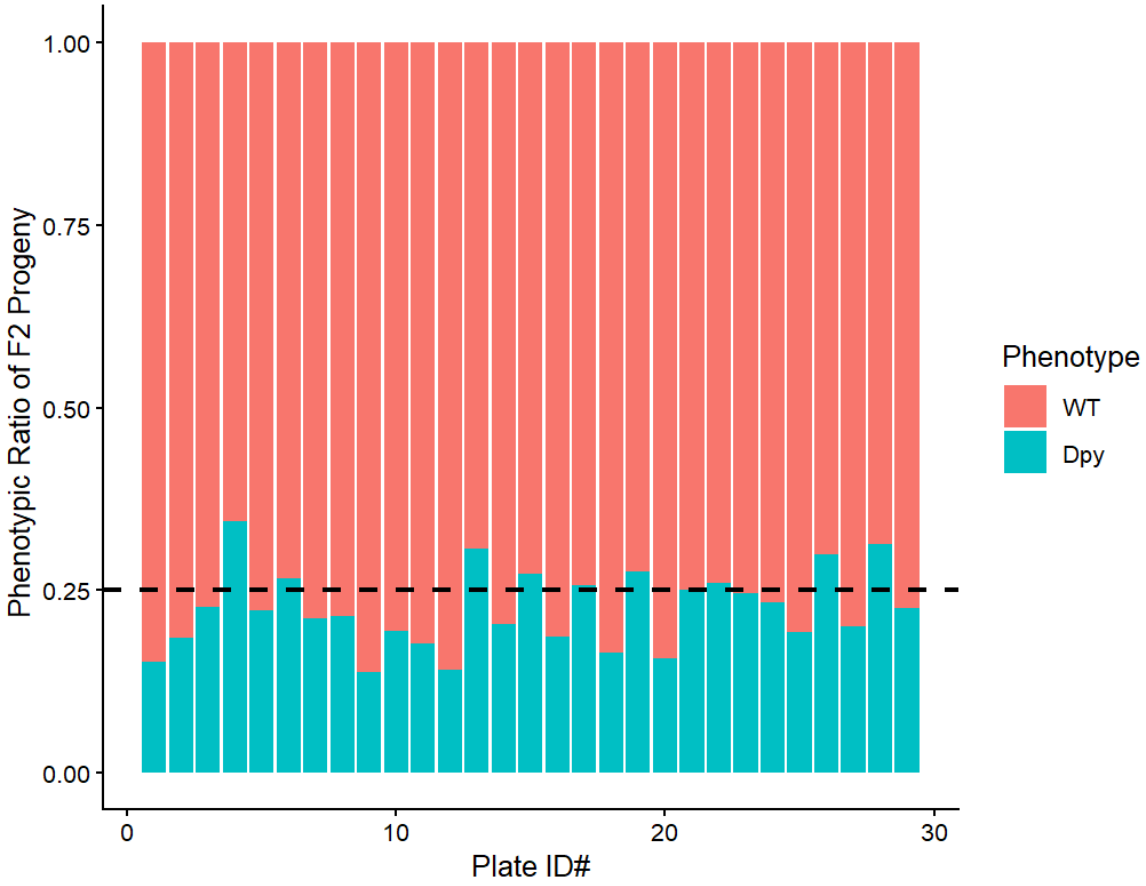


Figure 16. Ratio of F2 progeny wild type and Dpy phenotypes shows the *nok3* allele segregates recessively.

DISCUSSION

Research on body size variation across and within species has been prolific (Polly 2012, So *et al*. 2102, West *et al*. 2023, Woodward *et al.* 2005, Zimova 2023). Studies explore body size diversity to evaluate its role in evolutionary and physiological processes. Determining the genetic mechanisms that drive growth in nematodes has been explored extensively particularly in *C. elegans* which provides a foundational tool kit for investigating and characterizing genes and their phenotypes in *C. inopinata.* Associations between physiological attributes such as body size and growth and body size and fecundity in *C. elegans* and *C. inopinata* are an exciting avenue of interest for comparative evolutionary and developmental study (Woodruff *et al.* 2019). Correlations with other traits have the potential to reveal novel candidates that influence development and have the potential to expand biomedical research. Body size is a measurable trait that is regulated by individual or multiple genes and complex signaling pathways (Zhang *et al.*

2024). Studying phenotypic mutants is common practice in biological research to uncover the gene(s) underlying a given phenotype of interest and its function (Alberts *et al*. 2002). Targeted mutagenesis is a popular procedure to observe changes in an organism as a result of the gene deletion in addition to comparative phenotypic studies.

