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Transactivation and Mitochondrial Activity are Affected by High Temperature in *C. elegans* Sperm

A Thesis Presented

by

Jackie Rose McVay

To the Keck Science Department

Of Claremont McKenna, Pitzer, and Scripps Colleges

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Land Acknowledgement

The author of this thesis acknowledges that the research conducted in this thesis at Marquette University was conducted on the original homeland of the Potawatomi, Ho-Chunk, and Menominee Nations. The thesis is presented to Scripps College, on the original homeland of the Kizh and Tongva Nations. Please take a moment for gratitude and recognition for the indigenous peoples whose land you reside on. Colonialism is an ongoing process we must separate ourselves from and as guests taking up space on indigenous land we must do our part to support Indigenous Communities and land stewardship.

Abstract

Sexual reproduction has a conserved flaw in that it is temperature sensitive. Exposure to high temperature leads to male infertility, but little is known about the underlying mechanism. Understanding these mechanisms is important for agriculture and reproductive medicine. Using *C. elegans*, we investigated two potential aspects of male fertility that may be affected by high temperature conditions: activation of sperm by seminal fluid and sperm mitochondrial activity. There are two pathways for sperm activation in *C. elegans*: the SPE-8 pathway in hermaphrodites and the TRY-5 pathway in male seminal fluid. Hermaphrodite sperm with a mutation in *spe-8* can still be activated by TRY-5 in male seminal fluid, a process called transactivation. Preliminary data indicated that the function of the TRY-5 transactivation pathway was significantly reduced when males were exposed to high temperature. Second, we investigated if high temperature affects the abundance of sperm mitochondria. We observed a significant change in fluorescence intensity reflecting functioning mitochondria when sperm were exposed to 27°C. Our data suggest that both of our tested mechanisms may contribute to male infertility at high temperature.

Introduction

Sexual reproduction is ideal for species to produce unique, recombinant offspring that make populations more resilient to disease and environmental changes. However, there is a conserved flaw across sexually reproducing species: sexual reproduction is temperature sensitive, with dramatic drops in fertility to sterility outside of a defined temperature range (Müller and Rieu 2016; Rohmer et al 2011; Wang et. al 2007; Wolfenson et. al 2000). With rising global temperatures, it is vital to understand the mechanisms underlying the temperature

sensitivity of reproduction for agriculture and animal breeding (Wolfenson et. al 2000). One species vulnerable to rising temperatures is the nematode *Caenorhabditis elegans* (Harvey and Viney 2007; Petrella 2014; Poullet 2015; Prasad, 2011). *C. elegans* are an important decomposer of the bacteria that feed on fruits and vegetables (Kiontke et. al 2011). *C. elegans* are found throughout the globe with genetic variation between strains that is not linked to geographical isolation (Dolgin et. al 2007). *C. elegans* has a defined range of permissive temperatures for fertility; it is fertile between 20-26℃ but sterile at 27℃ (Begasse 2015; Petrella 2014; Poullet 2015). However, amongst different wild type *C. elegans* strains there is some variation in fertility at high temperature; some strains, like LKC34, irreversibly lose fertility with exposure to high temperature and some, like JU1171, can recover partial fertility when returned to lower temperatures (Petrella 2014). Unlike *Drosophila* and the related nematode *C. briggsae*, *C. elegans*' fertility response at high temperature is not related to the location from which strains have been isolated (Harvey and Viney 2007; Petrella 2014). Multiple mechanisms appear responsible for the temperature sensitivity of sexual reproduction, but the specific molecular pathways involved remain unknown (Begasse 2015; Petrella 2014; Poullet 2015; Nett et. al 2019).

C. elegans is an androdiecious species; hermaphrodites produce both eggs and sperm, while males produce sperm and mate with hermaphrodites to fertilize oocytes. In both hermaphrodites and males, sperm must be activated to become motile and fertilize an oocyte (Ellis and Stanfield 2014; L'Hernault 2009; Smith and Stanfield 2011; Ward and Shakes 1989). Hermaphrodites and males use sex specific sperm activation pathways (L'Hernault 2009; Smith and Stanfield 2011; Ward and Shakes 1989). Hermaphrodites use the SPE-8 pathway within the spermatheca (L'Hernault et. al, 1988) and males use the TRY-5 pathway released in the seminal

fluid (Smith and Stanfield 2011). *spe-8* mutant hermaphrodites are self-sterile, while *spe-8* males can still produce cross progeny (Nishimura and L'Hernault 2012; Ward and Shakes 1989). The SPE-8 pathway is mediated by zinc as a second messenger that signals sperm activation (Zhao et. al 2018). Exposure to zinc *in vitro* can activate sperm that have been isolated from males or *spe-8* mutant hermaphrodites (Liu et. al 2013; Nett et. al 2019). *In vitro* experiments show that sperm activation by zinc is moderately decreased in sperm from males raised at high temperature (Nett et. al 2019). TRY-5 is a serine protease found in male seminal fluid that serves as an extracellular activator (Smith and Stanfield 2011). TRY-5 is the only known protein in *C. elegans* seminal fluid and thus characterizes the seminal fluid activation pathway but additional proteases or downstream signaling components may be involved ((Stanfield and Villeneuve 2006; Smith and Stanfield 2011). *try-5* mutant males retain fertility because *try-5* mutant sperm can still be activated by hermaphrodite SPE proteins when released in the spermatheca (Smith and Stanfield 2011). Therefore, TRY-5 is not required for sperm activation but serves a redundant, extracellular role in signaling the initiation of male sperm activation (Smith and Stanfield 2011). The TRY-5 protease can also signal activation in hermaphrodite sperm. Exposure to TRY-5 in male seminal fluid leads to activation of the hermaphrodite sperm, a process called transactivation. TRY-5 activation is likely a remnant of when *C. elegans* was a gonochoristic species, and likely remains a redundant pathway to increase the number of both active male and hermaphrodite sperm (Smith and Stanfield 2011). Transactivation through mating in hermaphrodites that are mutant for the SPE-8 pathway results in a mix of hermaphrodite self-progeny and male fertilized cross-progeny (Smith and Stanfield 2011; Ward and Shakes 1989). Pronase E, a general protease, mimics the protease activity of TRY-5 (Stanfield et. al, 2011; Ward and Shakes 1989). *In vitro* experiments show that the level of sperm

activated by Pronase-treated sperm from males raised at high temperature is not decreased compared to that of sperm from males raised at lower temperatures (Nett et. al 2019). This suggests that sperm exposed to high temperature can still respond to the TRY-5 protease. However, it remains unknown how high temperature affects TRY-5 potency in male seminal fluid and transactivation.

