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RESEARCH ARTICLE

Defects in mating behavior and tail morphology are the primary cause of sterility in *Caenorhabditis elegans* males at high temperature

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ABSTRACT

Reproduction is a fundamental imperative of all forms of life. For all the advantages sexual reproduction confers, it has a deeply conserved flaw: it is temperature sensitive. As temperatures rise, fertility decreases. Across species, male fertility is particularly sensitive to elevated temperature. Previously, we have shown in the model nematode *Caenorhabditis elegans* that all males are fertile at 20°C, but almost all males have lost fertility at 27°C. Male fertility is dependent on the production of functional sperm, successful mating and transfer of sperm, and successful fertilization post-mating. To determine how male fertility is impacted by elevated temperature, we analyzed these aspects of male reproduction at 27°C in three wild-type strains of *C. elegans*: JU1171, LKC34 and N2. We found no effect of elevated temperature on the number of immature non-motile spermatids formed. There was only a weak effect of elevated temperature on sperm activation. In stark contrast, there was a strong effect of elevated temperature on male mating behavior, male tail morphology and sperm transfer such that males very rarely completed mating successfully when exposed to 27°C. Therefore, we propose a model where elevated temperature reduces male fertility as a result of the negative impacts of temperature on the somatic tissues necessary for mating. Loss of successful mating at elevated temperature overrides any effects that temperature may have on the germline or sperm cells.

KEY WORDS: Temperature stress, Male behavior, Sperm, Thermosensitivity

INTRODUCTION

Survival of populations is dependent upon the ability of individuals to produce progeny. Despite this necessity, fertility has a flaw observed across species: it is temperature sensitive. Organisms from fruit flies and worms to sheep and humans display a dramatic loss of fertility when encountering temperatures only a few degrees higher than their optimal fertile temperature range (Harvey and Viney, 2007; Prasad et al., 2011; Rohmer et al., 2004; Wang et al., 2007). Although there is evidence that female fertility can be affected by increased temperature (Gouvêa et al., 2015; Petrella, 2014; Poullet et al., 2015; Tusell et al., 2011), male fertility is more commonly the

principal cause for temperature-sensitive infertility (Cameron and Blackshaw, 1980; Harvey and Viney, 2007; Petrella, 2014; Poullet et al., 2015; Prasad et al., 2011; Shefi et al., 2007; Yaeram et al., 2006). The steps and cellular pathways central to male fertility that are disrupted at elevated temperatures remain largely unknown. Here, we used the model nematode worm *Caenorhabditis elegans* to identify specific aspects of male fertility that are disrupted at elevated temperatures.

Classical mutant genetic screens in *C. elegans* have uncovered several temperature-sensitive mutants that exhibit a lower threshold temperature at which they lose fertility (Conine et al., 2010; Coustham et al., 2006; Kawasaki et al., 1998). However, it is unclear whether these mutant phenotypes are the result of disruption in the pathways that are affected in wild-type organisms at elevated temperature or are an indirect acquisition of germline temperature sensitivity through disruption of other pathways. Therefore, to understand the cellular pathways that are disrupted at elevated temperature leading to a loss of fertility in wild-type organisms, experiments using wild-type animals are required. *Caenorhabditis nematodes* provide an ideal model to study temperature-sensitive sterility because germ cell development, sperm function *in vitro*, mating behaviors and sperm transfer can all be measured in real time in live animals or cells. In addition, there are >200 curated wild-type strains with genomic data that facilitate analysis of natural variation in the loss of fertility at elevated temperature (Cook et al., 2017). Loss of fertility in hermaphrodites as temperature increases has been analyzed in wild-type isolates of *C. elegans*, and the related *Caenorhabditis* species *C. briggsae* and *C. tropicalis* (Harvey and Viney, 2007; Petrella, 2014; Poullet et al., 2015; Prasad et al., 2011). In all three species, there are more thermotolerant isolates that are fertile at a temperature around 1°C above the optimal fertility temperature where more thermosensitive strains become sterile (Harvey and Viney, 2007; Petrella, 2014; Poullet et al., 2015; Prasad et al., 2011), and there are known effects of temperature on both sperm and oocyte function (Aprison and Ruvinsky, 2014; Gouvêa et al., 2015; Harvey and Viney, 2007; Petrella, 2014; Poullet et al., 2015; Prasad et al., 2011).

Analysis of the effects of elevated temperature on gametes leading to loss of fertility in hermaphrodites is complicated by the fact that they are self-fertile, producing both sperm and egg. Conversely, studying the effects of elevated temperature in *C. elegans* males allows the analysis to be limited to the effects on sperm. Previously, we found that the percentage of *C. elegans* males that produce progeny dropped to near zero in eight wild-type strains when males were raised at 27°C (Petrella, 2014). Similar consequences to male fertility have been seen in multiple wild-type strains of *C. briggsae* and *C. tropicalis* males at elevated temperature (Poullet et al., 2015). In *C. elegans*, differences

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between wild-type strains were seen when males were raised at elevated temperature and down-shifted (27°C→20°C) in the last larval stage. Fertility was partially restored in the thermotolerant strain JU1171 after down-shifting but not in the thermosensitive strains LKC34 and N2 (Petrella, 2014). Unlike in hermaphrodites, the cellular or behavioral consequences of elevated temperature that lead to male loss of fertility at elevated temperature have not been investigated.

Male reproduction in *C. elegans* requires production of both haploid spermatids through the meiotic divisions of spermatogenesis and functional motile sperm through maturation during spermiogenesis. Spermatogenesis begins during larval development, in early to mid-L4 stage, and fully formed spermatids are present by the end of the L4 larval stage (L'Hernault, 2006; Schedl, 1997). These non-motile spermatids then become activated through the process of spermiogenesis to become mature spermatozoa capable of movement and fertilization (Ellis and Stanfield, 2014). Activation of male spermatids occurs only after successful transfer to a hermaphrodite, which results in sperm motility via the formation of the pseudopod necessary for sperm crawling. Activation occurs through two redundant signaling pathways: the TRY-5 pathway in response to the TRY-5 protease in male seminal fluid, and the *spe-8* pathway in response to an unknown signal in the hermaphrodite reproductive tract (Ellis and Stanfield, 2014; L'Hernault et al., 1988; Smith and Stanfield, 2011). If male spermatids do not activate properly, they cannot crawl from the uterus to the spermatheca, the hermaphrodite sperm storage organ, and will fail to fertilize an oocyte, resulting in no progeny.

For males to produce progeny, sperm must be transferred to hermaphrodites through a complex male mating behavior process. These stereotypical mating behaviors involve male-specific neuronal circuits and require properly formed male tail structures. The initial steps of mating require males to successfully find a hermaphrodite and subsequently locate the hermaphrodite vulva. Sensory neurons in the head allow males to find hermaphrodites in response to mating hormones (Narayan et al., 2016; Srinivasan et al., 2008; Wan et al., 2019; White et al., 2007). This is followed by signals through the nine pairs of sensory rays found in male-specific tail structures, which are necessary for maintaining contact with the hermaphrodite and finding the vulva (Garcia et al., 2001; Koo et al., 2011; Liu and Sternberg, 1995; Liu et al., 2011). The final step before sperm transfer is insertion of the spicules into the vulva, which requires coordination between sensory neurons and male tail muscles (LeBoeuf et al., 2014; LeBoeuf and Garcia, 2017; Schindelman et al., 2006). Only once these mating steps are finished can males then successfully transfer sperm into the uterus. Whether loss of male fertility is due to the effects of elevated temperature primarily on the gametes, as is seen in hermaphrodites, or on somatic tissues required for mating behavior is unknown. Elevated temperature could affect one or all of these steps in males from the earliest steps of spermatogenesis to the final step of sperm transfer after spicule insertion.

