Discovery and Characterization of Novel Sperm Development Patterns in the Nematode Rhabditis Sp. Sb347

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Discovery and characterization of novel sperm development patterns in the nematode Rhabditis sp. SB347

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Master of Science

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ABSTRACT

Studies of gamete production in Caenorhabditis elegans self-fertile hermaphrodites have informed textbook descriptions of nematode reproduction and have significantly contributed to our understanding of fundamental cellular and developmental mechanisms. However, C. elegans is one species within a large phylum of mostly dioecious (male/female) species. Whether the C. elegans pattern of self-fertile hermaphroditism and gamete development is representative of other nematodes remains largely unexplored.

In Chapter 1, we describe an alternative pattern of sperm production in the trioecious (male/female/hermaphrodite) nematode Rhabditis sp. SB347 that differs from C. elegans in two striking ways. First, instead of making a one-time switch from sperm to oocyte production, R. sp. SB347 hermaphrodites produce sperm and oocytes simultaneously. Secondly, instead of limiting germ cell proliferation to germline stem cells (GSCs), sperm production in R. sp. SB347 includes an additional population of mitotically dividing cells that are a developmental intermediate between GSCs and fully differentiated spermatocytes. These cells, which are present in males and hermaphrodites but not females, exhibit key characteristics of spermatogonia, the mitotic progenitors of spermatocytes in flies and vertebrates. Specifically, they amplify germ cell numbers, exist outside the stem cell niche, and synchronously develop and proliferate within germ cell clusters (or cysts). We next discovered that spermatogonia are a feature of sperm production in other trioecious species of the Rhabditis genus, but not in the male/female species Rhabditis axei. The discovery of simultaneous hermaphroditism and spermatogonia in a lab-cultivatable nematode suggests R. sp. SB347 as a richly informative species for comparative studies of gametogenesis.

In Chapter 2, we assess whether C. elegans germ cell development genes have been conserved across species and identify homologs in R. sp. SB347. By comparing these homologs' roles and expression patterns between species, we may better understand differences between these two divergent mechanisms of hermaphroditism and sperm production. Such molecular comparative analyses may also inform our understanding of germ cell evolution. Here we begin to develop tools to investigate expression of two conserved homologs, FBF and SPE-44.

In Chapter 3, we consider what aspects about R. sp. SB347’s unique form of sperm development have not yet been fully explored. We report relevant preliminary data and recommend future directions for addressing these questions.
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Finally, I would like to recognize my parents for teaching me that anything is possible, to be resilient, and to do what I love.
This M.S. is dedicated to my mother and grandmother for being my role models and for their boundless support and enthusiasm throughout my education.
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Chapter 1. Germ cell cysts and simultaneous sperm and oocyte production in a hermaphroditic nematode

This chapter of my thesis is a draft of a manuscript to be submitted for publication. The manuscript reflects work done by several co-authors: Xiaoxue Lin, Maureen Farrell, Kathryn Rehain-Bell, and Diane C. Shakes.

Rehain-Bell made the first observation in her 2012 B. S. thesis of the unexpected cells "of unknown function" that are characterized here. Farrell initially led these studies and reported that these cells were hermaphrodite-specific clusters that produce sperm in her M. S. thesis in 2015. Lin and McCaig partnered in collecting the majority of the data reported here unless otherwise noted. McCaig analyzed the quantitative data and carried out the experiments characterizing actin patterns, while Lin carried out the experiments testing collagenase treatment and collected data from closely related species. Lin and McCaig co-wrote the first drafts of the manuscript, and then McCaig edited the manuscript with thorough input from Dr. Shakes. Rehain-Bell and Farrell have also provided helpful suggestions for the manuscript text.
Introduction

Most animal species are dioecious (male/female). However, when mates are scarce, selective pressures can favor the evolution of self-fertile hermaphrodites (Ghiselin 1969; Clark 1978; Jarne & Charlesworth 1993). Within the phylum Nematoda, evolutionary transitions from outcrossing males and females to self-fertile hermaphrodites have occurred in multiple, separate lineages (Kiontke et al. 2004; Kiontke & Fitch 2005; Denver et al. 2011). Hermaphroditic nematodes can be found across the phylum in both parasitic and free-living species (Castro 1996; Criscione et al. 2005; Kiontke & Fitch 2005; Kanzaki et al. 2013).

In *Caenorhabditis elegans*, studies of the developmental and molecular underpinnings of nematode gametogenesis have contributed significantly to our understanding of both germ cell biology and fundamental mechanisms in cell and developmental biology (Corsi et al. 2015). Anatomically, the *C. elegans* hermaphrodite gonad is organized as a linear tube with germline stem cells (GSCs) at the distal end and differentiated, meiotic gametes at the proximal end (Figure 1A). A single somatic cell at the distal end (the distal tip cell, DTC) serves as the stem cell niche and maintains a zone of ~200 mitotic, undifferentiated germ cells (Kimble & White 1981; Hansen, Wilson-Berry, et al. 2004; Crittenden et al. 2006; Fox & Schedl 2015). As germ cells move beyond the niche, they both enter meiotic prophase and begin differentiating into either spermatocytes or oocytes (Figure 1A-B) (Lui & Colaiácovo 2013). *C. elegans* hermaphrodites are somatic females which achieve self-fertility by producing...
sperm as larvae, storing their sperm in a pouch-like structure called the spermatheca, and then producing exclusively oocytes as adults (Figure 1A). As individual oocytes mature, they pass through the spermatheca in an assembly-line fashion where they are fertilized by the stored sperm (Figure 1A) (Ward & Carrell 1979; L'Hernault 2006).

This same pattern of “sperm first, then oocytes” gamete production has been observed in self-fertile hermaphrodites of the *Caenorhabditis* and *Pristionchus* genera (Rudel et al. 2005; Sommer 2005). Yet even within the same genus, *C. elegans* and *C. briggsae* hermaphrodites convergently evolved distinct molecular changes to support sperm production in an otherwise female body (Nayak et al. 2005; Hill et al. 2006; Hill & Haag 2009; Guo et al. 2009; Liu et al. 2012). Alternative patterns for achieving hermaphrodite self-fertility have been reported; for example, hermaphrodites in the parasitic species *Rhabdias ranae*, simultaneously produce both oocytes and sperm within a discrete zone of the ovatestis (Runey et al. 1978). However, current studies are too limited to assess whether the sequential hermaphroditism strategy adopted by *Caenorhabditis* and *Pristionchus* predominates within the larger phylum.

*Rhabditis* sp. SB347 is a free-living, trioecious (male/ female/ hermaphrodite) nematode from the same *Rhabditidae* family as *C. elegans* (Félix 2004; Kiontke & Fitch 2005), but its reproductive biology differs in several ways (Félix 2004; Shakes et al. 2011; Chaudhuri et al. 2011; Chaudhuri et al. 2015). First, whereas *C. elegans* is androdioecious (male/ hermaphrodite) (Maupas 1901; Corsi et al. 2015), *R.* sp. SB347 is trioecious (male/ female/
hermaphrodite), with XX animals developing into either females or hermaphrodites (Félix 2004). In R. sp SB347, hermaphroditism is specifically linked to passage through a stress-resistant and dispersive larval morph, referred to as “dauer” in free-living nematodes and “infective juvenile” in parasitic species (Félix 2004; Chaudhuri et al. 2011). Additionally, R. sp. SB347 exhibits non-Mendelian sex ratios (Shakes et al. 2011; Chaudhuri et al. 2015). More specifically, self-fertilizing hermaphrodites produce an unexpectedly high number of male offspring (~10%) (Félix 2004)(M. Farrell, unpublished), while males sire exclusively XX feminine progeny due to a modified spermatogenesis program in which only the X-bearing sperm are functional (Shakes et al. 2011) (D. Shakes, unpublished).

Given the many differences between the reproductive biology of R. sp. SB347 and C. elegans, we investigated whether R. sp. SB347 might also have evolved a distinct developmental solution to achieving self-fertility. Here we describe the unexpected discovery of germ cell clusters within the gonads of R. sp. SB347 hermaphrodites that enable the continuous and simultaneous production of sperm and oocytes in both larvae and adults. Characterization of these germ cell clusters revealed them as mitotically proliferative spermatogonia within germ cell cysts. Such cells are a feature of sperm production in most other taxa but have not been previously reported in a nematode, including C. elegans. Given this apparent novelty, we also examined near relatives of R. sp. SB347. Other trioecious Rhabditis species shared the R. sp. SB347 pattern of hermaphrodite sperm production, whereas males of Rhabditis axei and
hermaphrodites of the more distant *Oscheius tipulae* exhibited the
*Caenorhabditis* patterns. These data indicate that *R.* sp. SB347 evolved
heretofore undescribed mechanisms for both nematode sperm production and
achieving hermaphrodite self-fertility, distinct from those of the model nematode
*C. elegans*.

**Results**

*Rhabditis* sp. SB347 hermaphrodite gonads contain distinctive cell clusters
that have no *C. elegans* analog

To identify potential differences in germ cell patterning between *R.* sp.
SB347 and *C. elegans* hermaphrodites, we compared isolated gonads co-
labelled with DAPI and anti-α-tubulin antibodies. Surprisingly, *R.* sp. SB347
gonads contained isolated cell clusters positioned along the line of developing
oocytes (Figure 1B - cc1-3). These cell clusters were distinct from somatic
sheath cells in both their gonadal distribution (“s” in Figure 1B,C,E) and their
chromatin morphology (Figure 1D). The cell clusters were specific to *R.* sp.
SB347 hermaphrodites: they were almost always in the gonad arms of presumed
hermaphrodites (576/596 scored) but never in those of females (0/24 scored)
(Figure 1E). Analogous clusters were not present in *C. elegans* hermaphrodite
gonads (Figure 1C).
Cell clusters in *R.* sp. SB347 hermaphrodite gonads enable the production of sperm in oocyte-producing adults

Because the cell clusters were specific to *R.* sp. SB347 hermaphrodites and not females, we hypothesized that they might function in hermaphrodite sperm production. To test if they were spermatocytes, hermaphrodite gonads were co-labeled with antibodies against α-tubulin and the major sperm protein (MSP). MSP is first detectable within late spermatocytes, where it localizes to discrete structures called fibrous bodies (FBs). MSP remains in this pattern until it disperses cytoplasmically in spermatids and partitions specifically to the pseudopods of crawling spermatozoa (Figure 2A) (Ward & Klass 1982; Shakes et al. 2011). For these studies, a total of 1,428 discrete clusters from 518 hermaphrodite gonad arms were scored. Cells with microtubule spindles were scored as undergoing division (mitotic or meiotic). MSP was used as a stage-specific marker of spermatocytes (Smith 2006; Chu & Shakes 2013).

In *R.* sp. SB347 adult hermaphrodites, MSP was detected not only in crawling spermatozoa (Figure 2B-viii), but also in some of the most proximal cell clusters (Figure 2Bv-vii; 2C). Within these MSP-positive (MSP+) cell clusters MSP was organized in the FB pattern of spermatocytes (Figure 2Bv-vi). 43% of these MSP+ clusters (95/222) also exhibited microtubule spindles, characteristic of meiotically dividing spermatocytes (Figure 2Bv-vi; 2C). The proximal region of many gonads also contained clusters of haploid spermatids and DNA-containing residual bodies, the immediate products of spermatocyte meiosis in *R.* sp. SB347 (Figure 2A, 2B – vii) (Shakes et al. 2011)(DS, in press). Thus, in striking contrast
to the sequential “sperm first, then oocytes” pattern of gamete production in C. elegans hermaphrodites, adult R. sp. SB347 hermaphrodites simultaneously produce both sperm and oocytes within a single gonad.

Within these same gonads, numerous MSP-negative (MSP-) cell clusters were scored as either dividing (10% or 120/1206) (Figure 2B- iii; Figure 2C) or not dividing (90% or 1086/1206) (Figure 2Bii, iv) (Figure 2C). Since these dividing cells lacked MSP, we concluded that they could not be meiotically dividing spermatocytes, and must therefore be mitotically dividing. Dividing MSP- cell clusters were observed along the length of the gonad, with some adjacent to the most mature oocytes and thus physically distant from the stem cell niche (the DTC) (Figure 2C). Therefore, we hypothesized that these clusters were developmental intermediates between undifferentiated GSCs and late-stage MSP+ spermatocytes.

**MSP-negative clusters exhibit characteristics of spermatogonial cysts**

Although MSP-negative, mitotically dividing germ cell clusters have not been described in C. elegans or other nematodes studied to date (Morgan et al. 2010; Chu & Shakes 2013; Sommer 2005; Denver et al. 2011), sperm production in other animals, including Drosophila and vertebrates, typically includes cysts of interconnected, mitotically dividing cells called spermatogonia. Spermatogonia, distinct from GSCs, undergo a limited number of mitotic divisions that expand the germ cell pool before they differentiate into spermatocytes — a process known as transient amplification (Reviewed in (White-Cooper & Bausek 2010; Kimble
Given this broader context, we hypothesized that the MSP- clusters observed in *R. sp. SB347* hermaphrodite gonads were a nematode form of spermatogonial cysts.