Gene deletion through Crispr/Cas9 in *C. inopinata* is a feasible option

Many physiological and developmentally important *C. inopinata* genes and the roles they play are the subject of recent research. (Kawahara *et al.* 2023, Kanzaki *et al.* 2018, Woodruff *et al.* 2024) Crispr/Cas9 is an efficient and economical tool for gene editing in both C. elegans and non-model organisms (Kim *et al.,* Dickinson *et al*. 2020). This study demonstrates that Crispr/Cas9 is a tool that can be used to create knock-out mutations in *C. inopinata.* The gene Sp34_40141100 was successfully deleted after three attempts, following improvement in microinjection technique. Because the deletion did not produce the desired phenotype, this gene is not causative. While a causative lesion was not confirmed using Crispr/Cas9 in this study, this gene editing technology could be a useful tool in revealing cause and effect genes in this species in future studies.

Nearby orthologs were not found to harbor any obvious deleterious mutations in *nok3* homozygotes

Gene sequencing of known orthologs in mutant strains is a simple and effective way to rule out candidate genes. *cin-dpy-13 and cin-col-34* were selected for sequencing in WOU11 because they are the orthologs of *ce-dpy-13* and *ce-col-34* which cause a dumpy phenotype. They are also positioned within the area of interest that is believed to carry the *nok3* mutation. Here, sequence data alignments for the dumpy mutant strain did not show any difference between *cin-dpy-13* and *cin-col-34* with respect to wild type. Other genes within the area of interest can be identified in future studies for additional sequencing.

The *nok3* allele regulates body size

In this study, body measurements of adult NKZ35 worms show results consistent with prior body size comparisons between the average body length and width of adult hermaphroditic *C. elegans* (Cho *et al*. 2021). and the average body size length and width of adult female and male

wild type *C. inopinata* (Kanzaki *et al*. 2018). However, the body size measurements of WOU11 worms suggest *nok3* has a sex specific impact.

Previous research on adult *C. elegans* Dpy mutants found that a large number of known dumpy alleles tend to have an average body length of 676.49 μm. (Cho *et al*. 2021). The same study showed an average body width of adult *C. elegans* Dpy mutants to be 74.52 μm (Cho *et al*. 2021). Here, the average body length of adult female and male *C. inopinata* Dpy mutants is 1175.7 μm and 1068.1 μm, respectively (Fig. 6, Fig. 7, Fig. 10). The average female body width is 94 μm and the average male body width is 80.4 μm (Fig. 6, Fig. 7). Like the decrease in length and increase in width in *C. elegans* dumpy mutants, these size patterns are also seen in the female *C.* WOU11. However, the length differential between the adult male wild type *C. inopinata* and the adult male mutant is not statistically significant (Fig. 6, Fig. 7). Despite not displaying the shorter length, the difference in the body width of the adult male WOU11 and wild type is statistically significant (Fig. 7). The lack of a shortened body length in the male mutants is inconsistent with the traditional Dpy phenotype classification, while its wider width is consistent. The differences in length and width between the female and male mutants shown in this study is not unexpected. Female wild type *C. inopinata* are generally longer and wider than males (Kanzaki *et al.* 2018). When the worms presented with the *nok3* allele phenotype, the females still remained larger than the males. (Fig. 6, Fig. 7). This suggests that the body size sexual dimorphism seen in wild type is preserved in dumpy mutants. It should also be noted that as WOU11 females age they seem to approach the lengths of wild type females but remained wider than them throughout.

It is not possible based upon this study alone to draw a conclusion that a new mutant classification should exist for the wider-only male *C. inopinata* mutants. At least one study reported on a similar observation with the recessive mutant alleles at the *dpy-21* locus of *C. elegans*. In that study, the dumpy phenotype in hermaphrodite animals presented but not in male animals (Meneely and Wood 1984). A large sample size needs to be studied. A number of Dpy genes are implicated in the X-linked dosage compensation pathway. These genes function by regulating X chromosome transcript levels in females. When mutated, the expression of these X chromosome transcripts is excessive resulting in female exclusive lethality or a Dpy phenotype (Riddle *et al*. 1997). Given the *nok3* allele appears to still effect males, but only with regard to width, it may be the case that *nok3* increases X chromosome transcript expression only enough to

yield a larger width in males. In contrast, females having elevated expression of transcripts from both X chromosomes may be underlying the shorter length, larger width, and changes in the egg laying circuit.

nok3 does not affect the pace of growth for male WOU11 but does affect females

Body size in *C. inopinata* is associated with developmental rates (Woodruff *et al*. 2018). Prior studies have found that *C. inopinata* grows slowly, at about half the rate as *C. elegans* (Woodruff *et al*. 2019). Here, the data reflects no difference in when growth begins for NKZ35 and WOU11. Female NKZ35 grew more than female WOU11 females between 48 and 72 hours. Peak body length growth of the female NZK35 was reached during this interval, but WOU11 females appear to grow steadily across all intervals. While the body length growth of female NZK35 plateaued at 96 hours, in female WOU11 it did not. Male NKZ35 and male WOU11 body length grew uniformly relative to each other with both seemingly plateauing after 72 hours.