After activation, motility is necessary for fertilization in *C. elegans* because spermatozoa can be swept out of the spermatheca and into the uterus by passing oocytes (L'Hernault et. al 1988). High temperature is known to affect sperm motility in a wide variety of mammals, including boars, rabbits, and mice (Cameron and Blackshaw 1980; Maya-Soriano et. al 2015; Sabés-Alsina et. al 2016; Wechalekar et. al 2016). In rabbits, short exposure to hyperthermic conditions largely dampens sperm metabolic activity and motility (Sabés-Alsina et. al 2016). Motile sperm have higher mitochondrial activity and oxidative phosphorylation is pivotal for sperm activity (Guo et. al, 2017). Heat stress affects sperm motility through modulating mitochondrial activity and ATP synthesis yield (Gong et. al, 2017). Heat shock reduces mitochondrial activity and membrane potential in bovine oocytes, but addition of coenzyme Q10 rescues mitochondrial activity (Roth 2018). Heat stressing mitochondria also leads to the production of reactive oxygen species, damaging the respiratory chain and inhibiting ATP synthesis (Slimen et. al 2014; Zhao et. al 2006). It remains unknown if heat stress affects the abundance or function of mitochondria in *C. elegans* sperm.

Here, we characterized two unstudied mechanisms underlying male sterility at high temperature in *C. elegans*: the TRY-5 activation pathway in male seminal fluid and mitochondrial activity. Crossing *spe-8* hermaphrodites with a fatter appearance called the dumpy phenotype to wildtype males allowed us to quantify the number of self-progeny fertilized by

TRY-5 transactivated sperm. At high temperature there was a significant reduction in the potency of seminal fluid transactivation. In a second set of experiments, we used MitoTracker Red CMXRos to stain functioning sperm mitochondria in worms raised at different temperature conditions and measured the fluorescence intensity, which correlates to mitochondrial activity. There were significant differences in sperm mitochondrial activity that varied by strain. Together, this work suggests two potential mechanisms involved in fertility loss due to high temperature in *C. elegans* males.

Methods

Strains used

Caenorhabditis elegans were maintained using standard procedures at 20°C on AMA1004 *Escherichia coli*-spotted NGM plates. Male strains were maintained by continually crossing males and hermaphrodites of the same genotype at 20°C. All strains used, JU1171, LKC34, N2, AV125 *spe-8(hc40); dpy-4(e1166)* and BA524 *fer-1(hc1); him-5(e1490,)* were obtained from the *Caenorhabditis* Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). The location of the three wild type strains was as follows: JU1171 from Concepción, Chile; LKC34, from Madagascar; and N2, from Bristol, UK.

Temperature Treatments

Three temperature treatments were used in this study. (1) Continuous exposure to 20°C: experiments were performed on strains maintained continuously at 20°C. (2) Continuous exposure to 27°C: P0 males and hermaphrodites were up-shifted to 27°C at the L4 stage and experiments were done on F1 males that had experienced their entire lifespan at 27°C. (3) Up-shift from 20°C to 27°C: males were developed at 20°C until the L4 larval stage and then up-

shifted to 27°C (in the absence of hermaphrodites), and experiments were conducted after 24 hours at 27°C.

TRY-5 Transactivation Crosses

L4 males and hermaphrodites were selected and isolated from each other for 24 hours. Then one young adult male and one young adult hermaphrodite were placed together on a plate with 15 µl *E. coli*, to encourage mating, and allowed to mate and deposit embryos for 24 hours after which the P0 animals were removed. The upshifted pairs were moved to a 27°C incubator, while the control pairs were kept at 20°C. F1 embryos were allowed to grow for three days. The F1s were then counted and the hermaphrodites were visually assessed for carrying the dumpy phenotype. Each worm was removed from the plate after it was counted and scored.

Mitochondrial Fluorescence Assay

L4 males were isolated on AMA1004 plates without hermaphrodites and allowed to age for ~24 h. On the day of experimentation, males were transferred to plates with 15 µl E. coli and 50 µl of 0.05 mmol l-1 MitoTracker Red CMXRos (Fisher Scientific M7512) in 1× M9 buffer was dispensed onto the food lawn on the plate. After males were allowed to feed for 4 hours, they were then placed in a well glass plate filled with M9 buffer twice to remove residual food stained with MitoTracker Red. Washed males were transferred into 15 µl dissection buffer (11% 10X Egg Buffer, 10% 10 mM levamisole, 2.5% of 20% tween 20 solution, 76.5% Deionized water) on an 18 x 18 coverslip. A syringe was used to bisect males and release sperm onto the coverslip. Then 15 µl fixation buffer (11% 10X Egg Buffer, 2.5% 20% tween 20, 5.4% Formaldehyde, 81.1% Deionized water) was added to the dissection buffer and mixed by pipetting up and down. 15 µl of solution was then removed from the coverslip, taking care not to disturb the submerged males. A room-temperature gelatin-coated poly-L-lysine (GCP) slides (ddH2O, gelatin,