To test this hypothesis, the MSP- cells were assessed for three key characteristics of spermatogonia: (1) transient amplification, (2) proliferation independent of the stem cell niche, and (3) presence of cytoplasmic bridges between cells. First, to determine if the cells undergo transient amplification that increases germ cell numbers, we plotted the relationship between cluster location and the number of cells per cluster. This illustrated a trend where cells per cluster increased as the developing clusters shifted proximally (Figure 6). Second, consistent with spermatogonia having the capacity to proliferate independently of signals from the stem cell niche (Kiger et al. 2001; Tulina & Matunis 2001), mitotically dividing MSP- clusters were observed distant from the DTC (Figure 2B-C).

Third, we determined whether cells within the MSP- clusters were interconnected by cytoplasmic bridges. To first test whether the cells were being held together solely by extracellular matrix, isolated gonads were treated with collagenase. Under these conditions, the clusters dissociated from the oocytes, but cells within clusters remained connected (Figure 3A). Next, since spermatogonia in other organisms are linked by actin-rich cytoplasmic bridges (reviewed by (Greenbaum et al. 2011), hermaphrodite gonads were labeled with F-actin-specific rhodamine-phalloidin. Within clusters, phalloidin labeled both the cortical microfilaments (Figure 3B-arrowhead) of individual cells as well as actin
bridges between cells (Figure 3B-arrows). In contrast, the GSCs (Figure 7) and each developing oocyte (Figure 3B) were individually connected to a thin, shared rachis. The cell clusters were not attached to this rachis (Figure 3B).

If spermatogonia are connected by cytoplasmic bridges, cells within spermatogonial cysts should be synchronized in both their developmental and cell cycle timing (Guo & Zheng 2004). Cells within individual clusters were either uniformly MSP- or exhibited equivalent MSP+ patterns (Figure 2B), suggesting that they were developing synchronously. Cell cycle synchrony was tested in two ways. If cells within clusters were synchronously dividing, the number of cells within clusters should double after each division (illustrated in Figure 3C). As predicted, most MSP- clusters were scored with 1, 2, 4, or 8 cells (Figure 3C). In a more direct test of cell cycle synchrony, gonads were labelled with the monoclonal antibody MPM-2 that binds to the phosphorylated target proteins of M-phase cyclin-dependent kinases (Davis et al. 1983; Golden et al. 2000). Within the distal mitotic zone, MPM-2 labeled individual M-phase putative GSCs (Figure 3D- GSCs). In contrast, within cell clusters, MPM-2 either labeled all of the cells (Figure 3D- cc3) or, more typically, none of the cells (Figure 3D- cc1, cc2, cc4). This pattern suggests that cells within clusters were synchronously either in M phase or interphase. Consistent with previous descriptions of C. elegans mitotic cells (Crittenden et al. 2006; Maciejowski et al. 2006), MPM-2 labeling of comparable C. elegans hermaphrodite gonads was restricted to individual cells in the mitotic zone (Figure 3E).
Because the cell clusters in *R*. sp. SB347 hermaphrodite gonads exhibited these four key features of spermatogonial cysts — amplification of germ cell number, mitotic proliferation independent of the stem cell niche, cytoplasmic bridges, and developmental and cell cycle synchrony — we concluded that they are a nematode version of spermatogonial cysts.

**Spermatogonial cysts are also a feature of sperm production in *Rhabditis* sp. SB347 males**

To determine whether sperm production in *R*. sp. SB347 males likewise included spermatogonia-based germ cell expansion, male gonads were also co-labelled with DAPI and MPM-2 antibody (Figure 3F). In the distal region of male gonads were two DTCs and a line of putative GSCs (Figure 3F-GSCs) whose chromatin morphology was similar to putative GSCs in hermaphrodite gonads (Figure 2B-i). As in hermaphrodites, some male gonads included singlets or pairs of MPM-2+ GSCs (data not shown). More significantly, large clusters of MPM-2+ cells were also observed far away from the DTCs. MPM-2+ cells immediately adjacent to the haploid sperm were presumed to be meiotic M-phase spermatocytes (Figure 3F-meiotic), while those within the less proximal clusters (Figure 3F-spermatogonia) were synchronously in mitotic M-phase and appeared to be the male equivalent of hermaphrodite spermatogonia (Figure 3D-cc3).
**R. sp. SB347 hermaphrodites continuously simultaneously produce spermatocytes and oocytes production at all ages**

In *C. elegans*, the “sperm first then oocytes” pattern of gamete production (Ward & Carrell 1979) can be observed by comparing the gonads of hermaphrodites in their last larval (L4) stage with those that are transitioning to adulthood (Figure 1A). Gonads from *C. elegans* hermaphrodite L4 larvae contained a line of maturing and meiotically dividing MSP+ spermatocytes that give rise to haploid spermatids (Figure 4A) (Ward & Klass 1982), whereas hermaphrodites making the one-time switch to oogenesis during the L4-to-adult molt contain only a few remaining spermatocytes followed by developing oocytes (Figure 4B).

To determine whether the *R. sp. SB347* pattern of simultaneously producing sperm and oocytes begins during early gamete production, we also examined the gonads of L4 larval hermaphrodites. Even as L4 larvae, hermaphrodite gonads contained both spermatogonial cysts and developing oocytes (Figure 4C), indicating that simultaneous production of oocytes and sperm is not preceded by an extended period of only sperm production. Notably, though, the most proximal (most mature) germ cells were invariably a cyst of MSP- spermatogonia or MSP+ spermatocytes. Relative to *C. elegans*, the development of haploid spermatids was delayed, because only 25.4% (16/63) of the L4 gonads contained MSP+ spermatocytes and haploid sperm were never (0/63) observed.
To determine whether *R*. sp. SB347 hermaphrodites continue to produce both spermatocytes and oocytes as they age, we compared germ cell patterns at different ages (Figure 4D). Hermaphrodites maintained an average of 2-3 MSP+/− clusters per gonad arm (Figure 4D). Moreover, most (128/144) of the gonad arms in the most elderly (60-72 hours post-adult-molt) still contained both developing oocytes and spermatogonia (Figure 4E). Therefore, *R*. sp. SB347 hermaphrodite sperm and oocyte production is not only simultaneous but also continuous.

**Hermaphrodites in closely related species employ similar reproductive strategies**

To our knowledge, this is the first report in nematodes of either continuous, simultaneous hermaphroditism or spermatogonia. To begin to address how this particular reproductive strategy might have evolved, we examined patterns of gamete production in nematode species closely related to *R*. sp. SB347. The phylogenetic relationships of these species are depicted in Figure 5A (Kiontke and Fitch 2005; Kiontke and Fitch, unpublished 20s rRNA data). Two closely related trioecious *Rhabditis* species *Rhabditis* sp. JU1782 and *Rhabditis* sp. JU1783 exhibited a pattern of gamete production similar to that of *R*. sp. SB347 (Figure 5B-D). Adult hermaphrodite gonads of both species contained both spermatogonia and developing oocytes (Figure 5B), while adult females contained only oocytes (Figure 5C). Furthermore, L4 hermaphrodite gonads of both species also contained both spermatogonia and oocytes (Figure 5D).
To determine if a dioecious (male/female) species within the *Rhabditis* genus might also utilize spermatogonia, we labeled male gonads from the closest known dioecious relative, *Rhabditis axei*, with DAPI and MPM-2 antibody (Figure 5E). MPM-2 labelled single GSCs in the distal mitotic zone, yet no clusters of MPM-2+ mitotic cells were observed.

Outside the *Rhabditis* genus, we also investigated whether hermaphrodites of the androdioecious (male/hermaphrodite) species *Oscheius tipulae* would exhibit simultaneous hermaphroditism. However, much like *C. elegans*, *O. tipulae* hermaphrodites produced sperm as L4 larvae before making a one-time switch to oocyte production during the L4 to adult molt (Figure 5F).

**Discussion**

We discovered two novel features of gamete production in the nematode *Rhabditis* sp. SB347. First, the hermaphrodites simultaneously produce both sperm and oocytes throughout their reproductive lifetimes. Simultaneous and continuous production of sperm and oocytes within a single gonad had been reported in the parasitic nematode *R. ranae* (Runey et al. 1978), but never in a lab cultivatable nematode. Second, both *R. sp. SB347* hermaphrodites and males amplify sperm production via spermatogonial cysts. Spermatogonanial cysts have not been previously described in a nematode, yet these *R. sp. SB347* cysts share key features with those in other taxa (i.e. flies and mice): their cells divide mitotically to increase germ cell numbers, they proliferate without signals
from the stem cell niche, they are linked by cytoplasmic bridges, and, within individual cysts, they develop and divide in synchrony.

**Distinct features of R. sp. SB347 germ cell development**

Both *C. elegans* and *R. sp. SB347* hermaphrodites achieve self-fertility by producing sperm and oocytes within a single gonad, yet the two species evolved distinct solutions for producing two types of gametes from what we hypothesize to be a common pool of GSCs. In both cases, the GSCs themselves are linked through physical connections to a shared cytoplasmic rachis. In *C. elegans*, as germ cells move out of the niche, exit mitosis, and begin to differentiate, both the developing spermatocytes and oocytes maintain their linkage to the rachis (Gumienny et al. 1999; Amini et al. 2014; Lints & Hall 2009). The one-time switch from producing spermatocytes to producing oocytes is achieved through regulatory changes (reviewed in (Ellis & Schedl 2007; Zanetti & Puoti 2013). In *R. sp. SB347*, as germ cells move out of the niche, they follow two distinct paths. Developing oocytes exit mitosis and begin to differentiate while maintaining their linkage to a thin, shared rachis. The route towards sperm production differs in two ways: cells leaving the niche continue to divide mitotically and detach from the rachis. As they divide, they develop distinct cytoplasmic connections to their daughter cells, forming discrete spermatogonial cysts. Mitotic exit and meiotic entry occur only much later as the cyst reaches the proximal end of the germline.

In other organisms, mitotically dividing gametogonial cysts are a standard part of sperm and oocyte production and help to expand the germ cell pool
(reviewed in (Greenbaum et al. 2011; Kimble 2011; Spradling et al. 2011). In *R.* sp. SB347, this feature is specific to sperm production. In *C. elegans*, germ cell cysts are notably absent, although the mitotic zone contains some cells with a limited capacity to proliferate independent of the niche (Fox & Schedl 2015; Cinquin et al. 2010). However, the coordinated commitment of *C. elegans* germ cells to both meiosis and differentiation as they leave the niche (reviewed in (Kimble & Crittenden 2007; Hansen & Schedl 2013) makes it difficult to study these two events in isolation. During *R.* sp. SB347 spermatogenesis, but not oogenesis, these two events are separated in time and space. Key unanswered questions include how *R.* sp. SB347 spermatogonia maintain a proliferative state despite their distance from the niche and whether they can be experimentally manipulated to de-differentiate into GSC-like cells, as can occur in *Drosophila* and mouse (Sheng et al. 2009; Nakagawa et al. 2010).

Spermatogonial cysts in *R.* sp. SB347 share the hallmark features of those in other organisms. Within individual cysts, the *R.* sp. SB347 spermatogonia were interconnected by actin bridges extending between cells within cysts. While we did not observe actin shaped in circular structures like those of ring canals observed in *Drosophila* spermatogonial cysts, it is still possible that the actin-rich cytoplasmic bridges in *R.* sp. SB347 spermatogonial cysts are formed after incomplete cytokinesis, as occurs in flies and mice (reviewed in (Greenbaum et al. 2011)). Likely due to these interconnections, cells within individual cysts exhibit developmental and cell cycle synchrony. The same is true in flies and mice (Guo & Zheng 2004). The discovery of both cyst and
rachis based interconnections within *R*. sp. SB347 hermaphrodites begs the question: what is the functional difference between these two structural organizations? While both structures join individual cells with interconnecting cytoplasm, the extent that signals pass between cells through these connections may differ. In *C. elegans*, molecules injected into the rachis diffuse through the germline, yet cells maintain cell cycle independence (Crittenden et al. 2006; Maciejowski et al. 2006) and recent studies suggest that diffusion blockers slow diffusion between sub-regions of the germline (Cinquin et al. 2015). The structure and properties of the thin *R*. sp. SB347 rachis or how the spermatogonia might detach and initiate their distinct division patterns have yet to be fully investigated.

