REGENERATION OF THE GERMLINE IN THE ANNELID CAPITELLA TELLETA

By

LEAH DANNEBERG

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To my Grandparents, Agnes and Walter Dannenberg
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<td>MPCs</td>
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The germline is essential for sexual reproduction and survival of the species. Typically, the germline is separated from the soma early in embryogenesis. Previous studies suggest that germ cells cannot regenerate once removed from the embryo, but few animals have been experimentally tested. We investigated the ability of the germline to regenerate in the lophotrochozoan, *Capitella teleta*, by deletion the germline precursor (cell 3D) in early stage embryos using an infrared laser. Larvae and juveniles, resulting from germline deletions were examined for presence of multipotent progenitor cells (MPCs), stem cells that form the germ cells and somatic stem cells. Contrary to control deletions of a non-germline macromere, most larvae resulting from deletion of cell 3D lacked MPCs as assayed by expression of germline markers, *CapI-vasa*, *CapI-nanos* and *CapI-piwi*. However, 13% of experimental larvae had MPCs, indicative of germline regeneration. In contrast, by 2 weeks post-metamorphosis, all juveniles resulting from deletion of cell 3D had MPCs, as detected by *CapI-vasa* expression. Furthermore, when raised to adulthood, the majority of animals developed reproductive structures and were fertile. In another set of deletions, we removed both the D quadrant mesoderm and germline. Theses juveniles regenerated MPCs, but the
deletion caused a novel response in larval tissue. Our results indicate that *C. teleta* can regenerate the germline following removal of the germline progenitors in the early embryo. The dramatic difference in ability to regenerate the germline between the larval and adult stages suggests that there are two distinct compensation events during the life cycle: a regulative event in the early embryo and a stem cell trans-differentiation event after metamorphosis, when the animals are capable of substantial body regeneration.
CHAPTER 1
INTRODUCTION

The germline is necessary for sexual reproduction, which is imperative for the survival and evolution of species. In many well-studied bilaterian organisms, the germline separates completely from the somatic cells early in embryonic development, either by sequestration of proteins and mRNA in the cytoplasm of the zygote, or by induction via a cell-signaling event from other cells in the embryo to specify this lineage. These two distinct mechanisms are known as preformation and epigenesis, respectively (Nieuwkoop & Sutasurya 1979; 1981; Extavour and Akam, 2003). In some species, the segregated cells that will later form the sperm and egg are known as primordial germ cells (PGCs). In other animals, such as in the snail *Illyanassa obsoleta* and the sea urchin *Strongylocentrotus purpuratus*, multipotent progenitor cells (MPCs) are segregated and can generate both germline and soma (Juliano et al., 2010). It has been proposed that in many animals, germline and somatic lineages must separate early in embryonic development to avoid evolutionarily detrimental competition between different cell lineages within the organism (Buss, 1987). In addition, this segregation of the primordial germ cells from somatic lineages minimizes the possibility of passing on somatic mutations to the germline (Blackler, 1970; Drake et al., 1998; Milholland et al., 2017; Seydoux and Braun, 2006; Strome and Updike, 2015). A portion of the scientific community believes that, excluding fertilization and embryogenesis, there is no exchange between the somatic and germline lineages (reviewed in Weisblat, 2006).

Removal of germ cells by excision, irradiation, or deletion results in sterile adults in many organisms, including in *Mus musculus*, *Xenopus laevis*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Ambystoma mexicanum*, and *Gallus gallus* (Barnes
et al., 2006; Blackler, 1965; Buehr and Blackler, 1970; Dubois, 1962; Dulbecco, 1946; Everett, 1943; Fargeix, 1975; Nieuwkoop P.D., 1951; Reynaud, 1976; Sulston and Schierenberg, 1983; Züst and Dixon, 1975). These organisms develop reproductive structures with no gametes. The results of these experimental manipulations support the idea of separation between the germline and soma, and importantly, of a single embryonic origin for the germline. Once segregated, the germline cannot reform from other cell types. In contrast, more recent experiments in the ascidian *Ciona intestinalis* have uncovered an example of germline regeneration. In *C. intestinalis*, the germline is located in the tail during the larval tadpole stage, and when the larval tail is removed, the resulting juveniles lack germ cells. However, after 15 days, a few germ cells appear, and later, the adults produce sperm (Takamura et al., 2002). It is unknown if this example is a rare occurrence or whether additional sampling will reveal more cases of animals that can regenerate their germline. It is worth noting that experimental manipulations of the germline have only been performed on a small fraction of animal clades.

The superphylum Lophotrochozoa contains 14 highly diverse animal phyla and, relative to other taxa, little is known about the development of the germline, and its members have not yet been carefully examined for an ability to regenerate the germline (reviewed in Extavour and Akam, 2003). Many members of this superphylum have a shared developmental program called spiral development. Embryos that undergo spiral cleavage have a stereotypic cleavage pattern in which each embryonic cell is identified based on spatial relationships within the embryo, cell size, and time of division (Wilson, 1982; Henry and Martindale, 1999, 1998). The cleavage pattern of spiralian embryos
has enabled researchers to perform single cell blastomere deletions and carry out fate map studies on several species (Ackermann et al., 2005; Boyer et al., 1996; Damen and Dictus, 1994; Hejnol et al., 2007; Henry and Martindale, 1998; Maslakova et al., 2004; Meyer et al., 2010; Render and Render, 1997; Weisblat and Shankland, 1985). From these studies, it has been shown that several aspects of the fate map are conserved across species and even across phyla. One notable example is the apparent conservation of the embryonic origin of the germline. In all animals examined, the precursor of the germline is localized to a single cell in the 64-cell stage embryo, the cell 4d (Lambert, 2008).

One lophotrochozoan, the annelid C. teleta, has several advantages for studies of the germline. The embryonic origin of the germline in C. teleta appears to be conserved with that of other spiralians, in that descendants of cell 4d coincide with the position, size, and shape of cells within the coelomic cavity that express the germline and stem cell-specific genes piwi, nanos and vasa (Dill and Seaver, 2008; Giani et al., 2011; Meyer et al., 2010). However, unlike in other spiralians, the germline and the mesoderm do not arise from the same precursor cell, allowing for manipulation of the germline without also disrupting mesoderm formation (Meyer et al., 2010). In addition, C. teleta reproduces sexually with separate male and female sexes that can be successfully mated in the laboratory. Reproductive structures are visible and have been characterized in detail. Furthermore, like many other annelids, C. teleta can regenerate (Bely, 2006; Bely et al., 2014). For example, following a transverse amputation, C. teleta can regenerate its nervous system, musculature, and gut following an amputation event (de Jong and Seaver, 2016). Both somatic (ovaries) and germline (oocytes)
components of the reproductive tissues can also regenerate following amputation posterior of the 6th thoracic segment (Giani et al., 2011; Hill and Savage, 2009). The ability of C. teleta to regenerate multiple tissue types led us to hypothesize that this animal may have a unique stem cell program that allows trans-differentiation or dedifferentiation of somatic stem cells to germ cells, and potentially regeneration of the germline.