To determine why male *C. elegans* lose fertility at elevated temperature, we analyzed spermatogenesis, spermiogenesis, male tail morphology, mating behaviors and sperm transfer for defects at elevated temperature. Analyses were done in the more thermotolerant strain JU1171 and two thermosensitive strains, LKC34 and N2. We found that there was no decrease in sperm number in any of the three strains at 27°C. *In vitro* sperm activation assays showed that there were no defects in activation with compounds that mimic the TRY-5 activation pathway, but there were moderate defects in activation with compounds that change ion concentrations in sperm. However, we saw large effects of temperature on mating behavior, sperm

transfer and male tail morphology in males that experienced 27°C. Our data indicate that decreased male fertility at elevated temperature is primarily due to changes in somatic tissues that affect their ability to perform and complete mating, and that these somatic effects preclude identification of any effects that temperature may have on male sperm.

MATERIALS AND 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 and CB138 *unc-24(e138)*] 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
Four 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 (for tail morphology, F1 males were up-shifted at the L1 stage – see below). For both continuous 20°C and continuous 27°C exposures, L4 males were isolated from hermaphrodites and maintained at the experimental temperature for 24 h prior to each analysis. (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 18 or 24 h at 27°C. (4) Down-shift from 27°C to 20°C: F1 males developed at 27°C were down-shifted to 20°C at the L4 stage (in the absence of hermaphrodites), and experiments were conducted after 24 h at 20°C.

Spermatid counting
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 NGM plates without bacteria for 5 min before being placed in M9 on gelatin-coated poly-L-lysine (GCP) slides (ddH$_2$O, gelatin, chromium potassium sulfate and poly-L-lysine hydrobromide) and covered with a 18 mm×18 mm coverslip. Care was taken to keep from crushing males while wicking, which results in spermatids exploding from the tail. Slides were placed in liquid nitrogen for approximately 1 min before the coverslip was removed and the slide was then fixed in 100% cold methanol and acetone for 10 min each. Slides were incubated with block (1.5% BSA, 1.5% ovalbumin, 0.05% NaN$_3$ in 1× PBS) for 30 min and DAPI (0.2%) for 10 min, then washed with 1× PBS for 4×10 min before being mounted with a 22 mm×22 mm coverslip using gelutol. The posterior ends of individual intact males 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. For sperm counting, images of each male were imported into FIJI (Rueden et al., 2017; Schindelin et al., 2012; Schneider et al., 2012), merged into a stack, and trimmed to exclude any images within the z-stack

that did not contain sperm. The hyperstacks temporal-color code function was then used to create a projection of the stack where sperm in different planes were pseudocolored different colors. The multipoint tool within FIJI was then used to count sperm until either all of the sperm within the male were counted or until at least 200 sperm were counted. Each strain was analyzed using males that were raised at 20°C or 27°C continuously. Analysis of strains was performed in two or three separate biological replicates for each strain for a total of 12–18 males at each temperature condition.

Spermatid activation

Sperm activation assays were performed similar to the description in Shakes and Ward (1989). 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 NGM plates without bacteria prior to dissection. Spermatids were dissected from males in sperm buffer (5 mmol l^{-1} Hepes pH 7.0, 50 mmol l^{-1} NaCl, 25 mmol l^{-1} KCl, 5 mmol l^{-1} CaCl$_2$, 1 mmol l^{-1} MgSO$_4$ and 10 mmol l^{-1} dextrose) plus activator (Fenker et al., 2014). Activators used were 200 µg ml^{-1} Pronase E and 1 mmol l^{-1} ZnCl$_2$ (Fenker et al., 2014). Spermatids were incubated in sperm buffer alone, in sperm buffer with Pronase E for 10 min, or in sperm buffer with ZnCl$_2$ for 20 min. Incubation times for each activator were chosen based on preliminary experiments as the time point where the highest percentage of sperm showed activation for a particular activator. Replicates for different temperature treatments were done on subsequent days with the same sperm buffer, with activators added fresh each day. Spermatids were imaged on a Nikon Eclipse TE2000-S inverted microscope with Nomarski optics using Q Capture Pro 7 software with a Q imaging Exi Blue camera and Plan Apo 60×/1.25 numerical aperture oil objective. Spermatids were scored using ImageJ software as not activated (round, no pseudopod or spikes) or activated (having either a pseudopod or projecting spikes). Three biological replicates were carried out for each activator and each strain at both 20°C and 27°C, with total number of sperm between 234 and 1258 (see Table S1 for specific numbers of sperm scored per treatment).

Male mating interests

Mating interest assays were performed using a protocol modified from Chatterjee et al. (2013). L4 males were isolated on AMA1004 plates without hermaphrodites and allowed to age for ∼24 h. On the day of experimentation, 20 CB138 *unc-24*(*e138*) hermaphrodites were placed on a plate with only a small area of AMA1004 bacteria and allowed to acclimate for 10 min. A single male was placed on this plate, and its behavior was recorded for 4 min (Chatterjee et al., 2013). We recorded six behaviors within this period. (1) Response: a positive response was recorded when the male began to scan the hermaphrodite with its tail. (2) Response time: the time at which the male began scanning the hermaphrodite. (3) Failure to turn: a positive failing to turn response was recorded if the male reached the head or tail of the hermaphrodite but was unable to begin scanning on the opposite surface of the hermaphrodite. This appeared as the male trying to turn but eventually wandering away from the hermaphrodite. (4) Vulva located: a positive response was recorded if the male successfully found the hermaphrodite vulva while scanning and attempted to insert its spicule. (5) Number of passes of the vulva: the number of times the male passed over the vulva while scanning the hermaphrodite body. The vulva location efficiency (LOV efficiency) was calculated as: vulva location/number of passes. (6) Maintain contact: a positive response was recorded if the male successfully remained at the vulva during spicule insertion.

Three trials of 15 males each were conducted for all strains for each temperature treatment.

Male sperm transfer assays

L4 males and hermaphrodites were selected and isolated from each other for 24 h. After young adult males were moved to a plate spotted with 15 µl *E. coli*, 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 h, they were moved to new plates twice to remove the residual food with MitoTracker Red. Next, adult hermaphrodites were anesthetized in a 0.1% tricaine and 0.01% tetramisole in 1× M9 buffer anesthetic solution to reduce their movement and allow males to mate with them more easily. N2 worms were anesthetized for 15 min, JU1171 for 13 min and LKC34 for 10 min. These exposure times were used for each strain to optimize the level of anesthesia that resulted in hermaphrodites that move little but have enough muscle tone to allow successful mating. After anesthesia, hermaphrodites were transferred to new plates twice to remove residual anesthetic solution so that anesthesia would not be encountered by males. Twelve hermaphrodites were moved to new mating plates and arranged around the food spot like a clock face. Subsequently, 10 males were moved to the plates with hermaphrodites. Worms were allowed to mate for 45 min. Each plate was monitored for insemination events, and after a hermaphrodite was inseminated it was moved to a separate plate. After the mating period, the inseminated hermaphrodites were observed for an additional 45 min under the stereomicroscope to determine whether male sperm would migrate from the uterus to the spermatheca. Sperm were considered to have migrated if there were one or two distinct areas of fluorescence anterior or posterior of the vulva. For higher magnification analysis, each hermaphrodite that was inseminated was moved to an individual plate, allowed to recover for 45 min, then placed on a 2% agar pad, immobilized with 25 mmol l^{-1} sodium azide (NaN$_3$) in 1× M9 and covered with a coverslip. Images were taken on a Nikon Eclipse TE2000-S inverted microscope with Nomarski optics using Q Capture Pro 7 software with a Q imaging Exi Blue camera and Plan Apo 20× objective. Sperm migration was scored as being primarily in or adjacent to the spermatheca, distributed throughout the uterus and spermatheca, or absent, i.e. having been expelled from the hermaphrodite.

