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Decreased Brood-Size of Starvation-Induced Postdauer Adults is Inherited Transgenerationally via HRDE-1

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Decreased Brood-Size of Starvation-Induced Postdauer Adults is Inherited Transgenerationally via HRDE-1

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Abstract:

Environmental stress experienced *in utero* or in early life has profound effects on the behavior and physiology of an adult animal. Interestingly, genome-wide association studies in humans have shown that early-life stress not only affects the exposed individual, but also their future generations. However, the molecular mechanisms of how early-life stress can impact physiology transgenerationally in the absence of the original stress are not well understood. To study the mechanisms and consequences of environmental stress on adult phenotypes, we utilize the life cycle of the model organism *Caenorhabditis elegans.* In favorable conditions, the life cycle of *C. elegans* includes 5 stages – larval stages L1, L2, L3, and L4, and the adult stage. However, when worms experience starvation, heat, or crowding (high pheromone levels) in early life, they enter a stress-resistant 'dauer' stage. Our lab has previously shown the postdauer adults that transiently passed through the dauer stage due to starvation (postdauers, PD_{Stv}) had a significantly smaller brood-size (fewer progeny) than the worms that bypassed the dauer stage (controls, CON_{Stv}). In contrast, the postdauer adults that had entered the dauer stage due to crowding (PD_{Phe}) had significantly larger brood-sizes than adults that bypassed the dauer stage (CON_{Phe}). Here, we show that the decreased brood-size phenotype of PD_{Stv} worms is inherited for at least four generations, even though the progeny of PD_{stv} animals have not experienced starvation or passed through dauer stage. In contrast, the increased brood-size phenotype of PD_{Phe} is not transgenerational. We also show that the *hrde-1* mutants that transiently passed through the dauer stage due to starvation lose the ability to inherit the brood-size phenotype transgenerationally -- only the animals that had experienced starvation directly show the brood-size phenotype similar to that of the wild-type animals. This indicates that HRDE-1, a nuclear Argonaute protein, is required for inheritance of brood-size phenotype transgenerationally.

Executive Summary:

Early life experience of a human is very critical to his or her development. It has been widely reported that the stress experienced in early life does not only affect the exposed individual but also his or her offspring. For example, females who had experienced nutritional starvation *in utero* during the Dutch famine of 1944-45 were born with and gave birth to children with significantly lower birthweights than the females that did not experience nutritional stress *in utero* [Lumey L. H. 1992]. Therefore, we are investigating how stress induced phenotypes can be inherited over generations even when the offspring of an affected animal hadn't experienced any stress. In this research project, we used the model organism *C. elegans* to understand how early life stress can impact the exposed animals as well as their offspring. We used *C. elegans* because of the similarities the animal has with humans and their ability to exist as a hermaphrodite (animals that are able to self-fertilize). Thus, all the offspring of hermaphrodite animals has identical genome, similar to that of their parent. This allows us to investigate the reasons for the changes in gene expression caused by environmental stresses instead of changes in the DNA sequences.

In favorable conditions, *C. elegans* goes thorough three different developmental categories: embryonic stages, larval stages (L1, L2, L3 and L4), and the adult stage. They are only able to reproduce when they reach the adult stage. However, when this animal is exposed to environmental stresses in early life (L1 and L2), they enter into a stress resistant stage during their development [Fielenbach, *et. al.* 2008]. During this stress resistant stage, also known as the dauer stage, they can survive for months without eating and reproducing. However, when the animals experience favorable conditions again, they re-enter the continuous life cycle to reproduce. It has previously been shown by the Hall lab that animals that pass through the dauer

stage because of environmental stresses show different gene expression profiles than the animals that had not entered the dauer stage [Hall *et al.* 2010; Ow *et al.,* 2018]. Additionally, there is a significant difference between the gene expression of animals that passes through the dauer stage due to starvation or crowding (sensed by high concentration of pheromone in the animal's environment) [Ow *et al.*, 2018]. This suggests that the changes in gene expression due to environmental stress are regulated differently based on the type of stress an animal experience.

In addition, the Hall lab has shown that the changes in gene expression between control and postdauer adults that experienced different environmental stresses correspond to changes in the progeny number of the animals. Animals that pass through the dauer stage due to starvation produce significantly fewer offspring than the animals that bypass the dauer stage [Ow *et al.* 2018]. On the other hand, the animals that pass through the dauer stage due to crowding produce significantly more offspring than the animals that bypass the dauer stage [Hall *et al.* 2010; Ow *et al.* 2018]. The total number of progeny an animal reproduces over its entire life cycle is referred as the brood-size of the animal. Therefore, we asked whether this stress-induced brood-size phenotype of *C. elegans* is being inherited over generations like the inheritance of lower birthweight phenotype in humans due to the Dutch famine of 1944-45.