Historically, many scientists have observed morphological similarities between germ cells and stem cells in annelids (Faulkner, 1932; Potswald, 1972, 1969). Notably, both cell types have a large nuclear to cytoplasmic ratio and characteristic morphology of undifferentiated cells. More recent molecular studies, including studies in C. teleta, show that the markers vasa, nanos, and piwi are expressed in both the germline and somatic stem cell populations in many species, emphasizing the similarities between the germline and stem cells (Dill and Seaver, 2008; Fischer and Arendt, 2013; Giani et al., 2011; Lyons et al., 2012; Mochizuki et al., 2001; Raz, 2002; Rebscher, 2014; Rebscher et al., 2012; Shibata et al., 1999; Solana, 2013). Such genes are proposed to have a role in maintaining an undifferentiated state (Mochizuki et al., 2001). These cells have been referred to as ‘germline cell stem cells’, ‘germinal cells’, ‘pre-primordial germ cells (pre-PGCs)’, ‘presumptive primordial germ cells (PGCs)’ or ‘primordial stem cells (PriSCs)’ and ‘molecular progenitor cells’ depending upon the study. In previous studies, we referred to a cluster of cells with these characteristics in C. teleta cells as presumptive primordial germ cells (Giani et al., 2011), and we now refer to them as MPCs to better represent an additional potential role that the cells in this cluster may have during regeneration (de Jong and Seaver, 2017). The MPC cluster in C. teleta
larvae, juveniles, and adults can be visualized with the germline markers $Capl$-vasa, $Capl$-piwi1, $Capl$-piwi2, $Capl$-nanos (Dill and Seaver, 2008; Giani et al., 2011). These cells are either pluripotent stem cells capable of forming either germline or somatic cells, or a mixed population of cells with a subset destined to become germline stem cells and another subset destined to become somatic stem cells. We favor the later possibility, due to recent molecular evidence suggesting heterogeneity within the cluster. Specifically, only a small subset of cells in the MPC cluster expresses the marker $Ct$-myc (de Jong and Seaver, 2017).

To determine if $C. teleta$ can regenerate its germline, we performed single cell laser deletion experiments to remove the germline precursor cell. We then assessed MPC presence using molecular markers in larvae and juveniles. Adults resulting from embryonic deletion of the germline precursors were analyzed for the presence of reproductive structures, ability to mate, and viability of their offspring. In addition, we also investigated the cellular origin of the lineage capable of replacing the germline. To our knowledge, this work provides the first evidence of germline regeneration in a lophotrochozoan, and is one of only a few examples of germline regeneration in animals.

**C. Teleta Life History and Adult Anatomy**

During its life cycle, $C. teleta$ undergoes indirect development. Following embryogenesis, larvae begin to swim between at 5 and actively swim in the water column by 6 (Fig. 1-1A, adapted from (Seaver and Kaneshige, 2006)) ((Bhup and Marsden, 1982; Eisig, 1899; Reish, 1974; Werbrock et al., 2001). Metamorphosis from swimming larvae to juveniles occurs between 9 and 10 days post-fertilization, and adults develop reproductive structures around 8 weeks post-metamorphosis (Fig. 1-1A).
Adults reproduce sexually, and there are separate male, female and hermaphrodite sexes. Males and females can be differentiated from one another by structures that are easily visible in live specimens. The females have visible, paired ovaries (ov) on their ventral side of the anterior 10 to 12 abdominal segments (Fig. 1-1B). The males have genital spines (gs) on the dorsal side of the body in segments 8 and 9 as well as laterally positioned genital ducts (gd) between thoracic segments 7 and 8 (Fig. 1-1C) (Blake et al., 2009). Each male and female can reproduce multiple times. Hermaphrodites function only as females and develop when females are sparse in the population and nutrient levels are high (Holbrook and Grassle, 1984) and are genetically identical to males (Petraitis, 1985). Eggs are laid in a brood tube created by the female using sediment and secretions (Fig. 1-1D). The fertilized embryos develop into larvae and remain in the brood tubes until the larvae are competent to undergo metamorphosis (Fig. 1-1E) (Méndez et al., 2000).

**Fate Map and the Germline Lineage of C. teleta**

Early stage embryos of *C. teleta* cleave in a stereotypic fashion, and undergo spiral cleavage. Each cell can be identified based on a combination of spatial relationships, cell size, and time of birth. Similar to other spiralian, at the four-cell stage, the four cells in the embryo have a relationship to the future quadrants of the body, and are called A, B, C, and D. D is largest cell in *C. teleta* and the B cell is on the opposite side of the embryo, and the two cells contact each other at the vegetal pole, also known as the vegetal cross furrow. The micromeres are cells born from the macromere cells. Micromeres are indicated by a lowercase letter while the macromeres are given an uppercase letter. Each round of divisions is given a number, beginning with 1, and the micromeres and macromeres are identified with corresponding ascending
numbers after each division. The macromere cleavages begin with a clockwise division that results in the birth of the 1\textsuperscript{st} quartet micromeres (1a, 1b, 1c, and 1d), and the macromeres alternate between clockwise and counter-clockwise orientations of the mitotic spindle at each division (Eisig, 1898).

In addition to a conserved pattern of early cleavages, embryos that undergo spiralian development show conservation of fates among homologous cells. For fate mapping studies, an individual cell is labeled in the early embryo, and the descendants of that cell are followed to differentiated cell types, typically in larvae. A fate map of \textit{C. teleta} has been published (Meyer et al., 2010). From this fate map, it is known that the descendant cells of cell 4d make the multipotent progenitor cells (MPCs) as well as the anus (Fig. 1-2A). The germline arising from cell 4d is highly conserved in spirалиans (reviewed in Rebscher, 2014). In \textit{C. teleta}, the parent cell of 4d (3D) also makes the anus and a portion of the midgut (Fig. 1-2B). The majority of mesodermal tissue in the larvae is derived from the mesodermal bands, which arise from cell 3d (Fig. 1-2C) and cell 3c (Fig. 1-2D). Removal of blastomeres in the early stage embryo typically results in predictable loss of structures in larvae, and this is generally true in \textit{C. teleta} (Amiel et al., 2013).
Figure 1-1. Life cycle and reproductive anatomy of *Capitella teleta*. A. Timeline of indirect development of *C. teleta*. Asterisks indicate position of the mouth. B. Anterior end of adult female showing paired ovaries in abdominal segments. C. Adult male showing genital spines in segments 8 and 9, and genital ducts positioned at the boundary of segment 7 and 8. D. Brood tube containing embryos and adult female. E. High magnification of brood tube showing eggs inside. F. Schematic of *C. teleta* reproductive structures. gd, genital duct; gs, genital spines; ov, ovaries; PGC, primordial germ cells; pgz, posterior growth zone; met, metamorphosis.
Figure 1-2. Fate map studies helped determine the cell lineage for the germline and mesoderm. Single cells were injected with Dil and the cell’s lineage was traced to larval stages. The number and letter in the top right is the name of the filled cell in each panel. The larvae in the right of each panel are traced from the Dil labeled daughter cells for each injection (original data from Meyer et al. 2010). Asterisks indicate position of the mouth. A Cell 4d makes the multipotent progenitor cells and the anus. B Cell 3D is the parent cell of cell 4d, so it makes the MPCs, anus, and the midgut endoderm. C Cell 3d makes the left mesodermal band. D Cell 3c makes the right mesodermal band. Pt, prototroch; tt, telotroch.
CHAPTER 2
MATERIALS AND METHODS

Animal Care

Embryos were acquired by separating males from gravid females for 3-6 days and then combining them in a mating dish for 11 to 14 hours. Dishes were inspected for the presence of brood tubes created by the females (Seaver et al., 2005), and embryos were dissected from the brood tubes and placed in a dish of 0.2 µm-filtered seawater (FSW). All embryos and larval stages were raised in FSW with 60 µg/mL penicillin (Sigma-Aldrich) and 50 µg/mL streptomycin (Sigma-Aldrich) at 19 °C, which was exchanged each day until metamorphosis. Animals were staged according to a published staging chart (Seaver et al., 2005). All juvenile and adults animals were maintained in organically enriched mud.