Male tail morphology

For the three temperature treatments, males were raised continuously at 20°C, up-shifted from 20°C to 27°C at the L4 stage, or raised continuously from the L1 stage at 27°C. Day one young adult males were imaged live on 2% agar pads in 0.1 mmol l^{-1} levamisole in M9 to immobilize males and protract spicules for measurement, and 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. One or two half-tails depending on orientation were scored for ray morphology (missing, fused or extra). One spicule per tail was scored for spicule morphology and spicule length. Spicule length was measured in FIJI using the line and measure tools (Rueden et al., 2017; Schindelin et al., 2012; Schneider et al., 2012). The number of animals scored for each experiment is given in Tables 1 and 2.

Spicule protraction assay

Nine to 10 male worms were added to 200 µl 0.1 mmol l^{-1} levamisole in 1× M9 buffer in the bottom of a 3-well round-bottomed Pyrex

Table 1. Ray phenotypes at elevated temperature

Strain and treatment	% Malformed ray 1 (n)	% Fused ray 1–2 (n)	% Fused ray 3 (n)	% Fused rays 8–9 (n)	% Extra growths (n)	No. of sides
JU1171						
20°C	4.0 (2)	4.0 (2)	6.0 (3)	12.0 (6)	4.0 (2)	50
Up-shift	12.3 (7)	10.5 (6)	5.3 (3)	10.5 (6)	8.8 (5)	57
27°C	36.5 (19)	11.5 (6)	3.8 (2)	7.6 (4)	32.69 (17)	52
LKC34						
20°C	4.0 (2)	14.0 (7)	2.0 (1)	0.0 (0)	14.0 (7)	50
Up-shift	11.8 (6)	15.7 (8)	0.0 (0)	2.0 (1)	31.37 (16)	51
27°C	44 (22)	18 (9)	2.0 (1)	6 (3)	10 (5)	50
N2						
20°C	6.0 (3)	4.0 (2)	0 (0)	8.0 (4)	14.0 (7)	50
Up-shift	14.3 (8)	21.4 (12)	0 (0)	3.6 (2)	23.2 (13)	56
27°C	28.8 (15)	21.2 (11)	3.8 (2)	1.9 (1)	28.8 (15)	52

titer dish. Males were observed on a standard stereomicroscope for 5 min and a positive spicule protraction was scored if the spicule remained protracted for >10 s (Garcia et al., 2001).

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

RESULTS
Elevated temperature does not result in low sperm count
In many species, exposure to elevated temperature can result in low sperm count in males as a result of effects on spermatogenesis (David et al., 2005; Wang et al., 2007; Yaeram et al., 2006). We first determined whether reduced sperm count was the primary reason for lower fertility at elevated temperatures in *C. elegans*. We assessed sperm number in males raised continuously at 27°C compared with that of males raised continuously at 20°C. We counted sperm to determine whether young adult males had at least 200 sperm (normal range), 100–200 sperm (low range) or fewer than 100 sperm (extremely low range) (Murray et al., 2011). We found that the percentage of males raised at 27°C with a normal sperm count was the same as that of males raised at 20°C (Fig. 1). One JU1171 male and three LKC34 males raised at 27°C had a sperm count in the low range (100–200 sperm), but no males had fewer than 100 sperm (data not shown). For LKC34 males, two of the three males that had the lower sperm count had >190 sperm.

Thus, we concluded that, as found in *C. elegans* hermaphrodites (Harvey and Viney, 2007; Poullet et al., 2015), elevated temperature does not significantly impact male sperm count.

In vitro sperm activation by zinc is weakly affected by elevated temperature
We next assessed the effects of temperature on spermiogenesis, the formation of motile sperm through sperm maturation. Sperm are stored in males as round, non-motile spermatids (Fig. 2A). Upon ejaculation into the hermaphrodite, male sperm undergo spermiogenesis or sperm activation, forming a pseudopod resulting in motility (Fig. 2A). There are two main molecular pathways that lead to the activation of sperm: the TRY-5 pathway, which is activated by the TRY-5 protease in male seminal fluid, and the *spe-8* pathway, which is activated by an unknown signal present in hermaphrodites (Smith and Stanfield, 2011). Using two compounds known to activate sperm *in vitro* – Pronase E and zinc – we assessed whether these pathways were functional in sperm from males raised continuously at 27°C compared with sperm from males raised continuously at 20°C. Pronase E mimics the activation of sperm through the TRY-5 pathway in the male seminal fluid (Smith and Stanfield, 2011). Pronase E treatment resulted in a small but significant decrease (9%) in sperm activation in the JU1171 males that developed at 27°C versus 20°C, but there was no difference in activation in LKC34 and N2 males between the two temperatures (Fig. 2B). Zinc has been suggested to activate sperm through the *spe-8* pathway, which responds to signals that come

Table 2. Spicule phenotypes at elevated temperature

Strain and treatment	% Crumpled spicules (n)	Spicule length (μm)[a]	No. of tails	% Spicule protraction (n)[b]	No. of males
JU1171					
20°C	0 (0)	35.1±2.9	28	93.6 (73)	78
Up-shift	3.1 (1)	35.6±2.8	32	nd	nd
27°C	24.3 (9)	30.4±5.2	37	75 (63)	84
LKC34					
20°C	2.8 (1)	36.0±3.1	36	88.9 (72)	81
Up-shift	0 (0)	37.0±2.6	23	nd	nd
27°C	24 (6)	29.1±5.2	30	87.3 (69)	79
N2					
20°C	0 (0)	35.5±2.4	29	97.5 (77)	79
Up-shift	8.3 (3)	34.8±5.3	37	nd	nd
27°C	47.5 (19)	28.5±5.4	40	58.2 (46)	79

[a]For spicule measurements (means±s.d.), one spicule was imaged and measured from each tail. [b]A male was considered to have protracted spicules if they were protracted for ≥10 s in 0.1 mmol l^{-1} levamisole.

Fig. 1. Sperm count does not decrease in males raised at elevated temperature. Males were raised at 20 or 27°C, and sperm were counted in intact males to determine whether they had a minimum of 200 sperm as a cut-off for normal sperm number. There was no statistical difference in the number of males that had >200 sperm when raised at 20°C versus 27°C (*P*≤0.05, Fisher's exact test).
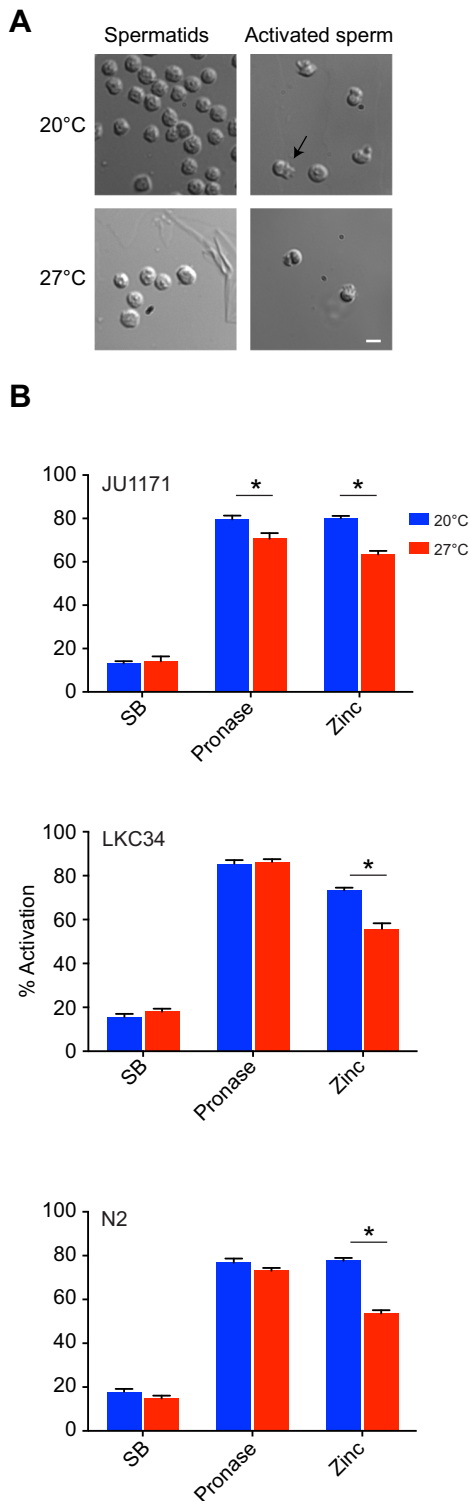
Fig. 2. Sperm from males raised at 27°C show decreased *in vitro* activation induced by zinc. (A) Sperm from males raised at 20°C or 27°C do not show differences in morphology. Arrow indicates an activated sperm pseudopod. Scale bar: 10 μm. (B) *In vitro* sperm activation induced by Pronase E was reduced only in JU1171 males raised at 27°C. Sperm activation induced by zinc was reduced in all three strains raised at 27°C. SB: sperm buffer without activator. Data are means and s.e. of the proportion. (*$P{\leq}0.01$, Fisher's exact test).