By identifying the mechanism via which stress-specific phenotypes are being inherited in *C. elegans*, we can gain insight into how stress-induced phenotypes in humans may be passed over generations. For the brood-size assay, we cultured the animals in small petri dishes and counted the number of offspring each animal produced during their reproductive life cycle. After completing the brood-size assay with the offspring of the animals that passed through the dauer stage due to starvation, we observed the inheritance of the brood-size phenotype until F5

generation. However, we did not see the inheritance of the brood-size phenotype in the crowding condition.

Given that the brood-size phenotype is being inherited in the starvation condition, we wanted to identify the mechanism via which the phenotype is being propagated. We examined the brood-size of a mutant animal, *hrde-1*, that lacks the germline specific nuclear Argonaute protein called HRDE-1. This Argonaute protein shuttles small interfering RNA (siRNA) into the nucleus to cause gene silencing, thereby changing the gene expression of the animal. It has previously been shown that HRDE-1 plays a role in maintaining the memory of initial stress experienced by an animal to promote transgenerational gene silencing, thus promoting the expression of stress-induced phenotype transgenerationally [Buckley, *et. al.* 2012; Rechavi *et al*. 2014; Devanapally *et al.* 2015]. Therefore, we wanted to investigate whether HRDE-1 is required for the inheritance of the brood-size phenotype upon passage through the dauer stage because of starvation.

After conducting brood-size assay with *hrde-1* mutant, we did not see the decreased brood-size phenotype over generations like the wild-type animals. Only the parent generation of *hrde-1* mutants that had experienced stress and passed through the dauer stage showed the phenotype. This indicates that the HRDE-1 protein is required for inheritance of the brood-size at least until F5 generation in starvation condition, but not in the parent generation. Therefore, we believe that the decreased brood-size phenotype in the parent generation is regulated differently than later generations. It has been shown by the Hall lab that the Argonaute protein CSR-1, that plays a role in maintaining and establishing euchromatic chromatin stage in *C. elegans*, is required for the expression of the brood-size phenotype in both starvation and pheromone condition [Ow *et al.* 2018]. Thus, we believe that the CSR-1 RNAi pathway modulates the

expression of the decreased brood-size phenotype in the parent generation of PD_{stv} animals whereas the HRDE-1 RNAi pathway modulates the decreased brood-size phenotype in later generations (F1 through F5).

The findings from this project are very important toward our understanding of how certain phenotypes, induced by environmental stresses, can be inherited over generations in humans. Since we have shown the nuclear Argonaute HRDE-1 plays a role in transgenerational inheritance of reproductive plasticity, it may be possible for the homolog of this protein (Piwi Argonaute protein family including AGO1) to cause transgenerational inheritance of stress induce phenotype in human. Therefore, it may be possible to prevent the propagation of stress-induced diseases caused by epigenetic mechanisms by targeting such proteins. Similar to HRDE-1, Piwi proteins binds to piRNA in the nucleus of germ cells [Juliano, *et al.,* 2011; Klenov, *et al.,* 2011; Siomi *et al.,* 2011], and participate in transposon (a DNA sequences can move from one location to another on the genome) silencing [Thomson & Lin, 2009]. The absence of Piwi proteins in mammal has shown to cause defect in germ cell development, thus causing sterility [Carmell, *et al.,* 2007]. Therefore, it is possible that transgenerational inheritance of stress-induced phenotype could be modulated via Argonaute/Piwi proteins family in human as well. If we are able to successfully understand the entire mechanism of transgenerational inheritance of stress-induced phenotypes, we will be able to stop the propagation of many diseases caused by environmental stresses.

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Advice for Future Students:

One of the best decisions I have made as an undergraduate was joining a research lab at the start of my sophomore year. I started searching for different laboratories during the second semester of my freshman year. I started off by visiting the office hours of my Introductory Biology professor, Dr. Wiles, who advised me on approaching different PIs (Principal Investigators). One of the best pieces of advice he gave me was reading through the journal articles published by different PIs. By reading through the published papers, I was able to understand what I like about different research labs and where I wanted to be for the next few years. After emailing more than ten professors and speaking to a few PIs, I decided to join the Hall lab at Syracuse University. One of the secrets to my success in the Hall lab is my determination to stay on top even when almost every experiment that I conducted failed. I continued to work hard and communicate with my PI about the problems I was having as an undergraduate. Our constant communication has allowed me to start a project that allowed flexibility, and thus allowed me to discover something new and interesting.

So, the best you can do is start searching for a research lab that you can join from your freshman year. Starting early will help you find a lab that suits you best. Additionally, the earlier you join a lab, the more time you have to collect data on your project; and the more data you have, the closer you are to getting your work published in a journal article. Also, please try not to switch laboratories as much because it will slow down your progress. Therefore, evaluate everything before you join a research lab. Finally, you need to love what you do. If you think you are not having fun with your research, then do something else. After all, college is the time you figure out what you want to do, and how you want to spend the rest of your life.