**Evolution of *Rhabditis* sp. SB347 hermaphrodite self-fertility**

It is possible, that within the *Rhabditis* clade, spermatogonial cysts may have originated with the evolution of simultaneous hermaphroditism since we observed them in three trioecious *Rhabditis* species, but not in the dioecious near relative *Rhabditis axei*. Certainly, the spermatogonial cysts enable simultaneous hermaphroditism in that they allow sperm-fated mitotic germ cells to develop independently from adjacent developing meiotic oocytes. Since the *R*. sp. SB347 clade is currently represented by only a few species, it is yet unclear whether the three species examined here arose from a common trioecious ancestor with these features or through independent acquisitions of convergently-evolved hermaphroditism.
Why might *R.* sp. SB347 male sperm production employ spermatogonial cysts while *R. axei* male sperm production does not? One possible explanation is that *R.* sp. SB347 sperm production utilizes spermatogonial mitotic divisions to compensate for having smaller pool of GSCs in the distal mitotic zone. Conversely, *C. elegans, O. tipulae,* and *R. axei* sperm production may not need spermatogonial cysts because their gonads contain much larger GSC pools. Fox & Schedl (2015) proposed a similar explanation for the lack of either gametogonial cysts or non-cyst forming, transient amplifying cells in *C. elegans* gonads, hypothesizing that the larger stem cell pool confers *C. elegans* a greater adaptability in rapidly producing gametes when environmental conditions are favorable.

Because the diversity of nematode reproductive strategies is poorly studied (Pires-daSilva 2007; Denver et al. 2011), it is possible that continuous simultaneous hermaphroditism and spermatogonial cysts are not limited to the *Rhabditis* species examined here. Simultaneous hermaphroditism has been described in a parasitic lungworm nematode *R. ranae* (Runey et al. 1978), and may be common in parasitic forms. The linkage of hermaphrodite self-fertility in *R.* sp. SB347 to passage through a behaviorally dispersive dauer phase (Félix 2004; Chaudhuri et al. 2011) combined with the observation that males mate primarily with females rather than hermaphrodites (Chaudhuri et al. 2015) suggest that hermaphrodite outcrossing is rare; a condition that would favor the continuous production of sperm to maintain self-fertility. More nematodes should be examined to determine if these features are limited to *Rhabditis* or are
perhaps more common within the phylum Nematoda. Outside of Nematoda, examples of simultaneous hermaphroditism have been documented in diverse phyla (Ghiselin 1969; Clark 1978). However, the ease of studying simultaneous hermaphroditism in the linearly organized gonad of a free-living and lab-cultivable nematode reveals *R*. sp. SB347 as a promising system for studying the cellular and molecular mechanisms required to simultaneously produce both sperm and oocytes within a single ovatetis.

**Materials and Methods**

**Strains**

All strains were cultured at room temperature using standard methods (Lambie, 1994). The *Rhabditis axei* strain (DF5006) and *Caenorhabditis elegans* strain Bristol N2 were obtained from the *Caenorhabditis* Genetics Center. The other *Rhabditis* strains were gifts from André Pires-daSilva. *R*. sp. SB347 was originally isolated from a dead deer tick in Connecticut by Walter Sudhaus, *R*. sp. JU1782 from a rotting stem in Ivry, France by Marie-Ann Félix, and *R*. sp. JU1783 from a star fruit in La Réunion by Melissa Doma. *Oscheius tipulae* was a gift from and isolated by Theresa Grana in Virginia.

To isolate hermaphrodites of the trioecious *Rhabditis* species (SB347, JU1782, JU1783), dauers were isolated and matured into adult hermaphrodites. Dauers and L3 females are similar in appearance, so human error likely occasionally resulted
in females in hermaphrodite samples. *Rhabditis* females were isolated by picking early L3 feminine progeny from early (first 12 hours) broods of selfing hermaphrodites, allowing them to mature in the absence of males, and monitoring for a lack of embryos.

**Immunocytochemistry and microscopy**

Hermaphrodite and male gonads were dissected in 6 µL of 1% levamisol in Edgar’s buffer (Boyd et al. 1996) on ColorFrost Plus slides (Fisher Scientific) coated with poly-L-lysine (Sigma Aldrich Co.). Anti-tubulin and anti-MSP labeling was done as previously described (Shakes et al. 2009) at 1:20,000 anti-MSP (G3197 polyclonal, gift from David Greenstein; (Kosinski et al. 2005)), 1:100 (0.025 mg/mL) FITC-conjugated anti-α-tubulin DM1A (Sigma). MPM-2 (EMD Millipore) labeling was done as described in (Kulkarni et al. 2012) but at a 1:200 dilution (0.005 mg/mL). Secondary antibodies were the same as described in (Kulkarni et al. 2012). Slides were mounted with Fluoro-Gel II (Electron Microscopic Science) containing 6-diamidino-2-phenylindole (DAPI).

For actin staining, slides with dissected gonads were fixed for 15 minutes in 4% fresh paraformaldehyde (Fisher) in 1x PBS. Samples were quenched in 1M glycine (Fisher) in PBS (Fisher) for at least 5 minutes, permeabilized in 0.1% Triton-X-100 (Fisher) in PBS for 1 minute, then dip washed in 1xPBS. Slides were incubated with rhodamine-conjugated phalloidin (Molecular Probes) diluted
1:100 in 1xPBS for 15 minutes in the dark before washing three times in PBS for 5 minutes each. Slides were mounted as above.

All images were acquired under epifluorescence using an Olympus BX60 microscope equipped with a QImaging EXi Aqua cooled CCD camera with an Olympus PlanApo 40x or 60x objective lens and IPLab software. Images were minimally processed to enhance contrast with IPLab software or Adobe Photoshop.

**Collagenase Treatment**

Hermaphrodite gonads were dissected as above and subsequently treated in a final concentration of 1% collagenase (Worthington Biochemical) in Edgar’s buffer (Boyd et al. 1996) for 15 minutes at room temperature before freeze-cracking and fixation in -20°C methanol.

**Cell cluster feature scoring**

Data shown in Figures 2C, S1, 3C, 4D are from a series of experiments where a total of 1,428 discrete clusters from 518 hermaphrodite gonad arms were scored. For each experiment, microtubule presence, MSP expression, the number of cells within each cluster, and the clusters' locations in the gonad were scored. Number of cells within clusters was counted according to DAPI-stained nuclei at all focal planes. Cluster location was scored according to the adjacent oocyte’s distance from the spermatheca, where the oocyte nearest the spermatheca is -1
and then counting backwards (-2, -3, etc.) distally. When a cluster resided between oocytes, the cluster was scored at the more proximal oocyte location.

<table>
<thead>
<tr>
<th>Germline stem cells (GSCs)</th>
<th>Undifferentiated, self-renewing stem cells that are maintained by a stem cell niche (the distal tip cell (DTC))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermatogonia</td>
<td>A proliferative, developmental intermediate between GSCs and spermatocytes. Exist in germ cell cysts.</td>
</tr>
<tr>
<td>Gametocytes</td>
<td>Germ cells that have committed to the meiotic program and are differentiating or have differentiated. Either spermatocytes or oocytes.</td>
</tr>
<tr>
<td>Cell cluster</td>
<td>An isolated group of cells physically associated together.</td>
</tr>
<tr>
<td>Germ cell cyst</td>
<td>A cluster of germ cells that are interconnected by cytoplasmic bridges.</td>
</tr>
</tbody>
</table>

Table 1. Terms used.

Figure 1. Organization of *Caenorhabditis elegans* and *Rhabditis* sp. SB347 oocyte-producing gonads. A) Schematic of germ cell development in *C. elegans* hermaphrodites. Description in text. One half of whole gonad (one of the two gonad arms) is shown. Spth= spermatheca. B-E) Isolated gonad arms labeled with DAPI (blue) and anti-α-tubulin antibody (green). *= location of distal tip cell (DTC). Spth= spermatheca. som= somatic sheath cell. Mt= distal mitotic region. cc= cell cluster. B) *R. sp. SB347* adult hermaphrodite gonad. C) *C. elegans* adult hermaphrodite gonad. D) Full-size DAPI images of cell clusters from (B) and somatic sheath cell nuclei from (C), (D), and (E). E) *R. sp. SB347* female gonad. All scale bars = 20 μm.
Figure 2. MSP and microtubule patterns during *Rhabditis* sp. SB347 hermaphrodite spermatogenesis. A) Top: schematic of MSP localization during *Rhabditis* sp. SB347 spermatogenesis. Blue= DNA. Red= MSP. FBs= fibrous bodies. Below: examples of spermatocytes and sperm from *R*. sp. SB347 males labeled with DAPI (blue), anti-α-tubulin (green), and anti-MSP (red) antibodies. Scale bars= 5 µm. Description in text. B) Isolated *R*. sp. SB347 hermaphrodite gonads stained as in (A). Scale bars= 20 µm. Arrows and roman numerals indicate regions corresponding to full-size images below, where scale bars = 5 µm. Locations of clusters are scored according to the adjacent oocyte, starting counting backwards from -1 at the most proximal oocyte, as shown. *= DTC location. C) Histogram of each cluster type at scored locations. Locations were scored by the adjacent oocytes, as in (B). All 1,428 scored clusters are represented, but the outer distal 5% of clusters’ data are shaded gray because very few hermaphrodite gonads contained such large numbers of oocytes.
Figure 3. Physical and cell cycle characteristics of spermatogonial cysts in *Rhabditis* sp. SB347 gonads. A) Portion of *R*. sp. SB347 hermaphrodite gonad after collagenase treatment, labeled anti-α-tubulin antibody (green). Arrow points to cell cluster separated from oocytes. B) *R*. sp. SB347 hermaphrodite gonad stained with rhodamine-conjugated phalloidin labelled actin microfilaments (red). *=DTC. Box indicates cell cluster shown at full size to the right, where arrowhead points to cortical microfilaments and arrows point to actin bridging between cells. C) Illustration of predicted mitotic doubling pattern of spermatogonia is shown above histogram of frequencies of numbers of cells scored in MSP-negative clusters. D) Two *R*. sp. SB347 adult hermaphrodite gonads labeled with MPM-2 antibody (green). Arrowheads indicate regions shown at full size below. cc= cell cluster. *= DTC. E) Distal mitotic region of a *C. elegans* adult hermaphrodite gonad labeled with MPM-2 antibody (green). Arrows indicate MPM-2+ cells. F) *Rhabditis* sp. SB347 adult male gonad labeled with MPM-2 antibody (green). All gonads are stained with DAPI (blue). *=DTC. Scale bars = 5 μm (A), 20 μm (B, D-F).
Figure 4. Sequence of gamete production over hermaphrodites’ lifetimes in *C. elegans* and *R. sp. SB347*. A-C) Isolated gonads co-labeled with DAPI (blue) and antibodies against α–tubulin (green) and MSP (red). Arrows (1), (2), (3) indicate primary spermatocyte, meiotic spermatocyte, and sperm respectively. A) *C. elegans* L4 hermaphrodite B) *C. elegans* hermaphrodite during the L4-to-adult
molt. C) *R.* sp. SB347 L4 hermaphrodite. D) Average number of (both MSP+ and MSP-) clusters per gonad arm scored at different ages. Bars = standard error. E) Older *R.* sp. SB347 adult hermaphrodite (60 hours after adult molt) gonad stained as in (A-C). cc= cell clusters. Spth= spermatheca. All scale bars = 20 μm.
Figure 5. Pattern of gamete production in closely related nematodes. A) Phylogenetic relationships between species examined and an outgroup *Pristionchus* ((Kiontke & Fitch 2005); Kiontke & Fitch, 20s unpublished rRNA data). Left column indicates sexes in that species and right column indicates mode of hermaphroditism. Seq= sequential, making sperm in L4 and then oocytes as adults. Sim= sperm and oocytes simultaneously. B-D) Isolated *R.* sp. JU1782 (left) and *R.* sp. JU1783 (right) gonads labeled with DAPI (blue) and antibodies against α-tubulin (green) and MSP (red). B) Adult hermaphrodite gonads. C) Adult female gonads. D) L4 hermaphrodite gonads. E) *R.* axei adult male gonad labeled with MPM-2 antibody (green). In the top right corner are full-size images of the distal mitotic region. F) Left to right: L4, molt, and adult *O. tipulæ* hermaphrodite gonads stained as in (B-D). cc = cell cluster. All gonads are stained with DAPI (blue). All scale bars = 20 micrometers.

Figure 6. Relationship between *R.* sp. SB347 hermaphrodite MSP-negative clusters’ location and the number of cells scored in that cluster. Box-and-whisker representation of the number of cells scored in MSP-negative clusters found at each location. Location was scored by adjacent oocyte, as illustrated in Figure 2B. Dots = outliers outside of 1.5 x IQR.
Figure 7. The GSCs in *R. sp. SB347* hermaphrodite gonads develop along a thin rachis. Isolated *R. sp. SB347* hermaphrodite gonad stained with DAPI (blue) and rhodamine-conjugated phalloidin specific to actin microfilaments (red). *=DTC.
Chapter 2. Assessment of conserved *C. elegans* germ cell development genes in *R.* sp. SB347

**Introduction**

*R.* sp. SB347’s pattern of sperm development is distinct from the model nematode *C. elegans* in two important ways. While *C. elegans* hermaphrodites sequentially produce only sperm and then only oocytes, *R.* sp. SB347 hermaphrodites are capable of continuously producing both sperm and oocytes in the same gonad. And while *C. elegans* GSCs differentiate directly into meiotic spermatocytes, the development of *R.* sp. SB347 sperm includes a proliferative intermediate stage that we characterized to be spermatogonia (Chapter 1). These novel patterns of sperm development have not been well characterized in any nematode before.