from the hermaphrodite (Liu et al., 2013; Zhao et al., 2018). Zinc treatment resulted in significantly decreased activation (17–24%) in all three strains from males that developed at 27°C versus 20°C

(Fig. 2B). *In vitro* sperm activation can lead to the formation of a full pseudopod or spikey projections that are thought to represent a step on the way to pseudopod formation, and spikes are seen more often with some activators *in vitro* than others (Singson, 2006). The decrease in activated sperm in all three strains at 27°C was primarily due to a decrease in sperm with pseudopods and not an increase in sperm with spikes (Fig. S1, Table S1). Finally, if sperm prematurely activate within a male, this can lead to male sterility (Liau et al., 2013). Because we did not see an increased level of sperm activation in sperm buffer without an activator for sperm from males raised at 27°C versus 20°C, there is no indication that elevated temperature results in premature sperm activation (Fig. 2B). Overall, the data support a model in which male growth at elevated temperature (27°C) does not affect the ability of sperm to be activated in response to the proteases in male seminal fluid as there was no or only a weak decrease in activation with Pronase E exposure. However, elevated temperature does negatively affect the ability of sperm to activate in response to changes in zinc ions.

Mating behavior is substantially impacted at elevated temperature

The ability of male *C. elegans* to produce cross-progeny requires both functional sperm and successful mating behavior. We wanted to assess whether elevated temperature affects mating behavior, thereby limiting the ability to have progeny. Stereotypical mating behavior in *C. elegans* has been well described, with a number of defined steps that can be observed (Fig. 3A) (Barr et al., 2018; Chatterjee et al., 2013). First, males will respond to the presence of a hermaphrodite by making contact with the hermaphrodite cuticle with their tail and beginning to scan for the presence of a vulva along the length of the hermaphrodite. During scanning, males will often have to successfully turn around the head or tail of the hermaphrodite. Finally, once the vulva is located, the male will move back and forth near the vulva to verify its location, and then maintain contact with it and insert the male mating organs, the spicules, into the vulva. We observed the stereotypical mating behavior of males using a standard 4 min mating assay (Barr and Garcia, 2006), where one male is placed with a number of uncoordinated (Unc) hermaphrodites for 4 min and monitored.

We performed experiments initially with males that were raised at 20°C or 27°C throughout development. In all three wild-type strains, males that were raised continuously at 27°C were significantly less likely to complete mating (Fig. 3E; Fig. S2). The primary step in mating that was affected by development at 27°C was the ability of males to respond to a hermaphrodite (Fig. 3B–E). Males that did contact a hermaphrodite often failed to commence scanning. For JU1171 and LKC34 males raised at 27°C that did respond to a hermaphrodite, there were no significant defects in the subsequent steps of mating compared with males raised at 20°C (Fig. 3B,C,E). However, none of the N2 males raised at 27°C completed mating even if they did respond to a hermaphrodite. This was primarily due to an inability to accurately find and maintain contact with the vulva, as indicated by a decrease in LOV efficiency and no males maintaining contact with the vulva (Fig. 3D).

The response of a male to a hermaphrodite is mediated through the male tail, which completes development during the L4 larval stage. To test whether this last stage of development is temperature sensitive, we performed temperature-shift experiments. Males were raised at either 20°C or 27°C until the L4 stage, and then shifted to the other temperature for 24 h before analysis. If the last stage of male tail development is temperature sensitive, such that it would affect male mating behavior, we would predict that up-shifting
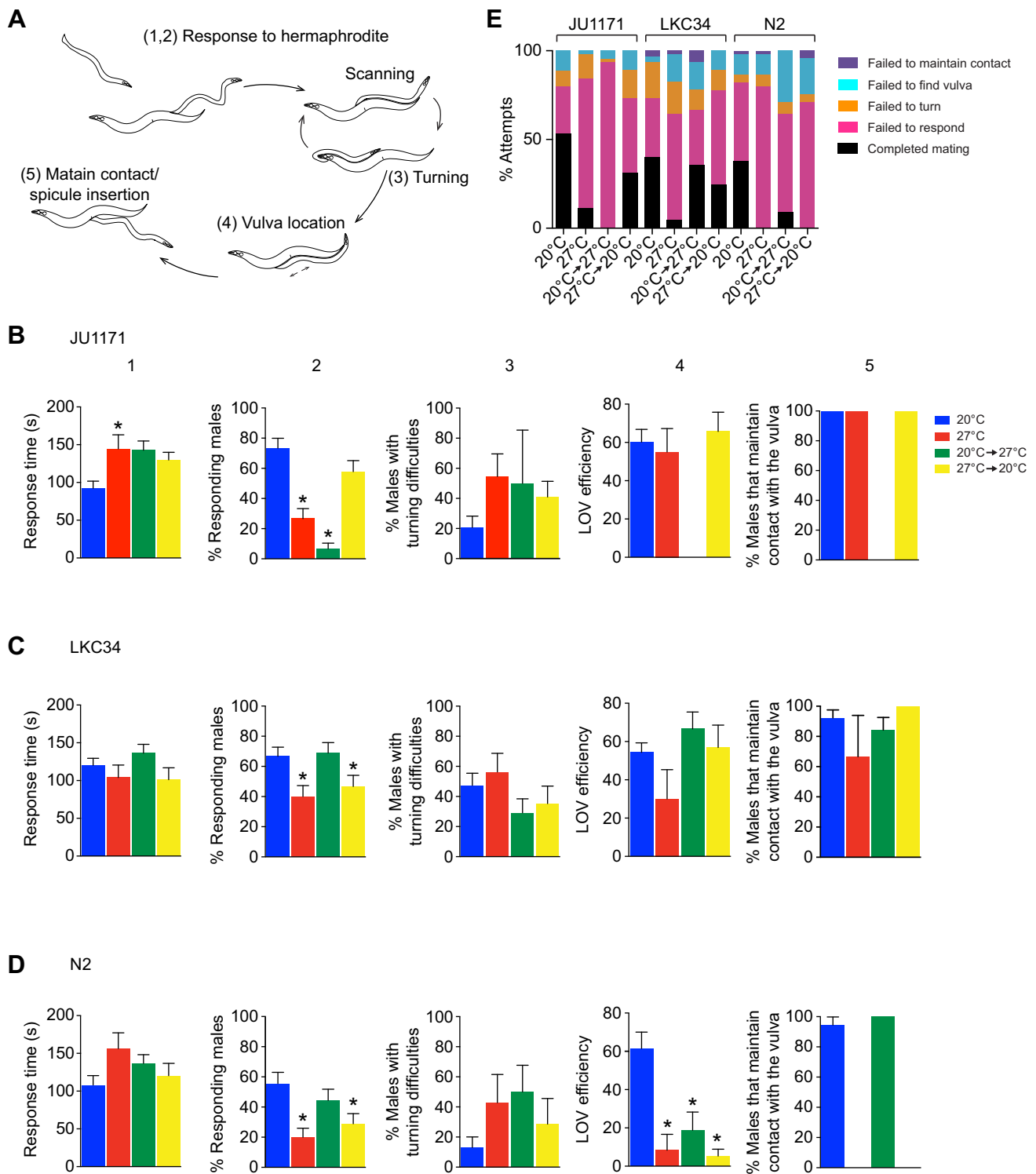
Fig. 3. See next page for legend.