Cell Deletions

Single blastomere deletions were performed using the XYClone system infrared laser (Hamilton Thorne) with the 20X objective fitted to a Zeiss Axioplan compound microscope as described in Yamaguchi et al., 2016. Embryos were placed on a Rainex-coated slide in FSW and oriented with the vegetal side up. A modified cover slip with two cover slip slivers on each end attached by dental wax was used to make a chamber to cover the embryos (Lyons et al., 2012). For all blastomere deletions, the power was set to 100% and the pulse length was adjusted based on the sensitivity of the brood, the size of the cell being targeted, and the stage of the cell cycle. The pulse range for cell 3D and 3B was one pulse between 150 and 250 µs, followed by a second pulse of 550 to 750 µs. Cell 2D and Cell 2C were deleted using two pulses between 350 and 450 µs. Following deletion, the targeted blastomeres were visually monitored to ensure that the
cytoplasm was leaving the cell. The embryos were then further sorted using a dissection microscope, to ensure complete removal of the cell with no damage to the surrounding cells. The embryos were subsequently monitored into the next cell division to ensure that the surrounding cells were dividing normally. At least 20 control embryos for each brood were raised at the same temperature to monitor overall health of the brood. Experimental animals were scored only if 90% of the controls had elongated bodies and appeared morphologically normally.

**Fixation and Whole Mount In Situ Hybridization**

Animals were fixed as either stage 9 larvae (approximately 9 days after fertilization) one-week (7 days post-metamorphosis) or two-week-old (14 days post-metamorphosis) juveniles. Fixation was carried out in larvae by first relaxing muscles in 1:1 FSW and 0.37M magnesium chloride for 10 minutes, followed by fixation in 3.7% paraformaldehyde (PFA) in FSW overnight at 4 °C. In juveniles, animals were removed from the mud and placed in dishes containing 0.5% corn meal Agar: FSW with 60 µg/mL penicillin and 50 µg/mL streptomycin to remove debris from the body and allow for clearing of gut contents. Juveniles were then placed in dishes containing 0.5% cornmeal agar with MgCl2 (1:1 FSW:0.37 M MgCl2) for 30 minutes, followed by fixation in 3.7% PFA overnight at 4 °C.

Following fixation, larvae and juveniles were washed in phosphate-buffered saline (PBS) and dehydrated through a series of methanol washes into 100% methanol, and stored at -20 °C for at least 24 hours prior to in situ hybridization experiments. The protocol for whole-mount in situ hybridization in *Capitella* is published in Seaver and Kaneshige, 2006. The digoxigenin-labeled riboprobe for *CapI-vasa* and *CapI-piwi1* were generated with the SP6 MEGAscript kit (Ambion, Inc., Austin, TX, USA), and the
digoxigenin-labeled riboprobe for *Capl-nanos* was generated with T7 MEGAscript kit (Ambion, Inc., Austin, TX, USA). Prior to the formal species description, *C. teleta* was known as *Capitella* sp. I (Blake et al., 2009). Genes named prior to the species description were named with a prefix ‘Capl’, and these include *Capl-vasa*, *Capl-nanos* and *Capl-piwi1*. The *Capl-vasa* and *Capl-nanos* probe sequences and length are published in Dill and Seaver, 2008. The *Capl-piwi1* probe sequence and length is published in Giani et al., 2011. All probes were diluted to a final concentration between 0.5 and 1 ng/µl. After hybridization of the probe at 65 °C for 48-72 hours, the probe was detected with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) for approximately 2 to 6 hours. The development reaction was terminated and the animals were washed by repeated exchanges of PBS + 0.1% Tween-20 (PTw) for at least 12 hours, and then were cleared by equilibration in 80% glycerol in 1X PBS with 0.125 µg/µL Hoechst 33342 (Life Technologies H3570) for at least 12 hours. Animals in glycerol were then placed on glass slides with coverslips for microscopic analysis.

**Scoring and Analysis**

For the -3D, -3B, -2D and -2C deletions, *Capl-vasa* expression was used to score larvae for presence or absence of MPCs, and for position of MPCs. Typically the germ cells are located anterior to the pigmented portion of the gut and posterior to the mouth along the ventral midline. Larvae were only scored if they had normally elongated bodies and *Capl-vasa* expression was detected in the posterior growth zone. Juveniles were scored for presence or absence of MPCs in the correct location, in the 4th and/or 5th thoracic segment along the ventral midline, as well as number of clusters and
number of countable cells. Cells were counted using the 20X and 40X objective of a Zeiss Axioplan compound microscope in juveniles viewed on the ventral side.

Adults were scored live between 8 and 12 weeks post metamorphosis for presence or absence of reproductive structures, and whether the animals were male, female, or hermaphrodite. Males were then mated to females, and hermaphrodites mated with males in individual dishes of FSW and mud. One male would be mated to either multiple or single females. In cases where not enough females or males of the experimental group were available, control animals would be used for mating. Fertility was determined by presence or absence of brood tubes with visible embryos inside. Larval viability was determined by ability of the larvae to swim when released manually from the brood tube.

**Imaging and Microscopy**

Larvae and juveniles were imaged using a SPOT FLEX digital camera (Diagnostic Instruments, Inc., SterlingHeights, MI) attached to an Axioskop 2 mot-plus compound microscope (Zeiss, Gottingen, Germany). SPOT imaging software (version 5.2) was used to capture images. Adults were imaged with a Ximea camera on a Zeiss Stemi2000 dissecting scope using the Ximea CamTool Software (QT version 5.6.1). All images were cropped and adjusted using Adobe Photoshop CS6 (version 13.0). All figures were generated in Adobe Illustrator CS6 (version 13.0).

**Statistical Analysis**

A one-way analysis of variance (ANOVA) was used to determine statistical difference in percent germline regeneration between different life stages. This was followed by Tukey’s post- HOC analysis. A specimen was considered to have compensated for loss of germline if it developed germ cells as indicated by Capl-vasa,
CapI-nanos, and CapI-piwi1-positive cells in the correct location in larvae, and CapI-vasa- positive cells in juveniles. In adults, the animals were considered to have regenerated their germline if they developed sex characteristics of males and/or females, and they successfully mated to show fertility. If the groups analyzed had a p value < 0.05 when compared to other groups, they were considered to be statistically different.
CHAPTER 3
RESULTS

Limited Regeneration of the Germline after Deletion in C. Teleta Larvae

To test for regeneration of the germline in C. teleta larvae, we deleted the precursor of the germline in cleavage stage embryos and analyzed larvae resulting from these embryos for presence of germline cells. Single cell laser deletions have been demonstrated to be successful in C. teleta (Amiel et al., 2013; Pernet et al., 2012; Yamaguchi et al., 2016). In C. teleta, the germline precursor cell 4d is extremely small, and therefore to improve accuracy and minimize damage to adjacent cells in the embryo, we deleted the larger, parent cell of 4d, cell 3D. Deletion of blastomere 3D removes the germline as well as the anus and a portion of the midgut in larvae (Fig. 1-2B). Large macromeres have been deleted previously, and larvae resulting from these deletions survived and can feed, indicating that a functional gut forms (Pernet et al., 2012). The embryos were raised to larval day 9, approximately 9 days after fertilization. These larvae were analyzed for presence or absence of MPCs using the previously characterized markers of the germline: Capl-vasa, Capl-nanos and Capl-piwi1 (Dill and Seaver, 2008; Giani et al., 2011). In C. teleta, all three of these genes are expressed in the MPCs, the stem cells of the posterior growth zone and also in the reproductive structures (gametes and gonads) of adults.