Project Body

Introduction:

We are living in a time where pollution, humanitarian crises and malnutrition are major issues people face around the world [Smith, n.d]. Evidence suggests that these environmental cues do not only affect the health of people who are exposed to these conditions, but also their offspring. For example, genome-wide association studies have shown that the good nutrition of an ancestor is associated with poor survival of the proband [Kaati, *et al.* 2007]. This lab has confirmed that ancestor's nutrition is the main driving force of intergenerational responses to stress [Kaati, *et al.* 2007]. There is also evidence of transgenerational inheritance of cardiovascular diseases in human due to excess food availability during the slow growth period before puberty [Kaati *et al.* 2002]. Although we can see the health effects these environmental stresses have on people, there is very little known about the mechanisms regulating the stress-induced phenotypes and how they are being inherited inter-generationally. Inheritance of epigenetic marks regulating gene expression is thought to account for the changes in offspring's phenotype; however, we still do not know as much about the mechanism by which epigenetic changes are being established and inherited.

In order to investigate the mechanisms of how environmental stress impacts adult phenotype and their offspring, our lab is examining inheritance of reproductive plasticity using the model organism *Caenorhabditis elegans (C. elegans)*. We use *C. elegans* as a model organism because they have a short life span, and all the progeny of the self-fertilizing hermaphrodites are genetically identical. Also, at least 38% of the protein-coding genes in *C. elegans* have orthologues in human [Shaye and Greenwald, 2011] and 40% of the disease-

causing genes in human have orthologue in *C. elegans* [Culetto and Satelle, 2000]. Therefore, by studying *C. elegans*, we can learn information about different biological pathways in humans.

Under favorable conditions, the life cycle of *C. elegans* includes the embryonic stages, four larval stages (L1, L2, L3 and L4), and the reproductive adult stage (controls, CON). However, previous work has shown that worms that experience starvation, heat, and/or crowding (detected as high pheromone concentration) go into a stress-resistant larval stage called the 'dauer' stage [Fielenbach, *et. al.* 2008]. In the dauer stage, a worm can survive a few months in unsuitable conditions without eating and reproducing, and when brought back to ideal growth conditions, they resume their continuous life cycle as L4 larvae (postdauers, PD) [Fielenbach, *et. al.* 2008]. In the laboratory, we can induce worms to enter the dauer stage by exposing them to various environmental stresses such as starvation, heat, or pheromone (signal for crowding).

In previous work, the Hall lab showed that *C. elegans* maintains a cellular memory of its developmental history through gene expression and chromatin remodeling in the postdauer animals compared to controls [Hall, *et. al.* 2010]. Furthermore, they showed "seasaw" expression of 512 genes in the PD_{stv} and PD_{phe} animals whereby 249 genes that are upregulated in PD_{phe} are downregulated in PD_{stv} and the other 263 genes that are upregulated in PD_{stv} are downregulated in PD_{phe}, and the expression of these genes are also significantly different from the control animals (CON_{stv} & CON_{phe}) [Ow, *et al.*, 2018]. One of the consequences of this differential gene expression is the change in the brood-size of the PD_{stv} and PD_{phe} animals: PD_{stv} animals produces significantly fewer progeny (smaller brood-size) than the CON_{stv} [Ow, *et al.* 2018], whereas PD_{phe} animals produce significantly more progeny (larger brood-size) than the CON_{phe} animals [Hall, *et al.* 2010; Ow, *et al.* 2018].

Here, we show that the brood-size phenotype of PD_{stv} animals is transgenerational: progeny of PD_{stv} animals produce significantly fewer offspring compared to the progeny of CON_{stv} animals for at least four generations. Unlike the starvation condition, the progeny of the PD_{phe} animals do not inherit the brood-size phenotype transgenerationally. We also show here that the nuclear Argonaute protein, HRDE-1, previously shown to be responsible for transgenerational gene silencing in *C. elegans* to promote germline immortality [Buckley, *et. al.* 2012], plays a role in determining whether PD_{stv} animals inherit the brood-size phenotype transgenerationally. In *hrde-1* mutants, the decreased brood-size phenotype of PD_{stv} animals is not inherited after the parent generation. Therefore, we propose the epigenetic changes in PD_{stv} animals are passed onto future generations by HRDE-1 activity. The outcome of this HRDE-1 induced epigenetic change is a smaller brood size, as noted for the progeny of the PD_{stv} animal, over four generations. It is possible that the interaction between HRDE-1 and siRNA increases following starvation and passage through the dauer stage [Ow *et al.* 2018], thus allowing the germ cells to retain the memory of initial stress. Additionally, the production of new HRDE-1 specific siRNA in the germ line following starvation could cause transgenerational inheritance.