males to 27°C at the L4 stage would result in similar disruptions in male mating behavior to continuous exposure to 27°C. Conversely, down-shifting to 20°C at the L4 stage may be sufficient to allow proper formation of male structures, enabling male mating behaviors to be restored. We found that up-shifting at the L4 stage did lead to a decrease in completed mating for both JU1171 and N2 males (Fig. 3E; Fig. S2). Like males raised continuously at 27°C, up-shifted males had difficulty responding to a hermaphrodite

(JU1171) and reduced LOV efficiency (N2) (Fig. 3B,D). LKC34 males showed similar levels of completed mating when up-shifted and when raised continuously at 20°C (Fig. 3E). We found that down-shifting at the L4 stage was able to restore completed mating to levels seen in males raised continuously at 20°C for both JU1171 and LKC34 males (Fig. 3E; Fig. S2). N2 down-shifted males still showed a significant decrease in completed mating compared with males raised continuously at 20°C (Fig. 3E; Fig. S2). Overall, males

Fig. 3. Males exposed to 27°C show a decline in mating behaviors. (A) Males demonstrate stereotypical mating behaviors that include responding to the presence of hermaphrodites leading to contact (1,2), scanning along the hermaphrodite cuticle, turning if reaching the hermaphrodite head or tail (3), locating the vulva (4), and maintaining contact with the vulva (5). Redrawn from Wormbook (Barr and Garcia, 2006). (B–D) Each stage of mating behaviors was recorded for JU1171 (B), LKC34 (C) and N2 (D) strains for males raised continuously at 20°C (blue) or 27°C (red), males raised at 20°C then up-shifted to 27°C (green), or males raised at 27°C then down-shifted to 27°C (yellow). Response time (1) was increased only in JU1171 males raised at 27°C compared with JU1171 males raised at 20°C ($P \leq 0.01$, Student's t-test). However, there were significantly fewer males that responded to the presence of hermaphrodites (2) in all three strains, although the temperature treatment that resulted in decreased male response differed between strains ($P \leq 0.05$, Fisher's exact test). No strains showed increased turning difficulty (3) with any temperature treatment compared with males raised at 20°C ($P \leq 0.05$, Fisher's exact test). Strikingly, only the N2 strains showed a decreased vulva location (LOV) efficiency, which reflects increased passes across the vulva (4), but did so with any exposure to elevated temperature ($P \leq 0.01$, Student's t-test). Finally, if males were able to find the vulva when exposed to elevated temperature, there was no significant decrease in their ability to stop movement and maintain contact (5) at the vulva ($P \leq 0.05$, Fisher's exact test). (E) Males were scored as having completed mating, or failed during the mating process by not responding to the presence of hermaphrodites (failed to respond), not maintaining contact with the hermaphrodite after trying to execute a turn (failed to turn), leaving the hermaphrodite or not finding the vulva within the 4 min time frame (failed to find vulva), or failing to maintain contact with the vulva once near it (failed to maintain contact). n=45 males per data point. Error bars are s.e. of the proportion, except for response time where error bars are s.e.m.

of all three strains exhibited reduced mating behavior when grown continually at 27°C and varying levels of reduced mating behavior when experiencing 27°C for only portions of development. These data indicate that behavioral changes leading to a failure to mate at elevated temperature are a significant factor in male sterility at elevated temperature.

Development at elevated temperature results in decreased sperm transfer

Our previous study indicated that males down-shifted from 27°C to 20°C had very few cross-progeny (Petrella, 2014), but in our mating assay both JU1171 and LKC34 down-shifted males showed behaviors very similar to those of males kept continuously at 20°C (Fig. 3E). We next assessed whether males that behaviorally complete mating also successfully transfer sperm to strain-specific hermaphrodites in a 45 min window (Fig. 4A). This assay allowed us to address two questions: (1) does a longer period for mating increase the number of successful matings?; and (2) does a male that behaviorally completes mating by maintaining contact with the vulva functionally transfer sperm to a hermaphrodite? We chose to do sperm transfer experiments using strain-specific hermaphrodites instead of Unc hermaphrodites, which are from the N2 background, to capture strain-specific mating ability and diminish known intra-strain mating issues (Bahrami and Zhang, 2013).

We found that when males were raised continuously at 20°C there was no significant difference between the percentage of males that could complete mating in our behavioral assay within 4 min and the percentage of males that successfully transferred sperm into a hermaphrodite within 45 min (Fig. 4B; Table S2). Thus, in ideal temperature conditions the behavioral assay and sperm transfer results are highly correlated. Increased time did not significantly increase the rate of successful matings as assayed by sperm transfer. Additionally, all three strains were able to mate with both strain-specific wild-type hermaphrodites and *unc-24* mutant hermaphrodites of an N2 strain background. Under conditions in

which males were raised continuously at 27°C and very few males were observed to complete mating, we also saw a very low percentage of males that could transfer sperm (Fig. 4B; Table S2). Even though both JU1171 and LKC34 males down-shifted from 27°C to 20°C could behaviorally complete mating, no down-shifted males transferred sperm in any of the strains. Similarly, significantly fewer LKC34 males up-shifted from 20°C to 27°C successfully transferred sperm than completed mating (Fig. 4B; Table S2). Overall, any exposure to 27°C resulted in significantly fewer males transferring sperm to hermaphrodites than for the same strain raised continuously at 20°C. Thus, even if a male can still behaviorally find a hermaphrodite and maintain contact with the vulva, exposure to elevated temperature limits its ability to successfully transfer sperm.

Male tail structure is affected by elevated temperature

Successful mating and sperm transfer require male-specific structures in the male tail, including sensory rays and spicules. There are nine sensory rays on each side of the male tail that demonstrate a typical morphology, which are needed for males both to respond to a hermaphrodite by starting to scan and to find the vulva. As we found defects in mating behavior, we analyzed ray morphology in 1 day old males that were raised continuously at 20°C or 27°C and in males that were up-shifted from 20°C or 27°C at the L4 larval stage for 24 h. For males raised at 27°C, there was a significant increase in the number of males with at least one defect in ray structure in all three strains (Fig. 5A,C). For males up-shifted from 20°C or 27°C, there was a significant increase in the number with defects in ray structure for N2 only. The primary defects that increased with temperature were around rays 1–2, with rays 1–2 fused or ray 1 highly reduced (Fig. 5A, Table 1). There were also extra ray-like growths that often looked brush-like in appearance, most commonly in the ray 1–2 area of the tail (Fig. 5A, Table 1).

We also analyzed the spicules, which are important for probing the vulva and holding it open for sperm transfer. We found that there were a significant number of males with malformed/crumpled spicules when raised continuously at 27°C versus 20°C or up-shifted to 27°C for all three strains (Fig. 5B,D, Table 2). Additionally, significantly more N2 males raised at 27°C had crumpled spicules compared with either JU1171 or LKC34 males under the same conditions. We also measured spicule length under all three temperature conditions and found that it was significantly shorter in males raised continuously at 27°C compared with that in males raised continuously at 20°C or in males that were up-shifted for all three strains (Fig. 5E, Table 2). Many of these shorter spicules had generally normal morphology but were considerably shorter in length. Finally, we scored for spicule protraction in males exposed to levamisole. When a male finds the vulva, the protractor muscles contract to allow the spicules to probe and enter the vulva. This action can be induced *in vitro* by exposure to levamisole. There were significantly fewer males that had protracted spicules in the group raised continuously at 27°C versus 20°C for JU1171 and N2 (Table 2). There was no difference in spicule protraction in LKC34 males between the two temperatures (Table 2).