chromium potassium sulfate and poly-L-lysine hydrobromide) was then touched to the coverslip to adhere the coverslip to the slide. The slide was then placed into liquid nitrogen for approximately 1 minute before the coverslip was removed and the slide was then fixed in 100% cold methanol for 10 minutes. The slide was washed in a coplin jar filled with 1X PBS for 3 ×10 min, then incubated in a coplin jar with 200 µl DAPI and filled with PBS for 10 minutes in the dark. The slide was then washed in a coplin jar filled with 1X PBS for 3 ×10 min before being mounted with a 22 mm×22 mm coverslip using gelutol. The sperm of the male worms sampled at each temperature condition were imaged in a z-stack. Images were acquired using Leica Application Suite Advanced Fluorescence 3.2 software using a Leica CTR6000 deconvolution inverted microscope with a Hamamatsu Orca-R2 camera and Plan Apo 63×/1.4 numerical aperture oil objective. Images were deconvolved. To calculate fluorescence, images were imported into FIJI (Rueden et al., 2017; Schindelin et al., 2012; Schneider et al., 2012). A standardized Region of Interest (ROI) tool was designed and used to measure the fluorescence of a specified region of the images. Fluorescence was calculated such that: Fluorescence = $(ROI_{sperm} - ROI_{background})/ROI_{background}$, where the background was a region adjacent to the measured sperm. Each strain was analyzed using males that were raised at 20°C, raised at 20°C until the L4 stage and then upshifted to 27°C during development or raised at 27°C continuously.

Statistical analysis

All statistical tests including Fisher's exact test and Student's t-test were done using PrismGraph Pad 6 or 7 software (La Jolla, CA, USA). A P-value less than or equal to 0.05 was considered significant.

Results

Transactivation is Reduced at High Temperature in Wildtype Crosses with a Male Competitive Advantage

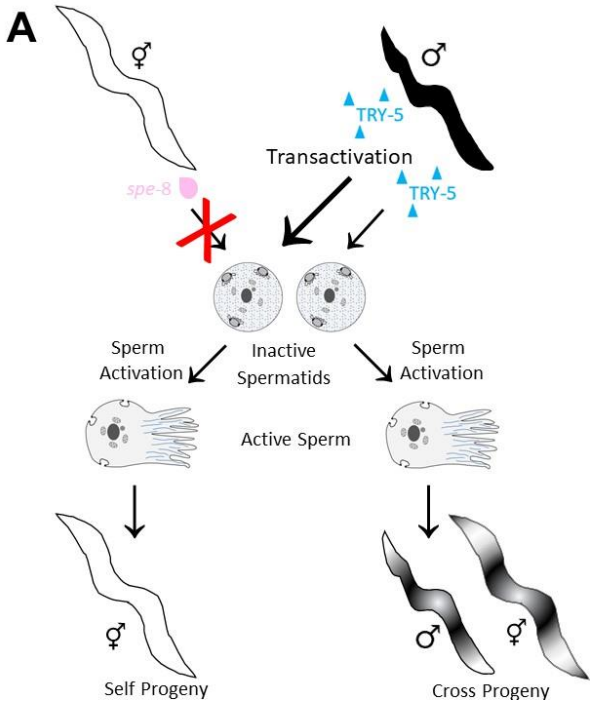

Figure 1. The transactivation pathway. (**A**) *spe-8* hermaphrodites are self sterile because they cannot activate their own sperm. TRY-5 in male seminal fluid can still activate *spe-8* hermaphrodite sperm, a process called transactivation. Both hermaphrodite self progeny and male/hermaphrodite cross progeny are produced.

To fertilize an oocyte, *C. elegans* spermatids must be activated to form motile sperm with a pseudopod. *C. elegans* utilize different, sex-specific pathways to activate their sperm. Hermaphrodites primarily utilize the SPE protein family of activation pathways (L'Hernault 2009), which is mimicked by *in vitro* zinc activation (Zhao et. al 2018). Males primarily utilize the TRY-5 pathway, a protease found in the seminal fluid, which is mimicked by *in vitro* Pronase E activation (Smith and Stanfield 2011). Previous work has shown that sperm from *C. elegans* males raised at high temperature showed low *in vitro* activation with zinc suggesting that SPE-8 activation is affected by high temperature, but the same effect was not observed for high temperature derived sperm treated with Pronase E (Nett et. al 2019). Thus, the effect of temperature on the activity of the TRY-5 protease in seminal fluid is unknown. To test if the TRY-5 protease can function at high temperature, we took advantage of a unique property of TRY-5 activation: transactivation. Hermaphrodites with a *spe-8* mutation produce functional spermatids that they cannot activate into motile sperm using the hermaphrodite SPE activation pathway, resulting in self-sterility

(Figure 1A, red X). When mated with males, *spe-8* mutant hermaphrodite sperm is activated by the TRY-5 protease in male seminal fluid. Seminal fluid activation of hermaphrodite sperm is called transactivation (Figure 1A, large arrow).

Three strains were tested that represent the diversity of thermal resistance in *C. elegans*: N2, the long standing laboratory wild type strain where males are sterile at high temperature (27°C) and only a small percentage recover fertility when downshifted to control temperature (20°C); JU1171, a wild type strain recently isolated from Chile, where males show some fertility at high temperature and had the highest recovery of fertility when downshifted from high temperature to control temperatures; and LKC34, a wild type strain recently isolated from Madagascar, where males show some fertility at high temperature but cannot recover fertility when downshifted to control temperatures (Petrella 2014).