Materials and Methods:

Laboratory Condition:

Wild-type *N2* Bristol strain of *C. elegans* was cultured in 20^{o}C with enough food (*E. coli*) in normal NGM (nematode growth media) plates to prevent the worms from being exposed to any unintended environmental stress. The *hrde-1* strain of *C. elegans* was cultured in 15^{o}C given that the worms of this strain are likely to become sterile over a few generations if they were

cultured at a higher temperature (i.e. 20ºC). However, during the brood-size assay, worms of both *hrde-1* and *N2* strains were cultured at 20ºC.

Formation of PD worms:

In order to form PD_{stv} animals, worms on NGM plates were left without food for at least 10 days. When worms entered the dauer stage due the lack of food, 1 ml M9 Buffer (3 g KH2PO4, 6 g Na2HPO4, 5 g NaCl, 1 mL 1 M MgSO4, H2O to 1 liter) was used to isolate the worms from the NGM plate and put into a centrifuge tube. The suspended worms were centrifuged at 3,000 rpm for 30 seconds. After centrifugation, the supernatant (M9 buffer) was removed and 1 mL of 1% SDS (Sodium Dodecyl Sulfate) was added to the worm pellet. Then, the tube was put to rotate in a tube rotator for 30 minutes, thus yielding enough time for SDS to lyse the non-dauer worms in the tube. Dauer worms are able to survive in SDS due to the thickened cuticle and buccal plug which isolate the animal from its environment [Cassada and Russell, 1975]. After 30 minutes, the tube was centrifuged again at 3,000 rpm for 30 seconds to pellet the worms. The supernatant (1% SDS) was removed and M9 buffer was added to the pellet. The worms with the M9 buffer were transferred to a NGM plate with enough food for the surviving dauer animals to return to L4 stage (PD_{stv}).

To recover PD_{phe}, wild-type *N2* worms were picked at the L4-stage and transferred to a 60mm NGM plate with enough bacteria (*E. coli*) to continue their life cycle. After 24 hours, the adults from the NGM plate were picked and soaked in Streptomycin to kill any bacteria that were attached to their body. The worms were then transferred to two 35mm experimental petri dishes -- once of which contained crude pheromone, usually secreted by *C. elegans* in response to crowing in their environment, and the other plate contained water (H_2O). Additionally, the petri

dishes, both pheromone and H_2O plates, contained heat-killed bacteria given that live bacteria prevent dauer formation [Neal, *et al.* 2007]. The adults were left for 24 hours to lay eggs, after which they were removed from the plates. The eggs in the plate that contained pheromone were left untouched for 6 - 7 days to allow the worms to enter the dauer stage. Approximately 48 hours after the dauer formation, the worms were transferred into a 60mm NGM plate with live *E. coli* for them return to L4-stage (PD_{phe}). For the plate that contained water, L1/L2 larvae were transferred into a 60mm NGM plate with live *E. coli* to continue their life cycle. Once the larvae had reached the L4-stage (CON_{phe}), they were transferred onto 35mm plates for the brood-size assay.

The dauer formation plates were made using a solution of Nobel Agar, NaCl, filtered H_2O, $CaCl_2$, $HgSO_4$, K^+ and cholesterol. To prepare the solution, a solution of NaCl, Nobel Agar and H_2O is autoclaved for an hour after which $CaCl_2$, $HgSO_4$, K^+ and cholesterol are added to the mixture. Once the solution is made, it is transferred to 35mm petri dishes with a drop of either pheromone or water. Finally, the plates are left to solidify at 25^oC for at least 12 hours.

Brood-size Assay:

The brood-size assay was carried out by manually counting the surviving progeny of both PD and CON worms. Given that *C. elegans* do not lay eggs before becoming adults, individual worms of L4 stage (parent generation) were transferred from a 60mm NGM plate to 10 - 12 different 35mm NGM plates -- each plate containing one L4-CON or PD worms. The worms from these 35mm NGM plates were moved into new 35 mm plates each day for 5-6 days to lay eggs until they finish laying eggs. The surviving progeny in these 35 mm plates were counted to determine the brood-sizes of parent generation worms.

To determine inheritance of the brood-size phenotype over several generations, individual progeny from each of the 35mm plates of parent generation were picked randomly and transferred to another 10 - 12 plates. Similar to the parent generation, these newly transferred worms, F1 generation, were moved into new plates for 5 - 6 days until they had finished laying eggs. Similar procedure was used for further generations (i.e. F2, F3, etc.).