Despite their differences, *C. elegans* and *R.* sp. SB347 belong to the same family, *Rhabditidae* (Kiontke & Fitch 2005). However, even within closely related nematodes, germ cell developmental pathways are known to rapidly evolve (Reviewed in (Haag & Liu 2013). Given this context, *C. elegans* and *R.* sp. SB347’s divergent germ cell patterning present an opportunity to compare which germ cell developmental pathways are or are not conserved between species.

The genetic players in *C. elegans* germ cell development have been well characterized (see Table 2 and Figure 9). In *C. elegans*, the stem cell niche is a large somatic cell with long cellular protrusions called the distal tip cell (DTC) (Byrd et al. 2014; Hall et al. 1999; Kimble & White 1981). The DTC maintains GSCs in a stem cell state through canonical Delta/Notch signaling. The DTC
expresses the transmembrane protein LAG-2 (a Delta homolog) that binds the GLP-1 (Notch homolog) receptor that is expressed in the GSCs’ cell membranes (Henderson et al. 1994; Crittenden et al. 1994). DTC signaling stimulates GSCs to express FBF, which translationally represses mRNAs of meiotic entry and differentiation genes, including those of a second RNA binding protein, GLD-1. As GSCs progress beyond the niche, FBF levels decrease, and GLD-1 translation increases. GLD-1 promotes the switch from mitosis to meiosis, in parallel with two other GLD proteins, by translationally repressing mitotic transcripts. As cells enter meiosis, they also begin differentiating into either spermatocytes or oocytes (Reviewed in (Kimble & Crittenden 2007). The sperm/oocyte decision is largely an inhibitory pathway between sperm fate (FEM and FOG) proteins and oocyte fate (TRA) proteins (see Figure 9 and Table 2). Coupling differentiation and meiotic entry, many proteins function in both processes, including GLD-1, GLD-2, GLD-3, FOG-1, NOS-3, and DAZ-1 (see Figure 9 and Table 2). Once cells have initiated meiosis, homologous chromosomes pair, the synaptonemal complex forms, homologs undergo meiotic recombination (Reviewed in (Lui & Colaiácovo 2013)). By late pachytene, the developing spermatocytes or oocytes have sufficiently differentiated that they express cell type-specific functional proteins.

In stark contrast to germ cell development in C. elegans, R. sp. SB347 have spermatogonia that continue to mitotically proliferate despite their distance from the DTC. The state of differentiation of R. sp. SB347 spermatogonia is currently unclear. One possibility is that R. sp. SB347 spermatogonia remain
uncommitted to the sperm fate until they enter meiosis, because the mitosis to meiosis switch is coupled with gamete fate decisions — as in *C. elegans* germ cells (Figure 8, Model 1). Alternatively, if *R. sp.* SB347 spermatogonia develop similarly to spermatogonia in *Drosophila*, we would expect them to commit to a sperm fate upon exiting the niche, distinguishing them from GSCs (Figure 8, Model 2). In this model, the sperm fate decision and the switch to meiosis would be separated in time and space. In order to distinguish between these possible models, we can characterize germ cell developmental stages using genetic markers. Such molecular analyses of germ cell developmental pathways in *R. sp.* SB347 will also determine whether the underlying pathways are shared with *C. elegans* and whether conserved proteins play similar roles.

Characterization of *R. sp.* SB347 germ cell development using genetic markers can clarify how *R. sp.* SB347 hermaphrodite germ cells are determined for either the sperm or oocyte fate, how spermatogonia are capable of proliferation outside the stem cell niche, and whether meiotic commitment is coupled with differentiation. For this reason, we assessed conservation of the following categories of *C. elegans* genes: GSC, sperm fate, oocyte fate, meiotic entry, meiotic machinery, sperm function, and oocyte function. Oogenic genes were included as negative controls of sperm fate genes and for the opportunity to assess whether oocyte differentiation occurs earlier than spermatocyte differentiation. The candidate genes’ roles and expression patterns in *C. elegans* germ cells are described in Table 2 and their relationships are illustrated in Figure 9.
Results

Identification of *C. elegans* homologs in *R. sp. SB347*

The *C. elegans* literature was searched for characterized genes with specific functions and specific mRNA and/or protein expression in germ cells (Candidates are described in Table 2 with citations). Genes that are not expressed in specific cell types or patterns were eliminated because investigating their expression in *R. sp. SB347* by *in situ* hybridization or immunofluorescence will not be informative. As expected, protein expression patterns were often more specific than mRNA expression patterns due to the large amount of translational repression by RNA binding proteins in *C. elegans* germ cell development (reviewed by (Nousch & Eckmann 2013)).

Next, because nematodes exhibit rapid sexual mode evolution and divergence of germ cell sex determination genes (Reviewed in Haag & Liu 2013; Stoithard & Pilgrim 2003), and because *R. sp. SB347*’s genome is not published, we investigated whether the remaining gene candidates were generally conserved in other nematodes. Fully sequenced nematode species were chosen as outgroups to examine sequence conservation across reasonably distant species. It is worth noting that most of the currently sequenced nematodes, including the outgroups chosen (*Strongyloides ratti, Ascaris suum*, and *Loa loa*), are parasitic species (Blaxter & Koutsovoulos 2014). Using BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1990) and COBALT (Constraint-based multiple protein Alignment Tool) (Papadopoulos & Agarwala 2007), sequence
homology between *C. elegans* candidate genes and these outgroups were examined (Table 3). 10/21 of the candidate genes were well conserved, while 6/21 were only somewhat conserved, and 5/21 were not conserved across species (Table 3). Curiously, *C. elegans* GLP-1/Notch and LAG-2/Delta protein sequences were not conserved in several species, despite that Notch and Delta are commonly conserved across phyla. This analysis also revealed that synaptonemal complex proteins were not generally conserved across species, with the notable exception of HIM-3. Genes that did not have homologs across species were eliminated as candidates (Table 3).

With a selective candidate gene list that excludes non-specific and non-conserved genes, we next tested whether sequences found in *R.* sp. SB347 transcriptome data were conserved homologs to the *C. elegans* genes (unpublished data kindly provided by Sophie Tandonnet, André Pires daSilva lab, University of Warwick). To assess whether the returned *R.* sp. SB347 sequence was a conserved homolog to the *C. elegans* sequence, E (expect) values were assessed (Table 4). An E < 10e−3 is statistically significant and indicates that the sequences are very likely homologous (Dalal & Atri 2014). For the aligning regions between the two species' sequences, the percentage of amino acid matches were considered (% identity in Table 4), in addition to the percentage of conserved amino acid characteristics (% conserved in Table 4). Amino acid % identity matches and % conserved of aligning regions, while they are less reliable indicators of homology than E values, are generally indicative of conservation at > 30% (Dalal & Atri 2014). Genes for which we were unable to identify an *R.* sp.
SB347 homolog included the signaling protein GLP-1/Notch, the sperm fate genes *fem-1* and *fog-3*, and the oocyte function gene *oma-1* (Table 4). For the remaining 10 candidates, a conserved homolog was identified in this analysis (bolded rows in Table 4).

**Preliminary comparisons of gene expression between sexes**

To begin to assess whether homologs identified in *R*. sp. SB347 function similarly, we can determine whether the genes’ expression patterns are consistent with their hypothesized functions. Specifically, expression patterns of sex determination genes can be compared between sexes to test whether they are sperm- or oocyte-enriched as we would expect.

For *C. elegans*, RNAseq experiments comparing hermaphrodite mutants that only make sperm (*fem-3(q96)*) or only make oocytes (*fog-2(q71)*) provided useful data for determining sex-specific expression (Ortiz et al. 2014). To assess whether gene candidates were expressed in a sex-enriched manner, we compared their expression in sperm- and oocyte-producing hermaphrodites as measured by (Ortiz et al. 2014) (data for all candidate genes are listed in Table 2; Figure 11A). When expression of the final candidates *fbf*, *gld-1*, *fem-2*, *fog-3*, *spe-44*, and *msp* are compared, the upstream sperm fate genes *gld-1* and *fem-2* are not sperm-enriched whereas the more downstream sperm fate genes *fog-3* and *spe-44* are.

To begin to assess whether *R*. sp. SB347 homologs are expressed in a manner consistent with their hypothesized function, we collected preliminary data
measuring \textit{fbf}, \textit{fem-2}, \textit{spe-44}, and \textit{msp} expression in \textit{C. elegans} and \textit{R. sp.}

SB347 hermaphrodites, males, and larvae by qPCR (Figure 11B-C). Due to technical problems with sample collection and RNA extraction, these data are unreliable and should be replicated in the future. For example, larvae should not contain spermatocytes and should therefore not express \textit{msp}. While \textit{C. elegans} larvae samples did not express detectable levels of \textit{msp}, \textit{R. sp.} SB347 larvae samples did express detectable levels of \textit{msp} – likely indicating that adults contaminated those samples. To avoid such problems in the future, qPCR samples could be collected from tightly synchronized populations grown in mass.

**Tests of antibodies against conserved meiotic machinery proteins**

The discovery that the meiotic recombination proteins HIM-3 and RAD-51 have conserved homologs in \textit{R. sp.} SB347 allows us to investigate whether the meiotic program is coupled with the gamete differentiation programs, as it does in \textit{C. elegans}. Unfortunately, pre-existing antibodies against \textit{C. elegans} HIM-3, \textit{C. elegans} RAD-51, and human RAD-51 did not cross-react with \textit{R. sp.} SB347 (data not shown). Future experiments may find more success by designing a species-specific antibody against meiotic machinery proteins.

**Test of antibody against \textit{R. sp.} SB347 FBF homolog**

To begin to assess whether \textit{R. sp.} SB347 spermatogonia are uncommitted to the sperm fate, we aimed to determine if the spermatogonia expressed the conserved GSC protein FBF by immunocytochemistry (Figure 12,
Model 1). We predicted that if spermatogonia are undifferentiated and express FBF, it is possible that FBF translationally represses both meiotic entry genes and sperm/oocyte differentiation genes, as FBF does in C. elegans. This pattern of FBF protein expression would explain the spermatogonia’s ability to proliferate outside the stem cell niche.

The sequence alignment of C. elegans FBF and its R. sp. SB347 homolog is shown in Figure 13, with the conserved PUF (Pumilio and FBF) functional domain highlighted in blue and the antigenic peptide for polyclonal antibody production in immunized rabbits highlighted in red. The PUF domain is an RNA binding domain shared with FBF and the Drosophila melanogaster RNA binding protein Pumilio (Zhang et al. 1997). PUF proteins appear to be conserved throughout germ cell evolution; Pumilio is expressed in GSCs in Drosophila melanogaster ovaries (Lin & Spradling 1997) and the human homolog PUM2 is also expressed in germ cells (Urano et al. 2005). Contrarily, FBF itself is not conserved in C. elegans’ close relative C. briggsae, though C. briggsae has different but similarly functioning PUF proteins (Haag & Liu 2013). While we identified an FBF homolog in R. sp. SB347, it should be noted that the sequence was even more homologous to C. elegans PUF proteins PUF-11 and PUF-3 (Table 4), which also function in germ cell development (Hubstenberger et al. 2012).

In order to test the specificity of the polyclonal antibody against R. sp. SB347’s FBF homolog, we determined whether anti-FBF antibody specifically bound a protein of the expected molecular weight of 61 kD. Western blots probed
with anti-FBF antibody bound a protein weighing approximately 61 kD, as expected (Figure 14A). Intriguingly, anti-FBF appeared to bind two proteins of slightly different molecular weights, which can be further resolved when the gel is run longer (Figure 14B). It remains undetermined whether the slight difference between the two weights is due to binding of two different FBF homolog proteins, two different splice variants, or reflects post-translational modification.

**Test of antibody against R. sp. SB347 SPE-44 homolog**

If *R. sp. SB347* spermatogonia are sperm-fated, then this implies that the sperm fate decision occurs before meiotic entry (Figure 8 and 12, Model 2). If spermatogonia are in fact sperm-fated, we predicted that they would express the key sperm fate gene, SPE-44. To assess SPE-44 expression patterns in *R. sp. SB347* germ cells, we designed an antibody against *R. sp. SB347*’s SPE-44 homolog.