Sperm migration is affected by elevated temperature in N2 and LKC34

Once the sperm is transferred to the hermaphrodite uterus, it must migrate to the spermatheca in order to fertilize an oocyte. Using our fluorescent sperm transfer assay, we isolated any hermaphrodites that had received sperm and waited a minimum of 45 min after sperm transfer to allow the sperm to migrate to the spermatheca (Fig. 4A). While we were able to detect migration to the
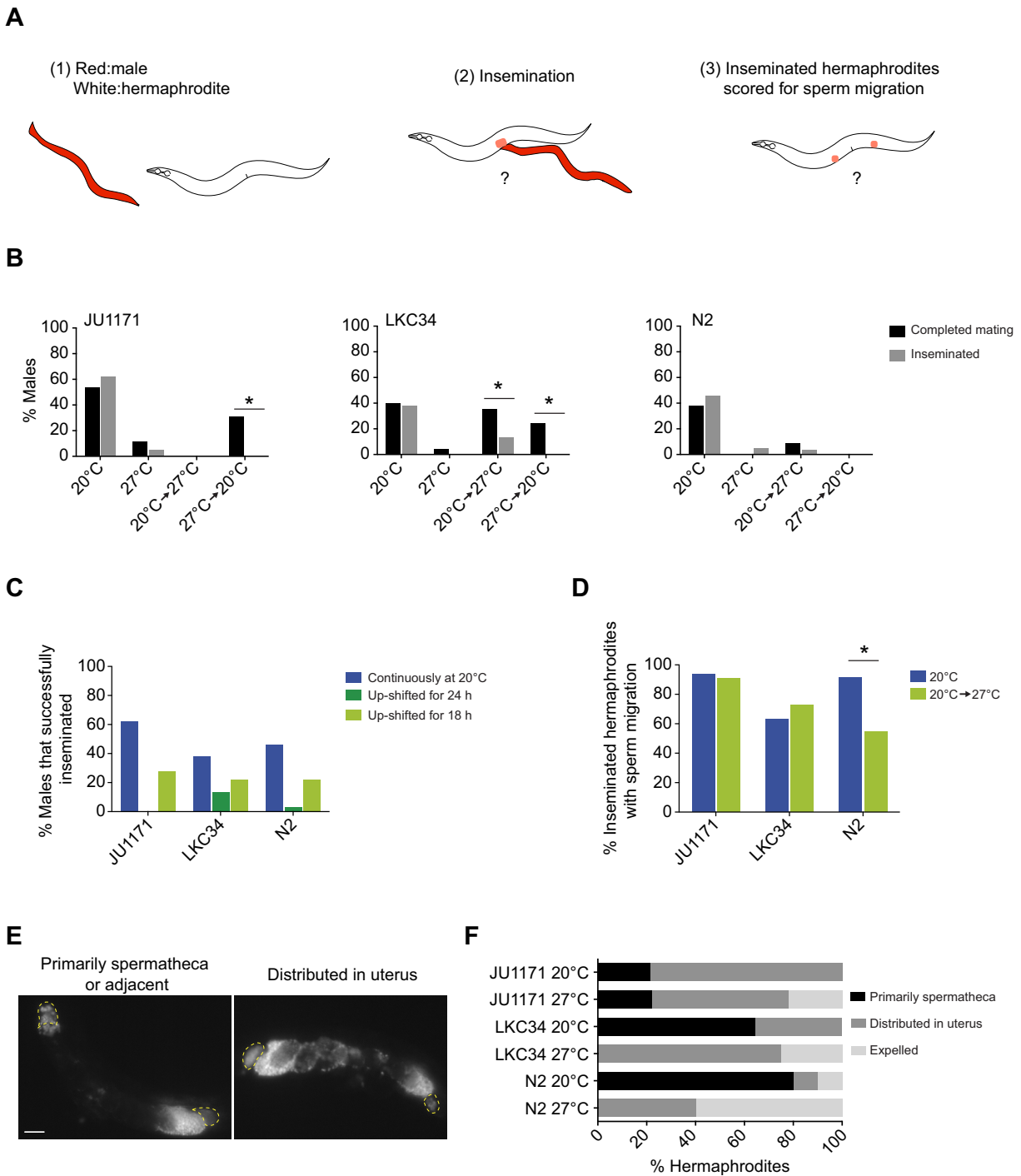
Fig. 4. Males exposed to 27°C show a decrease in sperm transfer to hermaphrodites. (A) Males were stained with MitoTracker Red CMXRos (1) and allowed to mate with anesthetized strain-specific hermaphrodites for 45 min (2). Inseminated hermaphrodites were removed and scored for sperm movement to the spermatheca after 45 min (3). (B) The percentage of males that completed all steps of mating using the 4 min mating assay compared with the percentage of males that could inseminate. There were significantly fewer males that could inseminate when shifted between temperatures as compared with continuous temperature treatment for JU1171 and LKC34 (*$P \leq 0.05$, Fisher's exact test). (C) More males could inseminate when up-shifted from 20°C to 27°C for only 18 h instead of 24 h. (D) The percentage of hermaphrodites that were inseminated where the sperm migrate to the spermatheca was lower for males up-shifted to 27°C for 18 h than for males kept at 20°C in N2 (*$P \leq 0.05$, Fisher's exact test). (E) Representative images of male sperm stained with MitoTracker Red CMXRos showing localization primarily within/adjacent to the spermatheca (left) or more distributed throughout the uterus. The location of spermatheca is indicated by dashed yellow outlines. Scale bar: 30 μm. (F) Percentage of hermaphrodites that show different distributions of male sperm within the reproductive tract. Hermaphrodites that no longer had any MitoTracker Red-labeled sperm were scored as having expelled sperm.

spermatheca under the stereomicroscope in hermaphrodites raised at 20°C, we were limited in our ability to assess sperm migration from males exposed to elevated temperature because so few male worms