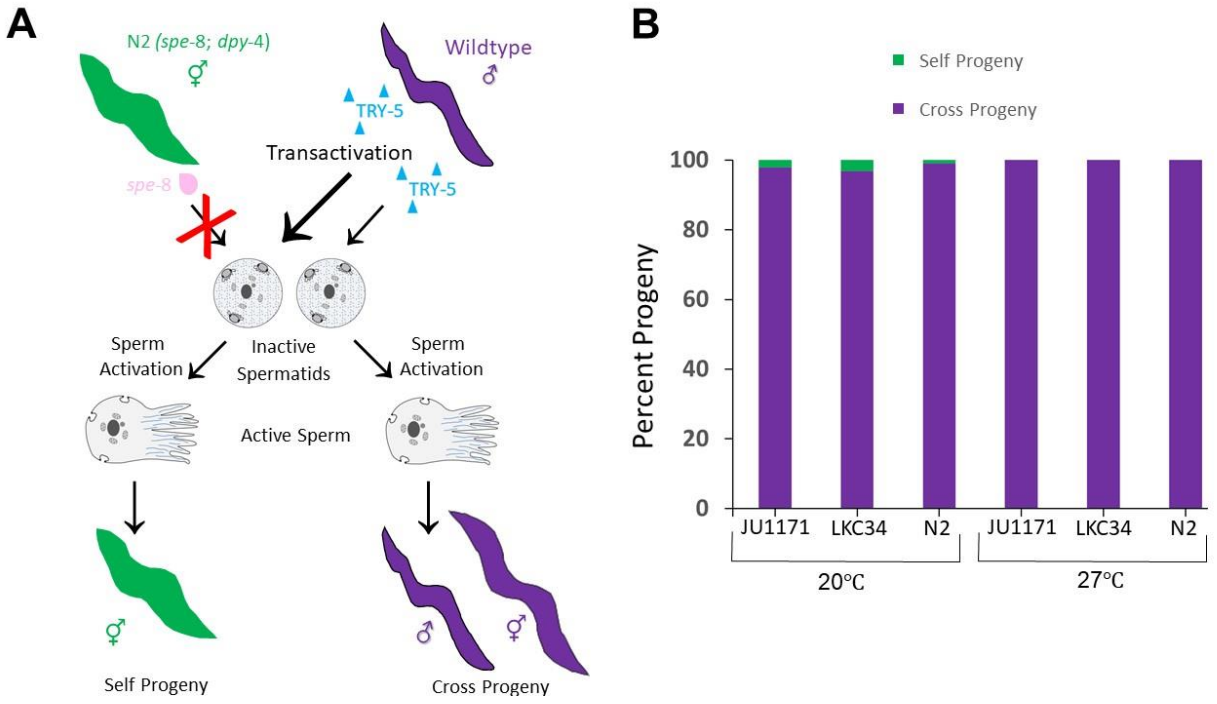

Figure 2. The transactivation pathway functions less at high temperature in crosses with wildtype males. (**A**) Crossing scheme for N2 (*spe*-8; *dpy*-4) hermaphrodites crossed with either JU1171, LKC34, and N2 wildtype males. F1 progeny can either be male/hermaphrodite wildtype cross progeny, fertilized by male sperm or shorter and fatter *dpy* hermaphrodite self progeny, fertilized by transactivated hermaphrodite sperm. (**B**) For each wildtype strain there was no self progeny are produced at 27℃ while a low percentage are produced at 20℃.

To test if the transactivation pathway is affected by high temperature, wild type males of each strain were crossed to *spe-8(hc40); dpy-4(e1166)* hermaphrodites (Figure 2A). *dpy-4* mutant hermaphrodites have a shorter and fatter appearance than wild type worms, called the dumpy phenotype (Figure 2A, green parent). *dpy-4(e1166)* is a recessive mutation; therefore, in this cross only self-progeny, produced by hermaphrodite sperm, would be homozygous for *dpy-4* mutation and would show the dumpy phenotype (Figure 2A, green progeny). Cross-progeny, produced by male sperm, would be heterozygous for the *dpy-4* mutants and would show a wild type appearance (Figure 2A, purple progeny). Therefore, the dumpy phenotype served as a visual phenotypic representation of successful transactivation of hermaphrodite sperm. A decrease in the percentage of self-progeny at high temperature would indicate a reduction in the ability of the

TRY-5 pathway to transactivate hermaphrodite sperm. Each strain of worms was raised separately at 20℃, the control temperature, until the L4 stage of development, then crossed at either 20℃ or 27℃. The P0 worms were allowed to mate and produce progeny for 24 hours, before being removed from the plate. The F1 progeny then developed into adults and were counted once they reached adulthood but before they produced their own progeny.

Crosses with wildtype males and *spe*-8; *dpy-4* hermaphrodites at 27℃ showed a reduction in transactivation compared to the same crosses performed at 20℃ (Figure 2B). At 20℃, 2.1% of JU1171 F1 progeny were self-progeny, 3.1% of LKC34 F1 progeny were self-progeny, and 0.9% of N2 F1 progeny were self-progeny. At 27℃, each strain produced 0 self-progeny, indicating a potential reduction in TRY-5 potency, however the differences are not significant. Due to the competitive advantage of male sperm over hermaphrodite sperm (L'Hernault 2009; Ward and Carrel 1979), very few self-progeny were observed, even at 20℃. Therefore, any changes in the level of transactivation were not determined to have statistical significance.

Transactivation is Reduced at High Temperature in Crosses with N2 *fer-1* Males, Indicating High Temperature Dampens TRY-5 Activation

Male sperm has a competitive advantage over hermaphrodite sperm: they are larger and greater in number (Ward and Carrel 1979). Previous work has shown that *fer-1* mutants can be used to correct for male competitive advantage (L'Hernault et. al 1988; Smith and Stanfield

2011). *fer -1* males are competent to mate but produce sperm with short, immotile pseudopods (Ward and Miwa 1978; Ward et. al 1981; Ward and Shakes 1989).