Both *C. elegans* SPE-44 and its homolog in *R. sp. SB347* contain the DNA-binding SAND domain (Figure 15), as SPE-44 is a transcriptional regulator of sperm genes (Kulkarni et al. 2012). It is worth noting that *R. sp. SB347*’s SPE-44 homolog was also homologous to the *C. elegans* SAND domain-containing GMEB protein (Glucocorticoid Modulatory Element Binding) (Surdo et al. 2003) (Table 4). The antigenic peptide for antibody production in immunized rabbits was a conserved region that falls outside of the SAND domain (Figure 15).

As previously described (Kulkarni et al. 2012), the binding pattern of the anti-*C. elegans* SPE-44 antibody within sperm-producing *C. elegans* germ cells
is very distinct: SPE-44 is first expressed in early meiotic spermatocytes (Figure 16A), and localizes precisely to chromatin, as is characteristic of transcriptional regulators, with the exception of the X chromosome (Figure 16B). The binding pattern of the anti-SB347 SPE-44 antibody exhibited a very different pattern within male and hermaphrodite gonads (Figure 16C-D). Instead of being expressed in sperm-fated cells following the distal mitotic region of GSCs and localizing to chromatin (Kulkarni et al. 2012), (Figure 16A-B), SB347 anti-SPE-44 bound proteins in the cytoplasm and exhibited particularly bright labeling of the rachis in all sexes (Figure 16C-D; female rachis pattern not shown). In western blots, the anti-SB347 SPE-44 antibody failed to bind a protein at the expected molecular weight of 41 kD (Figure 17). Instead, the antibody bound a protein at a higher molecular weight (approximately 70 kD) (Figure 17). From these data, we conclude that anti-SPE-44 antibodies do not bind to the predicted *R. sp. SB347* SPE-44 homolog, and might instead bind a rachis protein weighing approximately 70 kD.

**Discussion**

Here we identified 10 conserved homologs to *C. elegans* germ cell development genes in *R. sp. SB347* (Table 4 bolded rows). As expected, many *C. elegans* genes were either not conserved across nematodes (Table 3) or did not match *R. sp. SB347* sequences (Table 4). This is not surprising given that nematodes rapidly evolve reproductive modes (Pires-daSilva 2007; Denver et al. 2011). Even when comparing the genetic regulators of sex determination...
between *Caenorhabditis* species, there are instances of divergence (Reviewed in (Haag & Liu 2013)). Similarly, it was previously known that sequences of synaptonemal complex proteins vary greatly across species (Fraune et al. 2016; Hemmer & Blumenstiel 2016), so it was not surprising that SYPs1-3 were not conserved (Table 3). We were surprised, however, to find that the *C. elegans* Notch and Delta genes GLP-1 and LAG-2 were not conserved in several nematode outgroups (Table 3), and we were likewise unable to identify a GLP-1 homolog in *R. sp. SB347* (Table 4).

Intriguingly, analyses of germ cell development regulation between *Caenorhabditis* species have revealed that RNA binding proteins may play integral roles in facilitating germ cell evolution (Haag & Liu 2013). Haag & Lui (2013) propose that RNA binding proteins can facilitate developmental changes during evolution by simply targeting different mRNA transcripts or by translationally repressing their target mRNAs at different points in germ cell development. One example of this is presented with the RNA binding protein GLD-1, which has a homolog in the convergently evolved hermaphroditic species *Caenorhabditis briggsae*. RNAi experiments indicate that GLD-1 functions in oocyte fate determination (Nayak et al. 2005; Beadell et al. 2011), in contrast to its role in sperm fate determination in *C. elegans*. Therefore, it is hypothesized that in the convergent evolution of hermaphroditism in these two *Caenorhabditis* species, the mRNAs bound by GLD-1 differed to facilitate the sperm-to-oocyte switch (Nayak et al. 2005; Beadell et al. 2011; Haag & Liu 2013). For this reason, we recommend investigation into both FBF and GLD-1 homologs in *R. sp.*
SB347, as they are both RNA binding proteins that may have been co-opted differently in the evolution of hermaphroditism and sperm development in the *Rhabditis* clade.

While we have identified homologs in *R.* sp. SB347, it is unknown if these homologs serve similar or different functions as their *C. elegans* counterparts. Therefore, in order to draw parallels between the *C. elegans* and *R.* sp. SB347 genes, the homologs’ functions and expression must be compared. Ideally, as CRISPR gene editing techniques are developed in *R.* sp. SB347, knockouts and fluorescent fusion constructs would enable us to characterize the *R.* sp. SB347 homologs.

Because RNAseq has been done with *R.* sp. SB347 (unpublished data, Sophie Tandonnet, Pires daSilva lab, University of Warwick), in the future, those experiments could expand to compare expression between males, females, and hermaphrodites to determine whether genes are expressed in a sex-specific or sex-enriched manner. Conveniently, *R.* sp. SB347 populations contain naturally occurring females. Therefore, mRNA expression can be compared between females and hermaphrodites to determine which genes might be related to sperm development, as both females and hermaphrodites have feminine soma.

Ultimately, once the conserved homologs in *R.* sp. SB347 have been characterized, they can be used as genetic markers of types of germ cells. Using these markers, the developmental status of *R.* sp. SB347 spermatogonia can be investigated. Here we predicted two possible outcomes: either spermatogonia are uncommitted to the sperm fate and would express GSC genes (Figures 8
and 12, Model 1) or spermatogonia are committed to the sperm fate and would express sperm fate genes (Figures 8 and 12, Model 2). Testing these possible models and characterizing R. sp. SB347 sperm development in more detail will provide a unique perspective in comparing sperm development between nematodes and other animals.

**Methods**

**Candidate gene search**

Well-characterized *C. elegans* germ cell development genes were chosen and reviewed in the literature. For the most thorough literature review, references were checked with those on Wormbase (wormbase.org, release WS257). pBLAST (NCBI, [https://blast.ncbi.nlm.nih.gov/Blast.cgi](https://blast.ncbi.nlm.nih.gov/Blast.cgi)) (Altschul et al. 1990) searches were done with protein sequences for the most sensitivity to evolutionarily distant homologs. To check if proteins were conserved in other nematode species, the *C. elegans* protein sequence was BLAST searched into “Nematoda,” and results were queried for homologs from the sequenced representative species in Table 3 and Figure 10. Homologs were not necessarily characterized or determined to be orthologous to the *C. elegans* gene of interest. For alignments from multiple nematode species, NCBI’s COBALT ([https://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi](https://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi)) (Papadopoulos & Agarwala 2007) was used directly from the BLAST search results. The resulting multiple alignments were assessed by eye for regions of alignment. Genes that
were only present in two or fewer of the five species were not considered good candidates.

*R. sp.* SB347 transcriptome data were acquired by Sophie Tandonnet of André Pires daSilva lab, University of Warwick, by RNAseq. Homologous sequences were identified by BLAST searching for *C. elegans* protein sequences in *R. sp.* SB347 *in silico* translated sequences (BLASTx search function), and the resulting *R. sp.* SB347 mRNA sequences were kindly provided by Sophie Tandonnet. These sequences were then translated *in silico* using expasy translate tool (choosing the longest translated peptide) (Swiss Institute of Bioinformatics, [http://web.expasy.org/translate/](http://web.expasy.org/translate/)) (Artimo et al. 2012) and subsequently BLAST searched with *C. elegans* protein sequences. The resulting BLAST results of E values, % identity, and % conserved from the matching pairwise alignment were recorded in Table 4.

**Bioinformatics and antibody production for SB347 FBF and SPE-44**

The translated protein sequences of *R. sp.* SB347 FBF and SPE-44 homologs were aligned with the corresponding *C. elegans* protein using Geneious version 9.0.2 (Kearse et al. 2012). The text file of the pairwise alignments were then edited to highlight the conserved domains spanning the residues indicated by a NCBI CDD (Conserved Domains Database) search (Marchler-Bauer et al. 2009) on each protein sequence. To design antibodies against FBF and SPE-44, the full peptide sequences were given to Yenzym (South San Francisco, CA), who analyzed the peptides for antigenicity. The antibodies were produced by and
affinity purified by Yenzym. Two rabbits were immunized per peptide. Experiments shown here were from the rabbit that produced polyclonal antibodies that showed more specific binding by immunocytochemistry.

**SDS-PAGE and Western Blotting**

200 worms were picked to 20μL of M9 buffer with Roche cOmplete EDTA-free protease inhibitors (Sigma) on a 2mL screwcap tube cap, then centrifuged down, flash frozen in liquid nitrogen, and then stored at -80°C. Next, 20μL of 2x Laemmli SDS sample buffer (Biorad) + Beta mercaptoethanol were heated and added to frozen samples. Samples were then heated at 95°C for 5 min. Samples were next vortexed and then centrifuged at 15,000 r.p.m. at room temperature for 3 minutes.

For SDS-PAGE, pre-cast tris-glycine gels (Bio-Rad Mini-Protein TGX any kD, cat # 4569035) were used. 20 μL of sample, loading buffer (for blanks), or protein standards (Biorad Precision Plus Protein Dual Color cat # 1610374) were loaded into each lane. Gels were run at 100V. Next, proteins were transferred to a PVDF membrane (GE # 10600021) using a standard semi-dry transfer method at 19V for 30 minutes.

Following transfer, membranes were blocked overnight at 4°C in a solution of 4% Carnation instant skim milk in 1xTBST. Membranes were incubated for 1-2 hours with primary antibody diluted in 4% milk in 1xTBS-T. Anti-FBF was diluted 1:500 for a concentration of 0.00126 mg/mL. Anti-SPE-44 was diluted 1:250 for a final concentration of 0.0022 mg/mL. Following primary incubation, membranes
underwent 6x5min washes in 1xTBS-T. Next, membranes were incubated for 1-2 hours with HRP-conjugated goat anti-rabbit IgG secondary diluted 1:10,000 (for final concentration of 0.0002 mg/mL) in 4% milk in 1xTBS-T and then washed 6x5min in 1xTBS-T.

For detection, 1 mL of ECL (GE Healthcare Amersham ECL Prime RPN2232) was applied to the membrane. After 5 minutes, membranes were exposed to X-ray film in a dark room. Where necessary, contrast was increased of scanned images of films using Adobe Photoshop.

Immunocytochemistry

We tried two different anti-RAD-51 antibodies on R. sp. SB347 gonads. The first was a polyclonal antibody against the N-terminus (aa 1-100) of the C. elegans RAD-51 protein (Novus # 29480002). We tried this antibody with a 15 minute 4% PFA fixation followed by 0.1 M glycine in PBS, then 1 minute in 0.1% Triton-x-100 in PBS, a dip in PBS, block for 20+ minutes, and then primary antibody diluted 1:100 or 1:400. We also tested this antibody using a freeze crack and methanol fixation at -20 and varied the dilutions from 1:25 to 1:400. In these experiments, we also tested different antibody solvents, incubation lengths and incubation temperatures (2 hours room temperature or 4 degrees C overnight), and different wash lengths.

The second RAD-51 antibody was a polyclonal antibody against the full length human RAD51 protein (Abnova # H00005888-B01P). For this antibody, we tested two different fixation methods. The first method fixed slides for a minute in
cold methanol followed by 30 minutes in 2% PFA. The second method was to fix for 10 minutes in 4% PFA. The antibody was tested at both 1:50 and 1:100 dilutions after either fixation method.

*C. elegans* anti-HIM-3 antibody was kindly provided by Monique Zetka (Zetka et al. 1999) and was recommended to be diluted 1:200 for immunocytochemistry. We tried two different fixation methods. First, we tried a 5 minute 1.5% PFA fix followed by freeze crack, 1 minute in 95% ethanol, 5 minute wash in PBS-Triton-x-100 before blocking (adapted from (Howe et al. 2001)). Second, we tried freeze crack followed by methanol fixation at -20 degrees C.

For anti-SPE-44 immunocytochemistry with dissected *R. sp. SB347* gonads, we tried both methanol and aldehyde fixation methods (as described elsewhere in this thesis) and did not observe any differences in binding patterns. The most optimal dilution was 1:300 for a final concentration of 0.0018, which was determined from a range of dilutions from 1:150 to 1:600.

**qPCR**

RNA was isolated from 20 to 80 worms using RNAzol RT (Molecular Research Center) according to the manufacturer’s protocols, except it was done at half the volumes. RNA was resuspended in water and total RNA was measured by a Nanodrop. RNA was either stored at -80 degrees C or immediately used for cDNA synthesis. cDNA was synthesized from total RNA using iScript reverse transcriptase master mix (Bio-rad) according to the manufacturer’s protocols. qPCR was done using species-specific (*C. elegans* and *R. sp. SB347*) primers
that were designed using Primer3 software through
http://biotools.umassmed.edu/bioapps/primer3 www.cgi. Fluorescence emitted
from SyberGreen (Thermo Fisher) was detected by a StepOne real-time PCR
system (Thermo Fisher).
Figure 8. Above: schematic of germ cell developmental stages. Below: Proposed models of *R. sp. SB347* spermatogonia development. We hypothesize that either *R. sp. SB347* spermatogonia, like that in *Drosophila*, are sperm-fated (model 1) or, alternatively, develop more like *C. elegans* germ cells which have no intermediate between GSCs and spermatocytes and therefore the...
spermatogonia remain undifferentiated (model 2). In *C. elegans*, GSCs differentiate and commit to meiosis rapidly, but sometimes at different rates (Fox & Schedl 2015; Cinquin et al. 2010), so the region where different types of cells are observed, most of which are committed to meiosis, is referred to as the "transition zone" (TZ).