mated or transferred sperm. We found that if males up-shifted from 20°C to 27°C were only exposed to 27°C for 18 h instead of 24 h, significantly more males transferred sperm (Fig. 4C). Therefore, we
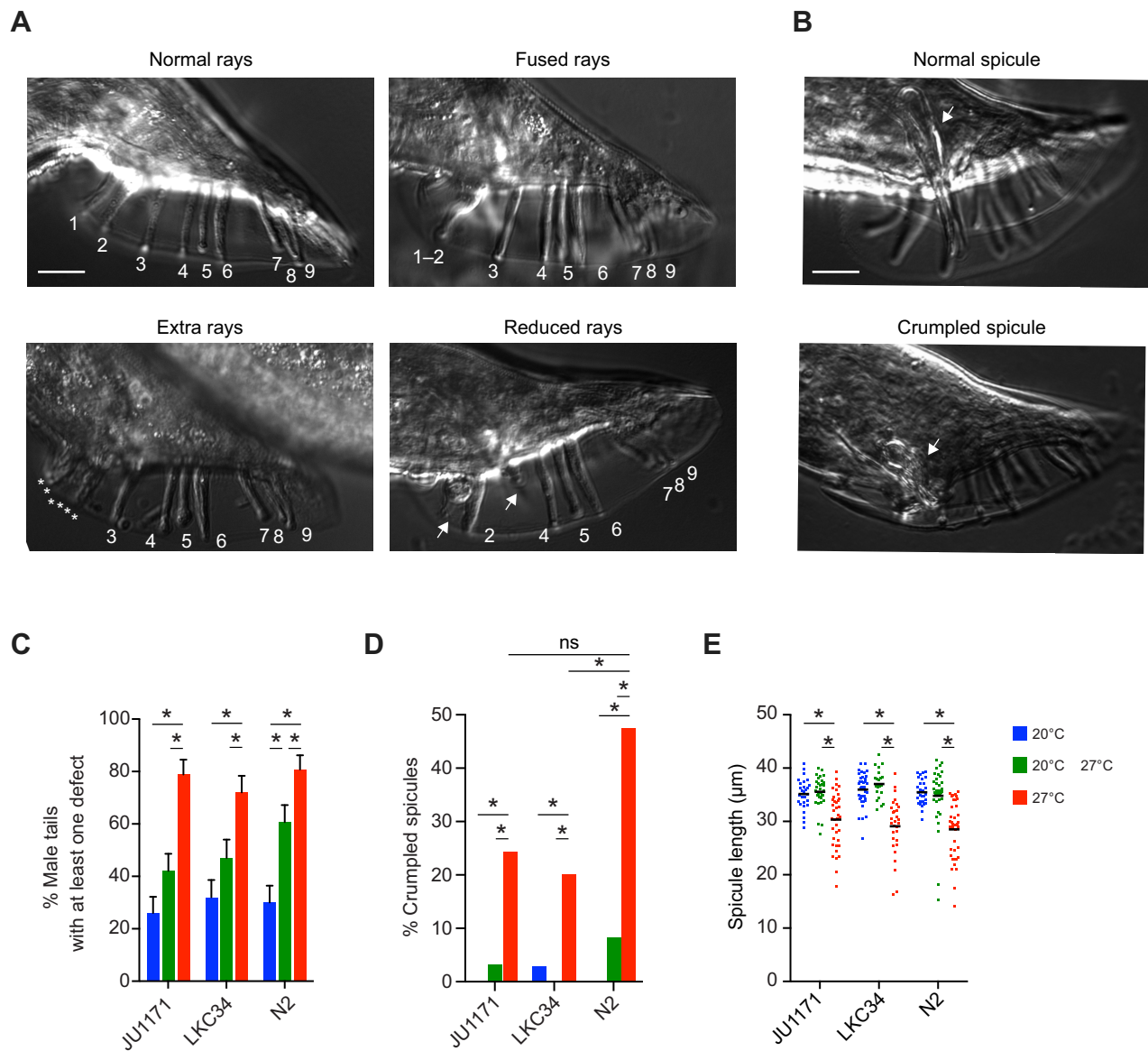
Fig. 5. Males exposed to 27°C have defects in sensory rays and spicules. (A) Representative images of ray morphology in JU1171 males raised at 27°C. Normal morphology shows nine rays along the length of the fan. In some males, rays are fused, extra rays form (asterisks), or rays are strongly reduced (white arrows). Scale bar: 10 µm. (B) Representative images of spicule morphology from LKC34 males at 27°C. The normal spicule is long, smooth and protracted out of the body of the male (arrow). The crumpled spicule is short and malformed (arrow). Scale bar: 10 µm. (C) Percentage of males that showed at least one defect in ray formation. Error bars are s.e. of the proportion (*$P \leq 0.05$, Fisher's exact test). (D) Percentage of males with crumpled spicules (*$P \leq 0.05$, Fisher's exact test). (E) Spicule length. Line represents mean, each dot represents a single spicule measured per tail (*$P \leq 0.05$, two-way ANOVA with Tukey's correction for multiple comparisons).

compared the ability of sperm to migrate to the spermatheca between males raised continuously at 20°C and males exposed to 27°C for ~18 h post-developmentally. For JU1171 and LKC34, sperm from a male exposed to 27°C for ~18 h migrated with the same efficiency as sperm from males that had only experienced 20°C when observed under the stereomicroscope (Fig. 4D). However, for the N2 strain, sperm from a male exposed to 27°C for ~18 h migrated significantly less often than sperm from males that had only experienced 20°C (Fig. 4D). To assess sperm migration in more detail, we imaged hermaphrodites that had been inseminated after 45 min and looked at the distribution of sperm in the uterus and spermatheca under higher magnification. Hermaphrodites inseminated by LKC34 or N2 males that had only experienced 20°C primarily had sperm either within the spermatheca or right

beside the spermatheca (Fig. 4E,F). In contrast, hermaphrodites inseminated by LKC34 or N2 males that were up-shifted to 27°C either expelled all sperm (data not shown) or showed a large proportion of sperm distributed throughout the uterus (Fig. 4E,F). JU1171 hermaphrodites inseminated by males showed a distribution of sperm to both the spermatheca and the uterus that was similar between temperatures. Thus, for the LKC34 and N2 strains, even a limited exposure to 27°C can affect male sperm migration within the hermaphrodite.

DISCUSSION

Temperature-dependent defects in fertility are seen across taxa when individuals are raised outside of their optimal reproductive temperature range. Here, we have completed the first analysis

determining which aspects of fertility are affected by elevated temperature in *C. elegans* males. Unlike in mammals and *Drosophila* (Kim et al., 2013; Rohmer et al., 2004), we did not find a significant decrease in spermatid number. We also only saw moderate effects of elevated temperature on sperm activation. Instead, the primary effect of elevated temperature was on mating behavior and male tail morphology, which limits the ability of males to respond to the presence of hermaphrodites and successfully transfer sperm. These defects override any potential effects of temperature on sperm function. Further experiments that can circumvent the behavioral effects of temperature will be needed to determine whether *C. elegans* male sperm are functional to fertilize oocytes at elevated temperature.

Temperature effects on the soma have a large impact on fertility

One striking finding of our study is the perturbation of mating behaviors exhibited by males raised at elevated temperature. Mating behaviors are primarily driven by interactions between somatic neurons and muscles, not by the function of the germline (Barr et al., 2018). Therefore, changes to somatic tissues at elevated temperature play a large role limiting fertility in males. The primary defect we saw in males that experienced 27°C continuously was a decreased mating response compared with that of males exposed to 20°C continuously. Male response to a hermaphrodite requires males to seek and find a hermaphrodite and then, after making contact with a hermaphrodite, scan the hermaphrodite. Scanning of the hermaphrodite is mediated through signaling from the sensory rays (Liu and Sternberg, 1995). There were significant defects in the rays in males that experienced 27°C continuously. However, we saw defects primarily in rays 1 and 2 and not a complete loss or malformation of all rays. Generally, male mating behavior has been shown to be normal as long as the tail has least three functional rays (Liu and Sternberg, 1995). Therefore, if only rays 1 and 2 were disrupted, we would expect male mating to be less affected. Further analysis of the neurons within the rays would be necessary to determine whether there are additional ray defects contributing to the mating behavior. The other aspect of the male response to hermaphrodites is their ability to find the hermaphrodite. Male attraction to hermaphrodites is mediated through neural input from head neurons responding to mating pheromones, including several amphid neurons and the male-specific cephalic neurons (Narayan et al., 2016; Srinivasan et al., 2008; Wan et al., 2019; White et al., 2007). The AWA neurons are one of the primary amphid neuron pairs that function in the perception of mating pheromones (Wan et al., 2019). Interestingly, the AWA neurons are the sole synaptic input onto the primary neurons that sense and respond to temperature in *C. elegans*, the AFD neurons (Kimata et al., 2012; Ramot et al., 2008; White et al., 1986). Thus, temperature and mating pheromone sensing in males are part of a single circuit. Changes in temperature cause a change in the signaling rate of AFD neurons (Kimura et al., 2004; Ramot et al., 2008). This allows worms to sense and move in response to changes in temperature, a response called thermotaxis (Goodman and Sengupta, 2019; Kimata et al., 2012). The temperature at which AFD neurons change their signaling rate leading to thermotaxis is dependent upon the temperature at which the worm developed (Clark et al., 2006; Kimura et al., 2004; Ramot et al., 2008). Therefore, the temperature to which a worm will migrate to along a temperature gradient is dependent upon the temperature at which it was raised (Goodman and Sengupta, 2019; Mori and Ohshima, 1995). Additionally, there is a universal increase in neuronal excitability with elevated temperature that occurs in all neurons, including both AFD and AWA neurons (Graham et al., 2008; MacIver and Roth, 1982; Ramot et al., 2008). Our data suggest that when males are raised continuously at 27°C, there could be changes to the neuronal circuit that senses or transmits the response to mating pheromones, leading to a decreased or absent mating drive.