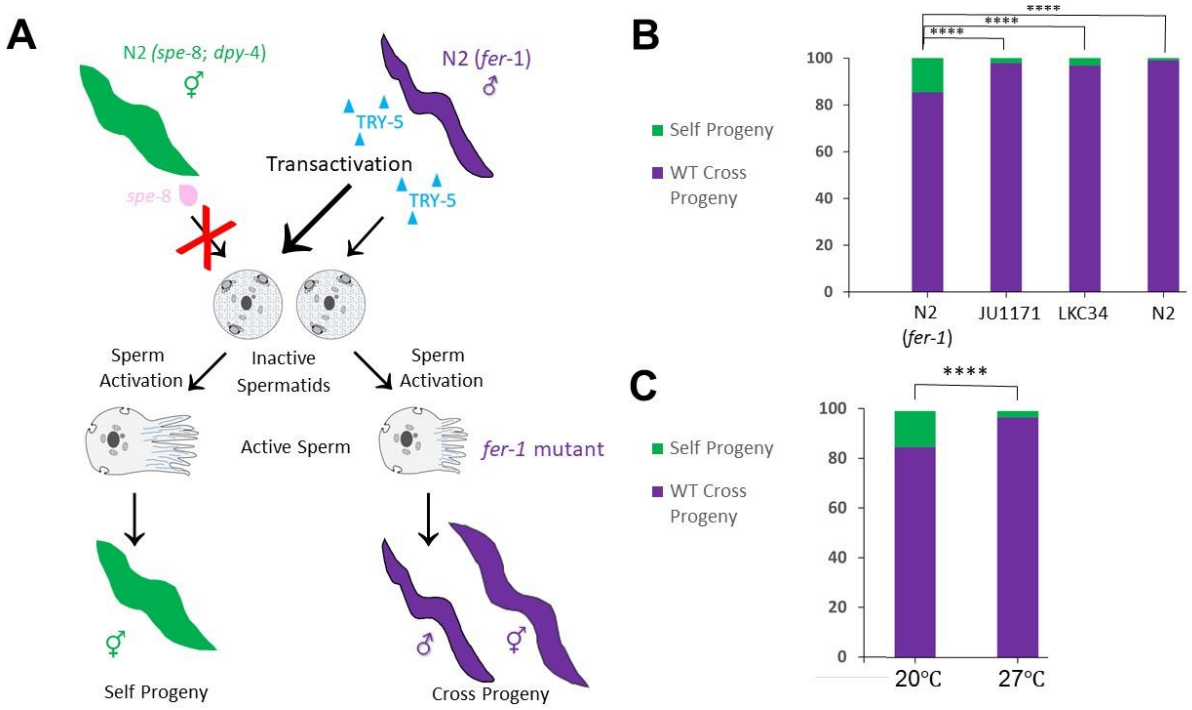

Figure 3. The transactivation pathway functions significantly less at high temperature in crosses with N2 *(fer*-1) mutants. (**A**) Crossing scheme for N2 (*spe*-8; *dpy*-4) hermaphrodites crossed with N2 (*fer*-1) males. F1 progeny can either be male/hermaphrodite wildtype cross progeny, fertilized by male sperm or shorter and fatter *dpy-4* hermaphrodite self-progeny, fertilized by transactivated hermaphrodite sperm. The *fer*-1 mutation produces sperm with a defective pseudopod, depicted as a shortened sperm. (**B**) Crosses with N2 (*fer*-1) males at 20℃ produce more self-progeny than crosses with wildtype strain males of JU1171, LKC34, or N2 background. (**C**) The percentage of *dpy-4* self-progeny produced at 27℃ is significantly less than the percentage of self-progeny produced at 20℃. (P< 0.0001, Fischer's Exact Test).

In order to test for a significant difference in transactivation at high temperature, the same cross experiment was repeated with *fer-1(hc1); him-5(e1490)* males in the N2 background to eliminate the competitive advantage of male sperm and produce more self-progeny (Figure 3A, male depicted in purple and *fer-1* mutation depicted by the shortened spermatocyte). Crosses with *fer-1* males and *spe*-8; *dpy-4* hermaphrodites at 20℃ produced significantly more self-progeny than wild type males crossed to *spe*-8; *dpy-4* hermaphrodites (Figure 3B). Thus, these mutant crosses were used to test for changes in TRY-5 activation due to high temperature.

Crosses of *fer-1* males with *spe*-8; *dpy-4* hermaphrodites showed a significant reduction in transactivation at 27℃ compared to the same crosses performed at 20℃ (Figure 3C). At 20℃, 14.6% of the F1 progeny were self-progeny, while at 27℃, 2.6% of F1 progeny were self-progeny. The reduction in F1 self-progeny that resulted from mating with males raised at 27℃ indicated fewer hermaphrodite sperm were transactivated at 27℃ than at 20℃. The reduction in transactivation indicated that high temperature dampens the TRY-5 pathway and is a likely contributing factor in *C. elegans* fertility loss at high temperature.

Exposure to High Temperature Severely Decreased the Total Progeny

It is important to note the complete effect of high temperature on *C. elegans* sperm and fertility. To understand the total effect, the raw total number of progeny was recorded at 27℃ and 20℃ for all male strains: JU1171, LKC34, N2, and N2 (*fer-1*) (Table 1). All Crosses between male strains and *spe-8; dpy-4* hermaphrodites produced significantly less progeny at 27℃ than the same crosses performed at 20℃ (Table 1). The reduction in total progeny indicated that high temperature dampens the TRY-5 pathway in both male and hermaphrodite sperm, reducing the total number of available active sperm from both sexes to fertilize hermaphrodite oocytes. The scale of the reduction in progeny indicated that confounding effects of high temperature: reduction in mating behavior, failure to transfer sperm, defects in spicule morphology, and oogenic germline effects (Nett et. al 2019), are present and contribute to the vast reduction in progeny. The confounding factors would not contribute the observed reduction in the percentage of transactivated self-progeny at 27℃ (Fig. 3C), indicating that TRY-5 activation is implicated in addition to the other known mechanisms.