To determine whether HRDE-1 is responsible for the inheritance of the brood-size phenotype, a brood-size assay with *hrde-1* mutants was conducted alongside wild-type animals. Therefore, each trial of the brood-size assay for *hrde-1* mutant involved CON_{stv}, PD_{stv}, *hrde-1* mutants that did not pass through the dauer stage (*hrde-1* CON_{stv}), and *hrde-1* mutants that had transiently passed through the dauer stage (*hrde-1* PD_{stv}).

Results:

Early-Life Starvation has a Transgenerational Effect on the Brood-size of C. elegans.

Given that the brood-sizes of the PD_{stv} and CON_{stv} animals differ significantly, we wanted to test whether this phenotype is being inherited in future generations. To determine whether the brood-size phenotype is being inherited transgenerationally after early-life starvation, we conducted brood-size assays with the animals that have passed through the dauer stage (PD_{stv}) due to starvation and have not passed through the dauer stage (CON_{stv}). Then, we isolated the progeny of the PD_{stv} and CON_{stv} animals (F1 generation) to determine whether the brood-size phenotype is inherited. As shown in Fig. 1, PD_{stv} animals had significantly fewer progeny than the CON_{stv} animals as observed previously [Ow, *et al.* 2018]. Interestingly, the progeny of the PD_{stv} animals (F1 generation) showed similar phenotype even though these animals did not experience any starvation or pass through the dauer stage themselves. Next, we

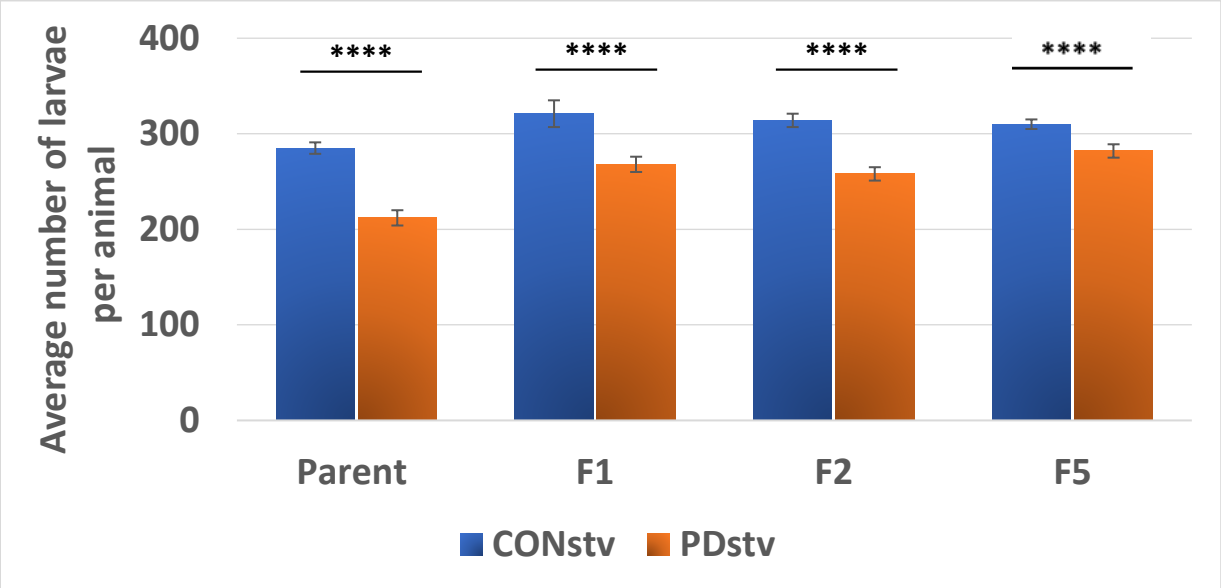

Fig. 1: Brood-size assay of PD_{stv} and CON_{stv} adults and their progeny.
The worms that had passed through the dauer stage due to starvation (parent-generation) had significantly fewer progeny than the control animals. The progeny of PD_{stv} animals inherit the reduced progeny phenotype from their parent in the F1- F5 generations.
At least 25 animals per experimental group were used over three independent trials for each generation. Student t-test *p*-values: ****p<0.0001.

also carried out the brood-size assay with the progeny of each generation up to the F5 generation to determine the extent of inheritance. We also observed the decreased brood-size phenotype in both F2 and F5 generations of the PD_{stv} animals. This result indicate that the brood-size phenotype is inherited for at least 4 generations when animals transiently pass through the dauer stage due to starvation stress.

The Brood-size Phenotype is Not Inherited in Pheromone Condition.

To determine whether the increased brood-size phenotype of PD_{phe} animals is inherited transgenerationally, we conducted brood-size assays with the animals that had transiently passed through the dauer stage due to pheromone (PD_{phe}) and the animals that had not passed through the dauer stage (CON_{phe}). Interestingly, the F1 generation of PD_{phe} animals did not show the increased brood-size phenotype. This result indicates that the brood-size phenotype is not inherited transgenerationally in the pheromone condition. However, there was only one trial

conducted for the pheromone condition and we are currently in the process of performing more trails.