At day 9 of larval development, the expression of Capl-vasa in un-manipulated controls is restricted to the MPCs, posterior growth zone, and some cases in the developing brain (Fig. 3-1A). The posterior growth zone expression of these markers serves as an internal positive control to ensure that probe detects endogenous Capl-vasa transcripts. At day 9, the larvae have a single cluster of MPCs located at the
ventral midline (Fig. 3-1A’). A control deletion of macromere 3B was performed. This cell is of a similar size to cell 3D and contributes to the gut, but not to the germline. Deletion of this cell resulted in larvae with Capl-vasa expression similar to that of the un-manipulated control, but with added expression in the head ectoderm. This control (3B) indicates that deletion of a large part of the gut alone does not interfere with germ cell formation (Fig. 3-1B, 3-1B’). When the germline precursor, cell 3D, was deleted and Capl-vasa expression was examined in day 9 larvae, the majority of cases had no germ cells present (n=25/30) (Fig. 3-1C, 3-1C’). However, 16% of cases had MPCs in the correct location at the ventral midline (n=5/30) (Fig. 3-1D, 3-1D’).

We also examined other markers of the germline in larvae resulting from embryos in which 3D was deleted. Capl-piwi1 mRNA expression in day 9 larvae is similar to that of Capl-vasa. Expression of Capl-piwi1 in unmanipulated controls is in the MPCs and the PGZ (Fig. 3-1E, 3-1E’). When cell -3D was deleted and the embryos raised to day 9 larvae, the majority of cases showed loss of the MPCs as assessed by expression of Ct-piwi1 (n=22/26)(Fig. 3-1F, 3-1F’). Similar to larvae resulting from -3D deletions and examined for Capl-vasa expression, 15% of the -3D deletions had MPCs present (n=4/26)(Fig. 3-1G, 3-1G’). Two of the larvae had no MPCs, as assessed by Capl-piwi1 expression, but there were labeled cell clusters with morphology similar to MPCs in the head and trunk (data not shown). In day 9 control larvae Capl-nanos expression is present in the PGZ and the MPCs (Fig. 3-1H, 3-1H’). When cell -3D was deleted and Capl-nanos expression was analyzed there were no detectable germ cells in the majority of the resulting day 9 larvae (n=30/33) (Fig. 3-1J, 3-1J’), although approximately 10% had detectable MPCs (n=3/33) (Fig. 3-1I, 3-1I’). Therefore, the
combined results of analyzing three distinct germline markers (*Capl-piwi1*, *Capl-nanos* and *Capl-vasa*) all show consistent expression patterns in -3D larvae: MPCs were not detectable in most larvae, but in a small proportion of larvae, MPCs are detectable. This proportion (approximately 13%) is similar for the three different markers used. These results show that a small percentage of larvae can compensate for loss of the progenitor of the germline.

**Germline Regeneration in Juveniles**

We used the *Capl-vasa* probe to assess the presence or absence of the germ cell cluster in two-week old juveniles arising from embryos in which cell 3D was deleted. Two weeks after metamorphosis, *Capl-vasa* is expressed in the MPCs of juveniles, which are located between thoracic segments 4 and 5 (Fig. 3-2A, A'). *Capl-vasa* is also expressed in immature oocytes, in the developing ovaries (Fig. 3-2E), in the PGZ (not shown), in cells in the coelomic cavity in segments posterior to segment 6 and in the abdominal segments of a portion of animals (not shown) (de Jong and Seaver, 2017). Juveniles were analyzed for presence or absence of a MPC cluster, the number of MPCs, total cluster number, and location of MPCs in each animal.

In unmanipulated control juveniles, the majority have one *Capl-vasa*+ cluster at the ventral midline (n=37/42), and the cell number within the cluster ranges from 30 to 55 cells between segments 4 and 5 (Fig. 3-2A, A'). The remaining controls have two clusters (n=5/42), closely spaced, in an hourglass shape, with 30-55 countable cells total (data not shown). In juveniles raised from embryos in which cell -3D was deleted (germline removed), 13/32 animals had clusters similar to the controls, with 30-55 cells in one cluster located at the ventral midline (Fig. 3-2B, B'). In 11/32 cases the juveniles resulting from the germline deletion had multiple MPC clusters located in the region of
the ventral midline, between segments 3 and 7 (Fig. 3-2C, C’). In 8/32 cases, the animals resulting from the 3D deletion had one small cluster, defined as containing fewer than 25 cells (Fig. 3-2D, D’). In addition, several of the animals in which 3D was deleted, also expressed Capl-vasa in immature oocytes in the developing ovaries (Fig. 3-2E’). Notably, following germline removal, all of the juveniles had Capl-vasa positive clusters at the ventral midline near segment 5 (n=32/32), demonstrating a dramatic ability to replace the germline in juveniles.

Regeneration of the Germline in Adults

Animals that had their germline removed (-3D) during embryogenesis, were examined for their ability to reproduce as adults (approximately 8 to 10 weeks post-metamorphosis). The animals were then scored for male and female-specific reproductive structures and were also mated to see if viable offspring were produced to demonstrate fertility. Approximately 41% of the -3D deletions resulted in worms with visible genital spines in segments 8 and 9, and genital ducts between thoracic segments 7 and 8, the same reproductive structures as morphologically normal control males (n=30/73) (Fig. 3-3A, B, C). Approximately 50% of the -3D deletions resulted in worms with ovaries in the anterior abdominal segments, typical of adult females (n=37/73) (Fig. 3-3D, E). The remaining adults were comprised of one hermaphrodite and five immature adults with genital ducts only (data not shown). In order to demonstrate fertility, single males were mated with up to three females, and assessed for presence of a brood tube with embryos or larvae. If any of the females produced offspring, the male was scored as fertile. The females were counted as fertile if they produced a brood tube with swimming larvae inside or embryos that later developed into swimming larvae. The hermaphrodite was mated with one male. All of the animals with
complete reproductive structures (males, females, and hermaphrodite) were fertile (n=68/68). These data show that the germline of *C. teleta* can fully regenerate after removal of the germline precursor in the early embryo.

**Regeneration of the Germline during Different Life-Stages**

Because we noticed differences in the fraction of individuals that showed presence of a germline at different stages of the life cycle, we determined whether there is a statistical difference in percent germline regeneration at different life history stages. The percentage of animals with a regenerated germline is statistically different between larval and juvenile stages (p<0.01), and larval and adult stages (p<0.01). There is no statistical difference between the proportion of animals that showed regeneration of the germline between juvenile and adults (p=0.44) (Fig. 3-4). The striking difference in the presence of *CapI-vasa* positive clusters between larvae and 2-week-old juveniles indicates that there may be multiple germline regeneration events: a compensation event in the early embryo that results in a small fraction of the larvae having MPCs, and a second more substantial regeneration event that occurs between larval and juvenile stages and results in all juveniles and adults with germline or germline descendants.