Table 1: Total Progeny from *spe-8* hermaphrodites crossed with males

Strain	20°C	27°C
JU1171	538	20
LKC34	302	70
N2	456	43
N2 (*fer-1)*	251	38

Exposure to High Temperature Led to Strain Specific Changes in Mitochondrial Activity

Previous experiments indicated that exposure to high temperature decreased *C. elegans* sperm motility (Nicholas Sepulveda, unpublished data). Mitochondria produce ATP required for sperm motility and are known to be affected by high temperature stress (Gong et. al, 2017; Guo et. al 2017; Roth 2018; Slimen et. al 2014; Zhao et. al 2006). To test for changes in sperm mitochondrial activity at high temperature, we stained functional sperm mitochondria, fixed the sperm to microscope slides, viewed the sperm on a deconvolution microscope, and analyzed the images for changes in fluorescence intensity at different temperature conditions.

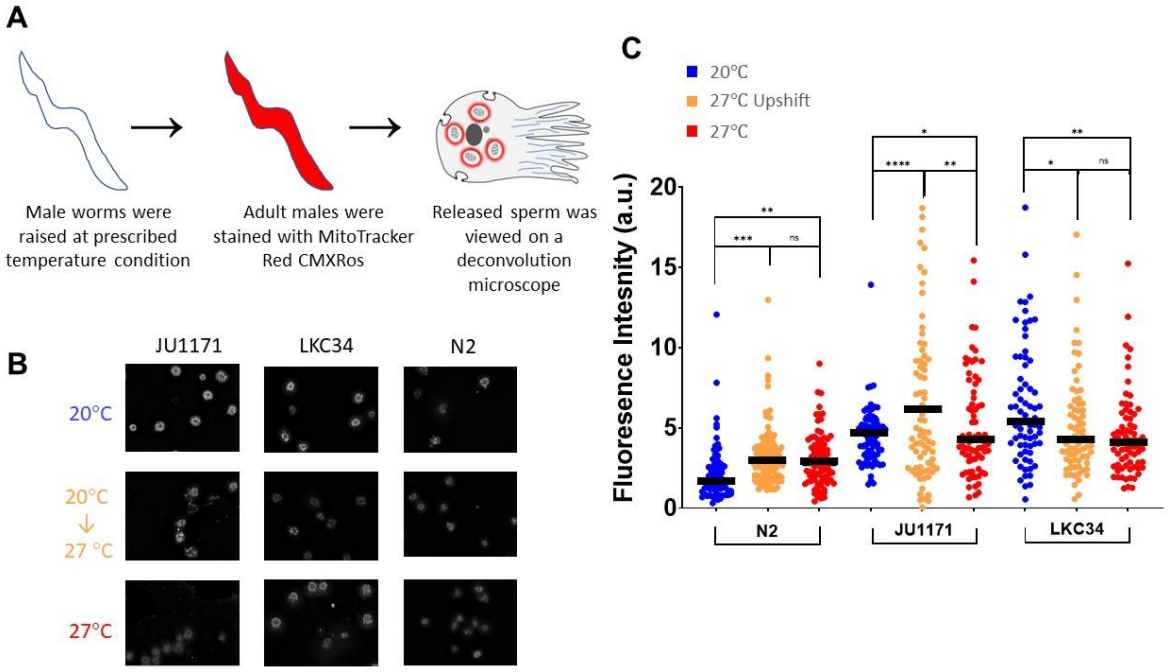

Figure 4. Exposure to high temperature changed mitochondrial activity in patterns that varied by strain. (**A**) Male worms were raised at either 20°C their entire lives, 20°C until the L4 stage and then upshifted (denoted by orange arrow in B) to 27°C during sexual development, or 27°C their entire lives. Young, adult males were then stained with MitoTracker Red CMXRos and bisected to release sperm onto microscope slides. The slides were then fixed with liquid nitrogen and imaged on a deconvolution microscope. (**B**) Sample images of JU1171, LKC34, and N2 sperm stained with from worms raised at either 20°C, 20°C to 27°C upshift, or 27°C MitoTracker Red. (**C**) For each strain, exposure to high temperature changed the normalized fluorescence intensity (FI), but the patterns of change varied depending on the strain. N2 males showed an increase in FI with developmental upshift (yellow) and lifelong exposure to high temperature (red) compared to worms raised at 20°C (blue). JU1171 males showed an increase in FI with developmental upshift to high temperature and a decrease in FI with lifelong exposure to high temperature. LKC34 males showed a decrease in FI with developmental upshift and lifelong exposure to high temperature. (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, Fischer's Exact Test).

Using the same three wild type strains as above, male worms were raised at three different temperature conditions: 20°C their entire lives, 20°C as larva and then upshifted to 27°C during the L4 stage when sexual development occurs, or 27°C their entire lives. L4 males were isolated for 24 hours to mature into young adults and stained with MitoTracker Red CMXRos, which stains the membranes of mitochondria undergoing oxidative phosphorylation (Figure 4A). The stained males were placed on microscope slides and bisected to release their sperm. The

stained sperm were fixed and viewed with fluorescence microscopy (Figure 4B). The adjusted fluorescence intensity was calculated for each sperm in the image and compared between temperature treatments.

Exposure to high temperature changed the average fluorescence intensity for each strain of worms, but the pattern of change varied between strains (Figure 4C). In N2 worms, males developmentally upshifted to 27℃ and males raised at 27℃ their entire lives had a significant increase in fluorescence intensity compared to males raised at 20℃ their entire lives. In JU1171 worms, males developmentally upshifted to 27℃ showed a significant increase in fluorescence intensity compared to worms raised at 20℃, while males raised at 27℃ their entire lives showed a significant decrease in fluorescence intensity compared to males raised at 20℃ their entire lives. In LKC34 worms, males developmentally upshifted to 27℃ and males raised at 27℃ their entire lives showed a significance decrease in the fluorescence intensity compared to males raised at 20℃ their entire lives. The changes in fluorescence intensity reveal either a difference in the absolute number of mitochondria or a change in the percentage of mitochondria undergoing oxidative phosphorylation. It is unclear how the differences in mitochondrial activity contribute to the thermal resistance of each strain or a mechanism of fertility loss due to high temperature. Further work is needed to understand if the negative relationship between thermal resistance and mitochondrial activity could explain the difference in sperm motility observed previously.