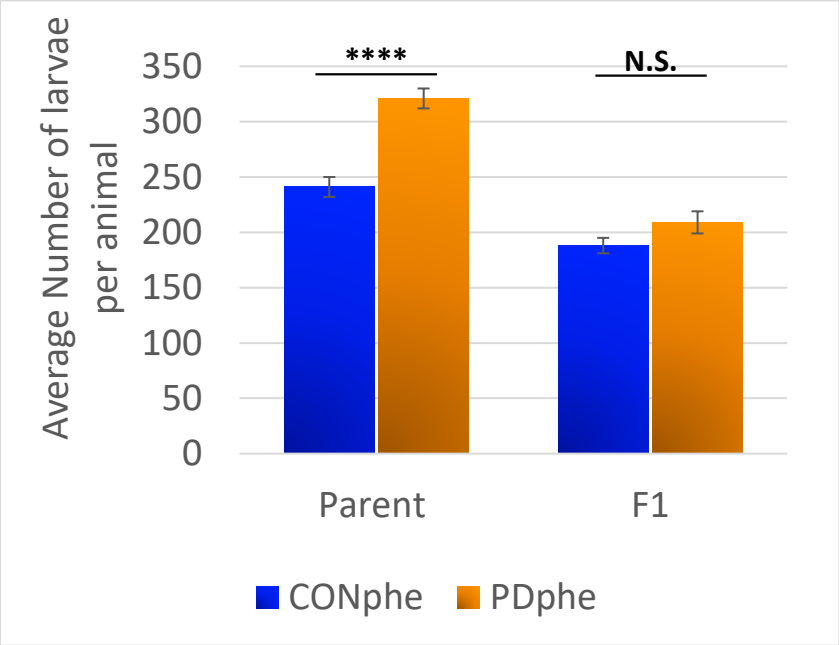

Fig. 2: The increased brood-size phenotype of PD_{phe} animals is not inherited transgenerationally.

The data represents one trial with at least 9 animals per experimental group. Student t-test *p*-values: ****$P<0.0001$ & N.S. = Not Significant

HRDE-1 is Required for Transgenerational Inheritance of the Brood-size Phenotype in Starvation Condition.

Given that we observed transgenerational inheritance of the decreased brood-size phenotype in the starvation condition, we used *hrde-1* mutants to determine whether this protein is required for the inheritance of the phenotype. HRDE-1 is a nuclear Argonaute protein that interacts with siRNA (small interfering RNA) in the nucleus of germ cells in *C. elegans* to promote trimethylation at histone H3 at Lys9 (H3K9me3) at target gene loci [Buckley, *et al.* 2012]. For the brood-size assay with the *hrde-1* mutant, we used four different experimental groups: 1. Wild-type CON_{stv}, 2. Wild-type PD_{stv}, 3. *hrde-1* mutants that bypassed the dauer stage (*hrde-1* CON_{stv}) and 4. *hrde-1* mutants that passed through the dauer stage (*hrde-1* PD_{stv}). As shown in Fig. 3, the parent generation of *hrde-1* mutants that was exposed to starvation stress (*hrde-1* PD_{stv}) showed the decreased brood-size phenotype as did the wild-type animals.

However, the *hrde-1* F1 generation did not show any differences in their progeny number between the control and postdauer animals, unlike the wild-type F1 generation. This result suggests that HRDE-1 is not required for the brood-size phenotype in the parent generation but is required for transgenerational inheritance of the decreased brood-size phenotype in starvation conditions.