**Expansion of *Capi-Vasa* and *Capi-Nanos* Trunk Expression Following Deletion of Cell 2D**

Our observation that a small proportion of larvae resulting from -3D deletion have MPCs suggests that another cell in the embryo that would usually generates somatic cells likely takes on a germline fate. We hypothesized that the cellular origin of the germline during its regeneration might be a mesodermal precursor cell, because in most bilaterians, the embryonic origin of the germline is mesodermal (Extavour, 2007). In *C. teleta*, cells 3d and 3c form the majority of the mesoderm (Meyer et al., 2010) (Fig. 2C,
D). Half of the trunk mesoderm (3d) and the germline (4d) can both be deleted at once by deleting their shared parent cell, 2D. 2D gives rise to the left mesodermal band, the anus, as well as the germline (Meyer et al., 2010). After the deletion of cell 2D, the embryos were raised to larval day 9 and expression of both Capl-vasa and Capl-nanos was analyzed.

At this stage in unmanipulated controls, Capl-vasa is expressed in the MPCs and PGZ, and in the developing brain of some animals (Fig. 3-5A). At day 9 in ventral view, the ganglia of the ventral nerve cord are visible with a nuclear stain (Fig. 3-5A’, bracket). When cell 2D is deleted in embryos, the larval expression of Capl-vasa changes dramatically. Expression of Capl-vasa in the MPCs was not present in any cases, whereas in the -3D embryos it was only present in a small percentage of embryos. In the -2D embryos, expression of Capl-vasa in the PGZ is faint. Surprisingly, the predominant expression of Capl-vasa is in a band of cells in the mesoderm on the left side that typically extends throughout the length of the trunk. This band of Capl-vasa+ cells is on the same side of the body that normally generates mesoderm from the deleted cell (n=23/25) (Fig. 3-5B, C). The medial edge of this band of cells abuts the lateral edge of the ventral nerve cord (Fig. 3-5B’, C’). Expression in these cells is perinuclear, and the cells are round with a large nuclear to cytoplasmic ratio, similar to undifferentiated cells. In the remaining two cases, there is mesodermal Capl-vasa expression on the left side of the trunk as in the majority of cases, but the cells are arranged in two distinct domains in the trunk with a gap in the mid-trunk, and more cells expressing Capl-vasa in the anterior trunk mesoderm (data not shown). To determine if this change in Capl-vasa expression is a response to the loss of a mesodermal band,
we performed a deletion of the precursor of the other mesodermal band precursor’s parent cell, cell 2C. This deletion resulted in larvae with expression similar to the brood controls, with Capl-vasa in the MPCs, and weak expression in the PGZ (n=69/71)(Fig. 3-5D, D’). The remaining two larvae resulting from the 2C deletion have MPCs, which are centered at the ventral midline like in the controls. They also have clusters of vasa+ cells dispersed throughout the anterior end of the larvae in the ectoderm (data not shown). This experiment indicates that the loss of the mesodermal band alone is not causing the dramatic change in Capl-vasa expression; instead, the combination of the precursors of the germline and one mesodermal band that leads to a abnormal, broad band of Capl-vasa expression in the trunk mesoderm.

To further understand this unexpected response, we deleted cell 2D and analyzed the resulting larvae for expression of Capl-nanos. In unmanipulated day 9 larvae, Capl-nanos is expressed in the MPCs and PGZ (Fig. 3-5G, G’). Capl-nanos expression in larvae resulting from the -2D deleted embryos changes dramatically, although the expression pattern is slightly different from expression of Capl-vasa in -2D larvae. Similar to the Capl-vasa pattern, Capl-nanos expression in the -2D deletions is in the left mesoderm in the trunk, and is positioned adjacent and lateral to the ventral nerve cord (Fig. 3-5H’, D’, J’). However, unlike the expression of Capl-vasa, the labeling only extends midway along the trunk (n=28/32) (Fig. 3-5I, J). In addition, Capl-nanos-positive cells can be seen distributed in the ventral ectoderm of several anterior segments (Fig. 3-5H). These cells are arranged in a segmentally repeated arrangement at the boundaries between segments in the ectoderm (Fig. 3-5J). Of the remaining -2D larvae, 2 out of 3 have Capl-nanos expression in a band of mesoderm that extends
throughout the trunk, like that of the -2D CapI-vasa larvae. One larva shows expression only in the PGZ (data not shown). These small differences in expression pattern between CapI-nanos and CapI-vasa following -2D deletion, suggest that CapI-nanos-expressing cells represent either a subset of the CapI-vasa there are distinct subpopulations of cells.

**Capi-Vasa in Juveniles Following 2D Deletion**

We investigated whether the dramatic change in expression of CapI-vasa and CapI-nanos in larvae following deletion of -2D persisted into juvenile stages. We also investigated whether juveniles could regenerate their MPCs following -2D deletion, like those resulting from 3D deletion, or if they were unable to regenerate the germline due to loss of the mesodermal cell 2d. We examined both one and two week post-metamorphic juveniles following deletion of cell 2D.

For the 1-week post-metamorphosis juvenile controls, a cluster of MPCs typically contain between 20 and 55 countable cells (Fig. 3-6A, A’). Approximately half of the juveniles 1-week post metamorphosis resulting from 2D deletions (n=8/17) had no MPCs present (Fig. 3-6B, B’). A proportion of cases had a small cluster of MPCs (defined as having 10 or fewer countable cells; n=5/17) (Fig. 3-6B). These cells were in one cluster with the typical MPC morphology, and were located in the correct position (between segments 4 through 6) (Fig. 3-6B’). Only 3/17 juveniles following deletion of 2D had a cluster similar in size to that of the controls. The remaining 1-week juvenile had 5 small clusters distributed across thoracic segments 3 through 7 (data not shown). None of these juveniles had an expanded CapI-vasa positive expression domain in the mesoderm comparable to that of the -2D larvae (Fig. 3-5).
For the 2-week post-metamorphosis juveniles, a typical cluster is one cluster of MPCs, containing between 30 and 55 countable cells (Fig. 3-7E, E’). A small percentage of controls have the same number of MPCs but are organized into two clusters, closely spaced (data not shown). A small cluster contains fewer than 25 countable MPCs, and multiple clusters are two or more clusters of MPCs. Unlike the one-week post-metamorphosis juveniles resulting from -2D deletion, the majority of 2-week juveniles following 2D deletion had multiple MPC clusters distributed across a number of thoracic segments (n=14/22) (Fig. 3-7F, F’). Others had a small cluster of MPCs (n=6/22) (Fig. 3-7 G, G’). One experimental 2-week juvenile had a single control-sized cluster, and another juvenile had no detectable MPCs (data not shown). These combined results indicate that there is regeneration of the germline post-metamorphosis, with the majority of regeneration events occurring between one and two weeks post-metamorphosis. These data also show that the expansion of Capl-vasa expression following 2D deletion disappears following metamorphosis. Furthermore, the multiple clusters spread throughout the thoracic segments in 2-week juveniles is likely unrelated to the gene expression pattern seen in larvae following 2D deletion.