Discussion

Across species temperature sensitivity is a conserved flaw in sexual reproduction; outside of a defined temperature range there is a dramatic drop in fertility leading to eventual sterility. Here, we characterized two mechanisms that may underlie male infertility due to high temperature in the nematode *C. elegans*. *C. elegans* males use the TRY-5 serine protease as a redundant, extracellular pathway in the seminal fluid to activate immotile spermatids into motile sperm. We found that TRY-5 potency is temperature sensitive, with decreased function at high temperature. *C. elegans* sperm must produce cellular energy, ATP, in the mitochondria to remain motile in the hermaphrodite spermatheca. We found that high temperature affects mitochondrial number or function in sperm in a strain dependent manner. More work needs to be done to understand the effect of high temperature on other sperm activators in the seminal fluid and to understand the strain specific differences in mitochondrial activity at high temperature.

High Temperature Decreases TRY-5 Extracellular Sperm Activation in Seminal Fluid

We found *C. elegans* males produce a significantly lower percentage of transactivated self-progeny when crossed with self-sterile *spe-8* hermaphrodites at high temperatures of 27℃ than males crossed at viable temperatures of 20℃. The reduction in transactivated self-progeny indicates that the potency of TRY-5 or additional uncharacterized activating factors in the seminal fluid are not functioning properly when males are raised at high temperature. High temperature also affected the total number of progeny, indicating that reduced potency of TRY-5 or additional activating factors in the seminal fluid affect male sperm in a similar manner to hermaphrodite sperm. TRY-5 is an extracellular serine protease in the seminal fluid that signals the initiation of sperm activation (Smith and Stanfield 2011). *In vitro* experiments indicate that *C. elegans* spermatids can be activated by Pronase E at high temperature (Nett et. al 2019).

Pronase E is a general protease that is thought to mimic the activity of TRY-5 (Stanfield et. al, 2011; Ward and Shakes 1989). TRY-5 could either be affected by high temperature, reducing its ability to cleave its target membrane protein and signal the initiation of activation, or high temperature could dampen a subsequent protein in the seminal fluid activation cascade. Sperm motility is acquired during sperm activation, a process that signals the growth of the pseudopod, making the sperm competent to fertilize an oocyte (Ward and Carrell 1979). Since high temperature reduces TRY-5 or subsequent protein activation in the seminal fluid, both male and hermaphrodite sperm are not signaled to activate in the hermaphrodite spermatheca and cannot crawl to fertilize an oocyte. Instead, the immotile spermatids are swept out of the spermatheca and into the uterus by passing oocytes (L'Hernault et. al 1988).

Differences in Mitochondrial Activity May Explain Strain Specific Differences in Fertility at High Temperature

We stained *C. elegans* sperm mitochondria with MitoTracker Red CMXRos, which stains mitochondria undergoing oxidative phosphorylation. We found that worms raised at the high temperature of 27℃ their entire lives and worms upshifted to high temperature during sexual development, showed significant differences in sperm mitochondrial fluorescence than worms raised at a viable temperature of 20℃. Mitochondrial fluorescence varied in a strain specific manner. We tested three different strains: N2, JU1171, and LKC34, that each represent a unique response in fertility to different high temperature conditions. N2, the longstanding laboratory wildtype strain isolated in Bristol, England, are sterile at high temperature and only a small percentage recover fertility when downshifted to 20℃ (Petrella 2014). N2 mitochondria increase fluorescence when upshifted to 27℃ and when raised at 27℃ their entire lives. In JU1171, a wildtype isolate from Chile, some males are fertile at 27℃ (Petrella 2014). When down shifted from 27℃ to 20℃, JU1171 exhibits the highest recovery of fertility (Petrella 2014). JU1171

mitochondria show increased mitochondrial fluorescence with developmental upshift and decreased mitochondrial fluorescence when raised at 27℃ their entire lives. LKC34, a wildtype isolate from Madagascar, show some fertility at high temperature but do not recover fertility when downshifted from 27℃ to 20℃ (Petrella 2014). LKC34 mitochondria show decreased fluorescence with both developmental upshift and when raised at 27℃ their entire lives.

Mitochondrial fluorescence could change due to a difference in the number of mitochondria present in the sperm or the amount in mitochondria undergoing oxidative phosphorylation, both measures of mitochondrial activity. Differences in mitochondrial activity may explain strain specific differences in fertility at high temperature. Oxidative Phosphorylation plays a pivotal role in sperm motility (Guo et. al 2017). Motile sperm have higher mitochondrial activity, transmembrane potential, respiratory chain activity, and biosynthesis than immotile sperm (Gong et. al 2017). The fluorescence results indicate that there may be an increase in JU1171 mitochondrial activity with short exposure to 27℃ and a decrease in JU1171 mitochondrial activity with prolonged exposure to 27℃, which could explain how JU1171 is infertile at 27℃ but can recover fertility when downshifted by increasing mitochondrial activity to improve sperm motility. Similarly, LKC34 could fail to recover when downshifted from 27℃ to 20℃ because the mitochondria cannot recover full activity and restore sperm motility. Differences in the number of mitochondria present could explain strain-specific differences as well. N2 does not align with this model however, because when raised at 27℃ and upshifted to 27℃ N2 mitochondrial fluorescence increased, indicating an increase in mitochondrial activity or greater number of mitochondria. To fit the described model, N2 should recover fertility when downshifted from 27℃ to 20℃ like JU1171, but it fails to do so. N2 has been found to be more affected by elevated temperature compared with newly isolated wild type strains, specifically

JU1171 and LKC34 (Nett et. al 2019). Therefore, it is possible that an additional mechanism, such as decreased mating behavior, is implicated specifically in N2, that has greater effects on fertility than mitochondrial activity. More wildtype isolates need to be tested to determine if mitochondrial activity leads to strain specific differences in fertility at high temperature.