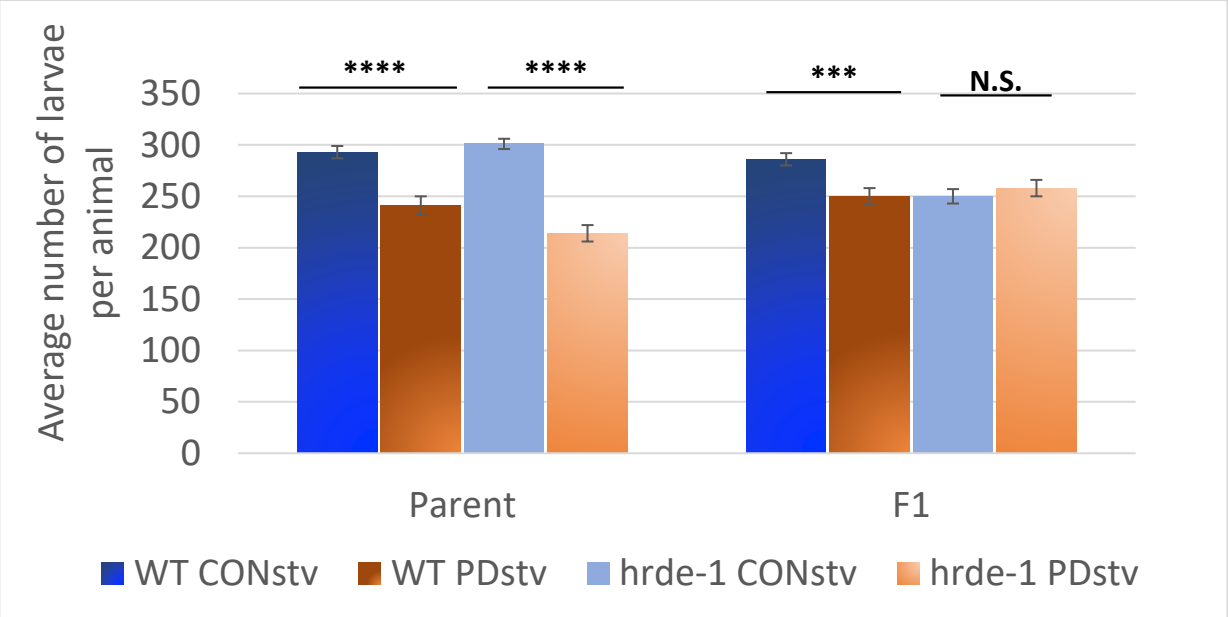

Fig. 3: HRDE-1 is required for transgenerational inheritance of the brood-size phenotype in starvation condition.
Wild-type animals exhibit the decreased brood-size phenotype upon passage through the dauer stage and in F1 generation. However, the *hrde-1* mutant only exhibited the decreased brood-size phenotype in the parent generation, and not in the F1 generation.
At least 39 animals per experimental groups were used over three independent trials for each generation.
Student t-test *p*-values: ***$p<0.001$, ****$p<0.0001$ & N.S. = Not Significant

Discussion:

To understand whether environmental stresses such as starvation and crowding have transgenerational effects, we utilized the brood-size assay to compare the number of larvae produced by CON and PD animals. Our results show that the number of progeny produced by PD_{stv} animals is significantly fewer than the number of progeny produced by CON_{stv} animals.

This result is consistent with the Hall lab's finding that the PD_{stv} animals produces significantly fewer number of progeny than the CON_{stv} animals [Ow, *et al.* 2018]. Next, we used the progeny of the PD_{stv} and CON_{stv} animals to determine whether the decreased brood-size phenotype is inherited in future generations. Our results show that the progeny of PD_{stv} animals express the decreased brood-size phenotype similar to that of their parents, and this phenotype is inherited for at least four generations.

However, when we carried out the brood-size assay with PD_{phe} animals, we did not see any inheritance of the increased brood-size phenotype. Only the parent generation that had experienced stress due to pheromone showed the increased brood-size phenotype. This result is consistent with the findings of the Hall lab [Hall *et al.* 2010]. Additionally, we were only able to carry out one trial, therefore more trials need to be done to make a reliable conclusion about the inheritance of the increased brood-size in pheromone (crowding) condition.

To investigate how the brood-size phenotype is being inherited in the starvation condition, we used *hrde-1* mutants to determine whether HRDE-1, a nuclear Argonaute protein, is required for the inheritance. It has previously been shown that HRDE-1 promotes gene silencing via RNAi pathways transgenerationally, thus allowing the germ-cells to retain a memory of the stress experienced by the parent generation [Buckley *et al.,* 2012; Rechavi *et al.,* 2014]. Our results with *hrde-1* mutants confirm our hypothesis that HRDE-1 is required for the inheritance of the decreased brood-size phenotype induced by passage though the dauer stage because of starvation.

Our findings are consistent with other studies investigating transgenerational inheritance of phenotypes after starvation. For example, Rechavi *et al.* showed that the longevity phenotype of the animals that had undergone a period starvation and arrest during L1-stage was inherited

for at least three generations [Rechavi *et al.* 2014]. They have also shown that the changes in heritable siRNA can persist over generations; these siRNAs may interact with HRDE-1 to promote transgenerational gene silencing, thus causing extended longevity for the descendants of L1 starved animals [Rechavi *et al*. 2014]. It is suggested that HRDE-1 is required for the maintenance of transgenerational gene silencing while mobile RNA importer SID-1 is required for the initiation of inherited silencing in *C. elegans* [Devanapally *et al.* 2015]. Therefore, we are currently investigating whether SID-1 plays a role in promoting transgenerational inheritance of the brood-size phenotype in starvation condition. Given that SID-1 is a dsRNA importer in germ cells [Devanapally *et al.* 2015], we hypothesize that the *sid-1* mutants will not be able to inherit the brood-size phenotype and will show similar phenotype as the *hrde-1* mutants. This result would confirm that the dsRNA is being imported into the germline via SID-1 and then interacts with HRDE-1 to promote transgenerational gene silencing. Furthermore, transgenerational inheritance of metabolic disorder due to diet-induced parental obesity in mice has shown that microRNA plays a crucial role in determining how certain genes are expressed transgenerationally [Fullston *et al.* 2013]. This result validates the hypothesis that the RNAi pathways of mammals and nematodes like *C. elegans* are similar, thus indicating that the RNAi pathways could be a potential target for preventing transmission of epigenetic diseases in human.