**Adults Following 2d Deletion Are Fertile**

Following 2D deletions, only 6% of animals survived from metamorphosis to adulthood, compared to 65% of cases in which -3D was deleted, and 83% of unmanipulated brood controls (Fig. 3-7A). This steep drop in survival is likely due to loss of substantial gut and mesodermal tissue, which could interfere with feeding and locomotion. Of the organisms that did survive, five were male and contained genital ducts and dorsal genital spines (Fig. 3-7B). Five other individuals were female and contained visible ovaries (Fig. 3-7C). The two remaining individuals were immature with
genital ducts only, and when allowed to develop longer, these immature animals later
died (Fig. 3-7D). The males and females were mated, and all were fertile (n= 10/10).
Therefore, removal of the left mesodermal precursor as well as the germline does not
prevent germline regeneration, meaning that the mechanism is not dependent on the
presence of cell 3d in the early embryo.
Figure 3-1. Limited regeneration of the multipotent progenitor cells in larvae following deletion of the germline precursor cell, 3D. All images are ventral views, with anterior to the left. The top right indicates the identity of the deleted cell or undeleted control (con). Numbers in the bottom right of panels indicates the number of cases for the results shown in the panel over total number of cases of the manipulation. The asterisks indicate the location of the mouth and the arrows indicate the MPCs. Larvae in panels A-D show Capl-vasa expression. A. Controls show Capl-vasa expression in the PGZ, the MPC cluster and the developing brain in a proportion of animals. Larvae in E-G show Ct-piwi1 expression. In controls Ct-piwi1 expression is present in the PGZ and MPCs at this stage. Panels H-J, Ct-nanos expression. H. Ct-nanos is expressed in the PGZ and MPCs in controls. Panels labeled with an apostrophe (for example A’ and B’) are a high magnification view of the region containing the MPC cluster from the corresponding larvae to the left with the same letter (for example A and B). Pt, prototroch; PGZ, posterior growth zone.
Figure 3-2. Regeneration of the multipotent progenitor cells in 2-week juveniles following removal of the germ line precursor (cell 3D). All images are juveniles 2 weeks post-metamorphosis with anterior to the left. The top right indicates identity of deleted cell. The numbers in the bottom right of panels indicates the number of cases for the category shown over the total number of cases. The asterisks indicate the location of the mouth. Panels labeled with an apostrophe (for example A’ and B’) are a high magnification view of the region containing the MPC cluster from the corresponding larvae to the left with the same letter (for example A and B). A Thoracic segments in an unmanipulated control showing expression of Capl-vasa in the MPC cluster. B Juvenile developed from -3D embryo with a MPC cluster similar to that of the control. C Juvenile developed from -3D embryo with multiple MPC clusters. D Juvenile developed from -3D embryo with a small MPC clusters. E Abdominal segments of a 2 week juvenile of a female or hermaphrodite. E’ Abdominal segments of a 2 week juvenile of a female or hermaphrodite that developed from an embryo following -3D deletion. The dotted lines mark the boundary between segments. The closed arrowheads indicate the MPCs and the arrows with tails show the immature oocytes that express Capl- vasa. Lines indicate cells expressing Capl- vasa in the coelemic cavity. Con, unmanipulated brood control; Ven, ventral view; lat, lateral view with ventral down.
Figure 3-3. Assessment of reproductive structures and fertility in adults following deletion of the germline (cell 3D). All images are adults developed following germline deletion (-3D) between 8 and 14 weeks post metamorphosis. Number of cases is shown in bottom right corner. Panels A-C are images of males. Panels D-F are images of females. A A reproductive male is depicted with the genital duct found between segments 7 and 8 and the dorsal spines seen in segments 8 and 9. B, C Magnification of the segments containing the gs. B DIC C Auto fluorescent features of the gs. D A reproductive female with pairs of ovaries in the abdominal segments. E High magnification of ov. F A brood tube containing embryos and an adult female. The dotted lines mark the boundary between the segments. gd, genital ducts; gs, genital spines; lat, lateral; lv; larvae; ov, ovary; ven, ventral.
Figure 3-4. Germline regeneration in *C. teleta*. The bars on the graph show the average percent regeneration for each life-stage. The error bars represent the standard error of the mean.
Figure 3-5. Expansion of Capl-vasa and Capl-nanos trunk expression after deletion of mesodermal and germ cell precursor, cell 2D. All images are stage 9 larvae, approximately 9 days post-fertilization, with anterior to the left. The top right indicates the identity of the deleted cell or un-manipulated control (con). Numbers in the bottom right indicates the number of cases for the deletion results over the total number of cases for that deletion. The asterisks indicate the location of the mouth, closed arrows indicate the MPCs, open black arrows indicate labeling of mesodermal band cells, black lines and ect indicated labeled cells in the ectoderm, white lines indicate the location of the ventral nerve cord (vn) labeled with Hoechst nuclear stain and square brackets mark the lateral edge of the ganglia in the vn. Panels labeled with an apostrophe (for example A’ and B’) are Hoechst nuclear labeling of the corresponding larvae to the left with the same letter (for example A and B). A, B, C, D Capl-vasa expression A brood control. B, C Larvae following -2D deletion showing a band of cells in the mesoderm to the left of the ventral midline. D Larvae following -2C deletion with expression similar to the brood controls. G, H, I, J Capl-nanos expression G Brood control. Expression is similar to that of Capl-vasa, but also present in the posterior growth zone at this stage. H, I, J Larvae following -2D deletion with mesodermal expression to the left of the ventral midline. Ectodermal cells can be seen in a segmental pattern anterior to the mesodermal expression. The border between the mesoderm and ectoderm is labeled with a black dotted line. ect, ectodermal; lat, lateral; pgz, posterior growth zone; pt, prototroch; ven, ventral.
Figure 3-6. Multipotent progenitor cell regeneration response after -2D deletion occurs post metamorphosis. All images are with anterior to the left showing the expression of Capl-vasa. The top right indicates identity of deleted cell or un-manipulated control (con). Numbers in the bottom right of these panels indicate the number of cases for the deletion results shown in the panel over the total number of cases. Asterisks indicate the location of the mouth, and arrowheads indicate the MPCs. Panels labeled with an apostrophe (for example A’ and B’) are a high magnification view of the region containing the MPC cluster from the corresponding larvae to the left with the same letter (for example A and B). The top 2 rows are juveniles 1 week post-metamorphosis and the bottom 2 rows are juveniles 2 weeks post-metamorphosis. A, E Brood control with one cluster of MPCs. B. Juvenile with no MPCs present following -2D deletion. C Juvenile with a small cluster of MPCs. D Juvenile following -2D deletion with a standard sized cluster. F 2 week juvenile following -2D deletion with multiple MPC clusters extending between thoracic segments 3 and segments 7. G Juveniles with a small cluster of cells following -2D deletion. Ven, ventral.
Figure 3-7. Deletion of the mesodermal and germline precursor, cell 2D, results in fertile adults but low percent survival. A Graph of the average percent survival to 8 weeks of all experimental replicates for the -2D deletions raised to adulthood, the -3D deletions raised to adulthood, and the brood-controls for the -2D and -3D deletion experiments. B,C,D The 3 categories of -2D adults: B Male C Female D immature. Asterisks indicate the location of the mouth. gd, genital ducts; gs, genital spines; lv, larvae.
CHAPTER 4
DISCUSSION

Regeneration of the Germline

Our data in *C. teleta* provide experimental evidence for regeneration of the germline, and provide an exception to the concept of a single origin of the germline. We deleted the presumptive germline in the early stage embryo, and showed that by 2 weeks post-metamorphosis, all animals had developed MPCs. Furthermore, most adults resulting from germline removal had developed reproductive structures and produced offspring. A few animals (n=5/73) only developed structures that resembled genital ducts by 13 weeks post-metamorphosis. We believe this to be a result of tissue loss in the gut from the -3D deletion. This is likely due to loss of gut tissue that could lead to a disadvantage in nutrient uptake, and thus growth when compared to controls.