TRY-5 Activation and Mitochondrial Activity Contribute to A Multi-Mechanism Model of Sterility at High Temperature

Our results indicate that two mechanisms that impact sperm motility, male sperm activation and male sperm mitochondrial activity, are affected by high temperature. These results align with other aspects of male mating ability that are affected by high temperature. High temperature substantially implicates male mating behavior; at high temperature males fail to respond to hermaphrodites and specifically N2 males fail to complete mating by failing to locate the hermaphrodite vulva (Nett et. al 2019). In addition, males raised at high temperature also fail to complete sperm transfer and have defects in their spicule morphology (Nett et. al 2019). High temperature does not solely affect male mating ability. High temperature leads to loss of both spermatogenic and oogenic germline function at high temperature (Petrella 2014). In totality, defects in mating behavior, tail morphology, incomplete sperm transfer, decreases in motility to decreased sperm activation and mitochondrial activity, and defects in oogenic germline function, lead to sterility at high temperature in *C. elegans*.

Future Directions: TRY-5

We have shown that TRY-5 mediated activation in the seminal fluid is disrupted at high temperature. TRY-5 serves a redundant role in sperm activation in the seminal fluid, either cleaving a membrane protein that signals sperm activation or takes part in a signaling pathway in the seminal fluid (Smith and Stanfield 2011). Thus, it is unclear if high temperature dampens TRY-5 protease activity, a different factor in the male activation pathway, or both. To parse out a

specific effect of high temperature on the male activation pathway, we propose an imaging experiment on the effect of high temperature on TRY-5 activity in the gonad of *swm-1* mutant males. SWM-1 is a secreted serine protease inhibitor in the seminal vesicle that negatively regulates sperm activation (Stanfield and Villeneuve 2006). SWM-1 prevents premature activation of spermatids in the male gonad by inhibiting TRY-5; after mating, SWM-1 is not present in the seminal fluid and the sperm are activated by TRY-5 (Smith and Stanfield 2011). To test the effect of high temperature on TRY-5 in the male gonad, we propose imaging the gonads of three groups of males: control wildtype males raised at 20℃, *swm-1* mutant males raised at 20℃, and *swm-1* mutant males raised at 27℃. The males will be imaged to identify the presence of activated sperm in the seminal vesicle and throughout the male gonad.

By testing for the presence of activated sperm in each group of males, we can determine if TRY-5 activity is specifically dampened at high temperature. The control wildtype males raised at 20℃ will show inactive spermatids in the seminal vesicle, because the TRY-5 present in the seminal vesicle will be inhibited by SWM-1. The *swm-1* mutant males raised at 20℃ have uninhibited TRY-5 protease activity in the seminal fluid, so motile sperm will be present in both the seminal vesicle and throughout the male gonad due to the activated sperm's motility. The *swm-1* mutant males raised at 27℃ could show either inactive spermatids or active sperm. If inactive spermatids are present in the seminal vesicle, this would indicate that TRY-5 protease activity is dampened by high temperature. Reduced TRY-5 activity at high temperature in the seminal vesicle, would provide a mechanism for our results of dampened TRY-5 activity in the seminal fluid. The presence of active sperm would indicate that TRY-5 protease activity is not inhibited by high temperature, leading to uninhibited TRY-5 protease activity, comparable to the *swm-1* mutant males raised at 20℃. This result would indicate that TRY-5 is not the component

dampened in our seminal fluid experiments but instead either a second protease is present in the seminal fluid that is specific to hermaphrodite sperm activation, a model suggested by Stanfield and Villeneuve in their 2006 discovery of SWM-1, and this second protease or a downstream signaling component in the seminal fluid is temperature sensitive.

Future Directions: Mitochondrial Activity

We have shown that mitochondrial activity is affected by high temperature in a strain-specific manner. Two recently isolated wildtype strains, JU1171 and LKC34, showed changes in mitochondrial activity that could explain their strain-specific differences in fertility at high temperature. N2, the common laboratory wildtype strain, had mitochondrial changes that inversely correlated with its fertility at high temperature. N2 has been found to be more affected by elevated temperature compared with newly isolated wild type strains, specifically JU1171 and LKC34 (Nett et. al 2019). Thus, it is unclear if N2 is an outlier in a strain-specific mitochondrial activity driven model of fertility at high temperature. To further understand strain specific differences in mitochondrial activity, we propose that more strains with strain-specific differences in fertility at high temperature be tested for strain-specific differences in mitochondrial activity. Twenty-two different wild isolates of *C. elegans* have been analyzed for fertility loss at high temperature (Petrella 2014). Both MY2, isolated from Roxel, Germany, and CB4856, isolated from Hawaii, show a similar partial recovery of fertility when downshifted from 27℃ to 20℃ akin to JU1171 (Petrella 2014). Both DR1350, isolated from Pasadena, California, and MY3, isolated from Roxel, Germany, show no recovery of fertility when downshifted from 27℃ to 20℃ akin to LKC34 (Petrella 2014). These four strains will be tested in the same procedure as our current experiments, with the same three temperature conditions: raised at 20℃, upshifted to 27℃ during sexual development, and raised at 27℃. If MY2 and

CB4856 show a similar increase in mitochondrial activity with developmental upshift and decrease in mitochondrial activity when raised at 27℃ as JU1171, while DR1350 and MY3 show a similar decrease in mitochondrial activity for both developmental upshift and when raised at 27℃ as LKC34, we can conclude that strain specific mitochondrial activity underlies strain specific differences in fertility.

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