**Table 2.** Descriptions of gene candidates. Reviewed in (Kimble & Seidel 2013; Lesch & Page 2012; Kimble & Crittenden 2007; Ellis & Schedl 2007; Zanetti & Puoti 2013). RNA binding proteins reviewed in (Nousch & Eckmann 2013). Synaptonemal complex proteins reviewed in (Page & Hawley 2004). Sperm-enrichment values are the log2 fold-change in normalized mRNA expression between sperm- and oocyte-producing worms (both with feminine soma) as measured by RNAseq in (Ortiz et al. 2014). Positive values are sperm-enriched and negative values are oocyte-enriched. Values between -1 and 1 were considered sex neutral. TZ = transition zone, as illustrated in Figure 1 and 2. “+” in “category” means there are more than two roles this gene plays, and it was only categorized by the primary role.
<table>
<thead>
<tr>
<th>Category</th>
<th>Gene</th>
<th>Protein description</th>
<th>mRNA expression</th>
<th>Protein expression</th>
<th>Sperm enrichment</th>
<th>Eliminate?</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSC</td>
<td>lag-2</td>
<td>Delta homolog (Tax et al. 1994). Ligand for GLP-1/Notch (Henderson et al. 1994). Maintains GSCs (see glp-1).</td>
<td>DTC (Henderson et al. 1994)</td>
<td>DTC (Henderson et al. 1994)</td>
<td>n/a</td>
<td></td>
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<tr>
<td>GSC, oocyte fate</td>
<td>fbf-1/2</td>
<td>RNA binding proteins with PUF domain homologous to Drosophila Pumilio (Zhang et al. 1997). Bind meiotic transcripts, including gld-1, keeping GSCs in mitosis (Crittenden et al. 2002). Bind fem-3 and fog-1 to promote the sperm-to-oocyte switch in hermaphrodites (Zhang et al. 1997; Thompson et al. 2005). There is no clear ortholog in C. briggsae, but there are homologous PUF proteins whose roles have not been characterized in C. briggsae germ cells (Haag &amp; Liu 2013).</td>
<td>fbf-1 throughout. fbf-2 is limited to GSCs. (Lamont et al. 2004).</td>
<td>GSCs, though FBF-1 persists a bit later (Lamont et al. 2004).</td>
<td>0.10/0.50</td>
<td>mRNA non-specific</td>
</tr>
<tr>
<td>Sperm fate, meiotic entry</td>
<td><strong>glg-1</strong></td>
<td>KH /STAR family RNA binding protein (Jan et al. 1999). Promotes the mitosis to meiosis switch (Hansen, Wilson-Berry, et al. 2004) by binding mitotic transcripts, including mex-3 and the Notch receptor glp-1 (Marin &amp; Evans 2003; Ciosk et al. 2004). Also involved in oocyte meiotic development (Francis et al. 1995; Jones et al. 1996). In sperm development, promotes the sperm fate by translationally repressing the oocyte genes tra-2, oma-1, and oma-2 (Jan et al. 1999; Lee &amp; Schedl 2004). Translationally repressed by FBF-2 (Crittenden et al. 2002). RNAi phenotype in <em>C. briggsae</em> is opposite to that in <em>C. elegans</em>, causing masculinization of the germline rather than feminization, suggesting that GLD-1 has different targets in <em>C. briggsae</em> (Nayak et al. 2005).</td>
<td>Throughout (Jones et al. 1996).</td>
<td>Mostly in the transition zone (Jones et al. 1996).</td>
<td>-1.60</td>
<td>mRNA non-specific</td>
</tr>
<tr>
<td>Trait</td>
<td>Gene</td>
<td>Description</td>
<td>TZ onwards</td>
<td>TZ onwards</td>
<td>Score</td>
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<tr>
<td>Meiotic entry+</td>
<td>gld-2</td>
<td>Poly(A) polymerase and translational activator of meiotic genes and fem-3 (Wang et al. 2002). Interacts with GLD-3 as they both promote the switch to meiosis and sperm fate (Wang et al. 2002). Also has reported role in oogenesis (Kim et al. 2010).</td>
<td>TZ onwards (Wang et al. 2002).</td>
<td>TZ onwards (Wang et al. 2002).</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Meiotic entry+</td>
<td>gld-3</td>
<td>KH-domain RNA binding protein homologous to Drosophila Bicaudal-C (Eckmann et al. 2002). Negatively affects FBF's binding to RNAs, thus promoting the switch from mitosis to meiosis (Eckmann et al. 2002).</td>
<td>Not reported</td>
<td>TZ onwards (Eckmann et al. 2002).</td>
<td>0.85</td>
<td></td>
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</tbody>
</table>

Not conserved in C. briggsae
| Oocyte fate | tra-2 | Transmembrane protein that is cleaved (by protease TRA-3) and inhibits the FEM proteins in the cytosol to promote global and germline female sex determination (Hodgkin 1986; Doniach 1986; Sokol & Kuwabara 2000; Mehra et al. 1999; Kuwabara & Kimble 1995; Hodgkin & Brenner 1977). Cleaved intracellular domain also co-activates the oogenesis-promoting (and feminine global sex determination) transcription factor TRA-1 in the nucleus (Lum et al. 2000; Wang & Kimble 2001). Translationally repressed by GLD-1 and FOG-2 in hermaphrodite sperm development (Lee & Schedl 2001; Clifford et al. 2000; Doniach 1986). In males, TRA-2 is inhibited by HER-1, a product of the XO male somatic sex determination pathway (Kuwabara et al. 1992; Kuwabara & Kimble 1995). | Oocytes (Goodwin et al. 1993). Also in soma. | Pattern in germline not reported. Also expressed in soma. | -1.91 |
| Sperm fate | fem-1 | Ankyrin-repeat secondary messenger in global male sex determination and germline sperm fate (Doniach & Hodgkin 1984; Gaudet et al. 1996; Spence et al. 1990). Targets TRA-1 for degradation (along with FEM-2 and FEM-3) to promote male and sperm fate (Starostina et al. 2007; Hodgkin 1986). | Soma and germline of males and hermaphrodites, but germ cell pattern not reported (Gaudet et al. 1996). | Soma and germline of males and hermaphrodites, but germ cell pattern not reported (Gaudet et al. 1996). | 0.55 |
| Sperm fate | fem-2 | Protein phosphatase 2C family (Pilgrim et al. 1995). Negatively regulates TRA-1 to promote the male and sperm fate (Hodgkin & Brenner 1977; Hodgkin 1986; Kimble et al. 1984). | Soma and germline, but pattern in germ cells not reported | Soma and germline but pattern in germ cells not reported | -1.11 | Oogenic mRNA expression?
<table>
<thead>
<tr>
<th>Sperm fate</th>
<th><em>fog-3</em></th>
<th>Novel member of Tob family proteins, which negatively regulate cell proliferation in other animals (Chen et al. 2000). Required for sperm fate determination (Ellis &amp; Kimble 1995). Expression is regulated by TRA-1 (Chen &amp; Ellis 2000).</th>
<th>Germline pattern not reported, levels are high during L3/L4 spermatogenesis and declines in oocyte-producing adult hermaphrodites (Chen &amp; Ellis 2000).</th>
<th>Begins in transition zone and increases in spermatocyte meiotic prophase (Lee et al. 2011).</th>
<th>8.19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meiotic entry</td>
<td>nos-3</td>
<td>RNA-binding protein; one of three Nanos homologs that are required for germ cell survival (Kraemer et al. 1999). In a redundant pathway to GLD-2 and GLD-3, NOS-3 and GLD-1 promote meiotic entry (Hansen, Hubbard, et al. 2004; Eckmann et al. 2004). Interacts with FBF-1 and promotes the sperm to oocyte switch (Kraemer et al. 1999; Arur et al. 2011).</td>
<td>Present during all larval stages, but greatest levels are during late L4, before the sperm-to-oocyte switch (Kraemer et al. 1999). Patterns in germ cells not reported</td>
<td>Enriched in germ cells, but also expressed in the soma (Kraemer et al. 1999). Most abundant in mitotic and early meiotic germ cells (Kraemer et al. 1999).</td>
<td>-0.30</td>
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<tr>
<td>Meiotic machinery</td>
<td>rec-8</td>
<td>Sister chromatid cohesion family homolog to yeast Rec-8. Meiosis-specific cohesin (Pasierbek et al. 2001). Phosphorylated by aurora-like kinase AIR-2 during metaphase I and is subsequently degraded (Rogers et al. 2002).</td>
<td>Not reported</td>
<td>Cytoplasmic throughout germline until pachytene, when it lines chromasomes (Pasierbek et al. 2001). Under alternative fixation conditions, antibody binds only in the mitotic cells (Hansen, Wilson-Berry, et al. 2004).</td>
<td>-0.30</td>
</tr>
<tr>
<td>Meiotic machinery</td>
<td>him-3</td>
<td>HORMA domain synaptonemal complex protein that is homologous to yeast synaptonemal complex lateral element Hop1 (Zetka et al. 1999). Involved in homolog pairing and synapsis, and recombination (Zetka et al. 1999; Couteau et al. 2004).</td>
<td>Not reported</td>
<td>Meiotic cells in both spermatogenesis and oogenesis. Localizes between chromosome s during pachytene and dissociates from chromosome s by anaphase I (Zetka et al. 1999).</td>
<td>0.79</td>
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<tr>
<td>Meiotic machinery</td>
<td>syp-1 and syp-2</td>
<td>Coiled coil synaptonemal complex transverse proteins required for chromosome synapsis (Colaiácovo et al. 2002; Colaiácovo et al. 2003). Proper localization of each is dependent on that of the other because RNAi knockdown of one leads to localization defects for the other (Colaiácovo et al. 2003). Localization to chromosomes is dependent on REC-8 and HIM-3 (Colaiácovo et al. 2003; Couteau et al. 2004).</td>
<td>Not reported</td>
<td>Begins in late transition zone and early meiotic cells. Localized between chromatids during pachytene (Macqueen et al. 2002).</td>
<td>0.35</td>
</tr>
<tr>
<td>Meiotic machinery</td>
<td><em>syp-4</em></td>
<td>Central element of the synaptonemal complex (Smolikov et al. 2009).</td>
<td>Not reported</td>
<td>Localized to chromosomes during meiotic prophase I (Smolikov et al. 2009)</td>
<td>0.43</td>
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<tr>
<td>Meiotic machinery</td>
<td><em>rad-51</em></td>
<td>Homolog to yeast Rad51 and E. coli RecA. Binds double-stranded breaks during meiotic recombination (Alpi et al. 2003; Martin et al. 2005).</td>
<td>Not reported</td>
<td>Foci appear during late TZ and localize on double-stranded breaks during pachytene (Martin et al. 2005).</td>
<td>0.44</td>
</tr>
<tr>
<td>Oocyte function</td>
<td><em>oma-1</em></td>
<td>Zinc-finger protein redundantly (with OMA-2) required for oocyte maturation (Shimada et al. 2002).</td>
<td>Expressed in all germ cells of oocyte-producing gonads (Shimada et al. 2002).</td>
<td>Expressed only in proximal (mature) oocytes (Shimada et al. 2002).</td>
<td>-1.16</td>
</tr>
</tbody>
</table>
Figure 9. Relationships between candidate genes involved. Interactions and roles that have been characterized in *C. elegans* germ cell development, as described in Table 1. Key denotes colors that represent roles in mitosis/meiosis and sperm/oocyte decisions in *C. elegans* germ cell development.
Figure 10. Phylogenetic relationships between diverse nematodes. These species are used as reasonably distant outgroups in BLAST searches for conserved C. elegans proteins in Table 2. Relationships are according to (Kiontke & Fitch 2005; Blaxter & Koutsovoulos 2014).
Table 3. Search for germ cell genes conserved across diverse nematodes. Homology of candidate protein sequences in diverse nematode species that are fully sequenced. The *C. elegans* protein sequence was BLAST searched to all nematode species, and then recorded if any homologs (with E < 10e-3) were found in each species (+). Next, multiple alignments for homologs found in each species were generated using COBALT. From the alignments, the amount of conserved regions across species was recorded (Conserved regions).