Our study is unique in that the germline was removed in the early stage embryo, whereas in most studies, the germline is removed in larval or adult stages, after tissue differentiation. To our knowledge, these results are the first example of germline regeneration in a lophotrochozoan, a large and diverse clade, and point to the need for further sampling in this clade. Our findings suggest that there may be more examples of germline regeneration in this clade once more sampling is done. Studies in many model species have supported the dogma that the germline cannot regenerate following removal, leading to the hypothesis of a single origin of the germline. This hypothesis is likely biased by animals that have been sampled, only representing a fraction of animal diversity.

Thorough experimental manipulations of the germline have been conducted in amphibians. In axolotls, the lateral plate mesoderm, the source of the primordial germ
cells (PGC), was surgically removed prior to PGC migration. The larvae that developed after PGC removal had genital ridges, somatic reproductive tissues, but lacked germ cells (Nieuwkoop, 1951). In *X. laevis*, the region of the endoderm that forms the germ cells was removed and replaced with an anterior region of the endoderm, and the resulting tadpoles developed normal gonadal anlagen, but the gonads lacked germ cells and the adults were infertile (Blackler, 1965). The ability of the germline to regenerate was also investigated by removing the vegetal pole germ plasm in early stage amphibian embryos, via both UV irradiation (Nieuwkoop and Suminski, 1959) and pricking (Züst and Dixon, 1975); both techniques resulted in sterile animals.

Experimental manipulations in birds and mammals also support the idea that the germline has a single origin. In birds, the founder germ cell population, the anterior germinal crescent at somite stages, was either surgically removed (Dulbecco, 1946), removed by irradiation (Dubois, 1962), or cauterized (Fargeix, 1975). All three manipulations resulted in infertile adults. A series of experiments were also carried out in the chick, where the germinal crescent was irradiated, and then suspensions of PGCs from a turkey were intravenously injected. All germ cells that ended up in the host chicken gonad anlagen were from the donor turkey’s germ cells (Reynaud, 1976, 1970; Reynaud et al., 1969). The most convincing evidence in mammals for the single germline hypothesis is from an experiment conducted in *M. musculus*. The gonadal analgen in embryos was irradiated and the resulting adult mice had differentiated reproductive structures, but lacked germ cells (Everett, 1943).

Results from several invertebrates also support the single origin of the germline hypothesis. In *C. elegans*, the ablation of P4, which divides to form germ cell primordia,
results in an infertile adult that lacks germ cells, but has structured gonads (Sulston and Schierenberg, 1983). In *D. melanogaster*, evidence is based on a fly line with mutant *germ cell-less* (*gcl*) and *tudor* genes, both of which are necessary for germ cell specification. The mutant adult flies developed reproductive structures, but no germ cells (Barnes et al., 2006). However, the mutations in these genes crucial for germline development would have likely prevented the regeneration of the germline by another cell lineage, which might also require these genes. In sea urchin embryos, the small micromeres that generate the coelemic sacs and are thought to make MPCs (Yajima and Wessel, 2011), were deleted at the 4th embryonic cleavage, and the resulting animals were fertile (Ransick et al., 1996). However, when the same cells were deleted one division later, the animals produced reproductive structures, but lacked gametes (Yajima and Wessel, 2011). This suggests that the germline is not specified until after the 4th division micromeres and cannot regenerate following germline removal.

A few studies in addition to ours are beginning to complicate the idea of a single origin of the germline. In the ascidian *C. intestinalis*, the larval tail, which contains the germ cells, was removed, and the resulting juveniles had no germ cells (Takamura et al., 2002). However, after approximately 15 days, using an antibody against the *C. intestinalis vasa* homologue, a few germ cells were detected, and the adults that developed produced sperm. Interestingly, recent molecular evidence suggests that these regenerated PGCs come from multiple somatic origins (Yoshida et al., 2017). In the colonial ascidian *Botryllus primigenus*, the germline is segregated from the soma in the early embryo. When all of the buds and zooids are removed, an entire animal can bud from the tunic vessels, and eventually leads to regeneration of the colony. This
process is called vascularization (Milkman, 1967; Oka and Watanabe, 1959, 1957; Sabbadin et al., 1975). Immediately after vascularization, the colony has no vasa-positive germ cells, but after 2 weeks of colonial regeneration, vasa-positive cells re-appear de novo (Sunanaga et al., 2006). In the flatworm, *Macrostomum lignano*, the germline is segregated early, but during regeneration, the gonads and germ cells were formed from the somatic stem cells, meaning *M. lignano* also intrinsically has two mechanisms for germline formation (Pfister et al., 2008). The planarian, *Dugesia japonica*, also exhibits a similar capability for somatic cells to begin expressing germline markers de-novo following amputation (Sato et al., 2006). In annelids, there is limited data, but *Pristina leidyi* provides some evidence for the complexity of germline origin. In this asexually reproducing animal, vasa-positive cells appear after the animals are induced to become sexual. These cells arise post-embryonically from a stem cell population (Özpolat and Bely, 2015). In summary, these studies provide a number of examples that support the idea of both an embryonic origin and a somatic stem cell origin for the germline, which allows for regeneration of the germline in adults.

Animals that use preformation to segregate their germline have been proposed to lack an ability to regenerate germ cells when the germline precursors or primordial germ cells are removed. However, data in *C. intestinalis* seems to contradict this theory (Extavour, 2007). Although data is currently limited, it has also been suggested that some species may use both preformation and epigenetic or post-embryonic mechanisms to specify their germline, and these animals might be capable of recovering the germline following removal (Extavour, 2007; Juliano et al., 2010). This scenario seems likely for *C. teleta*. Based on the observation that outgroups of
bilaterians do not have a clear distinction between germline and somatic cells, one theory suggests that all stem cells of the last common ancestor of bilaterians had germline potential (Buss, 1987; Extavour, 2007; Michod, 1996; Michod et al., 2003; West-eberhard, 2005). In contrast, some evidence suggests that a committed population of germline stem cells that help protect the germline from mutation evolved with more complex body plan organization, and may be a eumetazoan trait (Barfield et al., 2016; Littlefield and Bode, 1986). Whatever the case, it is likely that many bilaterian species have somatic populations that can contribute to the germline under certain circumstances and this might be more prevalent than previously thought.

**Timing of Regeneration of the Germline in *C. Teleta***

*C. teleta* undergoes indirect development, meaning it has larval, juvenile, and adult stages. We analyzed germline regeneration across all life-history stages. When the percentage of germline regeneration is compared between larvae, juveniles, and adults, regeneration is statistically different between larvae and adult and larvae and juveniles. Only a small proportion of animals (13%) have regenerated their MPCs by day 9 of larval development. At the 2-week-post-metamorphosis time point, all juveniles have regenerated their MPCs. We know the germline regeneration event occurs post-metamorphosis, because in the 1-week juvenile time point (-2D), some animals have not yet regenerated their MPCs. This process occurs after tissues have been specified, and is likely a stem cell to germ cell transition.