The ability to transfer sperm is primarily dependent on the proper function of somatic tissues: the sensory rays in the tail must respond to the location of the vulva and the spicules must be inserted prior to sperm transfer (Barr et al., 2018), both of which showed defects in males exposed to 27°C continuously. Locating the vulva and insertion of spicules are dependent upon coordination between male-specific tail neurons and muscles (Garcia et al., 2001; Koo et al., 2011; Liu and Sternberg, 1995; Liu et al., 2011). After spicule insertion is sensed, sperm transfer will occur through a second set of coordinated neuronal signaling and muscular contraction events (LeBoeuf et al., 2014; LeBoeuf and Garcia, 2017; Schindelman et al., 2006). The decrease in spicule length and spicule protraction we saw in males raised continuously at 27°C may explain the inability of males to regain fertility when down-shifted from 27°C to 20°C (Petrella, 2014). These defects would preclude males from successfully mating even if they had functional sperm that were made at the permissive temperature. In support of spicule defects affecting the ability of males to successfully transfer sperm, we observed that males exposed to 27°C occasionally ejaculated outside the hermaphrodite on the plate (N.B.S., data not shown), which we did not see with males raised continuously at 20°C. Sperm present outside the uterus can occur if sperm transfer is initiated before the completion of spicule insertion. Therefore, temperature effects on tail morphology and potentially neuronal function of either the head neurons or male tail circuit could have a profound influence on male mating ability.

N2 males are more affected by elevated temperature compared with newly isolated wild-type strains

In most of the assays we performed, N2 males were more affected by elevated temperature than either LKC34 or JU1171 males. N2 males showed reduced mating efficiency even with short exposure to elevated temperature, defects in locating the hermaphrodite vulva, and stronger effects of temperature on male tail morphology. These differences may be due to the laboratory adaptation of the N2 strain in contrast to JU1171 and LKC34, which were recently isolated (Liu et al., 2013; Zhao et al., 2018). N2 is known to contain a number of unique alleles in genes that change its response to stimuli including oxygen levels and heat avoidance in comparison to recently isolated wild-type strains (Andersen et al., 2014; Chang et al., 2006; Glauser et al., 2011; Sterken et al., 2015). Additionally, male mating efficiency for N2 is low compared with that of most other wild-type isolates at 20°C, as a result of both a decrease in the ability of males to mate and the receptiveness of N2 hermaphrodites to male mating (Bahrami and Zhang, 2013). The long adaptation of the N2 strain in the laboratory, where self-fertilization in hermaphrodites has been selected for, may have reduced the ability of N2 males to mate even at low temperatures. N2 males may then be even more susceptible to stresses such as elevated temperature compared with males from more recently isolated strains. Careful analysis of the behavior and the effects of stressors on males from a broader range of *C. elegans* strains will be needed to determine whether N2 males are a true outlier in their higher temperature sensitivity or whether there is a continuum of male susceptibility to elevated temperature. However, as mating behaviors are traits that can quickly evolve (Lande, 1981; Lopes et al., 2008; Miyatake and Shimizu, 1999; Palopoli et al.,

2015; Wilburn and Swanson, 2016), the lack of selective pressure to keep male mating robust in strains that are maintained in the laboratory for long periods of time seems a likely path leading to the elevated temperature sensitivity of N2 males.

Does temperature cause a decrease in male sperm function?

In other organisms, one of the major effects of elevated temperature on male fertility is a decrease in sperm count. However, in male *C. elegans*, we observed that most males had a sperm count within the normal range. This mirrors what is seen in *Caenorhabditis* hermaphrodites, where loss of fertility at elevated temperature is not associated with a decrease in sperm number (Harvey and Viney, 2007; Poullet et al., 2015). In *C. elegans*, although the number of sperm in hermaphrodites does not decrease as temperature increases, the number of self-progeny decreases substantially (Harvey and Viney, 2007; Petrella, 2014; Poullet et al., 2015). Similarly, in *C. briggsae* and *C. tropicalis*, two related hermaphroditic *Caenorhabditis* species, there is only a small decrease in sperm number at temperatures where there is almost complete sterility (Harvey and Viney, 2007; Petrella, 2014; Poullet et al., 2015; Prasad et al., 2011). When any of these three species are raised at elevated temperature, fertility is greatly enhanced when sperm are provided from males. Therefore, the loss of fertility seen in *Caenorhabditis* hermaphrodites with elevated temperature is primarily due to a loss of sperm function, not a decrease in sperm count.

Is there a decrease in sperm function at elevated temperature in male *C. elegans* like that seen in hermaphrodites? While hermaphrodite and male sperm have many similarities, they also have crucial differences. Male sperm are physically bigger, there are sex-specific gene expression patterns in sperm, and there are activating factors in seminal fluid that male sperm experience which hermaphrodite sperm do not (Ebbing et al., 2018; Ellis and Stanfield, 2014; LaMunyon and Ward, 1998; Ma et al., 2014; Reinke, 2003). These differences could result in male sperm maintaining function at elevated temperature, where hermaphrodite sperm function is lost. Because of the strong effects of elevated temperature on mating behavior, it is difficult to ascertain whether male sperm could be functional if they were transferred into a hermaphrodite. However, there are some indications that there may be functional changes to male sperm that could contribute to decreased male fertility at elevated temperature. First, sperm from both LKC34 and N2 males showed a reduced ability to reach the spermatheca after inseminating a hermaphrodite. Additionally, using *in vitro* sperm activation assays, we saw distinct differences between the ability of sperm exposed to 27°C to be activated by Pronase E and zinc. With Pronase E, there was no or only a slight decrease in *in vitro* sperm activation in sperm from males raised at 27°C. This indicates that male sperm exposed to elevated temperature are likely able to respond to and be activated by the male-specific seminal fluid activation pathway through the TRY-5 protease (Fenker et al., 2014; Smith and Stanfield, 2011). However, with zinc, there was a significant decrease in activation in all three strains. *In vitro* activation by zinc likely works through the *spe-8* pathway, which responds to a hermaphrodite-derived signal (Liu et al., 2013; Zhao et al., 2018). The recent discovery of the role of the zinc transporter protein ZIPT-7.1 highlights the important role that zinc plays in both male and hermaphrodite sperm activation (Zhao et al., 2018). ZIPT-7.1 helps maintain intracellular zinc levels within sperm and is necessary for full *in vivo* activation of sperm from both males and hermaphrodites. Classic *spe-8* pathway mutants result in hermaphrodite self-sterility, but *spe-8* pathway males are fertile unless the TRY-5 pathway is also compromised

(Ellis and Stanfield, 2014). However, *zipt-7.1* mutants have highly reduced fertility in both sexes (Zhao et al., 2018). Despite the very low fertility of *zipt-7.1* mutant males, sperm from *zipt-7.1* mutant males exhibit *in vitro* activation that is only weakly reduced with Pronase E treatment compared with wild-type male sperm. However, *zipt-7.1* mutant male sperm exhibit almost no *in vitro* activation with exogenous zinc treatment. The pattern we found for *in vitro* activation of sperm from males raised at 27°C looks like a dampened version of that seen in *zipt-7.1* mutants: high levels of activation with Pronase E treatment but reduced levels of activation with zinc treatment. Similar to findings in *zipt-7.1* mutants, we also noted a loss of both male and hermaphrodite fertility at 27°C, suggesting that changes in intracellular ion levels or fluxes, especially zinc, could lead to a loss of sperm function at elevated temperatures. Additional experiments are needed to determine whether male sperm that have developed at elevated temperature have defects in the *spe-8* pathway or zinc signaling.

Author contributions
Conceptualization: E.M.N., L.N.P.; Formal analysis: L.N.P.; Investigation: E.M.N., N.B.S., L.N.P.; Writing - original draft: E.M.N., N.B.S., L.N.P.; Writing - review & editing: E.M.N., N.B.S., L.N.P.; Visualization: L.N.P.; Supervision: L.N.P.; Project administration: L.N.P.; Funding acquisition: L.N.P.

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