<table>
<thead>
<tr>
<th><em>C. elegans</em> protein</th>
<th><em>C. remanei</em></th>
<th><em>Ascaris suum</em></th>
<th><em>Loa loa</em></th>
<th>Strongyloides ratti</th>
<th><em>Pristionchus pacificus</em></th>
<th>Conserved regions</th>
<th>Good candidate?</th>
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<tr>
<td>LAG-2</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>GLP-1</td>
<td>+</td>
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<td>+</td>
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<td></td>
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<td>FBF</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>TRA-2</td>
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<td>FEM-1</td>
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<td>FOG-1</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Many short regions conserved</td>
<td>Yes</td>
</tr>
</tbody>
</table>
**Table 4.** Homologous protein search matches in *R.* sp. SB347. Homology of candidate proteins to *R.* sp. SB347 proteins. *R.* sp. SB347 translated protein sequences were BLAST searched in *C. elegans* protein sequences. E (expect) value is significant and indicates a likely homolog if E < 10e-3. If there was a matching sequence, they were pairwise aligned and of that aligning region, the % amino acid identity matches and % amino acid conserved features were recorded. GLD-2 and GLD-3 sequences were not obtained, and were not able to be analyzed here, though they were good candidates. Final candidates for which we identified a homolog in *R.* sp. SB347 are in bold.

<table>
<thead>
<tr>
<th>Protein</th>
<th>E val</th>
<th>% identity</th>
<th>% conserved</th>
<th>notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1</td>
<td>6.5</td>
<td>48%</td>
<td>65%</td>
<td>Alignment is only one short region. SB347 sequence is much longer. May not be spliced, but more likely the alignment is just a common domain. High E value indicates this is not a homolog.</td>
</tr>
<tr>
<td>FBF</td>
<td>2e-100</td>
<td>50%</td>
<td>60%</td>
<td>Matches PUF proteins PUF-11 and PUF-3 with higher E vals than FBF (E = 2e-110 and 2e-108 respectively). Also homologous to PUFs 5-9. PUF-5 and PUF-6 are most homologous of these, with E = 3e-97 and 3e-93 respectively.</td>
</tr>
<tr>
<td>GLD-1</td>
<td>5e-114</td>
<td>56%</td>
<td>69%</td>
<td></td>
</tr>
<tr>
<td>FEM-1</td>
<td>1e-42</td>
<td>43%</td>
<td>62%</td>
<td>No FEM-1 match. Best match is TRP (transient receptor potential) channel protein, with E value of 0.0.</td>
</tr>
<tr>
<td>FEM-2</td>
<td>6e-20</td>
<td>34%</td>
<td>40%</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Homologous protein search matches in *R.* sp. SB347. Homology of candidate proteins to *R.* sp. SB347 proteins. *R.* sp. SB347 translated protein sequences were BLAST searched in *C. elegans* protein sequences. E (expect) value is significant and indicates a likely homolog if E < 10e-3. If there was a matching sequence, they were pairwise aligned and of that aligning region, the % amino acid identity matches and % amino acid conserved features were recorded. GLD-2 and GLD-3 sequences were not obtained, and were not able to be analyzed here, though they were good candidates. Final candidates for which we identified a homolog in *R.* sp. SB347 are in bold.
There were two sequence matches found in SB347 transcriptome. Similar results for both.

<table>
<thead>
<tr>
<th>Protein</th>
<th>E Value</th>
<th>Identity 1</th>
<th>Identity 2</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOG-1</td>
<td>3e-42</td>
<td>41%</td>
<td>60%</td>
<td>Top match is actually cpb-1 with E value of 1e-91.</td>
</tr>
<tr>
<td>FOG-3</td>
<td>2e-40</td>
<td>38%</td>
<td>54%</td>
<td></td>
</tr>
<tr>
<td>SPE-44</td>
<td>2e-28</td>
<td>54%</td>
<td>74%</td>
<td>Also matches GMEB (Glucocorticoid Modulatory Element Binding protein) transcriptional regulator homologs</td>
</tr>
<tr>
<td>REC-8</td>
<td>2e-09</td>
<td>32%</td>
<td>53%</td>
<td></td>
</tr>
<tr>
<td>HIM-3</td>
<td>1e-17</td>
<td>28%</td>
<td>47%</td>
<td>Four sequences in SB347 transcriptome matched. This data is for the best match. The others matched htp (him three paralog) better, suggesting <em>R. sp. SB347 also has several htp genes.</em></td>
</tr>
<tr>
<td>MSP</td>
<td>3e-68</td>
<td>78%</td>
<td>87%</td>
<td>Matches MSP-78 best.</td>
</tr>
<tr>
<td>OMA-1</td>
<td></td>
<td></td>
<td></td>
<td>No OMA-1 match. Matches MeNoRin dendritic branching protein mnr-1 with E value of 0.0.</td>
</tr>
</tbody>
</table>
Figure 11. Preliminary comparisons of mRNA expression between sexes in C. elegans and R. sp. SB347. A) Data reported in (Ortiz et al. 2014). RNAseq experiments comparing C. elegans hermaphrodite mutants, both with feminine soma, and either only produce sperm (fem-3(q96) mutants) or only produce oocytes (fog-2(q71) mutants). B-C) Preliminary qPCR data comparing candidate genes’ mRNA expression between either C. elegans (B) or R. sp. SB347 (C) hermaphrodites, males, and larvae. Mean CTs were compared between samples relative to beta tubulin.
Figure 12. Predicted expression patterns of SB347 FBF and SPE-44 homologs. Two predictions are made for whether R. sp. SB347 sperm production is consistent with either model 1 or model 2.
**Figure 13.** Protein sequence alignment of *C. elegans* FBF and *R. sp. SB347* homolog. PUF domain highlighted in blue and immunized peptide for antibody production in red.
Figure 14. Western blot tests of anti-FBF antibody. A) Western blot with anti-FBF and anti-SPE-44. Sample is a random mix of 200 *R. sp. SB347* male, female, and hermaphrodite adults. B) Western blot with sample of 200 *R. sp. SB347* hermaphrodites probed with anti-FBF where gel was run longer than in (A).
<table>
<thead>
<tr>
<th>Sequence</th>
<th>Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>sb347 spe44</td>
<td>1 --------------MSLWNPPFLVKTEPLDRSRRNLEED-DAAPQSDILE 35</td>
</tr>
<tr>
<td>c elegans spe44</td>
<td>1 MFQGD5VAASVLPSHPISGCDDFGQAEGLDSDDEHSTMTFQESQGQQYQILYGPAPADDLP 60</td>
</tr>
<tr>
<td>sb347 spe44</td>
<td>36 LFKNTRAGSPSSNHQFRSDDVLDVTGMLTGKLKRFRMLCPGIBHQRSCIQIDYTDMVSPK</td>
</tr>
<tr>
<td>c elegans spe44</td>
<td>61 ---RTITPLQITFEGDSAPTIVPVSUCLVNSMLNLFHCNPQGIROCIEVQG---DLLSPK 115</td>
</tr>
<tr>
<td>sb347 spe44</td>
<td>96 DFTIRACKRDQKMDKKSIRYKRSRMRMLEGLIDYFHQDFCSLACKGQRNH---147</td>
</tr>
<tr>
<td>c elegans spe44</td>
<td>116 QPITIRGDKNEQKMDKASIRVGRLRTIMEANTIDFYEIMNRCGQGCMQSNYVAFSEEEV 175</td>
</tr>
<tr>
<td>sb347 spe44</td>
<td>148 MKGFRPSDLRSKXSSAFN---ETTVPFLPSLISPISYGCTTNFTFLSPNSISHLDSGTVSFIPA 206</td>
</tr>
<tr>
<td>c elegans spe44</td>
<td>176 LQARSKKRSTSEQGLKYEINEMAGKEADNDRKSAKARFSRPGSFQKPRQVMKMEPO 235</td>
</tr>
<tr>
<td>sb347 spe44</td>
<td>207 ENVPHSNLNNN---NNTITVIDGIDFSSMSIPQFMNLHIDPVFSFWELHRVNI 263</td>
</tr>
<tr>
<td>c elegans spe44</td>
<td>236 DDFRFEFEDIFAPPLQSMSSNEETSSNKESECTPFDN2ILMCQNMPPMPMFWQMQTGV 295</td>
</tr>
<tr>
<td>sb347 spe44</td>
<td>264 ITDVBVRKIM---RLKDACAAHEIDAHAMNL-SRVTVALENQLYQVMFINQNXQTRNI 319</td>
</tr>
<tr>
<td>c elegans spe44</td>
<td>296 IGHFCDDTVSAINTLQSQSVMDFVPTTANHNLRTAFALIGPPVWVHQRWIENARYQQ 355</td>
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<tr>
<td>sb347 spe44</td>
<td>320 PMEAMCTYTRTRPSEQDEKIMINSQGGLRESQSKDDYFL 360</td>
</tr>
<tr>
<td>c elegans spe44</td>
<td>356 KHDENNDIQSTLAEHSSKYPQRTSSSSQSSLHATRETEKEVEVLEIVDCKYDDYPESE 415</td>
</tr>
<tr>
<td>sb347 spe44</td>
<td>416 CLPGFPHIQ 424</td>
</tr>
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</table>

**Figure 15.** Protein sequence alignment of *C. elegans* SPE-44 and *R. sp.* SB347 homolog. SAND domain highlighted in blue and immunized peptide for antibody production in red.
Figure 16. Test of *R. sp.* SB347 anti-SPE-44 by immunocytochemistry. A) *C. elegans* male gonad labeled with *C. elegans* anti-SPE-44 antibody. TZ = transition zone. Scale bar = 20 microns. Right: a different *C. elegans* male gonad labeled with *C. elegans* anti-SPE-44 antibody, cropped to only show spermatocytes. SPE-44 localizes to spermatocyte chromatin, with the exception of the one X chromosome (labeled as “x” where this is visible). Scale bar = 20 microns. B) Left: Epifluorescence image of *R. sp.* SB347 male gonad with anti-SPE-44 taken at a focal plane where DAPI-labeled nuclei were in focus. Scale bar = 20 microns. Below are full-size images of cells labeled a-e and scale bar = 5 microns. Right: a second image acquired where the rachis was in focus, with a full size image of anti-SPE-44 binding in the rachis below. Scale bars = 20 microns. C) *R. sp.* SB347 hermaphrodite labeled with DAPI and anti-SPE44. Scale bar = 20 microns. Sperm-producing clusters labeled 1-3 are shown at full size to the right where scale bars = 5 microns.

Figure 17. Test of ant- SB347 SPE-44 antibody by western blot. A sample of 200 feminine *R. sp.* SB347 worms was probed. The expected molecular weight of *R. sp.* SB347’s SPE-44 homolog is 41 kD. The band observed is heavier than predicted, at approximately 70 kD, and may reflect antibody binding to a larger protein present in the rachis (Figure 16).
The novel aspects of *R. sp.* SB347 germ cell development present many unanswered questions for future investigation. Further characterization of *R. sp.* SB347 germ cell development promises to continue to provide unique insights about developmental processes, which are discussed by topic below.

**Organization of germ cells**

*C. elegans* germ cells are organized within a syncytium, with each cell maintaining a small connection to a single common cytoplasm called a rachis (Gumienny et al. 1999). In contrast, *Drosophila* and mammalian germ cells are organized within multiple, discrete germ cell cysts (Reviewed in (Greenbaum et al. 2011)). Here we discovered that, within the gonads of *R. sp.* SB347 simultaneous hermaphrodites, sperm develop within germ cell cysts but oocytes develop along a thin rachis (Figure 3B). Thus *R. sp.* SB347 hermaphrodite gonads provide a unique system for comparing the differences between these two ways of connecting germ cells.

We found that spermatogonia are independent of the oocyte rachis and their development begins as one cell located in the distal region of the gonad, alongside immature oocytes (Figure 2B-C; Figure 6). Currently, these data do not distinguish between two possible origins of the spermatogonia. One model is that the one cell spermatogonium broke off the common rachis, which connects
GSCs (Figure 7), as it developed and it differentiated into a sperm-fated spermatogonium. Another model could be that the one cell spermatogonia are in fact a second type of GSC, distinct from those found near the DTC. Our current observations do not distinguish between these two possibilities. We have not observed a spermatogonium-like cell breaking off of the rachis, and we have not thoroughly tested which cells have a GSC-like identity. Future experiments that distinguish whether there are one or two types of GSCs in *R. sp. SB347* hermaphrodites may additionally reveal whether the GSCs are truly bipotential or if there are differences between sperm-producing and oocyte-producing GSCs.