It is interesting to note that the timing of the germline regeneration event coincides with the approximate time period that the animal is capable of posterior regeneration (Giani et al., 2011; de Jong and Seaver, 2016). Future studies are needed to determine whether or not the ability to regenerate somatic tissues is a requirement for
germline regeneration. It has been hypothesized that stem cells with dual germline and somatic potential are linked to the high regenerative capabilities of some metazoans (Rebscher, 2014). Thus far, animals that can regenerate their germline also have substantial regenerative ability. In the future, it would be intriguing to investigate mollusks for their ability to regenerate a lost germline, since they do not have regenerative abilities but are closely related to annelids.

**Evidence for Regulation in the Spiralian Embryo of C. Teleta**

Spiralians, particularly annelids and mollusks, have long been associated with mosaic development, meaning that each cell in the embryo is fated to form a specific structure, and if the cell is lost, the structure will also be lost. In the annelids *Lanice*, *Chaetopterus*, *Sabellaria*, *Tubifex* and *Nereis*, blastomeres were separated during early cleavage stages, allowed to develop in isolation, and each cell continued to divide as if it had not been isolated from the embryo (Costello, 1945; Hatt, 1932; Penners, 1926; Tyler, 1930; Wilson, 1904). In addition, experiments in which individual blastomeres were removed from the embryo in mollusks (Clement, 1967, 1962) have largely resulted in loss of structures. More recently, spiralian embryos have been found to be more regulative than once assumed. A few experiments show spiralian species to have a regulative ability when one or more cells in the 4 to the 8-cell stage embryo are deleted. In the snail, *Ilyanassa obsoleta*, when blastomeres A and C are deleted, 10% of larvae compensate and make eyes (McCain and Cather, 1989). When 1a and 1c are deleted at the 8-cell stage, both the gastropod *Lymnaea stagnalis* and the flatworm *Hoploplana inquilina* can compensate and form eyes (Arnolds et al., 1983; Boyer, 1987).
In a previous study in *C. teleta*, deletion of 13 different blastomeres were deleted in early stage embryos, and resulted in loss of the expected structures in larvae (Amiel et al., 2013).

Therefore, it is surprising that in our experiments, 13% of larvae had regenerated MPCs following the deletion of the germline precursor, cell 3D. Using three different markers, *CapI-vasa*, *CapI-nanos*, and *CapI-piwi1*, increased our confidence that the loss and gain of MPCs was a cellular response and not just a change in gene expression. It is notable that the percentage of compensation was consistent across all three genes. This percentage of regulation is similar to that seen when the blastomeres that generate the larval eyes in *C. teleta* are deleted at the 8 cell stage of embryonic division (Yamaguchi et al., 2016). We hypothesize that, following the deletion of the germline precursor, cell 3D, in a small percentage of embryos, another cell physically shifts its position and receives signals that induce it to become the germ cell lineage.

**Repression of Stem Cell Response by the Germline in *C. Teleta***

We deleted cell 2D in an attempt to determine the cellular origin responsible for the observed regeneration of the germline. Cell 2D is the parent cell of the germline lineage (cell 4d), a portion of the midgut (contributed by cell 3D) and the left mesodermal band (Meyer et al., 2010). When this cell was deleted, there was a drastic change in expression of *CapI-vasa* and *CapI-nanos*. Following deletion of the germline precursor, 3D, larvae expressed *CapI-vasa* and *CapI-nanos* in the stem cells of the growth zone in all larvae, and in the MPCs in 13.4% of larvae, the MPCs. Following 2D deletion, no MPCs were visible using these markers. In addition, we were surprised to find a large band of cells expressing these markers in the left mesodermal tissue, on the side of the body in which precursor lineage was removed in the embryo. These cells are
large, round, and have a large nuclear to cytoplasmic ratio, and as such, we interpret these cells to be stem cells. The large band of cells extends from the growth zone, a reservoir of stem cells, through most of the trunk of the larva. Interestingly, although found in the same general region of the body, Capl-vasa and Capl-nanos have different patterns of expression following 2D deletion. This suggests that the cells in the mesoderm are a heterogeneous population. The location of these cells, in the left mesodermal band, whose tissue was lost by deletion of cell 2D, suggests that they might form part of a regenerative response to the lost tissue. There are also cells expressing Capl-vasa and Capl-nanos in the ectoderm, that likely originate from cell 2d due to their position in the larvae. Perhaps 2d and/or its descendents receive a signal that induces expression of these germline/stem cell genes from a cell that would, under normal circumstances, induce cell 3D to become the germline. Cell 2d is a large cell and its position, directly adjacent to macromere D, could allow for it to contact cells that would usually be in contact with cell 3D.

We originally hypothesized that a subset of the large band of cells expressing the germline/stem cell markers following -2D deletion would become the MPC clusters. However, this large Capl-vasa and Capl-nanos expression domain in larvae is absent in 1-week post metamorphic -2D juveniles, and MPC clusters are only present in 9/17 animals at this stage. This suggests that the cells that formerly expressed these genes in the mesodermal band do not play a direct role in the MPC regeneration event. Remarkably, when the other mesodermal precursor cell 2C that makes the right mesodermal band, was deleted, the resulting expression of Capl-vasa in larvae was identical to expression in control larvae, and was expressed in the MPCs and faintly in
the PGZ. Therefore in this case, the right mesodermal band was removed without removing the germline lineage. From these results and results of previous embryonic deletions (Amiel et al, 2013), we hypothesize that the germ cell lineage normally inhibits mesodermal band regeneration. However, when both the mesoderm and germ cell precursors are removed, this inhibition is released, and the mesodermal band can regenerate, as indicated by the abnormal Capl-vasa and Capl-nanos expression along the trunk. In future studies, to explore this further, the 2C mesodermal band cell precursor and the 4d germline precursor could be deleted simultaneously.
CHAPTER 5
CONCLUSIONS: WORKING MODEL OF GERMLINE REGENERATION IN C. TELETA

Our findings led us to propose the following model of germline regeneration in C. teleta. When the precursor of the germline is removed in the early embryo, in a small portion of cases, another blastomere in the embryo is induced to generate the germline. In these cases, larvae possess the MPC cluster. However, in a majority of animals where the germline is removed, larvae lack MPCs. In juveniles, in a second regeneration event, a population of somatic cells trans-differentiate to become the new germline between one and two weeks after metamorphosis. The origin of the cells that become the new MPCs is currently unknown, but likely arise from stem cells, and may originate from multiple somatic stem cell populations. Almost all adults following embryonic germline deletion are fertile, demonstrating a robust ability of C. teleta to replace its germline. C. teleta males have a remarkable ability to form female reproductive structures and reproduce as females under environmental strain. In addition, C. teleta juveniles and adults can regenerate their full posterior ends (that includes reproductive structures in females) if it is lost. This ability to regenerate the germline is another example of developmental resiliency and an evolutionarily advantageous characteristic used to continue to reproduce, even if the cells fated to become the germline in the embryo are lost.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Leah Dannenberg completed her Bachelor of Science in biology with a minor in studio art from Georgia College and State University. During her undergraduate degree program, she worked in a yeast genetics lab at her home university under the mentorship of Dr. Ellen Yen Kang France. She also participated in the National Science Foundation’s Research Experience for Undergraduates program as a research intern at the Whitney Laboratory for Marine Biosciences under the mentorship of Dr. Elaine Seaver. These research experiences led her to pursue an advanced degree at the University of Florida.