Between female and male WOU11, the females outpaced the males in terms of body length growth. This data suggests that *nok3* affects the growth pattern of length for females worms but not males. As for body width, female mutants displayed greater growth in width than female NKZ35 at all intervals. Male NKZ35 and male WOU11 body width grew at a similar pace with each other. This data suggests that *nok3* affects the growth pattern of width for females worms but not males. Because the gene underlying the *nok3* allele affects male growth patterns differently than the females, the gene may be pleiotropic and/or requires an additional protein from a recessive, x-linked gene to fully be expressed in the Dpy males. A similar phenomenon was seen with *ce-dpy*-21 which is X-linked in *C. elegans* and regulates other genes that affect body growth which are dose dependent (Sternberg *et al.* 2024). An RNAseq study and/ or a proteomic assay could shed light on the role of the protein mechanisms underlying this trend.

Fecundity dynamics

Body size is known to correlate with brood size and embryo laying dynamics in animals including nematodes. (Blanckenhorn 2000, Hagmayer *et al* 2018, Morland and Sorci 1998, Norton and Bronson 2006, Woodruff *et al*. 2018) As seen here, the *nok3* allele dramatically alters the laying behavior of *C. inopinata.* WOU11 lays every 48 hours whereas NKZ35 lays every 24 hours.

This behavior is consistent with observations made during the laboratory cultivation of WOU11, as it typically takes longer to reach sufficient population densities as they are reared. Within *C. elegans* there are many mutations that result in deficiencies in egg laying (Shafer 2005). However, there are no reports that indicate any given mutation yields a large, days long oscillation in the egg laying circuit as seen here. Further, no Dpy genes are known to cause such a striking effect (Shafer 2005). Given that Dpy phenotypes tend to be driven by collagen mutations it is unlikely that it is affecting neurotransmitter pathways involved in the egg laying circuit (Shafer 2005). However, mutations of collagen in the vulval cells of *C. elegans* can cause egg retention though not to this extent (Shafer 2005). Conversely, the heterochronic gene *lin-28* in *C. elegans* does have a strong effect on the egg laying circuit. However, this gene directly impacts total fecundity by trapping embryos within the gonad and reducing laying, but not the timing of laying (Choi and Ambrose 2019). Further *lin-28* does not present with a dumpy phenotype. It is possible that *nok3* is in a cis-regulatory element where it is affecting the expression of genes controlling egg formation and egg laying timing. Further exploration of how *nok3* may affect embryogenesis timing and/or the female gonad is needed to conclusively explain the observed phenomenon.

Dominance of the *nok3* allele is consistent with the expected Mendelian ratio

Similar to many of the previously identified Dpy mutations in *C. elegans*, the *nok3* allele is recessive. It did not have any observable impact on the phenotype of the heterozygous females and segregated in the expected manner. Due to the recessive nature of *nok3,* it is likely to be a loss of function allele. While *nok3* itself is not likely to be a collagen encoding gene, it may be the case the *nok3* protein product is implicated in a collagen related pathway. This is possible because of the high proportion of dumpy causing genes connected to collagen function. To determine where in the animal this is occurring a northern blot or RT-PCR of tissues rich in collagen such as the cuticle could be performed.

CONCLUSION

The objective of this study is to report on new findings to help understand the genetic basis for the divergent body size in *C. inopinata*. We wanted to discover new genes and alleles that result in body size variation in *C. inopinata* with the aim of describing how they evolve across nematode species. Established protocols were used to create and select a dumpy mutant body size allele in an effort to shed light on what happens to *C. inopinata* when body size genes are not functioning or missing. Experimental evidence shows that targeted mutagenesis with Crispr/Cas9 technology is feasible in *C. inopinata.* Here, a body size effect on the fitness-related traits of growth rate and the egg laying circuit was found and a sex-specific dynamic was observed. Further studies will need to be undertaken to identify the specific gene(s) that control body size in *C. inopinata* and the role those genetic mechanisms play in these fitness-related traits. Overall, the results presented add to the growing body of research that seeks to understand the evolution of body size in *Caenorhabditis* nematodes.

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