When we scored the numbers of cells within spermatogonial cysts, cysts most frequently contained 1, 2, 4, or 8 cells (Figure 3C). However, we sometimes encountered cysts of odd numbers of cells (3, 5, 7, and also 6) (Figure 3C). This observation suggests that the connections within cysts may break or that cells might sometimes end mitotic divisions with complete cytokinesis. In addition, we sometimes scored cysts containing a very large number of cells, suggesting that the cysts may accumulate or merge together as has been observed in mice (Lei & Spradling 2013). In order to determine whether these atypical groups of cells within cysts were associated with old age, we performed the same frequency analysis as in Figure 3C, but separated young gonad data (L4→24h post-adult-molt) from elderly gonad data (36→72h post-adult-molt) (Figure 19). Comparing the patterns between young and elderly hermaphrodite gonads does not reveal dramatic differences. However, young hermaphrodites were more likely to contain cysts with small, odd numbers of cells (i.e. 3 and 5), while older
hormaphrodites were more likely to contain cysts with more than 8 cells (Figure 16). This analysis is a preliminary observation that may suggest that, as hermaphrodites age, the cysts accumulate into larger groups of cells. With further technological advances, this process could be investigated by live-cell imaging in hermaphrodites with labeled spermatogonia to determine whether two separate cysts can be observed merging. A more feasible, near-term experiment would be to use BrdU to label newly proliferating cysts for a short period, and then observe whether a group of both BrdU-labeled and unlabeled cells can be observed clustered together.

Currently, it is unclear whether R. sp. SB347 hermaphrodite spermatogonia are organized within cysts as a convenient mechanism of simultaneously producing sperm alongside oocytes or if, perhaps, germ cell cysts are a more optimal organization for sperm production than oocyte production. We determined that sperm production in R. sp. SB347 males is also organized within germ cell cysts because clusters of cells were synchronized within the cell cycle (Figure 3F). These data might suggest that the R. sp. SB347 lineage ancestrally organized sperm production within cysts before the acquisition of simultaneous hermaphroditism; however, the nearest male/female relative Rhabditis axei did not utilize spermatogonial cysts (Figure 5E). Preliminary investigation of R. sp. SB347 male germ cell connections by collagenase treatment followed by actin staining revealed that there is in fact a central rachis structure (Figure 18A). However, unlike the straight, large-diameter tube in C. elegans, the rachis in SB347 males appears to be thin and branched. Notably, in collagenase-treated
gonads, the male germ cells often broke apart from the gonad within isolated clusters while maintaining actin bridges between individual germ cells (Figure 18B).

In *C. elegans*, apoptosis is a common process during the oogenesis program, but not the spermatogenesis program (Reviewed in (Gartner et al. 2008)). Oocyte apoptosis occurs for two reasons. If a virus has infected the germline, then the germ cells undergo programmed cell death and are engulfed by somatic gonad sheath cells. But even in healthy oocyte-producing worms, developing oocytes undergo apoptosis, but in this case, cellular contents are donated to the central rachis and can be taken up by other oocytes (Gumienny et al. 1999).

It remains to be determined whether developing *R. sp. SB347* oocytes undergo apoptosis. Suggesting that developing oocytes may not undergo apoptosis, *R. sp. SB347* feminine gonads contain far fewer GSCs than *C. elegans* gonads do (an estimated 10-20 GSCs in SB347 (Pablo Ordonez, Shakes Lab, unpublished) compared with 220 GSCs in *C. elegans*), so developing germ cells may be less dispensable than in *C. elegans*. Furthermore, the rachis is much thinner in *R. sp. SB347* feminine gonads than the rachis found in *C. elegans* gonads, which may reflect a lack of cytoplasm sharing from apoptosed oocytes. A simple experiment that can determine whether developing germ cells in *R. sp. SB347* feminine gonads undergo apoptosis is to stain dissected gonads with a dye that labels apoptosing cells, such as acridine orange (Gartner et al. 2008).
A final open question regarding the structural organization of *R. sp. SB347* germ cells is whether the MSP+ spermatocytes send maturation signals to adjacent oocytes in hermaphrodite gonads. In *C. elegans*, MSP is both a sperm motility protein and a soluble signaling molecule that can initiate oocyte maturation and contractions of the somatic gonad leading to ovulation (Miller et al. 2001). MSP was observed in spermatocytes most typically located at the proximal end of the gonad, where oocytes would be expected to be maturing and ovulating (Figure 2C). However, we also occasionally observed MSP+ spermatocytes alongside oocytes that were further away from the spermatheca (Figure 2C). On the one hand, MSP-induced oocyte maturation from spermatocytes would help coordinate production of mature oocytes as spermatocytes are produced. On the other hand, MSP signaling from spermatocytes that are occasionally differentiated before reaching the proximal end of the gonad could induce maturation in oocytes that are not yet fully developed. It remains to be determined how sperm and oocyte production might be coordinated in *R. sp. SB347* hermaphrodites and whether MSP acts as a signal for oocyte maturation in this species.

**Germline stem cell niche**

In our immunocytochemistry experiments, we consistently observed DAPI-stained DTC nuclei at the distal end of *R. sp. SB347* gonads that resembled nuclei of *C. elegans* DTCs. In order to determine whether the DTC functions as the stem cell niche in *R. sp. SB347*, the DTC could be laser ablated and germ
cells monitored for meiotic entry and differentiation, which is how the DTC was determined to be the stem cell niche in C. elegans more than 30 years ago (Kimble & White 1981).

Assuming the DTC functions as the niche in R. sp. SB347, both the physical shape and molecular signaling mechanism of the R. sp. SB347 DTC remains uncertain. In C. elegans, GSCs are maintained in a stem cell state by juxtacrine Notch/Delta (GLP-1/LAG-2) signaling from the DTC. The DTC has long, tentacle-like protrusions that extend signaling for ~8 cell diameters (Byrd et al. 2014; Hall et al. 1999). Preliminary experiments examining the shape of the R. sp. SB347 DTC by scanning electron microscopy proved technically challenging but have not revealed long protrusions extending from the R. sp. SB347 DTC (unpublished data by Xiaoxue Lin and Pablo Ordóñez, Shakes Lab, data not shown). Curiously, our BLAST analysis found that GLP-1 and LAG-2 were not well conserved across nematodes and we did not identify homologs in R. sp. SB347 (Tables 3-4). Future experiments are needed to determine whether R. sp. SB347 DTCs have protrusions and to clarify whether R. sp. SB347 GSCs are maintained by juxtacrine Notch/Delta (GLP-1/LAG-2) signaling or if GSCs are maintained by a different mechanism altogether.

One striking difference between C. elegans and R. sp. SB347 germlines is the size of the GSC pool. While the precise number of GSCs should be determined in the future using a marker of GSCs, it is obvious that there are far fewer GSCs in R. sp. SB347 (220 vs. an estimated 10-20). This disparity suggests that the stem cell niche is smaller in R. sp. SB347. There are several
possibilities that could explain this. It is possible that the DTC has shorter protrusions, but regulates GSCs by juxtacrine signaling. Or, it is possible that the DTC regulates GSCs by direct contact, but does not have protrusions, and so only the most distal germ cell is a true GSC. A final possibility is that the DTC signals to GSCs by a paracrine signaling mechanism, which limits the niche to only the distal end of the gonad.

Regulation of germ cell development

There are two major developmental processes that must be carefully regulated in germ cell development: differentiation into either spermatocytes or oocytes and the switch from the mitotic cell cycle to the meiotic program. In *C. elegans*, regulation of these two processes is coupled. In several cases, the same RNA binding proteins repress translation of mRNAs that are involved in both processes (see Table 2 and Figure 9). The RNA binding proteins FBF and GLD-1 are conserved in *R. sp. SB347*. It is possible that FBF and GLD-1 function similarly in this species, coupling the sperm/oocyte decision with the mitosis/meiosis switch. It is also possible that these two processes are separated in *R. sp. SB347* germ cell development, and GLD-1 and FBF bind only either cell cycle or differentiation-related mRNAs.

As a starting point for assessing when and where within the gonad germ cells differentiate, we scored the locations and size of clusters that had begun to express MSP. MSP is a marker of fully differentiated spermatocytes, and the protein was typically only expressed in clusters at the proximal end of the gonad.
(Figure 2). However, there is a positive relationship between the location of clusters and the number of cells in a cluster (Figure 6). Given this tight relationship, it cannot be concluded which is a better determinant of whether a cluster expresses MSP - the number of cells within a cluster or the location of the cluster.

Because many genes involved in *C. elegans* germ cell development are translationally repressed by RNA binding proteins (see Table 2 and Figure 9), we tested whether *msp*, whose protein expression we had already characterized in *R. sp. SB347*, was translationally repressed. To test this possibility, we compared where *R. sp. SB347* male germ cells expressed *msp* mRNA and MSP protein. To determine *msp* expression, we designed custom small molecule fluorescent *in situ* hybridization (smFISH) probes using a free program available from Biosearch Technologies (Petaluma, CA) ([http://www.biosearchtech.com/stellarisdesigner/](http://www.biosearchtech.com/stellarisdesigner/)). The probes were designed using fewer than the recommended number of unique oligonucleotides for hybridization due to the short length of the *msp* mRNA sequence (21 instead of the recommended 30-48 probes). The fewer number of unique and specific probes may have resulted in higher background due to non-specific signals. After decreasing background signal in IPLab software, a reasonable expression pattern can be distinguished (Figure 20A). However, non-specific signal was still present in GSCs, which should not express any sperm genes at all. The most abundant signal coincides with the region of *R. sp. SB347* sperm development when MSP protein is expressed (Figure 20B). While these data are preliminary
due to these technical setbacks, we conclude that \textit{msp} mRNA does not seem to be translationally repressed.

\textbf{Significance}

The novel discovery of both simultaneous hermaphroditism and spermatogonia in \textit{Rhabditis} nematodes demonstrates that the already well-characterized nematodes are not necessarily representative of all nematodes. Specifically, these data refute the assumption that all hermaphroditic nematodes are sequential. Furthermore, in the highly disputed context of \textit{C. elegans} germ cell transient-amplification, here we have discovered a clear example of germ cell transient-amplification in the form of spermatogonia. Both novel features of \textit{R. sp.} SB347 germ cell development suggest the species as highly informative in comparisons of germ cell development across taxa and for understanding germ cell evolution.

\textbf{Methods}

Immunocytochemistry and cluster scoring was done as described in Ch 1 methods.

\textit{smFISH for \textit{R. sp. SB347 msp}}

Methods were adapted from (Lee 2006; Updike et al. 2014) and according to recommendations from Biosearch Technologies (Petaluma, CA). Custom small
molecule fluorescent in situ hybridization (smFISH) probes were designed for the 
R. sp. SB347msp mRNA sequence kindly provided by Sophie Tandonnet (André 
Pires daSilva lab, University of Warwick). Probe sequences were chosen using a 
free program available from Biosearch Technologies (Petaluma, CA) 
(http://www.biosearchtech.com/stellarisdesigner/). 

Gonads were dissected in Edgar’s buffer (Boyd et al. 1996) on poly-L-lysine 
slides and then fixed in 4% PFA in 1xPBS for 45 minutes. Slides were then 
washed twice for five minutes each in 1xPBS. Samples were then permeabilized 
in 70% ethanol at 4 degrees C overnight. Samples were surrounded in a square 
drawn with hydrophobic pen and ethanol removed. Samples were then washed 
in Biosearch Technologies’ proprietary Wash Buffer A for 3 minutes. Next, probes 
diluted to 125 nM in Biosearch Technologies’ proprietary hybridization buffer were 
added to samples. Hybridization was tested for variable times (30 minutes to 16 
hours) and was done at 37 degrees C in the dark. Longer hybridization times 
resulted in more distinguishable signal (12-16 hours). Samples were then quickly 
washed in Biosearch Technologies’ proprietary Wash Buffer A and then stained 
with DAPI diluted to 5 ng/mL in Wash Buffer A for 30 minutes at 37 degrees C. 
The DAPI solution was then removed and samples were washed in Biosearch 
Technologies’ proprietary Wash Buffer B for 2-5 minutes. Finally, samples were 
mounted with VectaShield (Vector Labs). Imaging was done as described in 
Chapter 1, but using IPLabs deconvolution software. Deconvoluted mages were 
adjusted using IPLabs software.
Figure 18. Germ cell connections in R. sp. SB347 males. A-B) Two different dissected male gonads that were treated with collagenase before fixation and phalloidin staining. Scale bars = 20 microns. Top = merge, middle = DAPI, bottom = actin.
Figure 19. Comparisons of cell numbers within cysts by hermaphrodite age. The same data that is presented in Figure 3C has been separated by young (top) and old (bottom) hermaphrodites. Young hermaphrodite data: n = 614 cysts from 224 gonad arms. Old hermaphrodite data = 814 cysts from 362 gonad arms.
Figure 20. Preliminary data investigating expression of *msp* mRNA and protein in *R*. sp. SB347 males. A) *msp* smFISH and DAPI staining in a dissected male gonad. B) A second male gonad labeled with DAPI, anti-tubulin, and anti-MSP antibodies. Scale bars = 20 microns.
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Clark, W.C., 1978. Hermaphroditism as a reproductive strategy for metazoans;