Therefore, we propose that HRDE-1 helps the germ cells of the PD_{stv} animals retain the memory of starvation, which is passed through generations to induce the decreased brood-size phenotype even when an animal is not experiencing any stress (Fig. 4). As suggested by Ow *et al.,* an unknown endocrine signal from the functional germline of PD_{stv} animals may be responsible for the distinct phenotype in the starvation-induced postdauer animals [Ow *et al.* 2018]. We hypothesize that this signal not only causes a distinct phenotype in PD_{stv} animals, but

it also promotes HRDE-1 interaction with siRNA in the nucleus of germ cells, thus causing transgenerational gene silencing. As a result, the progeny of PD_{stv} animals retain the memory of the stress experienced by the parent generation and express the decreased brood-size phenotype.

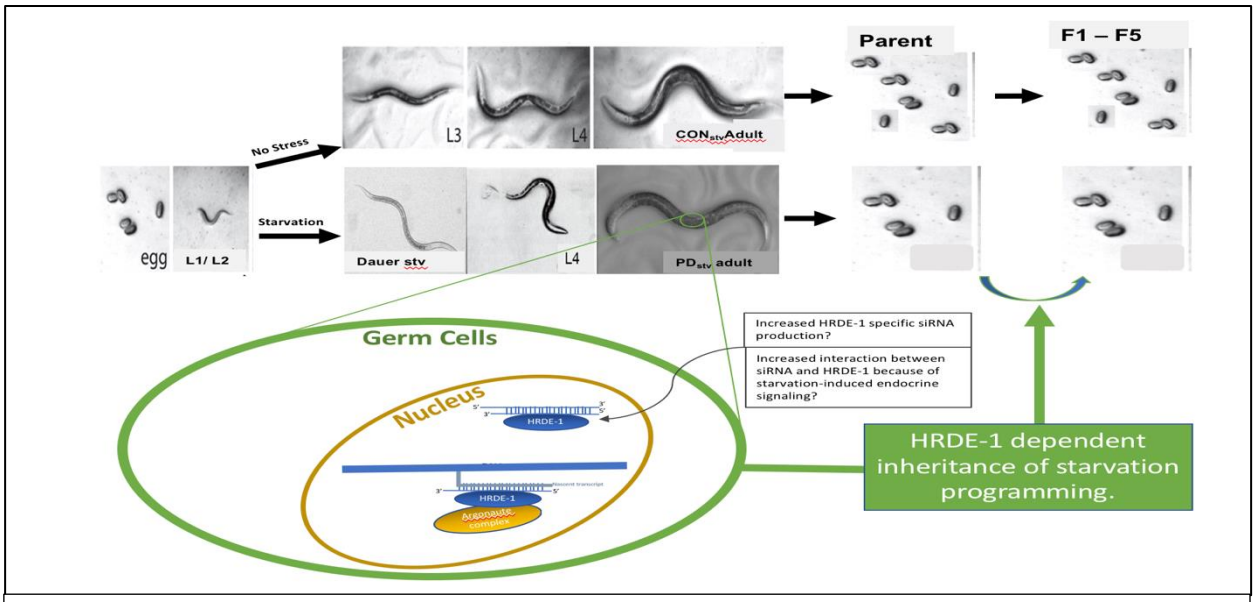

Fig. 4: HRDE-1 is required for the inheritance of the decreased brood-size phenotype in PD_{stv} F1 through F5 generations. See text for details.

This finding is very important toward our understanding of how certain stress-induced phenotypes are inherited transgenerationally in humans. We believe that the stress experienced in early life sends the stress signals to the germ cells which cause gene silencing transgenerationally. It is possible for similar Argonaute proteins of the Piwi family to be responsible for the maintenance of stress memory in humans as we have shown in this project. If we are able to completely understand the mechanism by which certain phenotypes can be inherited over generations in humans, it will be possible to prevent the inheritance of diseases caused by early life stress in human. Therefore, more research has to be done on how stress signals are sent to the germ cells and how proteins like HRDE-1 help the germ cells to retain the memory of ancestors' early life experiences.

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